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Therapeutic use of xenogeneic antibodies: approaches to reducing the anti-immunoglobulin response

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Abstract

Passively administered antibodies (polyclonal or monoclonal) with specificity for malignant cells, can successfully localize to tumours *in vivo;* hence they offer a potentially useful tool for the early diagnosis and treatment of neoplasia. However, extensive clinical studies have been limited by the fact that almost all anti-tumour antibody preparations are of non-human origin; thus, patients with cancer become sensitized to the therapeutic anti-tumour antibodies. The resultant anti-immunoglobulin (Ig) response leads to the formation of antibody/anti-antibody immune complexes *in vivo,* which dramatically reduces the effectiveness of this new therapy and may also lead to type III hypersensitivity. The aim of the work described in this thesis was to study ways by which antigen-specific unresponsiveness to xenogeneic Ig could be induced without impairing other immune functions.

Initially we developed a sensitive and reproducible indirect enzyme-linked immunosorbent assay, that enabled us to detect circulating host antibodies formed against the foreign Ig (chapter 2). Pharmacokinetic studies of mouse monoclonal antibodies (mAbs) administered i.v. to patients with cancer revealed that both whole and $F(ab')_2$ antibody fragments remain in the circulation for a relatively long time, eventually triggering an immune response. Mouse mAbs administered i.p. were equally immunogenic; they were not restricted by the peritoneum, but slowly migrated into the blood stream, reaching a peak at 50 hours post-injection corresponding to 22-28% of the injected dose. However, antibody dimers and polymers did not appear to leave the peritoneal cavity (chapter 3).

Our studies on the induction of unresponsiveness were performed in experimental animals and, when possible, in man. A variety of strategies were tested. i) Chapter 4 describes experiments where passive administration of anti-Ig antiserum was analyzed for its efficacy in reducing

the endogenous anti-Ig response. Patients with ovarian cancer who were undergoing i.p. mouse mAb therapy, were given an i.v. "chase" of affinity purified polyclonal human anti-mouse Ig antibodies to remove mAb molecules that had entered the blood stream. Although 18-20% of the injected mAb was catabolized within the first 40 hours, all patients mounted an anti-mAb response. ii) We next studied the possibility of inducing immunosuppression by deaggregation and impairment of antigen-processing (chapter 5). Deaggregation of human IgG (hIgG) significantly reduced its immunogenicity in CBA and C57BL/6 mice (tolerance "susceptible"), but did not affect the primary, and slightly reduced the secondary anti-hIgG response of tolerance "resistant" Balb/c mice. The response of Balb/c mice was significantly reduced only when 7-15 mg/kg cyclosporin A was administered (chapter 6). In clinical studies, patients with ovarian cancer who received radiolabelled (^{131}Iodine) monomeric mouse anti-tumour mAb for therapy, developed anti-mouse Ig antibodies at levels comparable with those of patients who received partially aggregated radiolabelled (^{90}Yttrium) mAb. iii) An alternative approach was to evoke the concept of antigen "suicide" for the induction of tolerance by clonal elimination of anti-Ig specific B-cells (chapter 7). A bispecific mouse mAb complexed with the drug vinblastine was found to be up to 80% less immunogenic than control preparations in rabbits. In contrast, when Igs were highly radiolabelled with either ^{111}Indium or ^{125}Iodine there was no significant reduction in their *in vivo* immunogenicity. iv) We next investigated the induction of immunosuppression by anti-lymphocytic antibodies, using a mouse mAb directed against an interleukin-4 receptor-related molecule in rabbits (chapter 8). However, we found that *in vivo* binding of this antibody onto immunocompetent cells did not lead to deletion or functional anergy, but instead enhanced the overall responses against itself and a hapten (FITC) molecule covalently linked to it. Finally the general significance and future usefulness of these various strategies are discussed in chapter 9.

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Abbreviations

Ab(s)	: Antibody(ies)
ABTS	: Azino-di-[3-ethylbenzthiazolinesulphonate]
Ag(s)	: Antigen(s)
αFP	: Alpha foetoprotein
A-hIgG	: Aggregated human IgG (partially)
ALP(s)	: Alkaline phosphatase(s)
APC(s)	: Antigen presenting cell(s)
BCG	: Bacillus Calmette-Guérin
BSA	: Bovine serum albumin
°C	: Degrees Celsius
CEA	: Carcinoembryonic antigen
CSA	: Cyclosporin A
Cy	: Cyclophosphamide
D-hIgG	: Deaggregated human IgG
DTPA	: Diethylenetriamine pentaacetic acid
EBV	: Epstein-Barr virus
EGF(R)	: Epidermal growth factor (receptor)
ELISA(s)	: Enzyme-linked immunosorbent assay(s)
FITC	: Fluorescein isothiocyanate
FPLC	: Fast protein liquid chromatography
GM-CSF	: Granulocyte-macrophage colony stimulating factor
HA-hIgG	: Heat-aggregated human IgG
HAMA	: Human anti-mouse antibodies
Hb	: Human haemoglobin
HCG	: Human chorionic gonadotropin
HH	: Hybrid-hybrid (a bispecific mouse monoclonal antibody)
hIgG	: Human IgG
HMFG	: Human milk fat globule
HRP	: Horseradish peroxidase
HSA	: Human serum albumin
HTLV	: Human T-cell leukaemia/lymphoma virus
I	: Iodine
ICAM	: Intercellular adhesion molecule
ICRF	: Imperial Cancer Research Fund
IEF	: Isoelectric focusing
Ig(s)	: Immunoglobulin(s)
IFN(s)	: Interferon(s)
IL(R)	: Interleukin (receptor)
In	: Indium
i.p.	: Intraperitoneal(ly)

i.v. : Intravenous(ly)
LAK-cell(s) : Lymphokine-activated killer cell(s)
LFA : Lymphocyte function-associated antigen
LR(s) : Lymphokine receptor(s)
mAb(s) : Monoclonal antibody(ies)
MHC : Major histocompatibility complex
MR : Molar ratio
mϕs : Macrophages
NBS : N-bromo succinamide
NK-cell(s) : Natural kiler cell(s)
OD : Optical density (optical absorbance)
OVA : Ovalbumin (egg albumin)
PBS : Phosphate buffered saline
PDGF(R) : Platelet-derived growth factor (receptor)
PLAP(s) : Placental alkaline phosphatase(s)
RES : Reticuloendothelial system
RIA(s) : Radioimmunoassay(s)
RPMS : Royal Postgraduate Medical School
RT : Room temperature
SDS : Sodium dodecyl sulphate
SDS-PAGE : SDS-polyacrylamide gel electrophoresis
TCA : Trichloroacetic acid
T_c-cell(s) : T-cytotoxic cell(s)
T_d-cell(s) : Delayed-type hypersensitivity T-cell(s)
T_h-cell(s) : T-helper cell(s)
T_s-Cell(s) : T-suppresor cell(s)
TcR(s) : T-cell receptor(s)
TD : Thymus dependent (antigen)
TI : Thymus indepependent (antigen)
TIL(s) : Tumour infiltrating lymphocytes
TNF(s) : Tumour necrosis factor(s)
UV : Ultra violet
VLB : Vinblastine
Y : Yttrium

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Chapter 1

General introduction

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Γυναικί ὁκόταν αἱ ὑστέραι σκληραί γένωνται καί ἐς τά αἰδοῖα ἐξίθοι, καί οἱ βουβῶνες σκληροί γίνωνται, καί καῦμα ἐν τοῖσοιν αἰδοίοισιν ἐνῇ, καρκινοῦσθαι φιλέει πάντα. "ΓΥΝΑΙΚΩΝ ΦΥΣΙΣ" . . . . . Γυναικί, ἐν Ἀβδήροισι, καρκίνωμα ἐγένετο περί τό στῆθος, καί διά τῆς θηλῆς ἔρρεεν ἰχώρ ὕφαιμος· ἐπιληφθείσης δέ τῆς ρύσιος, ἔθανεν. "ΕΠΙΔΗΜΙΑΙ", Ἱπποκράτης, 5ος αἰών π.χ.

When the uterus of a woman becomes indurated and protrudes to the external genitalia and the groins become hardened and there is burning in the genitals, all tend to become cancerous. "NATURE OF WOMEN" . . . . . A woman from Abdera developed carcinoma on the breast, and through the nipple there was sero-sanginous discharge; when the discharged ceased, she died. "EPIDEMICS", Hippocrates, 5th century B.C. (translation by Retsas, 1986).

Indeed, cancer is not a disease of the 20th century. There is evidence of carcinomatous abnormalities since the middle pleistocene period: a femur belonging to *Homo erectus* (Pithecanthropus) was discovered bearing an unusually irregular bony protuberance (Brothwell, 1967). Tumours of the head (mainly osteomata) were not uncommon among Incas in pre-Columbian South America (Moodie, 1926; Moodie, 1929). Detailed study of Egyptian mummies revealed evidence for the presence of solar keratosis (Brothwell, 1967), basal cell cutaneous carcinoma (Satinoff and Welsh, 1969), myoma (Strouhal, 1976), malignant cystadenocarcinoma of the ovaries (Brothwell, 1967), meningioma/angioma (Rogers, 1949), sub-epicranial dermoid tumour (Brothwell, 1967) and osteosarcomas (Rowling, 1961). A skull belonging to the Roman period was thought to represent a case of ivory osteoma (Brothwell, 1967), carcinomas of the ethmoid and the rectum was suggested as the cause of death of two bodies belonging to the Byzantine period (Smith and Dawson, 1924). Secondary carcinomatosis of the cranium was diagnosed in a mediaeval

Danish skull (Møller and Møller-Christensen, 1952), a Saxon skull of the same period showed destruction resembling fibroma or extensive polypi (Brothwell, 1961), and a skeleton from Hungary, dating from around the 11th century A.D., was found to have multiple osteolytic lesions (Nemeskeri and Harsány, 1959).

Despite the rarity of ancient human remains and the difficulty in accurately diagnosing the cause of death, there seems to be little doubt that a variety of malignancies existed in antiquity. Although the life expectancy was short, there is convincing evidence that individuals suffered from tumours of skeletal parts; however this bias is almost certainly due to the source of material, since, with the exception of Egyptian mummies, no soft tissues were available for examination.

Both malignant and benign neoplasia were referred to in many ancient Egyptian papyruses that are older than 4,000 years. But it was during the ancient Greek period (8th-4th century B.C.) that cancer was first scientifically studied and documented. Hippocrates (5th century B.C.) laid the foundations of modern oncology (derived from the greek word ὄνγκος (óncos) = irregular mass, volume, and λόγος (lógos) = an ending denoting "the science of") with his attentive studies and endeavour to treat this difficult and most often incurable disease. His essays (5 books are known to us today, that covered all known diseases at the time) became the "bible" for later physicians, and most of his accurate observations and suggestions for treatment remained unchallenged for centuries. Rightly Hippocrates is regarded as the father of medicine. His principle "Ὠφελέειν ἢ μή βλάπτειν" "be useful and do not harm", and his paradigm to observe and record, was followed by many Greek physicians throughout the Hellenistic, Roman, Byzantine and later period such as Demosthenes (1st century A.D.), Dioscurides (1st A.D.), Aretaeus (2nd A.D.), Galen (Galenus, 2nd A.D.), Leonidas (2nd A.D.), Paulus Aegineta (7th A.D.), Theophanes Nonnos (10th A.D.), Michael Psellos (11th A.D.) and Ioannes Actuarios (14th A.D.), to name a few. The immense significance of

their work lies not only in the everyday comfort offered to fellow people, but in their eagerness to observe, study, record and impart their knowledge to later generations (what better paradigm for "hatching" scientists of today!). The foundations of oncology and the basis of cancer manipulation had been set.

1.1 Molecules associated with malignant transformation

Today we know that malignancy begins at a site where alterations in the genome of a cell (or cells) result in uncontrolled growth. This can be caused by viral infection, or other exogenous and endogenous factors such as radiation, chemical or biological substances. Despite the great progress of medicine in the past 100 years, our knowledge is still limited as to how cancer is initiated in apparently healthy individuals. Each person's idiosyncrasy together with parameters unsuspected by or still unknown to us (eg. electromagnetic fields, nutrition, environmental pollution) may interfere with, promote or facilitate tumour growth. Although neoplasia is initially restricted to a particular site of a tissue, it may easily affect neighbouring cells, as well as metastasize (often specifically) to distant organs, even after radical excision of the primary tumour. Thus, this syndrome that we call cancer is a complex disease associated with a wide spectrum of effects ranging from direct tissue damage to abnormalities in metabolism, hormone- and immune-system regulation.

1.1.1 Oncogenes and their products

One question that has dominated the work of generations of investigators, is in which way malignant cells differ from their normal counterparts, since detailed analysis of abnormalities observed in cancer cells is necessary for better understanding of tumourigenesis. We now know from animal studies that certain viruses (oncoviruses) by infecting cells, introduce genes, termed viral oncogenes, which through overexpression, and in some cases mutation,

induce uncontrolled growth in the host cell (Bishop, 1983). The irony is that oncoviruses have most probably acquired their oncogene from host cellular RNA after a previous infection of normal cells (Bishop, 1983). It is increasingly becoming evident that protooncogenes (the cellular homologue of viral oncogenes) are normally present in a variety of cells, and that their products are involved in normal cell growth and proliferation. Thus, protooncogenes encode a variety of molecules that are involved at different stages in the cell growth cycle: growth factors, growth factor receptors. protein kinases and nuclear binding proteins (Bishop, 1983; Weinberg, 1985; Duesberg, 1985; Bishop, 1986). However, the tumourigenic derivatives of the normal genes, the oncogenes, are altered in such a way that cell growth is uncontrolled. Recent work in man indicates that this change in the genome can occur in many ways in addition to viral transfer (*v-onc*): point mutation (eg. *c-ras*; Reddy et al., 1982; Tabin et al., 1982), gene translocation (eg. *c-myc*; Nishikura et al., 1983; Stanton et al., 1984), amplification of the protooncogene, or deletion of a controlling ("suppressor") gene (Hunter, 1986), can be involved.

A variety of oncogenes have been shown to be involved in human neoplasia. Among the most extensively studied cancer-associated proteins is platelet-derived growth factor (PDGF), a product of the *c-sis* oncogene, which binds to the PDGF-receptor that it itself encoded for by the *c-src* and *c-abl* protooncogenes (reviewed by Stiles, 1985). It is found that a variety of tumour cell-lines secrete PDGF (or PDGF-like) molecules (Heldin et al., 1986; Betsholtz et al., 1986). Likewise, the receptor for epidermal growth factor (EGF) is the product of the *v-erb B* oncogene (Downward et al., 1984; Ullrich et al., 1984; Stoscheck and King, 1986), being found at elevated levels on many cell types (Scheving et al., 1981; Carpenter, 1981) including human carcinomas of cervical, ovarian and vulvual origin (Gullick et al., 1986) as well as gliomas (Epenetos et al., 1985a; Kalofonos et al., 1989a). In addition, interleukin 2 (IL-2, formerly T-cell growth factor) receptors are overexpressed on T-cell leukaemias after infection of T-cells by an oncovirus (HTLV-1) (Uchiyama et

al., 1977; Poiesz et al., 1980; Waldmann et al., 1985).

Therefore, a malignant cell would be characterized by the presence of a new or overexpressed gene (oncogene), secretion of large quantities of growth factor(s) and/or expression of growth factor receptors. In addition, protooncogenes may be associated with the induction of proteins that seem to be essential for their action. It has been shown that *c-myc*, *c-myb* and *c-fos* encode cytoplasmic proteins that bind to nuclear chromatin; it is thought that these molecules interact with DNA and play an important role in RNA processing (reviewed by Hunter, 1986). Perhaps the most investigated direct oncogene product is a protein with molecular weight of 21KD (p21) encoded by the *ras* genes. As molecular analysis has revealed, p21 is a family of similar proteins with single aminoacid differences, since they are encoded by *ras* genes characterized by point mutations. Activated p21s are found in a variety of solid tumours, as well as leukaemias (reviewed by Carney, 1988).

1.1.2 Virally encoded molecules

Cells transformed by DNA or RNA oncoviruses may express surface proteins and glycoproteins characteristic of the transforming virus (Lilly and Steeves, 1974; Rigby, 1979; Ernberg and Klein, 1979). Such molecules are most often virally encoded (Biggs, 1975; Essex, 1977; Kassel et al., 1977; Lennox, 1980), but may also bear no direct relationship to the virions themselves (Rogers et al., 1978; Karande et al., 1979). In addition, in experimental animals it was found that tumours induced by chemical carcinogens developed surface molecules that did not exist before transformation (Natori et al., 1978). However, these new moieties are not specific for the chemical reagent used or the cell-target. As shown in *in vitro* and *in vivo* experiments, chemically induced tumours are characterized by a huge diversity of surface-molecules (Basombrio, 1970; Basombrio and Prehn, 1972; Embleton and Heidelberger, 1972), many of which are identical to viral products (De Leo et al., 1977; Brown et al., 1977).

1.1.3 Oncofoetal antigens

The most extensively studied tumour markers are the oncofoetal antigens. These molecules are normally expressed or released during foetal life only, but are abnormally found in many neoplasias. Carcinoembryonic antigen (CEA) and α-foetoprotein (αFP) are two of the most common ones. Gold and Freedman were the first to show an association between neoplasia and embryonic gastrointestinal tissues through a molecule, later termed CEA, with a molecular weight of 180-200KD (Gold and Freedman, 1965). CEA is a cell-surface glycoprotein present in normal foetal gut, liver and pancreas (reviewed by Taylor-Papadimitriou and Griffiths, 1984), as well as in malignant adult tissues (retrogenetic expression). It is now clear that CEA is not a single molecular component, but a heterogeneous group of glycoproteins, expressed on a variety of epithelial malignancies such as colonic, gastric, cervical, ovarian and lung carcinomas (Ford et al., 1983; Griffin et al., 1984). However, CEA or CEA-like molecules were also found on normal adult tissues (eg. lung, liver; von Kleist et al., 1972), faeces (Kuroki et al., 1981), meconium (Primus et al., 1983a), and even granulocytes (Wagener et al., 1983). αFP is a globular protein found in foetal and maternal serum during pregnancy, foetal brain and bowel (Abelev, 1971). It was shown to be synthesized and released by the cells of hepatocellular carcinomas, germ-cell tumours, stomach, colon and lung carcinomas (Abelev, 1971). Elevated levels are also found during liver regeneration in hepatitis and liver cirrhosis, as well as in aplastic anaemia.

Placental alkaline phosphatase (PLAP) belongs to a group of "oncodevelopmental isoenzymes". Alkaline phosphatases (ALPs) are glycoproteins that physiologically catalyze the hydrolysis of monophosphate esters at high pH. In man there are three isoenzymic forms of ALPs, namely the placental, intestinal and liver/bone/kidney forms, named after the organ in which they were first identified (McKenna et al., 1979). At least three gene

loci appear to encode the protein moieties of the three different ALPs (Seargeant and Stinson, 1979). PLAP can be distinguished from the other two major forms of ALP by its unusual heat stability, differential inhibition, immunological reactivity and electrophoretic pattern (Fishman et al., 1968a; Nakayama et al., 1970; Fishman, 1974; Mulivor et al., 1978). It is a cell-surface associated enzyme, composed of two subunits with molecular weights of approximately 65KD each (Tokumitsu et al., 1979). PLAP is normally synthesized by syncytiotrophoblasts from the 12th week of pregnancy under control of the foetal genotype (Fishman et al., 1976; Doellgast and Benirschke, 1979).

The interest in PLAP stems from the observation by Fishman et al., that an ALP isoenzyme similar to PLAP (also referred to as the Regan isoenzyme) was ectopically expressed on cancer cells (Fishman et al., 1968a; Fishman et al., 1968b). The ectopic appearance of PLAP and PLAP-like isoenzymes has been described in testicular tumours (Paiva et al., 1983; Epenetos et al., 1984; Jeppsson et al., 1984), as well as other neoplasias such as lung (Nakayama et al., 1970), bladder (Herz and Koss, 1979) and breast carcinoma (Wada et al., 1979). However, molecules with similar enzymatic and immunological properties have been identified at low levels in normal testicular tissues, cervix, lung, and thymus (Mulivor et al., 1978; Goldstein et al., 1982).

Another group of oncofoetal molecules are the I/i blood group antigens and the related stage-specific embryonic antigen (SSEA-1). Among several glycopeptides that are present on the epithelium, there are some oligosaccharides containing the sequence GlcNAcβ1 -----> Gal in their structure. Blood group I and i antigens are expressed on a type of oligosaccharide precursor chain which is found on glycoproteins, glycolipids and/or polyglycosyl structures (Kapadia et al., 1981). The i antigen consists simply of a linear sequence of repeating N-acetyllactosamine units (Niemann et al., 1978), while I antigen possesses a N-acetyllactosamine branch on its

middle galactose residue (Watanabe et al., 1979; Feizi et al., 1979). Further glycosylation leads to blood group (B, A, H, Le^a, Le^b) active structures (Kapadia et al., 1981). Incompletely glycosylated I and i antigens have been detected on many tumours (reviewed by Taylor-Papadimitriou and Griffiths, 1984). Furthermore, it was found that I/i-like antigens have stage-specific expression in early embryos (Gooi et al., 1981). Thus, the SSEA-1 characterizes mouse embryos at a certain stage of differentiation, and is formed by $\alpha 1$ -----> 3 fucosylation of blood group I and i (Gooi et al., 1981; Shevinsky et al., 1982). SSEA-1 was found to be expressed on choriocarcinomas, differentiated hepatomas, colorectal carcinomas (Gold and Freedman, 1965), and most adenocarcinomas (von Kleist and Burtin, 1979), although its distribution is rather heterogeneous and not absolutely tumour-cell specific (von Kleist and Burtin, 1979).

With progress in immunobiochemistry a number of new foetal products that are also related to cancer have been described. Stavrou et al., have recently reported new tumour-associated oncofoetal molecules present on gliomas, neuroblastomas and melanomas, but absent from non-neurogenic tumours (Stavrou et al., 1987). These appear to be similar to the astrocytoma-associated molecules that were reported in the early 1970s (Coakham, 1974). It is obvious therefore, that in many cases, cell-surface markers which appear at some stage during differentiation, may also be present on malignant cells. This is also supported by other studies where it was observed that T-cell acute lymphoblastic leukaemia expresses a phenotype found only on immature cells in the thymus (Bradstock et al., 1980).

1.1.4 Other tumour-associated antigens

Human milk fat globules (HMFGs) are surrounded by membranes that are representative of the differentiation stage of the secretory mammary epithelial cell. The cream fraction of human milk provides a ready source of this

membrane which contains more than 15 components, of which a large glycoprotein (molecular weight >300KD), consisting of at least 50% carbohydrate, appears to be the most "antigenically" active. Smaller molecules are most probably precursors of this large mucin (Shimizou and Yamauchi, 1982; Taylor-Papadimitriou and Griffiths, 1984). Antisera to membrane preparations of the HMFG were used in the past to identify normal mammary epithelial cells in culture, and also to demonstrate the presence of differentiated antigens on mammary carcinomas (Ceriani et al., 1977; Heyderman et al., 1979). Apart from breast, a variety of neoplasms of epithelial origin express similar moieties, such as ovarian (Hammersmith Oncology Group, 1984; Epenetos et al., 1985b) and cervical cancer (Epenetos, 1985c), and carcinomas of the lung, uterus, thyroid and gastrointestinal tract (Hammersmith Oncology Group, 1984). However, HMFG-associated large mucins are also found in small amounts in resting breast and non-neoplastic mammary tissues, as well as in normal epithelium with secretory function, such as sebaceous and salivary gland, exocrine pancreas, bile duct, cervix, ovary, endometrium and kidney (Foster et al., 1982; Burchell et al., 1983; Taylor-Papadimitriou and Griffiths, 1984)

Elevated levels of hormones may also be associated with neoplasia. In this context, bombesin and bombesin-like peptides (such as gastrin releasing peptide, GRP) are important, since they were found in small cell lung cancer. They act in an autocrine fashion, and it has been suggested that such molecules may play an important role during normal foetal growth and development (Moody et al., 1985; Cutitta et al., 1985). Human chorionic gonadotropin (hCG) is another tumour-associated antigen, found in patients with trophoblastic neoplasms and germ-cell tumours (Bagshawe and Searle, 1977). HCG is secreted only by syncytiotrophoblastic cells, and its abnormal presence at elevated levels is almost certainly indicative of choriocarcinoma (Kurman et al., 1977).

Finally, the receptors for transferrin are expressed at high levels on many neoplasias. However, such elevated expression may be the result rather than the cause of malignancy, since transferrin receptors are upregulated on normal proliferating cells. The role of transferrin is to transport iron (Fe^{3+}) from absorption (intestine) and storage sites (liver, spleen, bone marrow), to sites of iron utilization. Transferrin is capable of carrying two Fe^{3+} ions, and binds to its receptor, a homodimeric glycoprotein (molecular weight 180KD), each chain of which can bind one transferrin molecule (Schneider et al., 1982). Iron is essential for cell proliferation, thus it was found that choriocarcinomas, nasopharyngeal and mammary tumour cells (Hamilton et al., 1979; Larson et al., 1980), B- and T-cell lymphoblastoid cells (Sutherland et al., 1981; Trowbridge and Domingo, 1981; Schneider et al., 1982), melanomas (Sutherland et al., 1981) and fibroblasts (Octave et al., 1979; Sutherland et al., 1981; Octave et al., 1981) all express elevated levels of the transferrin receptor, most probably an essential requirement for their unusually high metabolic rate.

1.2 Natural defense mechanisms against cancer

1.2.1 Normal immune responses

This section is a general and relatively brief overview of the immune responses. A more detailed description of many particular aspects of the natural defense mechanisms against foreign molecules will be given in the following chapters.

The introduction of a foreign molecule to a host, either in soluble form or associated with cell-membranes, evokes a cascade of reactions that involve antigen-specific and non-specific cells and soluble factors. There is a wide

diversity of immunocompetent cells characterized by individual activation pathways and mechanisms of action (Fig. 1.1); however, they basically fall into two categories: a) B- and T-cells (cytotoxic, helper, suppressor, delayed-type hypersensitivity), that express specific membrane-receptors for a given antigen, and after sensitization produce immunological memory, and b) accessory cells (macrophages, killer/natural killer cells, eosinophils, basophils, mast cells, neutrophils, etc.) that attack any antigenic target guided by T- and B- cell products (lymphokines, antibody). It must be emphasized however, that all the above cells are necessary for effective defense against potential pathogens, since they act synergistically, and directly or indirectly regulate each other's activities (Fig. 1.1). Improper function of a small subpopulation of immunocompetent cells can dramatically reduce, or even abolishe, an immune response.

B- and T-cells both have predefined specificities for a particular antigen. However, the way of recognition of the foreign immunogenic structure (epitope) is distinct for these two lymphocyte populations. B-cells are capable of binding to soluble and cell-surface antigen via their membrane immunoglobulins (Igs). The Ig molecule is composed of four polypeptide chains, two identical heavy (H) and two identical light (L), chains linked by disulphide bonds (detailed in Fig. 1.2). The N-terminal of both H and L units is characterized by the presence of three distinct regions with high structural variability among Igs (hypervariable region). When H and L chains are associated, these six hypervariable sequences form the antigen binding site, with a unique specificity for every B-cell clone.

T-cells on the other hand, do not recognize native antigen. Foreign molecules must be first processed (cleaved) by target cells, and presented as small peptidic fragments in association with the major histocompatibility complex (MHC) molecules. Thus, T-cells can only see immunogenic peptides that reside in the groove of "self" MHC, via their T-cell receptor (TcR). The TcR is composed of

Figure 1.1: Soluble and cellular components of the immune system.

B : B-cells

T_h : T-helper cells

T_c : T-cytotoxic cells

T_s : T-suppressor cells

T_d : Delayed-type hypersensitivity T-cells

K : Killer-cells

NK : Natural killer-cells

C3b(R): Complement component C3b (R: its receptor)

C3a(R): Complement component C3a (R: its receptor)

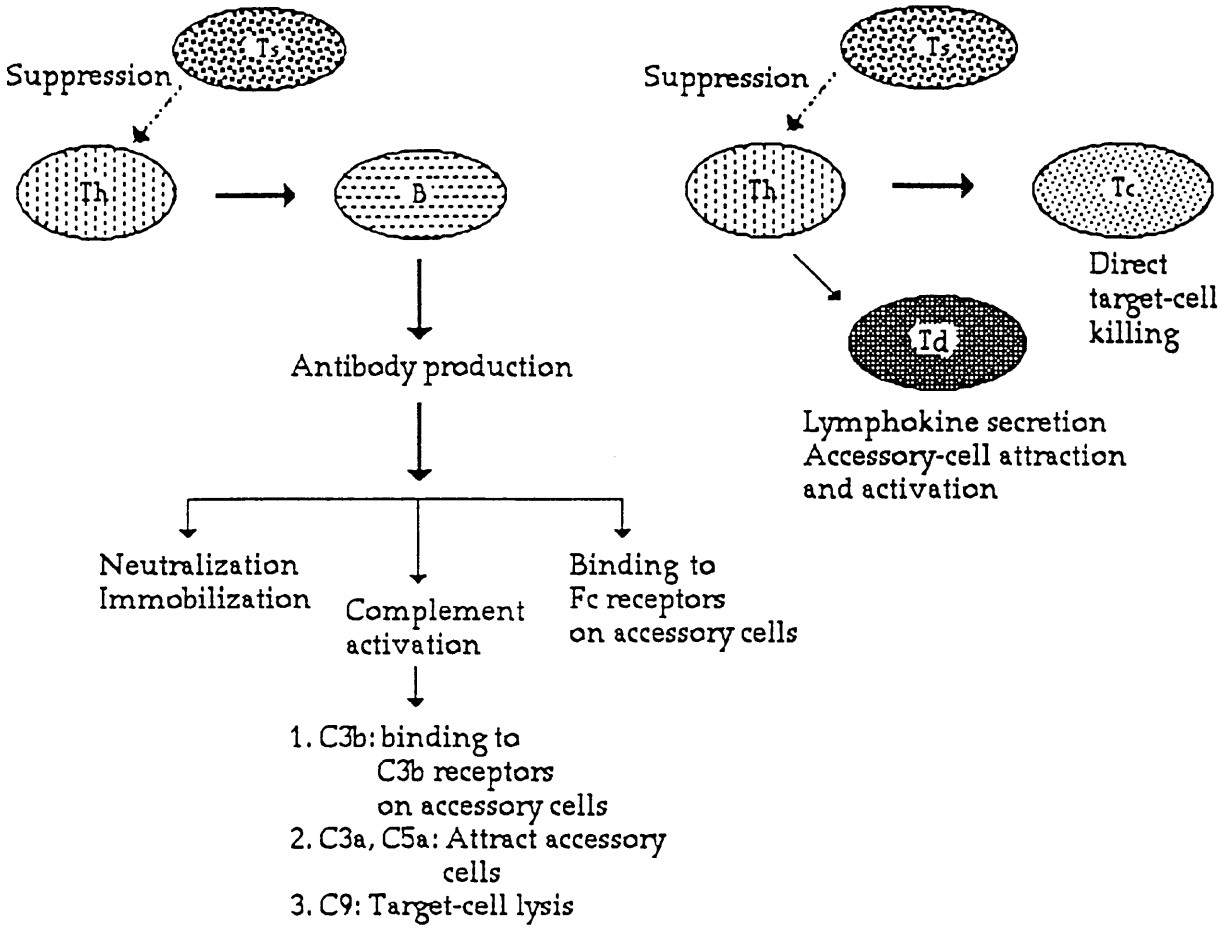
C5a(R): Complement component C5a (R: its receptor)

C9 : Complement component C9

FcγR : Receptor for IgG

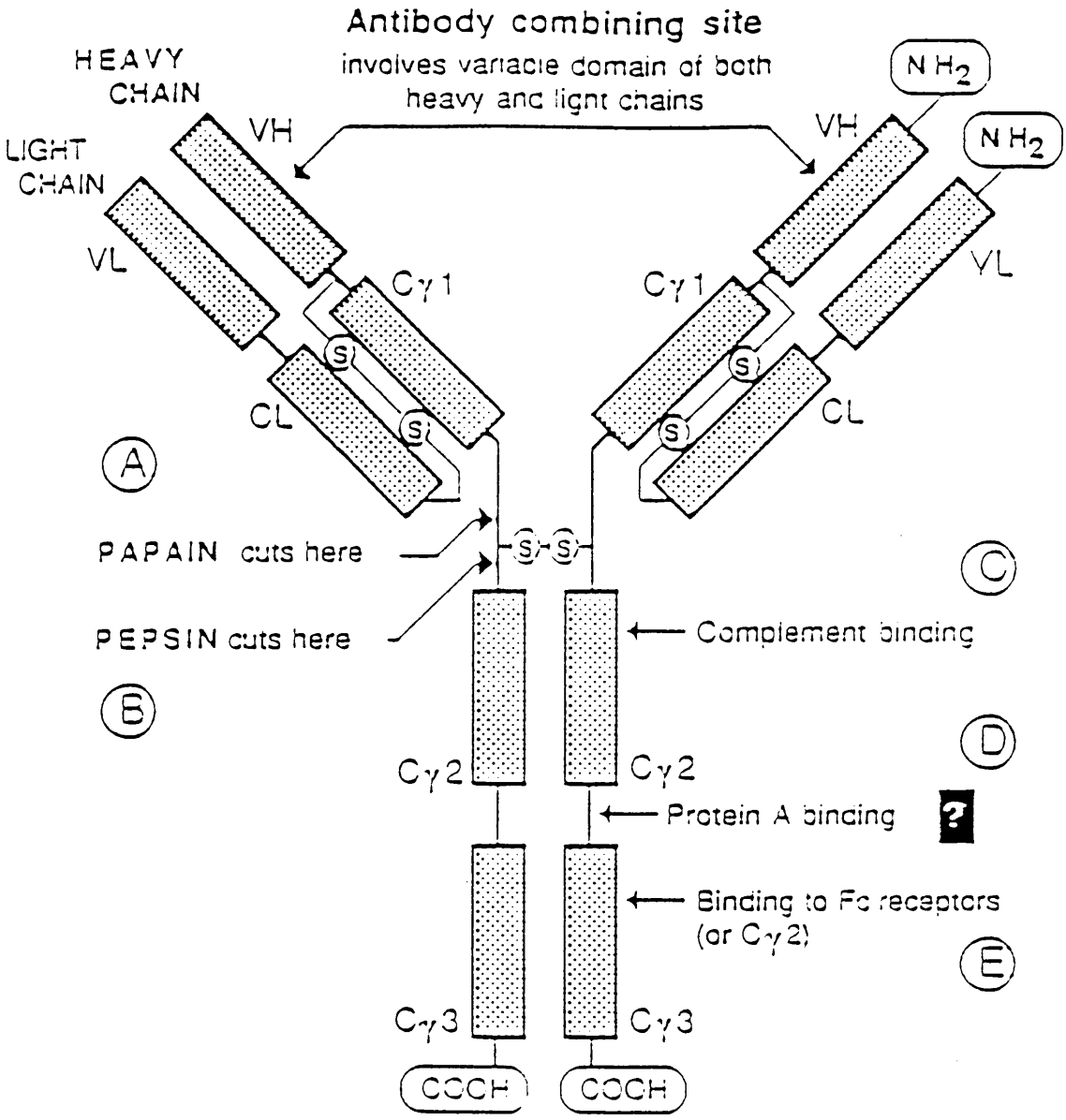
FcεR : Receptor for IgE

LR : Receptor for lymphokines



ACCESSORY CELLS:

Macrophages	:	$Fc_\gamma R$, $Fc_\epsilon R$, C3bR, LR	- phagocytosis
K/NK cells	:	$Fc_\gamma R$, LR	- direct killing of target cell
Basophils and mast cells	:	$Fc_\epsilon R$, C3aR, C5aR, LR(Basoph.)	- secrete vasoactive amines and chemo-attracttants for neutophils and eosinophils
Neutrophils	:	$Fc_\gamma R$, C3bR, LR	- phagocytosis, degradation, tissue damage
Eosinophils	:	$Fc_\gamma R$, C3aR, LR	- secretion of compounds toxic to parasites
Platelets	:	$Fc_\gamma R$	- secretion of vasoactive amines
Red cells	:	C3bR	- clearance of immune complexes



A Papain cuts here to give two univalent Fab fragments which bind antigen but do not crosslink it on the cell surface

B Pepsin cuts here to give one divalent F(ab')2 fragment which retains the antigen binding properties of the original IgG

C Complement binding (classical pathway) is initiated by binding C1q to Cγ2 domain; later C4 binds to Cγ1 or V_H

D Protein A binding probably occurs here

E Binding to Fc receptors on cell surface is usually via the Cγ3 domain; Cγ2 domain is sometimes also involved

Figure 1.2: Diagrammatic representation of the IgG molecule

two disulphide bond-linked polypeptide chains (Tiα and Tiβ) that resemble Igs, since they are both members of the same supergene family. There are T-cells that express different types of Ti chains, namely the Tiγ and Tiδ, but their role in immune responses is not clear. Some may be ontogenetically "early" T-cells, while others may be involved in mucosal immunity. Ti molecules bind to the antigen/MHC complex via their N-terminal variable domain; these are hypervariable regions comparable to those found in Ig molecules. However, Ti is structurally and functionally associated with another cell-surface polypeptide complex, termed CD3, which appears to be responsible for signal transmission after Ti-antigen/MHC interaction. T-cells usually also express a surface glycoprotein that is directly involved in binding to different classes of MHC. Thus, the presence of CD4 (ligand: MHC class II) or CD8 (ligand: MHC class I) on the surface of T-cells determines whether they will be class II or I restricted.

Two major stimuli signal B- and T-cell activation: a) binding of the antigen to the antigen-specific surface receptors, which results to lymphokine-receptor upregulation, and, for T-helper cells, lymphokine secretion, and b) binding of lymphokines to specific surface receptors, an action that promotes lymphocyte growth and maturation.

Thus, B-cells, after antigen stimulation, proliferate and mature to eventually become large Ig-secreting plasma cells. The secreted Ig possesses the same specificity as for the membrane-bound receptor, and act in a variety of ways to destroy the target-antigen (Fig. 1.1). Alone, antibodies can immobilize pathogens and neutralize hazardous compounds, such as bacterial toxins. Antibody/antigen immune complexes may also bind to Fc receptors (FcRs) on accessory cells. Cross-linking of the FcR via the Ig/antigen complex results in accessory cell activation and development of a variety of destructive properties that characterize each FcR^+ immunocompetent cell, such as phagocytosis from

macrophages or release of granules containing vasoactive amines and chemoattractants from mast cells (Fig. 1.1). Alternatively, antibody/antigen complexes can also activate the complement cascade via the classical pathway. In this system, C3b-coated antigenic targets recruit C3b receptor-bearing accessory cells, and initiate their toxic functions. In addition, the role of the soluble C3a and C5a complement fragments is to increase vascular permeability, and attract a wider range of accessory cells. Moreover, the complete complement cascade from C1 through to C9 leads to direct target-cell lysis (Fig. 1.1).

T-cells are also driven to multiplication and maturation upon recognition of the antigenic fragment within the appropriate MHC molecule. However, each of the four T-cell subpopulations possesses its own "private" properties and functions. Cytotoxic T-cells (T_c) are $CD8^+$ and primarily respond to viral peptides presented on MHC class I on the surface of virally infected cells. Helper T-cells (T_h) are $CD4^+$ and recognise antigenic peptides expressed on the surface of "professional" antigen presenting cells (APCs), such as macrophages, dendritic cells and, in secondary responses, B-cells, always in the context with MHC class II. T-helper cells would see antigenic determinants on virtually any MHC class II^+ cell, such as epithelial and endothelial cells, where class II expression can be induced or enhanced by interferon γ (IFN-γ). T-helper cells play an important role in immune responses, since the lymphokines they secrete (following antigen binding) are essential proliferation and maturation signals for T- and B-cell populations, as well as many other non-lymphoid cells. Among the most extensively studied lymphokine-products of T_h-cells, are IL-2, IL-4 and IFN-γ. Interleukins 2 and 4 are growth factors for both T- and B-cells. Interleukin 4 in addition, is a maturation factor for lymphocytes, causing cytotoxic T-cell precursors to mature into functional peripheral T_c on the one hand, and B-cell Ig class-switching to IgE on the other. Moreover, IL-4 induces MHC class II expression on B-cells and macrophages, and activates mast cells to release their granules. IFN-γ is also involved in upregulation of

MHC class II by a variety of cells that do not usually express class II and do not possess antigen presenting function. Several other lymphokines have also been identified and shown to be highly pleiotropic.

Delayed-type hypersensitivity T-cells (T_d) are $CD4^+$, and their main function is to recognise peptides derived from intracellular pathogens (presented with MHC class II), such as *Mycobacterium tubercolosis* after infection of macrophages. T_d-cells also secrete lymphokines, though of a different spectrum from those produced by T_h-cells. These lymphokines are responsible for the attraction and activation of many accessory cells, such as macrophages, neutrophils and eosinophils (Fig. 1.1). Thus, by recruiting and sensitizing such cells at the site of immunogenic stimulus, they essentially contribute to the defense mechanisms for effective target-destruction. Suppressor T-cells (T_s) are $CD8^+$, and their exact nature and way of action is still an enigma. It is believed to act via secretion of specific factors, or anti-idiotypic interactions with TcRs on other T-cells (mainly of T_h). However, T_s-cells are clearly associated with negative regulations of immune responses, although the particular conditions of their activation are still under investigation.

Finally, as mentioned above, a major property of antigen-specific lymphocytes is the induction of immunological memory, where second and subsequent exposure to the same antigen leads to enhanced immune responses. Sensitization of memory cells by antigen is associated with functional and structural changes of their state. Thus, they clonally expand, and this antigen-specific proliferation coincides with the expression of accessory molecules on the cell-surface, that facilitate adhesion (eg. leukocyte-functional antigen 1, LFA-1) and activation (eg. lymphokine receptors). Furthermore, for B-cells, it is during during the development of memory in the germinal centres that hypermutation in the Ig loci is thought to occur, leading to affinity maturation of the immune responses.

1.2.2 Immune responses to tumours

As discussed earlier, tumours may specifically express cell-surface molecules that are produced by a new (as in viral infection) or altered (as in chemically-induced tumours) genome. If these neoantigens are immunogenic, antibodies will be directed against them. Humoral anti-tumour responses have been demonstrated in chickens and cats infected with oncoviruses that infect T-cells; the antibodies formed were directed against virion-associated antigens, and were found to be capable of inducing immunological memory in vaccinated animals, thus preventing the onset of disease (Biggs, 1975; Essex, 1977). Anti-tumour antibodies were also detected in experimental animals, directed against chemically-induced neoplasms (Sikora, 1977), and in man, against incompletely glycosylated blood group (Lachmann and Mitchison, 1982) or brain glioma-associated antigens (Kornblith et al., 1979; Coakham et al., 1980; Kornblith et al., 1983). Such antibodies may kill malignant cells by activating complement components: C3b binds to C3b-receptor bearing cells which will effectively lyse the target-cell (Kassel et al., 1977). Alternatively, Fcγ-receptor bearing cells would "lethally" bind the antibody-coated tumour cell (Roitt et al., 1985).

Tumours often directly activate the alternative complement pathway. This has been shown in several *in vitro* experiments employing animal and human cell-lines (Okada and Baba, 1974; Budzko et al., 1976; Schorlemmer et al., 1977; McConnell et al., 1978). This phenomenon was mostly associated with virus-induced membrane changes (McConnell et al., 1981). Thus, the C3b that accumulates on the tumour surface will activate macrophages even in the absence of antibodies. However, it has been suggested that a soluble factor was also responsible for triggering macrophages to anti-tumour activity (Currie and Basham, 1975; Sethi and Brandis, 1975). This property was attributed to C3a (Schorlemmer et al., 1977; Ferluga et al., 1978).

Natural Killer and NK-related cells (large granular lymphocytes, LGL) are particularly important and effective against malignancy. Their exact lineage is still uncertain, since it has been suggested that they may be related to both macrophages and T-cells (reviewed by Ortaldo and Herberman, 1984). However, although their antigen recognition is not MHC-restricted, and they lack certain T-cell markers, it seems more likely that NK-cells are closer to the T-cell lineage (Grossman and Herberman, 1986a). This is supported by the observation that NK-cells are non-phagocytic, mostly non-adherent and lack typical macrophage surface markers (Ortaldo and Herberman, 1984). Their cytotoxic activity is similar to T-cytotoxic cells (Podack and Köningsberg, 1984), they express the typical T_c phenotype CD8 (Reynolds et al., 1981; Ortaldo et al., 1982), they secrete and are activated by IL-2 and IFN-γ (in an autocrine and paracrine fashion) (Domzig et al., 1983; Kasahara et al., 1983a; Ortaldo et al., 1984). In addition, under some circumstances, T-cells may develop NK-like activity (Grossman and Herberman, 1986a). The specificity of NK-cells for transformed, viral-induced and rapidly proliferating cells *in vitro*, first observed in the early 1970s (Rosenberg et al., 1972; Kiessling et al., 1975), is still not clearly understood (Herberman and Holden, 1978; Harfäst et al., 1978; Ortaldo and Herberman, 1984). It has been suggested that they "anchor" either indirectly to the antibody-coated malignant cell via their FcRs, or directly by binding to the transferrin receptor expressed in elevated amounts on many neoplastic cells (Roitt et al., 1985). It is thought that *in vivo* NK-cells may be related to immune surveillance and resistance to tumour development, growth and metastasis (Karre et al., 1980; Talmadge et al., 1980; Baumann et al., 1986).

Finally, the direct or indirect T-cell involvement in the defense against cancer has been shown in numerous experimental systems (Hellström and Hellström, 1974; reviewed by Lachmann and Mitchison, 1982). The secretion of anti-tumour antibodies, described above, implies the presence of anti-tumour T-helper cells. Macrophages that have processed tumour-associated antigens

(shed or after direct cell:cell contact) would present it in association with MHC class II, and hence stimulate T_h-cells. Anti-tumour T_h-cells have been demonstrated in various studies (Fujiwara et al., 1976; Shimizu et al., 1984), and their function was associated with certain HLA-DR molecules (Hayashi and Ochiai, 1986). T_h-cells have also been shown to specifically provide "help" to tumour-specific T_c-cells (Fujiwara et al., 1986) that infiltrate solid tumours, and are capable of direct killing and hence potentially tumour "rejection" (Tötterman et al., 1980; Eremin et al., 1981; Holmes, 1985). In addition, activated T-cells "enrich" the tumour microenvironment with factors that either enhance the activity of NK-cells (IFNγ), or attract (migration inhibitory factor, MIF) and amplify (macrophage activating factor, MAF) the cytotoxic action of macrophages (reviewed by Lachmann and Mitchison, 1982 and Roitt et al., 1985).

1.2.3 Failure of the natural immune response to tumours

Since the immune system can be pleiotropically stimulated to act against malignant cells, it is very possible that during an individual's life various single-cell transformations occur, and that these are successfully "dealt" with by macrophages, NK-, B-, T-cells and their soluble products. However, why are the natural defense mechanisms often unable to prevent or suppress growth of cancer? An obvious reason is that tumours may have an extremely high metabolic rate and doubling time, so that production of malignant cells may far exceed the natural rate of wastage. In addition, cells within the tumour mass become less accessible to immunocompetent cells (reviewed by Lachmann and Mitchell, 1982). Nevertheless, the real problem is how the tumour cells "escaped" immune surveillance in the first place, before their number "overshadowed" even the most potent immune responses. There are many explanations for this, the most likely of which will be discussed below.

Firstly, in many cases it has been demonstrated that tumours release molecules with an immunosuppressive effect. *In vitro* experiments have shown that αFP (Kamo and Friedman, 1977) or glioblastoma-derived factors belonging to the transforming growth factor-β (TGFβ) family (Brooks et al., 1972; Fontana et al., 1984; Siepl et al., 1988; Kuppner et al., 1989) inhibited anti-tumour cellular cytotoxic activity. At early stages in cancer, generalized immunosuppression is not evident. However, it is possible that the tumour is surrounded by a localized "defense zone" within which cellular immunity is suppressed.

Secondly, transformed cells may not exhibit any neoantigens that can "trigger" immune responses. As discussed earlier, oncogene products may remain intracellular (eg. p21), are shed (eg. αFP, hCG), or are simply quantitatively elevated versions of a normal counterpart. A large number of tumour-associated cell-surface antigens, such as PLAP, CEA, HMFG-related mucins, EGF-, PDGF- and transferrin-receptors, accomplish physiological functions during the normal cell-cycle. If these molecules are not somehow altered (in contrast to the partially glycosylated I/i blood group antigens) it is impossible for them to be recognised as foreign, and hence there is no T-cell or antibody-mediated response. NK-cells may be theoretically still able to attack the rapidly proliferating tumour cells, but their inability to fully inhibit the growth once again reflects the nature of immune responses, based on the cooperation of all immunocompetent cells through cell:cell and cell:lymphokine interactions (Baumann et al.,1986; Grossman and Herberman, 1986b).

Thirdly, it has been shown in experimental animals that tumour cells, which are clearly antigenic to a host and are rejected upon inoculation, develop into a cancer when given at low doses (Humphreys et al., 1962; Old et al., 1962; Bonmassar et al., 1974; Grossman and Berger, 1980). This phenomenon, termed "sneaking through", may be linked to "low zone" tolerance (Grossman, 1982; Grossman, 1984; De Boer and Hogeweg, 1985), since it appears

that the fully potent anti-tumour immune system adapts to cancer instead of destroying it.

Fourthly, it is generally believed that antibody responses against malignant cells are not particularly effective. In many animal studies it has been found that immunity to tumours can be transferred by cells but not by serum (reviewed by Lachmann and Mitchell, 1982). It seems therefore that antibody-dependent cell mediated cytotoxicity (ADCC), or complement activation via the classical pathway are only secondary defense mechanisms in tumour immunity. In addition, antigen modulation, which is often observed on malignant cells (Joseph and Oldstone, 1974; Roitt et al.,1985), would prevent antibodies from binding to the tumour. Moreover, when tumour-associated antigens are shed, apart from increasing the chances of metastasis (Currie and Alexander, 1974), they would also bind to and block the circulating anti-tumour antibodies. High levels of soluble antigen (especially in the tumour microenvironment) may also induce "high zone" tolerance. Paradoxically, it was also found that anti-tumour antibodies often enhance instead of preventing cancer growth (Kaliss, 1958; Prehn, 1976; Herberman, 1983). This may be due to the activation of suppressor cells by immune complexes (Hellström and Hellström, 1969; Sjögren et al., 1971; Baldwin et al., 1973); alternatively, if some of these antibodies recognize and bind to antigenic determinants on growth factor-receptors, they may mimic the action of the growth factor, thus stimulating instead of suppressing the target-cell.

Finally, apart from antibody responses, cytotoxic T-cell anti-tumour activity may be also impaired. It is known that many transformed cells do not express MHC class I molecules on their surface (Roitt et al., 1985). Hence, even if they bear tumour neoantigens on their cell-surface, T_c-cells are unable to recognise them when unassociated with MHC class I. Generally, tumour infiltrating lymphocytes (TILs), of which a significant proportion are T-cells, often fail to respond against the tumour, as shown by *in vitro* cytotoxicity experiments

(Vose et al., 1977; Vose and Moore, 1979; Eremin et al., 1981; Rabinowich et al., 1987). This may be attributed to tumour-related immunosuppressive factors, presence of T_S cells (Miescher et al., 1986), or may be simply due to "engulfing" and "isolation" of the leukocytes by the tumour mass. Lastly, as suggested by Prehn and Prehn, cellular responses may in fact facilitate and enhance tumour growth (Prehn and Prehn, 1987).

1.3 Adoptive immunotherapy

Unfortunately the vast majority of patients with cancer only present when the tumour mass has grown to an easily detectable size. The conventional method of treatment is surgical tumour excision, often accompanied by local radiotherapy in order to eliminate the tumour cells that may have escaped surgery. If other organs are thought to be involved, the above treatment is complemented by cytostatic and cytotoxic drugs. Cancer manipulation has radically changed in the last 70 years, aided by the technological "explosion" of the 20th century. Operating theatres are now equipped with sophisticated equipment; radiotherapy departments prove routinely that radiation can be beneficially used; the list of anti-cancer drugs geometrically increases, and combined protocols have optimized the conditions for better treatment, while minimizing side-effects. However, tumours do metastasize, and even after complete remission at one site, they return more potent and aggressive than before, in the same or other tissue. At that stage medicine can do little for the patient.

"... *the early cancer we have cured, but the one that rose to considerable size without surgery nobody has cured ..... deep seated (cancers) are not treatable with this approach (resection and cautery) and I have seen that all those who endeavoured to cure this kind of cancer, have exacerbated it and swiftly brought about the death of the patient; and these who dissected and*

cauterized the palatal or anal cancer and the hidden one in a female, were unable to heal the ulcers and the misery of the treatment caused the patients to fade away until their death, whereas without treatment they could have lived much longer ..." This excerpt is from Galen's thesis on the treatment of cancer, written the 2nd century A.D. (translation by Retsas, 1986), indicating that the situation has changed relatively little over the past 18 centuries. What can medicine do before the turn of the 20th century?

1.3.1 Bacillus Calmette-Guérin

An alternative to the conventional treatment of neoplasia, is to provide "help" to amplify the immune response to autochthonous malignant cells. This so called cancer immunotherapy looked very encouraging when it was found that administration of bacillus Calmette-Guérin (BCG) was associated with better prognosis in patients with cancer (Vogler and Chan, 1974). Thus, immunotherapy with BCG had a dramatic effect on the survival of patients with acute myelogenous leukaemia (Vogler and Chan, 1974; Powles et al., 1977), or with gastric (Ochiai et al., 1983), bladder (de Kernion et al., 1985; Lamm et al., 1986) and postoperative lung cancer (McKneally et al., 1977).

1.3.2 Tumour necrosis factor

It is now known that non-specific general activation of monocytes, macrophages, NK-cells and even TILs accompanies specific anti-BCG responses (Niitsuma et al., 1981; van der Meijden et al., 1989). Upon injection of BCG, a factor is released by mononuclear cells with cytolytic and/or cytostatic activity for a wide range of tumours, hence its name: tumour necrosis factor (TNF) (Carswell et al., 1975; Phillip and Epstein, 1986; Ostensen et al., 1987; Scheurich et al., 1987). Apparently there are two forms of TNF, the macrophage-derived TNF-α, and the lymphocyte-derived TNF-β, with similar activities (Nedwin et al., 1985). Before its full characterization, the latter was previously described

under the more general name of lymphotoxin, a family of cytotoxic compounds secreted by activated T-cells (Hiserodt et al., 1978; Harris et al., 1981). In fact, protein purification and gene cloning have now revealed that TNF-β and lymphotoxin shoe strong homology to each other (Pennica et al., 1984; Gray et al., 1984a; Shirai et al, 1985), and that they most likely bind to the same cell-surface receptor (Rubin et al., 1985). Successful TNF-mediated cancer treatment depends solely on whether malignant cells express TNF-receptors in adequate numbers (Kull et al., 1985; Rubin et al., 1985; Tsujimoto et al., 1985); this is critical, since it has been reported that a large proportion of tumour cell-lines do not (Williamson et al., 1983). However, despite successful *in vitro* experiments, results from clinical studies using BCG or TNF for the treatment of cancer, are met with more scepticism than excitement (reviewed by Gutterman, 1988).

1.3.3 Interferons

Interferons were first described by Lindenmann in 1957 as cell products that "interfered" with viral replication. We now know that interferons are a family of glycoproteins, synthesized by a variety of cells, and that their activity is not limited to protection against viral infection, but extends to stimulation of T-, NK-cells and macrophages (Zarling et al., 1979; Herberman et al., 1980). Their potential enhancement of natural anti-tumour defense mechanisms soon became apparent, after crude extracts of interferon were used in animal experiments (Gresser, 1977) and clinical studies (Strander, 1977; Mellstedt et al., 1979). Detailed biochemical analysis and gene cloning revealed that there are three main types of interferons: IFN-α (originally, leukocyte-derived), IFN-β (originally, fibroblast-derived) and IFN-γ (lymphocyte-produced) (Bunn and Foon, 1985; Gutterman, 1988).

The anti-tumour activity of IFN-γ is often related to its activity on the induction of TNF-receptors on the surface of malignant cells (Ruggiero et al.,

1986; Tsujimoto et al., 1986; Kuhl et al., 1989). In addition, it has been shown that it pleiotropically effects macrophages (by activating FcRs and increasing production of superoxide) and NK-cells (Herberman et al., 1980; Ortaldo et al., 1983a; Ortaldo et al., 1983b; Biron et al., 1983; Kleinerman et al., 1986; Sayers et al., 1986). IFN-γ also upregulates MHC class II expression (Schreiber et al., 1985; Adams and Hamilton, 1987), an action that facilitates antigen recognition by T_h-cells (Unanue and Allen, 1987), which subsequently triggers T-cytotoxic activity (Palacios et al., 1982). Natural (T-lymphocyte derived) or recombinant IFN-γ has been used in numerous clinical trials as an anti-cancer agent, for example, against chronic myelogenous leukaemia (Silver et al., 1987; Kurzrock et al., 1987), ovarian carcinoma (Welander et al., 1988), renal carcinoma (Rinehart et al., 1986; Garnick et al., 1988), and melanoma (Kirkwood and Ernstoff et al., 1985). In general, a positive response (partial and complete) occurred in 20-50% of the treated patients. Successful treatment greatly depended upon the dose and frequency of IFN-γ administration, since it was found that intermediate (Kirkwood and Ernstoff et al., 1985; Kleinerman et al., 1986) and daily doses (Garnick et al., 1988) appeared to be the most effective. Interestingly, extremely high doses appeared to have the opposite effect, inducing immunosuppression (Kleinerman et al., 1986).

Interferons α and β are secreted by virally-infected cells, bind to neighbouring cells and protect them from further viral infection (Borden, 1979; Kirkwood and Ernstoff, 1984). Their properties include direct inhibition of cell growth (Rubin and Gupta, 1980; Willson et al., 1984), binding and activation of NK-cells (Ortaldo et al., 1983a; Biron et al., 1983; van de Griend et al., 1986), and facilitation of T-cell cytotoxicity by enhancing MHC class I expression (Rosa et al., 1983; Ohlen et al., 1989); hence their immense usefulness as anti-cancer reagents.

IFN-α and β probably recognize the same receptor, which is different from that for IFN-γ (Celada et al., 1984), and have very similar anti-tumour activities. However, IFN-α is more often used in clinical trials, especially after it became available in large quantities by recombinant techniques. IFN-α is derived from a multigene family, hence the large number of subtypes (for both natural and recombinant IFN-α), with minor differences in their structure (eg. the commercially available IFN-α2a and IFN-α2b differ by one aminoacid) (Gutterman, 1988). Clinical studies using IFN-α in patients with malignant lymphoma yielded very encouraging results. The total response reported (partial and complete) was 25-55% (Louie et al., 1981; Foon et al., 1984; Stevenson et al., 1984); the action of IFN-α may be attributed to its anti-viral properties, since the onset of malignant lymphoma is often associated with infection by a retrovirus (human T-cell leukaemia/lymphoma virus I, HTLV-I) (Wong-Staal and Gallo, 1985). IFN-α was also very effective against hairy-cell leukaemia, with a total response of over 90% (Quesada et al., 1984; Jacobs et al., 1985; Thompson et al., 1985; Retain, 1985; Foon et al., 1986; Golomb et al., 1987; Golomb et al., 1988). Hairy-cell leukaemia, a B-cell disorder, is associated with infection by a human retrovirus (HTLV-II) (Wachsman et al., 1984); thus, the anti-viral properties of IFN-α may again explain the dramatic retreat of the disease. Similarly, the total responders among patients with acquired immune deficiency syndrome-related Kaposi's sarcoma (AIDS-KS), treated with IFN-α, was 20-50% (Krown et al., 1983; Groopman et al., 1984) presumably via the interferon's direct activity against HTLV-III (Ho et al., 1985). The anti-viral properties of IFN-α also became evident in the treatment of patients with chronic active hepatitis B, a disease caused by the hepatitis B virus, especially after it had been shown that these patients lack sufficient amounts of endogenous interferons (Abb et al., 1985). The total response observed was up to 40% (Greenberg et al., 1976; Scullard et al., 1981; Dusheiko et al., 1985; Lai et al., 1987). In addition, IFN-α has given promising results in patients with multiple myeloma (Quesada et al., 1986; Ohno and Kimura, 1986; Costanzi and Polard, 1987), chronic myelogenous leukaemia (Talpaz et al., 1983; Talpaz et al.,

1986; Niederle et al., 1987; Talpaz et al., 1987), melanoma (Retsas et al., 1983; Creagan et al., 1984; Kirkwood et al., 1985a), renal (Edsmyr et al., 1985; Kirkwood et al., 1985b; Umeda and Niijima, 1986; Muss et al., 1987), ovarian (Einhorn et al., 1982; Abdulhay et al., 1985; Einhorn et al., 1988) and bladder carcinoma (Ikic et al., 1981; Oliver et al., 1986). Finally. IFN-β has been successfully used, and has yielded results similar to those observed with IFN-α, in patients with renal carcinoma (Rinehart et al., 1986).

1.3.4 Interleukin 2

Interleukin 2 is one of the major lymphokines and a product of activated T-cells. Its role is to activate immunocompetent cells by binding (in an autocrine or paracrine fashion) to a cell-surface receptor, a heterodimer consisting of a chain with relative molecular weight of 70-75KD and a chain of 55KD (Sharon et al., 1986). Cells bearing IL-2 receptors include T-, B- and NK-cells, and may express either both chains (high affinity receptor), or either the 70-75KD (intermediate affinity) or the 55KD (low affinity) alone, depending on the state of activation (Nakanishi et al., 1984; Sharon et al., 1986; Loughnan et al., 1987). In numerous *in vitro* experiments it has been shown that IL-2 provokes MHC-restricted (T-cell) and -unrestricted (NK-cell) anti-tumour cytotoxicity (Henney et al., 1981; Handa et al., 1983; Lattime et al., 1983; Ortaldo et al., 1984; Svedersky et al., 1984; Ades et al., 1984; Malkovsky et al., 1987; Itoh et al., 1987; Belldegrun et al., 1989). In addition, IL-2 also stimulates production and release of other cytokines such as TNF-α and β (Svedersky et al., 1985; Nedwin et al., 1985), and IFN-γ (Kasahara et al., 1983b; Ortaldo et al., 1984; Sverdesky et al., 1985). Upon release, apart from MHC class II expression, IFN-γ also enhances IL-2 receptor expression (Holter et al., 1986), and thus has an additive effect on IL-2-induced TNF production.

Because of the multiple immunoregulatory activities of IL-2, *in vivo* administration of this lymphokine is currently being investigated. Many

studies are under way involving IL-2 alone, IL-2 in conjunction with various *in vitro* activated cells, or IL-2 in combination with other lymphokines and chemotherapeutic drugs.

Systemically administered IL-2 has shown some very encouraging results in the treatment of a variety of malignancies, such as melanomas, lymphomas, neuroblastomas, ovarian, renal and colorectal carcinomas (Rosenberg et al., 1987; West et al., 1987; Sondel et al., 1988; Sosman et al., 1988; Benedetti Panici et al., 1989; Lindemann et al., 1989; Stoter et al., 1989; Thatcher et al., 1989). In general, most of these trials are not sufficiently advanced to draw conclusions regarding the effectiveness of the treatment. The initial aim has been to define the optimal dose and administration schedule, and to assess toxicity. IL-2 has a rather short half-life in the circulation (Lotze et al., 1985; Thompson et al., 1987), thus relatively large bolus injections were necessary in order for the treatment to be effective. However, this was associated with severe toxicity (Lotze et al., 1986; Rosenstein et al., 1986; Rosenberg et al., 1987; Christiansen et al., 1988; Rosenberg, 1988). Nevertheless, daily doses or constant infusion of lower doses appeared to compromise the desirable anti-tumour effect and the non-specific side-effects (Cheever et al., 1985; Thompson et al., 1987; West et al., 1987).

1.3.5 Lymphokine-activated killer cells

Alternatively, peripheral blood mononuclear cells (PBMCs) from patients with cancer were incubated *in-vitro* with IL-2, and reinfused into the patient. It was found that *ex-vivo* IL-2-treated PBMCs exhibited an unusually high, MHC unrestricted anti-tumour activity (reviewed by Grimm, 1986). These cells, named lymphokine-activated killer cells (LAK-cells), proved to be effective when they were administered to patients with cancer, either alone or in combination with systemic IL-2 (Mule et al., 1985; Rosenberg et al., 1987; Phillips et al., 1987; Hsieh et al., 1987; Boldt et al., 1988; Hosokawa et al., 1988;

Schoof et al., 1988; Dutcher et al., 1989). Since the administered cells are already activated, lower amounts of IL-2 can be used, thus reducing the incidence of side-effects (Schoof et al., 1988). However, comparative studies between IL-2 and IL-2/LAK-cell treatment has not revealed significant differences between these two regimes (Rosenberg et al., 1987; Philip et al., 1989). The nature of LAK-cells is not yet clear (Phillips and Lanier, 1986). It is generally believed that they simply are IL-2-activated NK-cells (Itoh et al., 1985; Ibayashi et al., 1986; Phillips et al., 1987; McMannis et al., 1988). Nevertheless there is such heterogeneity among cells with LAK activity, that it is also possible that LAK cells are IL-2-activated "precursor" or immature lymphocytes (Gray et al., 1984b).

Interleukin 2-induced LAK activity may be enhanced by the use of other lymphokines, such as TNF-α (Owen-Schaub et al., 1988; McIntosh et al., 1988; Yang et al., 1989; Matossian-Rogers et al., 1989), granulocyte-macrophage colony stimulating factor (GM-CSF) (Singh et al., 1989), IFN-γ (Kuribayashi et al., 1981; Ricardi et al., 1983; Kassanara et al., 1983), IFN-α (Brunda et al., 1986; Cameron et al., 1988; Iigo et al, 1988) and IFN-β (Paolozzi et al., 1989). Nevertheless, despite the very promising results from animal experiments, the use of LAK-cells in man has proved to be less beneficial. This may be due to the fact that only a small proportion of LAK-cells actually reach the tumour site (Sasaki et al., 1989), whereas the majority appear to accumulate in the lungs (Reynolds et al., 1984; Rolstad et al., 1986; Maghazachi et al., 1988).

1.3.6 Tumour-infiltrating lymphocytes

Tumour-infiltrating lymphocytes on the other hand, may offer an alternative to adoptive cellular immunotherapy. As discussed earlier, TILs are often functionally deficient (Vose and Moore, 1979; Moy et al., 1985; Rabinowich et al., 1987), probably due to factors released by the tumour. Isolated TILs however, treated *in vitro* with IL-2, can exhibit potent anti-tumour activity

often greater than that of LAK-cells (Rosenberg et al., 1986; Spiess et al., 1987), although conflicting results have also been reported recently (Bosnes and Hirschberg, 1989). In addition, IL-2-activated TILs do not necessarily require systemic IL-2, and show a particularly specific cytotoxic effect against autologous tumours (Rosenberg et al., 1986; Spiess et al., 1987).

It has been shown that IL-2-expanded TILs release various lymphokines including TNF (Belldegrun et al., 1989) and, like LAK-cells, comprise a heterogeneous population, the majority of which are cytotoxic T-lymphocytes (Kurnick et al., 1986; Rosenberg et al., 1986; Spiess et al., 1987; Muul et al., 1987; Kradin et al., 1987; Itoh et al., 1987; Itoh et al., 1988), although macrophages (Rossi et al., 1988) and cells with NK activity have also been identified (Belldegrun et al., 1988; Anderson et al., 1988). Despite the difficulty in obtaining sufficient numbers of TILs, clinical trials are under way with encouraging results for tumour diagnosis (Fisher et al., 1989) and treatment using IL-2/TILs (Topalian et al., 1988; Kradin et al., 1989) or IL-2/TILs combined with IFN-α (Parkinson et al., 1989).

In conclusion, the data on adoptive cancer immunotherapy so far, is far from complete, but has provided impetus to research into both how to optimize this treatment, as well as how to make immunotherapy in general more successful. As the question of how to optimize the dose, schedule, cell culture conditions, cytotoxic-cell number and activity, as well as other specifics of biological response modifier-therapy, are addressed in clinical trials, other approaches suggested by data from *in vitro*, animal and clinical studies are emerging as future developments. These include other cytokines such as GM-CSF (Donahue et al., 1986; Groopman et al., 1987; Vadhan-Raj et al., 1987; Kleinerman et al., 1988), combination of lymphokines with drugs (Vetto et al., 1987; Stoter et al., 1989; Paciucci et al., 1989; Lindemann et al., 1989), or regional administration of lymphokines, LAK-cells and TILs (Okuno et al., 1986; Thatcher et al., 1989; Kamada et al., 1989).

1.4 Antibodies in the manipulation of cancer

... τίς οὖν ἥ τε κοινὴ καί ἰδία τῆς θεραπείας ἔνδειξις ἐπί καρκίνου, καιρός ἤδη λέγειν. Γαληνός, 2ος αἰών μ.χ.

... *it is now time to discuss which are the indications for* common *and for* selected *treatment of cancer.* Galen, 2nd century A.D. (translation by Retsas, 1986).

Galen very laconically underlined the aim for effective management of cancer eighteen centuries ago. Only at the end of the last century, was the question of specific cancer treatment re-opened (Baas, 1889), and later promoted by the great German physician Paul Ehrlich, who proposed the construction of "magic bullets" that would preferentially affect the target-cells (Ehrlich, 1904). A big step in this direction was taken 15 years ago, when the technique of producing unlimited amounts of antibodies with single specificity became available (Köhler and Milstein, 1975). This rekindled the hopes for specific tumour eradication. But have antibodies met our expectations so far in the management of cancer? Are monoclonal antibodies ever going to provide the panacea for cancer?

1.4.1 Cancer diagnosis

1.4.1.a *In vitro* diagnosis

During the last two decades, the development of specific and sensitive immunohistochemical techniques have made remarkable contributions in the diagnosis and management of patients with cancer. Immunostaining methods have the potential to demonstrate specific cell and tissue antigens, and their distribution and density on the cell or tissue.

Studies attempting to distinguish and identify different types of neoplasms have been reported in the past using polyclonal antisera (Coakham and Lakshmi, 1975). The advent of monoclonal antibodies (mAbs) however, enabled better characterization of tumour-associated molecules, and allowed unequivocal and reproducible differentiation of normal from neoplastic tissues.

Numerous tumour-associated antigens have now been defined by mAbs. CEA is the archetypal example of tumour antigen and, as described earlier, is widely present on carcinomas, including colorectal, lung and breast cancer (Griffith, 1984). Despite its heterogeneity (Gold et al., 1973; Rogers, 1976) and expression on non-cancerous tissues (von Kleist et al., 1972; Primus et al., 1983a), mAbs were developed that could distinguish normal from malignant CEA-expressing cells (Primus et al., 1983b; Primus et al., 1983c). Positive staining of breast tumours by anti-CEA antibodies was found to be related to possible involvement of regional lymph nodes (Shousha et al., 1979), whereas absence of CEA was associated with better prognosis (Primus et al., 1983c). Similarly, levels of CEA on colon carcinoma, as defined by mAbs, was relevant to the state of tumour differentiation (O'Brien et al., 1981; Primus et al., 1981).

Monoclonal antibodies have been developed that react with breast cancer and recognize mammary carcinoma cell-membrane glycoproteins (Soule et al., 1983; Edwards et al., 1986) or mucins associated with the milk fat globule membrane (Taylor-Papadimitriou et al., 1981; Burchell et al., 1983). Distinct glycoproteins have been found in malignant ovaries (Bhattacharya et al., 1984), and various antigens offer useful targets for mAbs in prostate cancer (Starling et al., 1986). Carcinoma of the lung has attracted the interest of many workers, who attempted to distinguish non-small cell lung carcinoma from small cell lung carcinoma (Hellstrom et al., 1986; Pettijohn et al., 1987). Likewise, a panel of mAbs enabled the differentiation of intraocular malignant melanoma from similar lesions (Bomanji et al., 1987).

Ectopic expression of PLAP has been reported in neoplasms of the testes (Millan et al., 1982; Paiva et al., 1983; Millan and Stigbrand, 1983; Epenetos et al., 1984), ovary, lung, stomach and pancreas (Stolbach et al., 1965; Nathanson and Fishman, 1971; Fishman et al., 1976). Here mAbs helped to distinguish different closely related isoenzymes (Millan et al., 1982; Millan and Stigbrand, 1983). On carcinomas of the colon, in addition to CEA, the SSEA is also expressed (Solter and Knowles, 1978), as well as the sialylated Lewis antigen (Juhl et al., 1986), the gastrointestinal antigen (Herlyn et al., 1979), and many others (Bleday et al., 1986). Lymphomas and leukaemias (Epenetos et al., 1985d; Ritter et al., 1985) were some of the first diseases to be phenotyped using antibodies, and a great deal of progress in the characterization of leukocyte antigens has been achieved through the organization of the International Leukocyte Antigen Workshops (McMichael, 1987).

In addition to membrane molecules, nuclear antigens may prove useful markers for cancer (Davis et al., 1978; Freeman et al., 1985; Freeman et al., 1986). Nuclear oncogenes and their products, as shown by mAbs, were found to be activated or amplified in colon (Der and Cooper, 1983; Alitalo et al., 1983), breast (Kozbor and Croce, 1984), lung (Little et al., 1983; Der and Cooper, 1983) and stomach carcinomas (Shibuya et al., 1985).

Tumour antigens may be secreted or shed from cancer cells into the blood stream. Measurement of these serum levels can be useful in monitoring the patients' condition. αFP and the β subunit of hCG have proved very useful markers for germ-cell tumours (Lange et al., 1976). PLAP serum assays have been shown to be useful in the monitoring of testicular seminoma (Epenetos et al., 1985e; Tucker et al., 1985), although false positive results have been reported in smokers (Tucker et al., 1985). An important new tumour marker CA 125 is proving to be useful in the detection and monitoring of most cases of epithelial origin ovarian cancer (Halila et al., 1986; Dhokia et al., 1986a).

Measurement of blood CEA levels can aid in the prognosis and monitoring of therapy of patients with colorectal and breast carcinomas (Lokich et al., 1978; Mughal et al., 1983; Haglund, 1986). Antigens related to pancreatic (del Favero et al., 1986; Sakaharane et al., 1986; Haglund, 1986), lung (Shinkai et al., 1986) and breast carcinomas (Dhokia et al., 1986b) have been detected in patients' sera, but their role in tumour detection and monitoring needs further evaluation. Oncogene products have recently been detected in the serum of patients with cancer (Dhokia et al., 1986c), but again, their significance in terms of tumour diagnosis and prognosis needs to be defined.

Thus, tumour markers could play a major role in the early detection of malignant disease, particularly in the screening of an asymptomatic population, although no tumour-marker has yet been proved useful in this context.

1.4.1.b *In vivo* diagnosis

Monoclonal antibodies may also be used *in vivo* for the accurate localization of primary neoplasms, but their role is more significant in the recognition of metastatic lesions, for which early detection critically affects management of the cancer. Thus, antibodies can be labelled with a variety of radionuclides and administered to animals or patients; the radioactivity from the suspected body areas is subsequently monitored using a γ-camera. This technique, termed immunoscintigraphy, has proved effective in numerous animal and clinical studies for cancer diagnosis.

Ideally, radionuclides used for tumour imaging with radiolabelled antibodies should be characterized by a reasonable physical half-life (12 hours - 3 days), γ-energy range of 20-240 KeV, high single γ-energy abundance per decay, small abundance and low energy particulate radiation, and reasonable radiolabelling chemical properties and stability (Wessels and Rogus, 1984). There is no

radioisotope that fulfills all the above requirements. However, ^{123}Iodine has proved very useful for antibody-mediated tumour localization. The 13 hour half-life of ^{123}Iodine may restrict its use to short-term studies, but excellent images can be obtained from its high quality γ-emission. ^{131}Iodine can be also used, although its β-radiation and 8-day half-life severely limit diagnostic doses, despite its low cost and ready availability (Keenan et al., 1985).

Effective radiolabels other than iodines have been also developed. ^{111}Indium produces imaging of very high quality, and together with ^{99m}Technetium is currently under extensive investigation (Childs and Hnatowich, 1985; Hnatowich et al., 1985). ^{77}Bromine is being used as a cyclotron produced imaging radionuclide (Scheinberg, 1982), together with ^{55}Cobalt (Larson, 1985) and ^{67}Copper (Cole et al., 1983). Other radioisotopes suitable for immunoscintigraphy include ^{46}Scandium, with its useful γ-radiation (Anderson and Strand, 1985), and ^{67}Gallium (Anderson and Strand, 1985; Koizumi et al., 1989). The latter offers little advantage over ^{111}Indium for single photon imaging, but ^{68}Gallium may be useful for studies with positron emission tomography. Finally, various stable elements such as manganese and gadolinium may be of potential value in nuclear magnetic resonance imaging (MRI) (Courtet et al., 1987).

The method of radiolabelling chosen must allow controlled incorporation and optimal preservation of radionuclide and antibody activities. A coupling reaction specific for aminoacid residues may lead to the antigen binding site being blocked, and loss of the antibody's immunoreactivity. Two moieties in immunoglobulins may provide linkage sites that are unlikely to interfere with the antigen binding site. One is the carbohydrate group usually present in the constant regions of heavy-chains, and only occasionally in the variable region. The other linkage site can be provided by free sulfhydryl groups formed on reductive cleavage on the interchain disulfide bridges of immunoglobulins.

Antibodies can be labelled with iodines using several electron acceptors (oxidizing reagents). Chloramine T (Hunter and Greenwood, 1962) and iodogen (Fraker and Speck, 1978; Solocinski et al., 1979) have been in use for a long time. Other radioiodination techniques have recently been employed, such as the mild oxidant N-bromosuccinamide (Reay, 1982), or linkers like the Bolton-Hunter reagent (Bolton and Hunter, 1973). The actual number of iodine atoms that can be attached per antibody molecule is dependent on the number of tyrosine moieties available for iodination. Proteins like antibodies are very sensitive to radioactivity, therefore special care must be taken regarding the oxidizing agent and the specific activity for fear of loss of the antibody's immunoreactivity.

The attachment of metal ions (such as ^{111}Indium, ^{67}Copper) to antibodies by means of chelating agents provides an alternative system for the preparation of new cancer imaging agents. Diethylenetriamine pentaacetic acid (DTPA) is the bifunctional reagent that is most commonly used. It chelates with metallic cations and is easy to use because there is no need for intermediates, and the chemistry is not complex. There are many forms of DTPA that have been employed, such as the cyclic dianhydrate (Hnatowich et al., 1983; Paik et al., 1985), the carboxycarbonic anhydrate (Paik et al., 1983a; Fawwaz et al., 1985), the o-acylisourea (Paik et al., 1983b), or the N-hydroxysuccinamide active ester (Paxton et al., 1985). Ethylenediamine tetraacetic acid (EDTA), used as bromoacetamidobenzyl EDTA (Goodwin et al., 1985; Gobuty et al., 1985) is equally effective in radiolabelling antibodies.

Many investigators have used antibodies to image transplantable tumours in animals. Research on tumour localization using radiolabelled polyclonal antiserum was first introduced in 1948 by Pressman and Keighley, who showed that anti-tumour antibodies specifically targeted to Wagner osteosarcoma in rats. Six years later the same group successfully visualized rat

Murphy lymphosarcoma (Korngold and Pressman, 1954), and the following year Bale et al. observed tumour uptake using antiserum against Walker sarcoma (Bale et al., 1955). Similar animal experiments were carried out in the 1960s and 1970s, imaging xenografted sarcomas in rats (Day et al., 1959; Spar et al., 1959; Bale et al., 1964), spontaneous tumours in dogs (Spar et al., 1960), and transplantable colonic neoplasms in hamsters (Goldenberg and Hansen, 1972; Primus et al., 1973; Goldenberg et al., 1974) and nude mice (Mach et al, 1974).

Although initially there had been limited success in the use of radiolabelled polyclonal antiserum, the production of monoclonal antibodies significantly improved tumour radioimmunolocalization. Monoclonal antibodies successfully localized *in vivo* in animals bearing human colon (Buchegger et al., 1983; Jakowatz et al., 1985; Fand et al., 1986; Rowlinson et al., 1988a), gastro-intestinal (Pimm et al., 1987), breast (Ceriani and Blank, 1988) and ovarian carcinomas (Murray et al., 1988), as well as B-cell lymphomas (Letvin et al., 1987; Buchsbaum et al., 1988), sarcomas (Bruland et al., 1987), gliomas (Takashi et al., 1987), teratomas (Yeh et al., 1988), and choriocarcinomas (Mano et al., 1986). Maximum tumour uptake was reached between 1 and 2 days after injection, whilst radioactivity gradually cleared from the rest of the body over the following days. However, radioactivity often remained in organs such as the liver, spleen and reticuloendothelial system, making the diagnosis of tumours at these sites difficult.

The plethora of animal experiments showed that anti-tumour antibodies can specifically and sufficiently recognize neoplastic lesions *in vivo,* and hence paved the way for clinical studies. In the last decade, the number of immunoscintigraphic studies in patients with cancer has increasingly grown. Antibodies have been used to detect both primary and metastatic lesions, since the presence of metastases affects the choice of therapy. Certainly conventional radiology, such as computer tomography (CT) scanning, have improved the accuracy of staging. However, in many cases metastatic disease is undetected by

conventional means at the time of initial therapy. Administration of radiolabelled antibodies have also proved beneficial in many other cases, such as the accurate detection of minor residues of ovarian cancer during second-look laparotomy (Jägger et al., 1989), or determination of liver involvement in patients undergoing surgery for colorectal cancer (Sickle-Santanello et al., 1987). In addition, radioimaging can be used post-operatively in order to detect small lesions that have possibly escaped surgery.

The first reports on the use of radiolabelled antibodies for tumour imaging in patients with cancer date back to the early 1960s, when polyclonal anti-fibrinogen antibodies successfully localized to primary and secondary brain tumours (DiChiro et al., 1963; Spar et al., 1963; Dewey et al., 1963; McCardle et al., 1966), as well as to cervical and ovarian carcinomas (McCardle et al., 1966). Later studies by Goldman et al. and Mach et al. showed high tumour uptake using polyclonal anti-CEA antiserum (Goldman et al., 1980; Mach et al., 1980).

With the advent of mAbs, new momentum was added to the field of radioimmunolocalization. Mach et al., were the first to publish successful tumour imaging in patients with colorectal cancer using ^{131}Iodine-labelled mAbs against CEA (Mach et al., 1981). The following year successful tumour localization of colon (Farrands et al., 1982), breast and ovarian carcinomas (Epenetos et al., 1982) was reported using ^{123}Iodine-labelled mAbs. Several other tumour imaging studies followed by many investigators, using radioiodines or ^{111}Indium-labelled mAbs in patients with gastrointestinal tract carcinomas (Moldofsky et al., 1983; Mach et al., 1983; Chatal et al., 1984; Delaloye et al., 1986; Douillard et al,., 1986; Riva et al., 1988; Beatty et al., 1989). breast (Rainsbury et al., 1983; Rainsbury, 1984; Hayes et al., 1986; Kalofonos et al., 1989b), ovarian (Epenetos et al., 1985b; 1985f; 1986a) and testicular carcinomas (Epenetos et al., 1985f; Critchley et al., 1986), brain tumours (Epenetos et al., 1985a; Richardson et al., 1986; Kalofonos et al., 1989a; Skarlos et

al., 1989; Zalutsky et al., 1989), melanomas (Larson et al., 1983; Larson, 1985; Halpern et al., 1985; Rosenblum et al., 1985; Buraggi et al., 1985; Murray et al., 1985) and non-small cell lung cancer (Kalofonos et al., 1988).

1.4.2 Cancer therapy

1.4.2.a Unmodified antibodies

Radioimaging experiments have shown that antibodies with specificity for tumour-associated antigens recognize and bind to transformed cells *in vivo*. Thus, it is theoretically possible that sufficient amounts of antibodies could kill the malignant cells they bind to. The concept of using specific antibodies for therapy of neoplasia was apparently first reported by Hericourt and Richet in 1895, who used antiserum against a human osteogenic sarcoma.

Administration of anti-tumour antibodies (polyclonal or monoclonal) is expected to activate the host's natural defense mechanisms. As described above, antibodies that have "coated" a malignant cell could attract monocytes that kill the target cell after binding the Fc part of the antibody by their FcRs (ADCC). Alternatively, the antibody may activate the classical complement pathway: C3b facilitates adherence by C3b-receptor bearing cells (monocytes, macrophages, neutrophils), which results in phagocytosis of the target cell. C3b may also augment ADCC. Moreover, formation of the complement complex C5-C9 disrupts the cell membrane leading to lysis. Another function that the administered antibodies may have, is that, if they are directed against growth factor receptors, they could block the target-cell's activation, and drive it to anergy.

Mouse monoclonal antibodies directed against tumour-associated antigens have been used for tumour-cell killing *in vitro*, in the presence of either

human complement or effector cells (eg. monocytes and macrophages) (Koprowski et al., 1978; Herlyn and Koprowski, 1982b; Johnson et al., 1986; Weiner et al., 1989). Antibodies were also found to be effective *in vivo* in attacking malignant cells in animals bearing human tumours (Herlyn et al., 1980; Herlyn and Koprowski, 1981; Badger and Bernstein, 1983; Adams et al., 1984). These preclinical studies led to the administration of monoclonal antibodies in patients with resistant forms of cancer, such as metastatic gastrointestinal and colorectal cancer (Sears et al., 1982; Sears et al., 1985), acute lymphoblastic (Ritz et al., 1981), T-cell (Miller et al., 1981) and chronic lymphocytic leukaemias (Dillman et al., 1982), cutaneous T-cell (Nadler et al., 1980; Miller and Levy, 1981) and B-cell lymphomas (Miller et al., 1982; Meeker et al., 1985).

Although some initial clinical studies produced encouraging results (Miller et al., 1982; Sears et al., 1982; Sears et al., 1985; Meeker et al., 1985), they were not substantiated when a larger number of patients was studied. The reason why the use of unmodified antibodies had been disappointing so far, may be because mouse mAbs are relatively ineffective at activating human immune mechanisms. In such a case, perhaps polyclonal anti-tumour antiserum would be preferable, despite the loss of specificity. Another reason could be that the amount of antibody that reaches and "coats" the tumour-cell may not be sufficient to cause substantial damage to a very rapidly proliferating cell. Antibody internalization will further reduce the effectiveness of the treatment.

1.4.2.b Immunotoxins

To overcome these problems, the antibodies can be "armed" with a toxic compound that will kill the target-cell upon contact or after internalization. Antibodies can be conjugated to various toxins of bacterial or plant origin to form "immunotoxins". Toxins that are most commonly used are the bacterial

exotoxin from *Corynobacterium diphtheriae*, the pokeweed antiviral protein, ricin (from the plant *Ricinus communis*), abrin (from the plant *Abrus precatorius*) and gelonin (from the plant *Gelonium multiflorum*), since they are more easily available, the chemistry of linking to the antibody is not very complicated, and they are "lethal" to the target-cell.

With the exception of gelonin and pokeweed antiviral protein, all the above toxins comprise two polypeptide chains, the A- and B-chain, which are linked by a disulfide bond. The B-chain has a receptor-binding site, so that the toxin binds on the cell-surface: abrin and ricin recognize galactose and lactose terminating glycoproteins and glycolipids on the cell membrane, whereas diphtheria toxin receptors are not fully identified yet. The toxin is endocytosed; the A-chain is then transported across the endosomal membrane into the cytoplasm (most often with the help of the hydrophobic region of the B-chain) and terminates cell protein synthesis. Abrin and ricin damage the 60S subunit of ribosomes, whereas diphtheria toxin inactivates the elongation factor 2 (reviewed by Thorpe and Ross, 1982, and Collier and Kaplan, 1984). Although the B-chain of toxins binds indescriminantly to any cell, specific binding and killing can be achieved by replacing the B-chain with an antibody molecule that has specificity for an antigen on the surface of the target-cell.

Antibodies have also been covalently linked to other toxic reagents including the aspergillus toxin (Conde et al., 1989), the exotoxin of *Pseudomonas aeruginosa* (FitzGerald et al., 1987; Kondo et al., 1988), saponin (from the plant *Saponaria officinalis*) (Tazzari et al., 1988; Blakey et al., 1988c), bryodin (from the plant *Bryonia dioica*) and momordin (from the plant *Momordica charanthia*) (Stirpe et al., 1988). Successful immunotoxin-mediated cytotoxicity has been obtained in many *in vitro* studies, using cell-lines derived from osteogenic sarcoma (Embleton et al., 1986), breast (Le-Maistre et al., 1987), ovarian cancer (Pirker et al., 1985), retinoblastoma (Merriam et al., 1984), liver-cell carcinoma (Oladopo et al., 1984), B- and T-cell leukaemias and

lymphomas (Raso et al., 1982; Uckun et al., 1985; Ghetie et al., 1988; Bacha et al., 1988; Stirpe et al., 1988; Bregni et al., 1988), squamous carcinomas (Ozawa et al., 1989), and very interestingly various drug-resistant cell-lines (FitzGerald et al., 1987).

However, it appears that immunotoxins are not very effective *in vivo*, as revealed by animal (Leonard et al., 1988; Hara et al., 1988; Hertler et al., 1989) and clinical studies (Spitler, 1987). One reason for the poor *in vivo* results is that the coupling procedure, and addition of toxin moieties, can reduce the antibody's immunoreactivity. Another important factor is that the linkage between the antibody and the toxic compound is very sensitive to *in vivo* enzymatic cleavage. In the future, more stable linkers, or recombinantly engineered antibody-toxin chimaeras (Chaudhary et al., 1989), may increase the immunotoxin's cytostatic efficacy *in vivo*. Another limitation is that target cytotoxicity depends on antibody-toxin internalization, and efficient toxin exit from the endosome into the cytoplasm (Calafat et al., 1988). The degree of internalization varies significantly among malignant cells (Preijers et al., 1988). In addition, the toxin may not necessarily leave the endosome in its native form, firstly because conjugation to the antibody may have altered vital determinants on its structure (Marsh and Neville, 1988), and secondly because the B-chain (which has been removed from the immunotoxin) is important for A-chain translocation from endosome to the cytosol. Antibody-whole toxin conjugate has been used in *in vitro* experiments and found to be far superior to antibody-A-chain, but in order to retain its antigen specificity, the former had to be used in the presence of excessive concentrations of sugars (Youle and Neville, 1980; Thorpe et al., 1981).

1.4.2.c Antibody-drug conjugates

Monoclonal antibodies can be also conjugated to chemotherapeutic drugs in order to specifically deliver them onto tumour-cells. Drugs most often used

include vinca alkaloids, methotrexate, adriamycin and daunomycin. Appropriate conjugation of the drug to the mAb should allow for the retention of both antibody immunoreactivity and drug effectiveness (Kulkarni et al., 1981; Umemoto et al., 1989). The number of drug molecules per antibody determines the cytotoxic properties of the conjugate (Garnett et al., 1983; Shen et al., 1986).

Promising *in vitro* results have been generated by coupling monoclonal antibodies to methotrexate (Uadie et al., 1983; Garnett et al., 1983; Shen et al., 1986; Deguchi et al., 1986), daunomycin (Ballou et al., 1983), idarubicin (Pietersz et al., 1988a), vindesine (Embleton et al., 1983) and adriamycin (Oncona et al., 1986; Shouval et al., 1988). Preclinical studies in tumour-bearing mice demonstrated tumour retention and growth suppression after administration of methotrexate (Embleton et al., 1984), adriamycin (Dillman et al., 1986; Yang and Reisfeld, 1988) or vindesine (Embleton et al., 1984) conjugated antibodies.

Preliminary clinical studies have shown that antibody-drug conjugates have the potential to target onto the tumour cells (Ballantyne et al., 1988). Whether they are also effective in cell killing, remains to be proved. Like immunotoxins, *in vivo* instability of the drug-conjugate and inefficient internalization or transport into the cytosol, may limit their use. Nevertheless, the recent use of acid-labile bonds between the drug and the antibody, to ensure proper cleavage only in the low pH environment of the lysosomes (Durrant et al., 1989), or the development of bispecific antibodies, with one binding site for a drug and the other for a tumour-associated antigen (Corvalan et al., 1987a; Corvalan et al., 1987b), have significantly improved the antibody-drug conjugate cytotoxicity. Such molecules appear to be very promising for future clinical development.

From the above data, we can conclude that antibodies can be clinically useful for the diagnosis of cancer, since they successfully localize onto neoplastic

lesions *in vivo*. Moreover, this encounter may become toxic to malignant cells if the host's immune defense mechanisms are activated; this activity is often insufficient, but it can be greatly improved by conjugating toxic reagents, such as chemotherapeutic drugs or plant toxins, to the antibody molecules. However, in this case the anti-tumour effect entirely depends on efficient conjugate internalization, and transfer of the toxic compound into the cytoplasm in a biologically active form. As discussed earlier, this does not always occur. In addition, immunotoxins and drug conjugates are subject to relatively fast *in vivo* degradation, which results in early inactivation of a large proportion of the anti-cancer reactivity.

1.4.2.d Radiolabelled antibodies

An alternative approach is to use therapeutic antibodies labelled at high specific activities with a suitable radioisotope. The advantage of this approach is that antibodies can carry a large number of isotope-ions without significant alteration of their immunoreactivity, catabolic rate and *in vivo* behaviour. Labelling techniques are usually simple, and the radioactivity delivered can irreparably damage the target cell upon binding, without necessarily the need of secondary manifestations, such as internalization. Moreover, radiation will only affect rapidly proliferating cells, eg. malignant cells. Thus, apart from a degree of temporary myelosuppression due to BM irradiation, depending on the total dose used, accumulation of radiolabelled antibodies in catabolic sites such as spleen and liver should not cause considerable side-effects. In addition, after of *in vivo* degradation of the antibody-radiolabel conjugate, the isotope is expected to be rapidly removed from the circulation, and cleared out through the kidneys, due to its small size; in contrast, larger toxic compounds may reside in catabolic organs for long periods. Finally, radiolabelled antibodies have a greater range of cytotoxicity, since they will irradiate the target-cells to which they bind together with neighbouring malignant cells

within a certain radius, whereas the toxicity of immunotoxins or drug conjugates is restricted to the target-cell.

Radionuclides suitable for antibody-guided irradiation should in general have properties similar to those of imaging radioisotopes, described above. However, their decay should be characterized by a large component of particulate radiation (α and β-emission) and little or no accompanying γ-rays. ^{131}Iodine is most widely used because of its availability, simple labelling method and β-emission. In addition, its γ-radiation is often regarded as an advantage, since it also permits tumour imaging. However, ^{131}Iodine is not the ideal isotope, and nuclides with higher and more "lethal" β-ray activity can be used, such as ^{90}Yttrium, ^{109}Palladium, ^{47}Scandium, ^{32}Phosphorus, ^{186}Rhenium, ^{69}Copper and ^{211}Astatine.

^{90}Yttrium and ^{186}Rhenium have been found to be among the best radiolabels for therapy, since they possess a sufficiently long half-life necessary for tumour localization, little or no γ-radiation, stable daughter products and reasonable chemistry of linkage to antibodies (Beirwalters, 1985). ^{109}Palladium (Keenan et al., 1985) and ^{32}Phosphorus (Foxwell, 1986) are β-emitting radionuclides that should be also superior to ^{131}Iodine because of their high energy and suitable half-life. In addition, ^{212}Bismuth has been proposed as a therapeutic radiolabel due to the extremely high linear energy transfer of its α-emission (Scheinberg et al., 1982). Finally, ^{10}Boron neutron-capture therapy combines the attractive features of both external beam and internal radioisotope therapy, to deliver a large differential dose to antibody-boron "loaded" tumour cells interspersed within healthy tissue (Fairchild et al., 1984).

In *in vivo* experiments, therapeutic monoclonal antibodies were labelled with ^{131}Iodine and administered to mice bearing human tumour xenografts. Tumour retention (Goldenberg et al., 1981; Sugiyama et al., 1988) and even cure (Buchegger, 1989) was observed for colonic carcinomas. Radiolabelled

antibodies were found to be effective in many other cases such as xenografted bladder carcinomas (Yeh et al., 1988), melanomas (Denardo et al., 1982) and neuroblastomas (Cheung et al., 1986). In addition, complete remission of the tumour was observed after administration of ^{90}Yttrium-labelled mAbs to nude mice with leukaemic spleens (Anderson-Berg et al., 1987).

The idea of using radiolabelled antibodies for the treatment of malignancies is not new. In 1966, McCardle et al. reported very encouraging results after treating two patients (one with a metastatic epigastric and the other with a brain tumour) with ^{131}Iodine-labelled polyclonal rabbit anti-fibrinogen antiserum. Similarly, iodinated polyclonal antiserum appeared to be beneficial when administered to patients with hepatocellular carcinoma, showing prolonged survival time in >40% of the patients (Ettinger et al., 1982; Liu et al., 1983; Order, 1985). The introduction of mAbs initiated more clinical studies. Patients with pleural and pericardial effusions responded well to a panel of ^{131}Iodine-labelled mAbs (Hammersmith Oncology Group, 1984; Pectasides et al., 1986). Very promising results were also obtained in Phase I and II studies in patients with brain gliomas (Epenetos et al., 1985a; Kalofonos et al., 1989a), lymphomas (Rosen et al., 1987; Zimmer et al., 1988) and ovarian carcinomas, where radiolabelled antibodies appeared to be effective in controlling small residual disease and accumulation of ascites (Epenetos et al., 1986a; Epenetos et al., 1987a; Ward et al., 1988). Nevertheless, results from treatment of liver metastasis (Delaloye et al., 1986; Epenetos et al., 1987b), pancreatic cancer (Schultz et al., 1988) and melanoma (Carrasquillo et al., 1984) were less encouraging. Clinical studies using ^{90}Yttrium-labelled mAbs have only recently started, and initial data from patients with ovarian cancer (Stewart et al., 1990) and hepatoma (Leichner et al., 1988) revealed that radiolabelled antibodies could potentially control the disease, but there is still much room for improvement.

1.4.3 Limitations

For the past 40 years antibodies have been used in patients with cancer for *in vivo* tumour localization and treatment, but their success was below our expectations. It is encouraging however, that a large number of investigators have shown that problems can be overcome, and early tumour detection or effective cancer treatment using antibodies may one day reach its full potential. The introduction of the monoclonal antibody technique played the major role in recent important developments, since it enabled the production of an unlimited number of monospecific antibodies in large quantities. Thus, antibodies today can be produced *in vitro,* and selected for properties that suit individual needs, such as affinity and isotype.

Nevertheless, *in vivo* experiments have shown that even if the ideal antibody is given to patients with cancer, only a very small percentage (<0.01%) of the antibody reaches the tumour (Epenetos et al., 1986b; Coakham and Kemshead, 1987; Zalutsky et al., 1989). However, it has been shown that regional administration can enhance tumour uptake in animals (Lee et al., 1987; Rowlinson et al., 1987; Wahl et al., 1988) and in clinical studies (Colcher et al., 1987; Malamitsi et al., 1988; Haisma et al., 1988). Moreover, antibody fragments that penetrate the tumour mass more deeply than do whole immunoglobulins can be used (Bergmann et al., 1987; Sutherland et al., 1987; Endo et al., 1988a; Colapinto et al., 1988). If the antibody alone is ineffective in triggering the host's immune defense mechanisms, toxic compounds can be easily linked to it, dramatically enhancing its anti-tumour activity. Moreover, the effect of the antibodies can be further improved when used in combination with various biological response modifiers, such as IFNs and TNF (Matsui et al., 1988; Smyth et al., 1988; Rosenblum et al., 1988). We are in the fortunate position now of being able to choose among toxin/drugs, linkers, radioisotopes, antibodies, routes of administration and doses that would suit

our particular requirements, and improve the effectiveness of the antibody-mediated tumour diagnosis and treatment.

However, there is unfortunately one further limitation to the *in vivo* clinical application of mAbs, resulting from the fact that the majority of diagnostic and therapeutic antibodies are of rodent origin, and hence are recognised by the host as foreign. Patients therefore mount an immune response against the xenogeneic antibody (Shawler et al., 1985; Schroff et al., 1985; Schultz et al., 1988; Goldman-Leikin et al., 1988), which is dose dependent (Sears et al., 1988), initially directed against the constant region (Chatenoud et al., 1986; Courtenay-Luck et al., 1986), but later extended against the whole molecule including the idiotope (Herlyn et al., 1985; Chatenoud et al., 1986; Courtenay-Luck et al., 1988). Even if a successful treatment protocol is devised, the human anti-mouse immunoglobulin antibodies that develop will limit the efficiency of the administered therapeutic antibody.

It has been shown that a large proportion of the injected therapeutic antibody is rapidly removed by anti-Ig antibodies that have developed in the host due to previous treatments (Pimm et al., 1985; Zimmer et al., 1988). As a consequence, the amount of the therapeutic antibody that reaches the tumour is very low, and continuously decreases upon further injections, as the anti-mouse Ig response rises. The *in vivo* antibody/antibody immune complexes may also lead to type III hypersensitivity (serum sickness), that can cause an inflammatory response with severe tissue damaging consequences (Steward, 1987).

The development of human anti-tumour antibodies may solve this problem, but until now these are not readily available. Until such a time, xenogeneic antibodies will continue to be in extensive use in a variety of clinical studies. It

is therefore of paramount importance to reduce or abolish the patients' response against the xenogeneic antibodies administered, if we wish antibody-mediated manipulation of cancer to be successful.

1.5 The induction of tolerance

The induction of immune suppression and immunological tolerance has been one of the earliest goals of immunology. Paradoxically, the mechanisms of immune responses could be analyzed, and the function and role of each immunocompetent cell identified, through unresponsiveness. Upon administration of a molecule with established antigenic properties, the host will either mount an immune response against it or not. At first this statement may sound very simple. However, the mechanisms that account for both immunity and unresponsiveness are very complicated, and are not yet fully understood. Thus, it is not surprising that a large number of theories have arisen, and hypotheses suggested, on the induction of immunological tolerance, that are often conflicting even when they describe the same phenomenon. Furthermore, it is appreciated that data from individual *in vitro* experiments and studies in experimental animals and man, using a wide range of antigens, may significantly differ, and hence cannot always be generalized.

There are two reasons why an antigen may not evoke an immune response: a) if it is recognised as "self", and b) if it directly or indirectly negatively affects immunocompetent cells. This unresponsiveness can be anything from short-term to permanent, and we now know that as it can be induced under particular conditions, it can also be "broken" under some other circumstances. This can be observed even in the most stringent tolerance situation, the tolerance to "self" antigens, that, fortunately very rarely, is disrupted leading to severe autoimmune diseases.

The exact mechanism(s) of "self"-tolerance are still to some extend a mystery, although it is increasingly becoming evident that T- and B-cells are under two distinct mechanisms of "self"-tolerance induction. This results from the fact that their requirements for activation by antigen are totally different (MHC restriction or not), and because whereas mature T-cells do not undergo further mutation (Fink et al., 1988), B-cells are subject to continuous antigen-dependent hypermutation in their Ig gene loci during adult life (Berek and Milstein, 1988).

It is generally accepted that immature T-cells are "educated" in the thymus to distinguish "self" from "non-self", through a process that involves positive and negative selection. Initially, $CD4^-8^-$ lymphoblasts enter the thymus and start to rearrange their TcR genes for the β, γ, δ and, later, α chains. Two populations of thymocytes emerge, the $CD4^-8^-$, TcR $\gamma\delta^+$ and $CD4^+8^+$ with very low density and affinity TcR $\alpha\beta$ (von Boehmer, 1988). A proportion of this latter population binds firmly to MHC molecules on the thymic cortical epithelium (stroma) and undergo a positive selection: thymocytes that bind MHC class II become $CD4^+8^-$ and those that bind class I become $CD4^-8^+$. The rest, and vast majority, of $CD4^+8^+$ that do not recognise "self" MHC molecules are programmed to die. The surviving cells migrate towards the medulla, where they meet macrophages and dendritic cells expressing auto-MHC with peptides of self antigens. This encounter proves to be "lethal" for potentially autoreactive T-cells (negative selection), whereas the rest make their way through the medulla to the periphery. From then on, engagement of the TcR by MHC (+ antigen) will only result to T-cell activation. The above notion of T-cell "self"-tolerance is supported and confirmed by various independent studies in experimental normal and transgenic mice (Kappler et al., 1988; Kisielow et al., 1988; Teh et al., 1988; Fowlkes et al., 1988; MacDonald et al., 1988; Sha et al., 1988; Zuniga-Pflucker et al., 1989; Bill and Palmer, 1989).

Although the majority of "self" antigens are already present, or able to migrate into the thymus (Nieuwenhuis et al., 1988), there may often be auto-antigens that never reach the thymic microenvironment. In this case a mechanism other than negative selection is proposed. Animal experiments with transgenic mice have revealed a state of clonal anergy for peripheral $CD8^+$ (Allison et al., 1988; Lo et al., 1988; Morahan et al., 1989) and $CD4^+$ (Rammensee et al., 1989; Mueller, 1989) auto-reactive T-cells.

"Self"-tolerance in B-cells is less well understood. It is clear that normally there is no antibody secretion against auto-antigens. This may be simply explained by the fact that all auto-reactive T_h-cells had been depleted in the thymus. However, T_h-cells with reactivity to foreign antigens have the potential under some circumstances to trigger autoantibody responses. It has been also suggested that the presence of T_S-cells may regulate this B-cell (and sometimes T-cell) unresponsiveness (Knight, 1982; Gibson et al., 1985). This is perhaps unlikely, since T_S-cells must always be present in all organs, in order to sustain B-cell suppression and protection from developing auto-reactivity during the unlimited number of antigen-driven mutations. Two theories appear to fulfill all the requirements for the apparent absence of auto-antibody production, which, not surprisingly, are diametrically opposite. Clonal deletion was proposed by Nemazee and Buerki, who found that "self"-specific B-cells against MHC class I were deleted in transgenic mice (Nemazee and Buerki, 1989), whereas clonal anergy was put forward by Goodnow et al., who found, also in transgenic mice, that B-cells against lysozyme ("self"), were apparently present as IgM^{+low}, IgD^{+high} cells but were unable to be activated and mature (Goodnow et al., 1988).

Tolerance can be also induced in adults, although it is not as easy and "natural" as in neonates, and it greatly depends on the nature of the antigen. In general, antigens fall into two categories, the T-dependent (TD) and T-independent (TI), according to whether T-cell participation is an essential

requirement for stimulation of antibody production (Miller, 1972). It is clear therefore that delivery of a negative signal to B-cells that are specific for TI antigens will totally abrogate the immune response, whereas with TD antigens similar phenomenon will be observed only after T-cell (T_h) anergy or depletion. In the latter case (i.e. TD antigens), antigen-specific B-cell inactivation, will not fully abolish, but certainly impair the immune response.

At the T-cell level, and of course for TD antigens, tolerance can be induced after T_h- and T_c-cell depletion or "blockage". One way to achieve this is by using anti-lymphocytic antibodies. As shown by many investigators, under these conditions animals fail to respond to various TD antigens given simultaneously or just after antibody-induced T-cell malfunction (Benjamin and Waldmann, 1986; Benjamin et al., 1986; Coronzy et al., 1986; Cobbold et al., 1986; Gutstein et al., 1986). Unresponsiveness may be observed to highly soluble, aggregate-free antigens that are not properly processed by antigen presenting cells (mainly macrophages). Thus, no cellular (and subsequently humoral) responses occur, since the antigen is not expressed in association with MHC. This phenomenon has been demonstrated in experimental models using xenogeneic albumin (Bell and Shand, 1973) and γ-globulin (Dresser, 1962; Golub and Weigle, 1969; Lukic´ et al., 1975a; Lukic´ et al., 1975b; Grebenau and Thorbecke, 1978). In addition, small molecules when conjugated with auto-antigens may be processed and presented to T-cells associated with auto-determinants that may deliver a negative signal to the lymphocytes. This was demonstrated when a hapten was linked to homologous IgG (Borel and Kilham, 1974; Borel, 1976a), red cells (Battisto and Bloom, 1966; Borek and Battisto, 1971; Naor et al., 1974) and lymphocytes (Miller and Claman, 1976; Long and Scott, 1977). Interestingly, in many of the above experiments, the suppression of cellular responses was also associated with direct B-cell inhibition, especially when the antigen was xenogeneic Ig, or autologous Ig coupled to a foreign hapten (Pan et al., 1976; Lee and Sehon, 1978; Grebenau and Thorbecke, 1978). This effect may be explained by the unique ability of the

Ig molecule to cross-link FcRs on B-cells with their surface anti-Ig (or anti-hapten) antigen receptors, resulting in specific B-cell "blockage" or deletion (Abas and Klaus, 1978; Tite and Taylor, 1979). This was also supported by similar experiments where antibody fragments were totally ineffective in tolerance induction (Lee and Sehon, 1976).

Alternatively, immune suppression to TD antigens is often explained by the presence of T_S-cells (Bencerraf, 1977), that can be activated upon stimulation of various antigens, as long as they are in the appropriate form and microenvironment. Their inhibitory effect may be due to cytokine interactions, such as IFN-γ and interleukin 4 (Knight, 1982). Induction of T-cells with suppressive activity was suggested in many experimental situations when sheep red blood cells, foreign Ig and other TD antigens were used as immunogens (Gershon and Kondo, 1970; Scott, 1978). It was found that such T_S-cells were negatively interfering with T_h (Basten, 1974; Tada and Takemori, 1974) and B-cells (Doyle et al., 1976; Aldo-Benson, 1979). Activation of suppressor cells was also suggested in experiments were tolerance was obtained against antigens given in amounts well below the optimal immunizing range ("low-dose" tolerance) (Mitchison, 1968; Andersson et al., 1974).

Induction of tolerance against TI antigens is theoretically simpler, since the only target is the B-cell. It has been shown that typical TI antigens, such as synthetic polypeptides or polysaccharides, when administered to experimental animals in excessive amounts, induced unresponsiveness ("high dose" tolerance) (Mozes et al., 1974; Klaus, 1976). In addition, polymeric antigens were found to be potent tolerogens in many *in vitro* and *in vivo* experiments (Desaymard and Feldmann, 1974; Desaymard and Howard, 1975; Moreno et al., 1976). The most likely explanation for the observed "high dose"- and polymer-induced tolerance, is the cross-linking of the surface Ig on the antigen-specific B-cells, that leads to functional neutralization or "blockade"

(Klaus et al., 1976; Abbas and Klaus, 1978). In addition, it seems that the long *in vivo* half-life of TI antigens with repeating antigenic determinants facilitates B-cell tolerance, since it has been shown that administration of enzymes can reverse the *in vivo* tolerogenic effects of polymeric antigens, such as dextrans (Fernandez and Möller, 1978) and polysaccharides (Brooke, 1964).

Interestingly, experiments with TI antigens have revealed that IgE responses are easier to abrogate than IgG responses (Desaymard, 1977), and both are easier to block than IgM responses (Klaus and Humphrey, 1975). Paradoxically, in many experiments induction of tolerance to TI antigens was associated with the presence of T-cells with suppressor activity, since it was found that polysaccharides were tolerogenic in normal mice, but not in nude ones (Baker and Prescott, 1979).

Antigen-specific unresponsiveness is also observed *in vivo* when homologous or heterologous antigen-specific antiserum is co-administered. This phenomenon was attributed to "coating" and "masking" of certain epitopes (Uhr and Möller, 1968), but it seems more probable that it is due to faster catabolism via the reticuloendothelial system, and disappearance from the system (Kölsch, 1980; Hoffman, 1980; Heyman and Wigzell, 1984). Antigens may also be chemically modified in order to make them less immunogenic. This approach was first shown by Parish in 1971, who reported that acetoacetylation of flagellin led to a reduction in immunogenicity, and induction of tolerance (Parish, 1971a; 1971b; 1973). Many *in vivo* and *in vitro* experiments followed, demonstrating the tolerogenic effect of chemical modification of antigens (Bach and Brashler, 1975), such as denaturation and separation of the polypeptide chains of ragweed antigen E (Takatsu et al., 1975), enzymatic digestion of albumin (Dosa et al., 1979), and attachment of polyethyleneglycol (PEG) to egg (Wie et al., 1981), bovine (Abuchowski et al., 1977) and dog albumin (Wie et al., 1981), L-asparginase and L-glutaminase-

asparaginase (Abuchowski et al., 1979), bovine pancreatic ribonuclease (Wie et al., 1981) and various allergens (Wie et al., 1981). PEG-linked adenosine-deaminase was also found to be tolerogenic in clinical studies, as reported by Hershfield et al., who treated children with adenosine-deaminase deficiency (Hershfield et al., 1987).

Immune unresponsiveness can also be induced by the use of immunosuppressive drugs. Cyclophosphamide (Cy) was the first one to be extensively studied. It has been shown that Cy suppresses the response against TI and TD antigens; it preferentially acts against B-cells (Poulter and Turk, 1972; Turk and Poulter, 1972), although an effect on T-cells was not excluded (Owens and Santos, 1971). It has also been reported that Cy facilitates induction of tolerance to TI (eg. levan, dextran) (Howard and Courtenay, 1976) and TD antigens (eg. sheep red blood cells) (Aisenberg and Davis, 1968; Many and Schwartz, 1970a). Other drugs that affect immunocompetent cells include glucocorticoids (reviewed by Steinberg and Williams, 1978), purine analogues (Santos, 1967; Campbell et al., 1976; Thomas et al., 1977) and cyclosporin A (Borel et al., 1977; Shevach, 1985). The latter is widely used today in the clinic due to its direct and potent activity against T-cell activation and absence of non-specific BM toxicity (reviewed by Shevach, 1985).

1.6 Aim of this thesis - Induction of tolerance to xenogeneic immunoglobulin

Immunoglobulins are classical TD antigens (Benjamin et al., 1986; Durrant et al., 1989), and their immunogenicity has always been a problem in clinical studies in patients undergoing antibody therapy. Various attempts have been reported so far for the induction of tolerance against the foreign Ig, with very successful *in vitro* and *in vivo* animal experiments, but with limited success in clinical applications. Avoidance of the unwanted anti-xenogeneic Ig responses have been obtained by: Ig deaggregation (Dresser, 1962; Peppys and

Taussig, 1974; Lukic'et al., 1975a), immunosuppressive drugs (Ledermann et al., 1988a; Ledermann et al., 1988b), chemical modification by conjugation to PEG (Wilkinson et al., 1988), to a chemotherapeutic drug (Durrant et al., 1989) or radiolabelling (Basten et al., 1971), and administration of anti-lymphocytic serum (Benjamin and Waldmann, 1986).

In the work described in this thesis we have attempted to induce tolerance against xenogeneic anti-tumour Ig administered to patients with cancer. This involved studies in experimental animals, and when possible in man. Our major aim was to specifically immunosuppress the host against the foreign antigen (Ig) leaving the rest of the immune system intact. All animal experiments were planned bearing in mind that possible success should be compatible with the clinical situation and directly applied to patients undergoing antibody-therapy.

At the beginning of this study it was essential to establish a sensitive, reliable, reproducible and sensitive method, in order to assess the host's response against the foreign Ig administered. Our assay is discussed in detail in the following chapter. We then studied the *in vivo* behaviour, pharmacokinetics and rate of catabolism of the antigen (mouse Ig) that we wished to render non-immunogenic, when administered in man. This is discussed in chapter 3.

For the induction of tolerance the strategies that we followed were: a) reduction of the foreign immunoglobulin's immunogenicity by accelerating its *in vivo* clearance in man using anti-Ig antiserum; b) interference at the antigen presenting level (macrophages) by using deaggregated Ig in an animal model, in parallel with clinical studies. The possible synergistic effect of deaggregation and an immunosuppressive drug, cyclosporin A, was also studied in animals; c) interference at the B-cell level by specifically eliminating anti-Ig B-cells in experimental animals, using chemically modified

(radiolabelled or complexed to a chemotherapeutic drug) Ig; and d) interference at the T-cell level by inhibiting the activation of lymphocytes using an anti-lymphocytic monoclonal antibody in animal studies.

Chapter 2

The ELISA method

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2.1 Introduction

The development of a technique for the measurement of the *in vivo* anti-immunoglobulin (Ig) response, was essential for this thesis. The assay had to be simple, sensitive, reliable and reproducible. In this chapter the method used is described in detail. Naturally for the completion of this thesis a variety of other techniques were employed; these will be discussed in their appropriate chapters.

Immunoglobulins are antigens (Ags) that elicit a T-cell dependent response in the recipient (Benjamin et al., 1986). The antibody (Ab) level in the host's serum (humoral response) is routinely accepted as a reliable and convenient means of measurement of the overall immune response against such antigens.

Various assays for the detection of anti-xenogeneic Ig antibodies have been described. The passive haemmaglutination method was one of the first assays to be used. Based on the adsorption of antibodies onto sheep red blood cells, this assay was developed in the late 1950s (Jandl and Simmons, 1957), and used by many investigators since then (Taussig, 1971; Pepys and Taussig, 1974; Carpenter et al., 1983).

Another, very popular method for demonstrating the presence of anti-Ig antibodies in animals and in patients, was the rate of blood clearance of radiolabelled Ig (Das and Leskowitz, 1970; Rossen et al., 1971; Pepys and Taussig, 1974; Lukic´ et al., 1975a). This was performed either by obtaining whole body counts or by simply measuring the radioactivity in blood samples using a γ-counter.

Immunohistochemical techniques are frequently used for a variety of assays. It is not surprising, therefore, that immunohistochemistry has also been used for

the detection of anti-Ig antibodies, especially after the advent of monoclonal antibodies (mAbs) (Köhler and Milstein, 1975): the administered Ig (playing the role of the Ag) is allowed to bind to its Ag *in vitro*, the unknown serum is applied and finally a fluoresceinated antiserum is added (Chatenoud et al., 1986) (Fig. 2.1a). An alternative to this, is the inhibition of binding of the administered Ig to its target cells, by preincubation with the unknown serum (Chatenoud et al., 1986) (Fig. 2.1b).

Over the last 30 years, new immunoassays have been developed to add to the above mentioned methods. Radioimmunoassay (RIA) was first described in 1956 (Berson et al., 1956), and was based on the inhibition of binding of a radiolabelled substance to its ligand, in the presence of unlabelled substance. The presence of anti-Ig antibodies in the serum was shown by mixing the blood sample with a known quantity of radiolabelled Ig. Complexed and unbound radioactivity were separated by size exclusion chromatography (Zimmer et al., 1988), or by precipitation after addition of formalin fixed *Staphylococcus aureus* cells (Carrasquillo et al., 1988). When the technique of adsorption of substances to a variety of solid supports was introduced (Catt et al., 1967; Catt and Tregear, 1967; Cambiaso et al., 1975), RIAs became the standard method for most assays, mainly because of their reproducibility and sensitivity (Minden et al., 1968).

There were some reservations concerning the use of radioactivity; however, these were overcome by replacement of the radioactive compound with an enzyme. Thus, the enzyme linked immunosorbent assay was developed (ELISA) (Engvall and Perlmann, 1971; Engvall and Perlmann, 1972; Graves, 1988), and supplemented if not supplanted RIAs in a growing number of applications.

The first report on Ig immobilisation (as antigen) on an insoluble support was reported in 1971 by R. D. Rossen et al. (Rossen et al., 1971). Serum

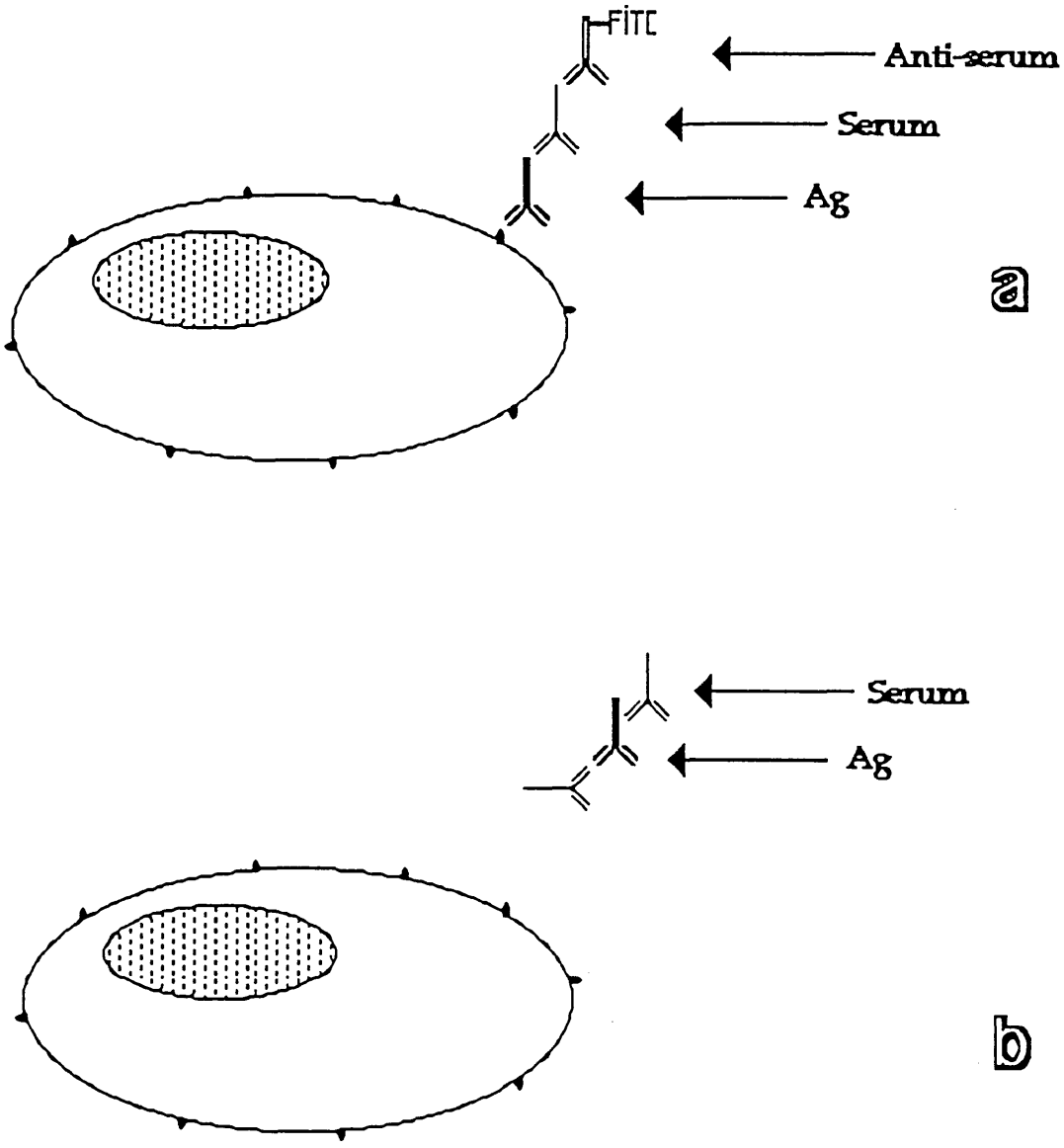


Figure 2.1: Immunohistochemical detection of anti-immunoglobulin antibodies by a) indirect immunofluorescence and b) inhibition of binding of the administered antibody onto its target-cell after preincubation with the host's serum. **Ag**: the xenogeneic immunoglobulin administered. **Serum**: the host's test serum containing anti-immunoglobulin antibodies. **Anti-serum**: commercially available fluoresceinated antibodies that recognise host's antibodies.

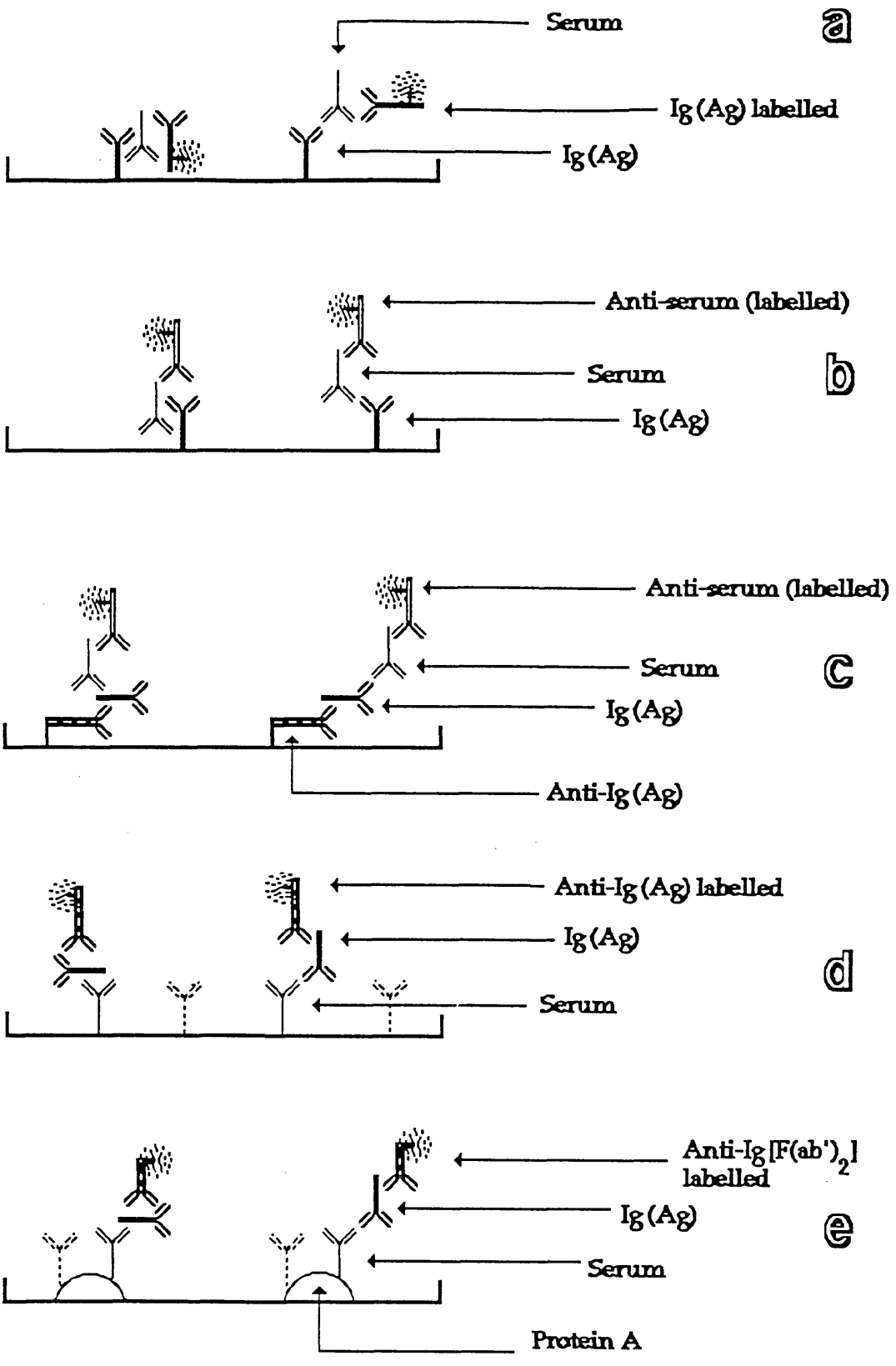
containing anti-Ig antibodies was applied, adsorbed, eluted (using a low pH buffer), and quantitated by spectrophotometry. Hence, in this case there was no need for a radioisotope or enzyme tracer.

However, the majority of investigators now immobilize the Ig onto 96-well polystyrene plates. Unknown serum is then applied, and anti-Ig antibodies are detected by either the addition of the same Ig that was used as antigen (Fig. 2.2a) (Miller et al., 1983; Herlyn et al., 1985; Sears et al., 1987; Goldman-Leikin et al., 1988), or anti-antiserum antibodies (Fig. 2.2b) (Shawler et al., 1985; Schroff et al., 1985; Courtenay-Luck et al., 1986; Courtenay-Luck et al., 1987; Ledermann et al., 1988a); both preparations for the secondary layer being labelled with either a radioisotope (for RIA), or an enzyme (for ELISA). A variation of the latter method is the adsorption of a well characterized anti-Ig antibody on the plate first, followed by the addition of the antigen (Ig), unknown test serum and labelled anti-"serum" (with specificity for Ig of the test serum, Fig. 2.2c) (Hafler et al., 1986; Hafler and Weiner, 1988).

Alternatively many investigators have chosen to immobilize first the unknown serum on the plate, and apply the antigen (Ig) later. The detection of the Ig is achieved with the help of a labelled anti-Ig antiserum. Unknown serum can be adsorbed either directly onto the microtitre plate (Fig. 2.2d) (Chatenoud et al., 1986), or via Protein A already bound on the plastic (Fig. 2.2e) (Moseley et al., 1988).

For our assays, we have chosen to use the indirect ELISA diagrammatically shown in Fig 2.2b. This was based on the following considerations. a) Tests depending entirely on the primary binding of antibody to antigen (eg. ELISA, RIA) are more sensitive than those requiring secondary manifestations of binding (eg. agglutination, precipitation) (Minden et al., 1968).

Figure 2.2: Detection of anti-immunoglobulin antibodies by RIA or ELISA.
Ig: the administered immunoglobulin used as antigen (Ag), employed either unlabelled (Figs. 2.2a, b, c, d, e) or labelled with a radioisotope or enzyme (Fig. 2.2a).
Anti-Ig: commercially available anti-serum that recognises the foreign immunoglobulin administered (used either unlabelled, Fig. 2.2c, or labelled with a radioisotope or enzyme, Figs. 2.2d, e).
Serum: the host's test serum containing anti-Ig antibodies.
Anti-serum: commercially available antibodies that recognise host's antibodies (labelled with a radioisotope or enzyme).


a
Serum
Ig (Ag) labelled
Ig (Ag)
b
Anti-serum (labelled)
Serum
Ig (Ag)
c
Anti-serum (labelled)
Serum
Ig (Ag)
Anti-Ig (Ag)
d
Anti-Ig (Ag) labelled
Ig (Ag)
Serum
e
Anti-Ig [F(ab')2] labelled
Ig (Ag)
Serum
Protein A

b) Plasma clearance of radiolabelled Ig is generally not accepted, basically because only antibodies of higher affinity are detected (Rossen et al., 1971), and the anti-Ig response is measured indirectly by calculating another parameter, namely the radioisotope. In addition this technique requires the administration of radiolabelled Ag to animals and patients. c) Immunohistochemistry (Fig. 2.1a, b) requires all Igs to have binding sites for tissue antigens and that positive cell lines are available to the user. d) Size exclusion chromatography and *Staphylococcus aureus* precipitation are difficult to standardize, and analyze quantitatively the response. e) Affinity chromatography is not very sensitive and requires relatively large amounts of serum. f) Adsorption of the whole test serum (or its immunoglobulin fraction) onto the microtitre plate (Fig. 2.2d, e) reduces sensitivity, because only few sites on the plastic will be occupied by specific anti-Ig antibodies. Finally g) although methods like those diagrammatically described in Figures 2.2a and 2.2c are considerably better than the others mentioned above, they may be hindered by higher cross reactivity (2.2c) and lower sensitivity (2.2a).

The ELISA technique used in this study has already been outlined in theory (Fig. 2.2b). However, despite the fact that many investigators use this method, the conditions must always be optimized and adjusted to meet individual needs. Various parameters characterize a reliable ELISA assay, namely: the type of microtitre plate, the amount of antigen (immunoglobulin in our case) coated onto the plastic, the serum and anti-serum concentrations. Finally, the expression and evaluation of the results will be discussed, together with attempts to increase the sensitivity of the assay.

2.2 Choosing the microtitre plate

Antigens attach to the plastic surface of microtitre plates by hydrophobic interactions (Van Oss and Singer 1966; Kochwa et al., 1967). The degree of binding depends on the type of plastic used (Lee et al., 1974), as well as the source of microtitre plates (Kenny and Dunsmoor, 1983). Generally, there are plates have high and others have low binding capacity (Kenny and Dunsmoor, 1987). A plate should be chosen to compromise between the highest possible adsorption of antigen, but the lowest non-specific binding of first and second layer antibodies (Fig. 2.2b).

Three U shape 96-well plates were tested for non-specific binding of 10 commercially available anti-Ig reagents conjugated to horseradish peroxidase (HRP). All 3 plates - from: Sterilin, UK, Costar, UK and Flow Laboratories, UK, - were coated with antisera at dilutions from 10^{-1} to 10^{-8} in phosphate buffered saline (PBS), pH 7.4, containing 0.05% tween 20 (Sigma). The sera: A: sheep anti-human Ig (Amersham, UK), B: rabbit anti-human IgM (DAKO, Denmark), C: donkey anti-rabbit Ig (Amersham), D: swine anti-rabbit Ig (DAKO), E: goat anti-rabbit IgG (Sigma, UK), F: sheep anti-mouse Ig (Amersham), G: rabbit anti-mouse Ig (DAKO), H: sheep anti-rat Ig (Amersham), I: rabbit anti-rat Ig (DAKO), J: sheep anti-rat IgM (Serotec, France), were incubated for 1hr at 37°C, and washed three times with PBS/0.05% tween 20, pH 7.4 (washing buffer). After the substrate 2,2' azino-di-[3-ethylbenzthiazolinesulphonate] (ABTS, Behringwerke, W. Germany), was applied, the absorbance in each well was measured at 405 nm, using a multiscan spectrophotometer (Titertek, Flow Laboratories). The enzymatic reaction was not stopped and the optical density (OD) was taken at various time points.

As shown in Figs. 2.3a and b, the non-specific binding in the Sterilin plate was significantly lower than for the Flow Laboratory one (Figs. 2.4a, b, c, d) at

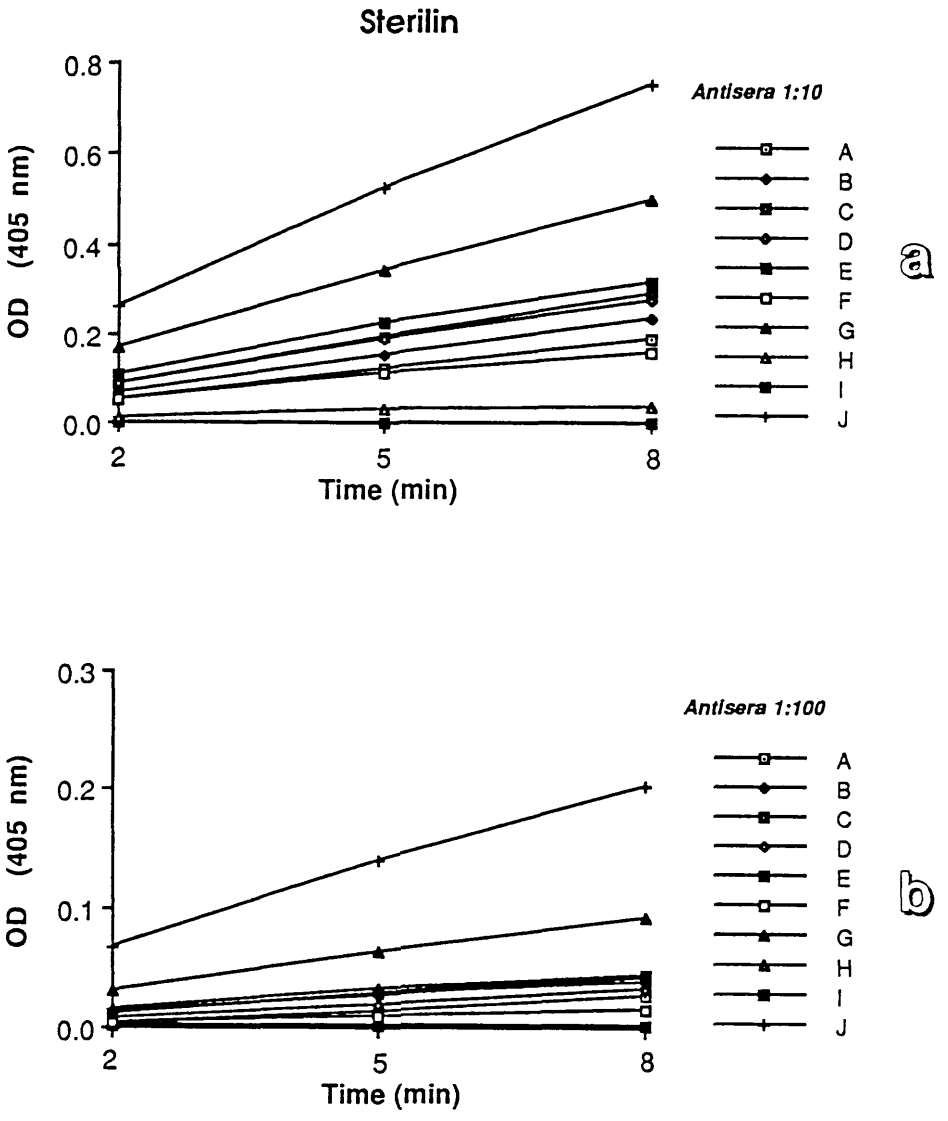


Figure 2.3: Non specific binding of ten commercially available antisera (directly conjugated to HRP) after incubation in Sterilin ELISA plates, at a) 1:10 and b) 1:100 dilutions. On the vertical axis is the absorption measured at 405 nm, over a period of 8 minutes (horizontal axis). A: sheep anti-human Ig (Amersham), B: rabbit anti-human IgM (DAKO), C: donkey anti-rabbit Ig (Amersham), D: swine anti-rabbit Ig (DAKO), E: goat anti-rabbit IgG (Sigma), F: sheep anti-mouse Ig (Amersham), G: rabbit anti-mouse Ig (DAKO), H: sheep anti-rat Ig (Amersham), I: rabbit anti-rat Ig (DAKO) , J: sheep anti-rat IgM (Serotec).

Figure 2.4: Non specific binding of nine commercially available antisera (directly conjugated to HRP) after incubation in Flow Laboratories ELISA plates, at a) 1:10, b) 1:100, c) 1:1000 and d) 1:10000 dilutions. On the vertical axis is the absorption measured at 405 nm, over a period of 8 minutes (horizontal axis).
A: sheep anti-human Ig (Amersham), B: rabbit anti-human IgM (DAKO), C: donkey anti-rabbit Ig (Amersham), D: swine anti-rabbit Ig (DAKO), E: goat anti-rabbit IgG (Sigma), F: sheep anti-mouse Ig (Amersham), G: rabbit anti-mouse Ig (DAKO), H: sheep anti-rat Ig (Amersham), I: rabbit anti-rat Ig (DAKO).

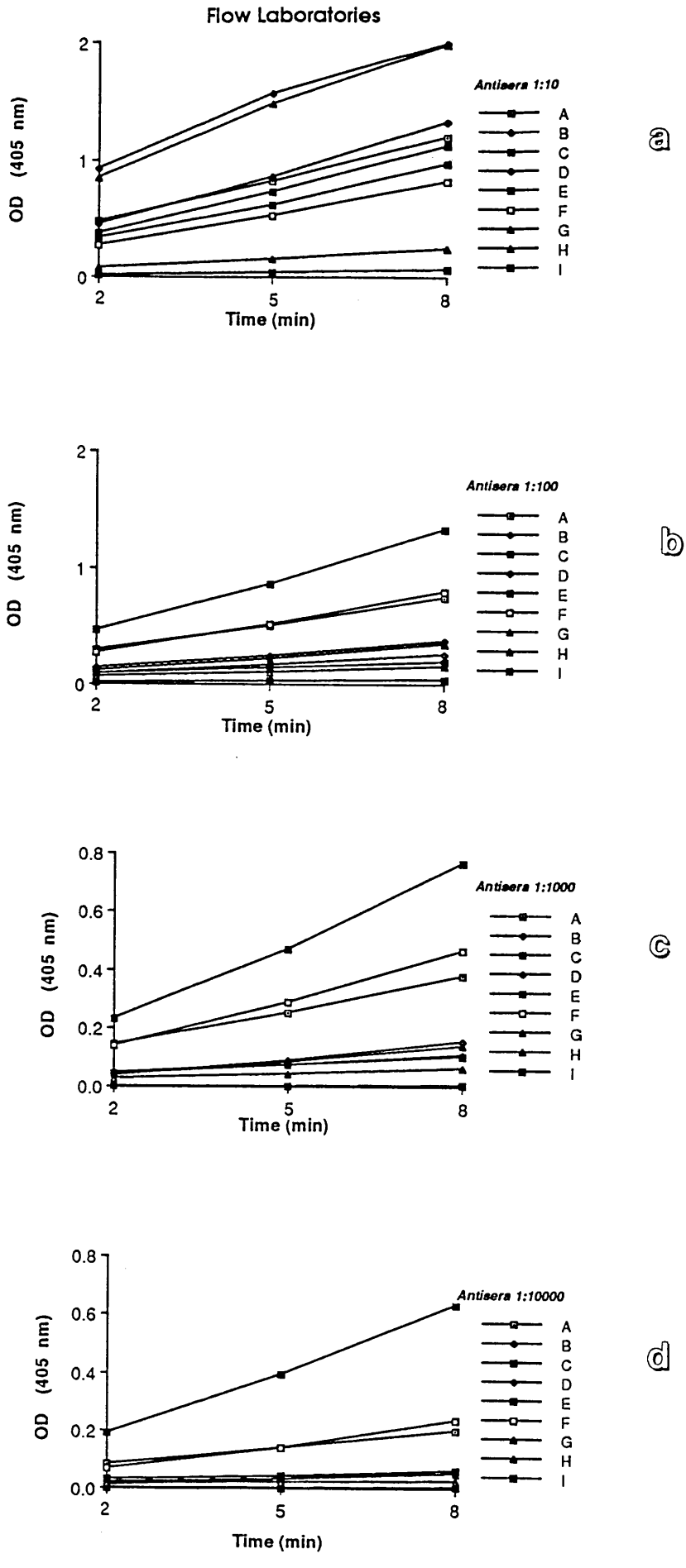
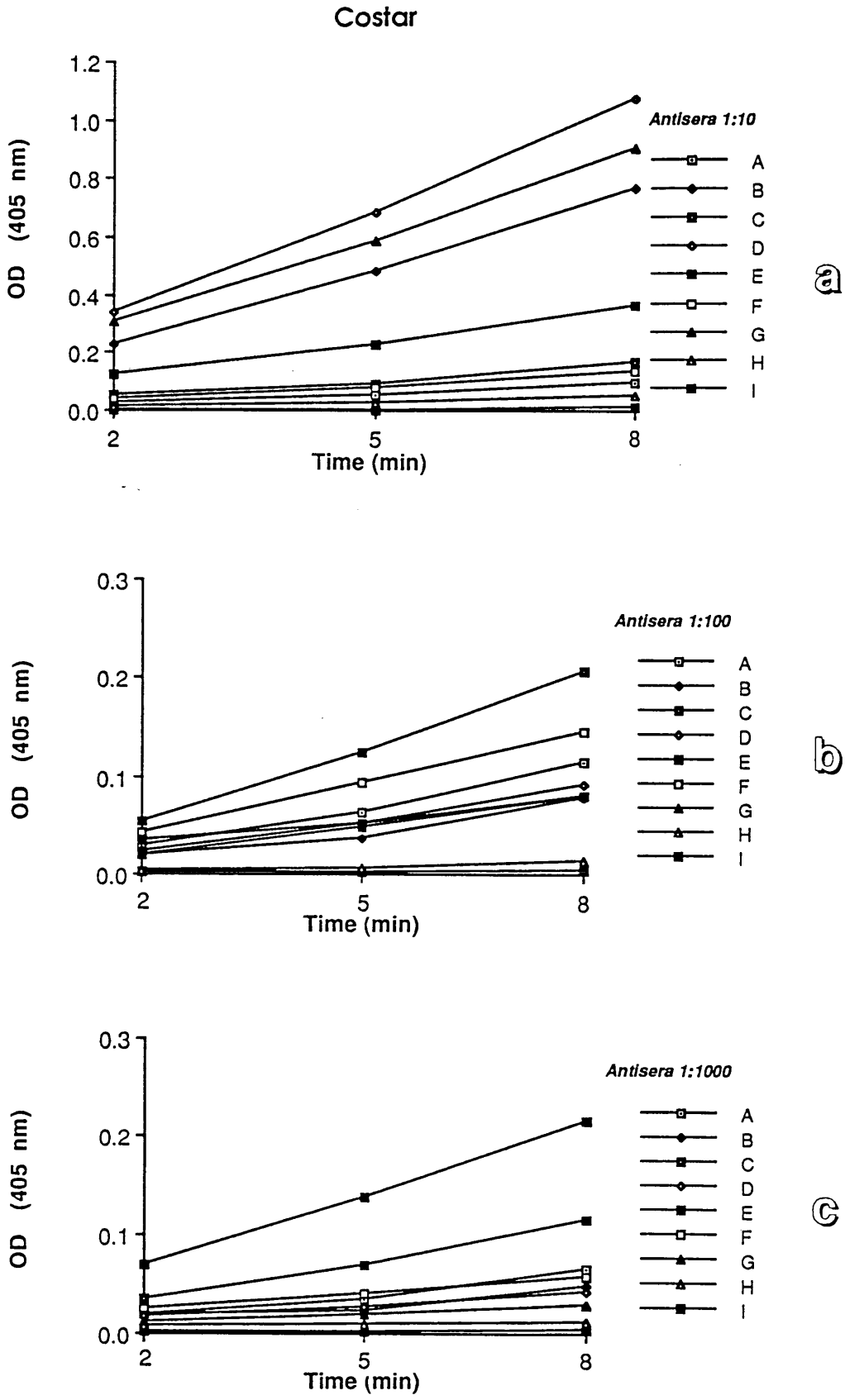

Flow Laboratories
OD (405 nm)
Time (min)
Antisera 1:10
Antisera 1:100
Antisera 1:1000
Antisera 1:10000
A
B
C
D
E
F
G
H
I
a
b
c
d

Figure 2.5: Non specific binding of nine commercially available antisera (directly conjugated to HRP) after incubation in Costar ELISA plates, at a) 1:10, b) 1:100 and c) 1:1000 dilutions. On the vertical axis is the absorption measured at 405 nm, over a period of 8 minutes (horizontal axis).
A: sheep anti-human Ig (Amersham), B: rabbit anti-human IgM (DAKO), C: donkey anti-rabbit Ig (Amersham), D: swine anti-rabbit Ig (DAKO), E: goat anti-rabbit IgG (Sigma), F: sheep anti-mouse Ig (Amersham), G: rabbit anti-mouse Ig (DAKO), H: sheep anti-rat Ig (Amersham), I: rabbit anti-rat Ig (DAKO).


Costar
OD (405 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2
5
8
Time (min)
Antisera 1:10
A
B
C
D
E
F
G
H
I
a
OD (405 nm)
0.3
0.2
0.1
0.0
2
5
8
Time (min)
Antisera 1:100
A
B
C
D
E
F
G
H
I
b
OD (405 nm)
0.3
0.2
0.1
0.0
2
5
8
Time (min)
Antisera 1:1000
A
B
C
D
E
F
G
H
I
c

all serum dilutions. At 1:100 dilution in the Sterilin plate, the background was close to zero. Likewise, Sterilin performed better than the Costar plate (Figs. 2.5a, b, c), which although itself better than that from Flow Laboratory, still showed high non-specific binding even at a dilution of 1:1000. We therefore used Sterilin plates for all subsequent experiments.

2.3 Determination of antigen concentration coated onto the microtitre plate.

The quality of an ELISA depends on many parameters; adsorbed antigen concentration and stability are crucial factors determining the sensitivity and reliability of the assay (Vos et al., 1987). Inappropriate amount of antigen could cause false results (Munoz et al., 1986). Stoichiometrically, the adsorption of an Ag on plastic shows a so called "region of independence" (Cantarero et al., 1980). Within this region there is a relationship between the amount of protein added and the amount adsorbed. The Ag-polystyrene interaction appears to be an example of Langmuir isotherm adsorption. The equation describing the maximal surface concentration of adsorbed molecules is:

$$\frac{1}{m} = \frac{1}{b} + \frac{1}{bKc}$$

where c is the Ag concentration in equilibrium with m grammes of adsorbed Ag/cm^2, b is a constant corresponding to the maximum amount of adsorbed Ag and K is the association constant for the reaction between the surface active sites and the protein to be adsorbed (Nieto et al., 1986). The lower the protein concentration needed to obtain similar surface coating, the higher will be the association constant. However it is not always correct to assume that the reaction is at equilibrium, and that adsorption follows the Langmuir isotherm. Binding of antigens can be diffusion limited. A theoretical model has recently been described by M. Stenberg et al. (Stenberg et al., 1988), where a diffusion limited scheme is applied to cylindrical microtitre wells, and a theoretical

diffusion limited reaction model of non-stirred solution is developed. The maximum amount of surface bound antigen, relative to the total amount present in the well, is estimated in relation to time, taking into account the diffusion constant of the Ag, the well radius and the Ag volume and concentration (Stenberg et al., 1988).

Adsorption within the "region of independence" may be more stable, but desorption of Ag occurs at a relative constant rate even within this region (Peterman, 1986). In addition, at extremely high Ag concentrations the coating efficiency decreases further, probably due to formation of intramolecular hydrophobic complexes (a common phenomenon with Igs). Thus, the hydrophobic adhesion of the protein to the plate is prevented (Pesce et al., 1981; Sorensen and Brodbeck, 1986).

Since adsorption follows a non-covalent, equilibrium-dependent reaction, different proteins will have different affinities for the plastic. Binding can be improved by using UV treated (Zouali and Stollar, 1986) or swollen (Causse et al., 1988) polystyrene plates, by cationising the Ag (Pesce et al., 1986), using methylated bovine serum albumin (Kavai et al, 1985), the appropriate detergent (Gardas and Lewartowska, 1988), or finally by utilizing a linker between the plastic surface and the Ag (Brennand et al., 1986; Smith and Wilson, 1986; Nygren and Karlsson, 1988; Jitsukawa et al., 1989). Ionic strength and pH of the coating buffer are also of great importance (Brock et al., 1984; Sorensen and Brodbeck, 1986; Geerligs et al., 1988).

In order to achieve the optimal antigen concentration for our ELISA plates (Sterilin), we have performed a chequer-board ELISA (Munoz et al., 1986): immunoglobulin was titrated to the point that gives the maximum absorbance using a control HRP conjugated anti-antibody reagent. Thus, polyclonal human IgG (Sigma) was coated onto the plate at 8 μg/well, and titrated down to 0.06 μg/well (1:2 dilutions) in triplicate. Three different coating buffers were

used in this experiment under identical conditions: carbonate/bicarbonate pH 9.6, PBS pH 7.4 and acetate pH 4.5. The plate was incubated for 4hr at 37°C, washed 3 times with washing buffer and a sheep anti-human Ig-HRP (Amersham) was applied at 1:500 dilution. After 1hr incubation at 37°C the plate was washed again, and the substrate ABTS was added.

The results, expressed as mean absorbance from each well at 405 nm, are shown in Figure 2.6a. Statistical analysis for each Ag concentration (using the Student's t test), revealed no significant difference between PBS and carbonate/bicarbonate buffers for concentrations within the range 8-0.5 μg/well. Effectively that part of the graph shows a plateau. However, from that point on, carbonate/bicarbonate buffer performed better. Similarly, the latter is superior to acetate buffer, although for low Ag concentrations (0.5-0.06 μg/well) there is no difference. Thus, carbonate/bicarbonate buffer pH 9.6 appeared to be the best of the three buffers used, for coating ELISA plates with Igs. This was therefore used as coating buffer in all subsequent experiments. Moreover, an Ig concentration of 0.5 μg/ml seemed to be optimal.

The same experiment was repeated using a mouse monoclonal antibody as Ag (EGFR1) in carbonate/bicarbonate buffer pH 9.6, and 2 batches of sheep anti-mouse Ig-HRP (Amersham) and a rabbit anti-mouse Ig-HRP (DAKO) as primary antibodies (at a constant dilution 1:500). In all cases (Fig. 2.6b) we confirmed the results of the previous experiment, showing an antigen concentration of 0.5 μg/well to be optimal for coating immunoglobulins onto our ELISA plates. This concentration was used in all further experiments.

In addition, when a mouse monoclonal antibody (HMFG1) was incubated for either 4hr or overnight (20hr) (Fig. 2.7) at 37°C in carbonate/bicarbonate buffer at 0.5 μg/well, and 8 different antisera applied (each at 1:10 serial dilutions), no change in the results was observed due to the prolonged incubation time,

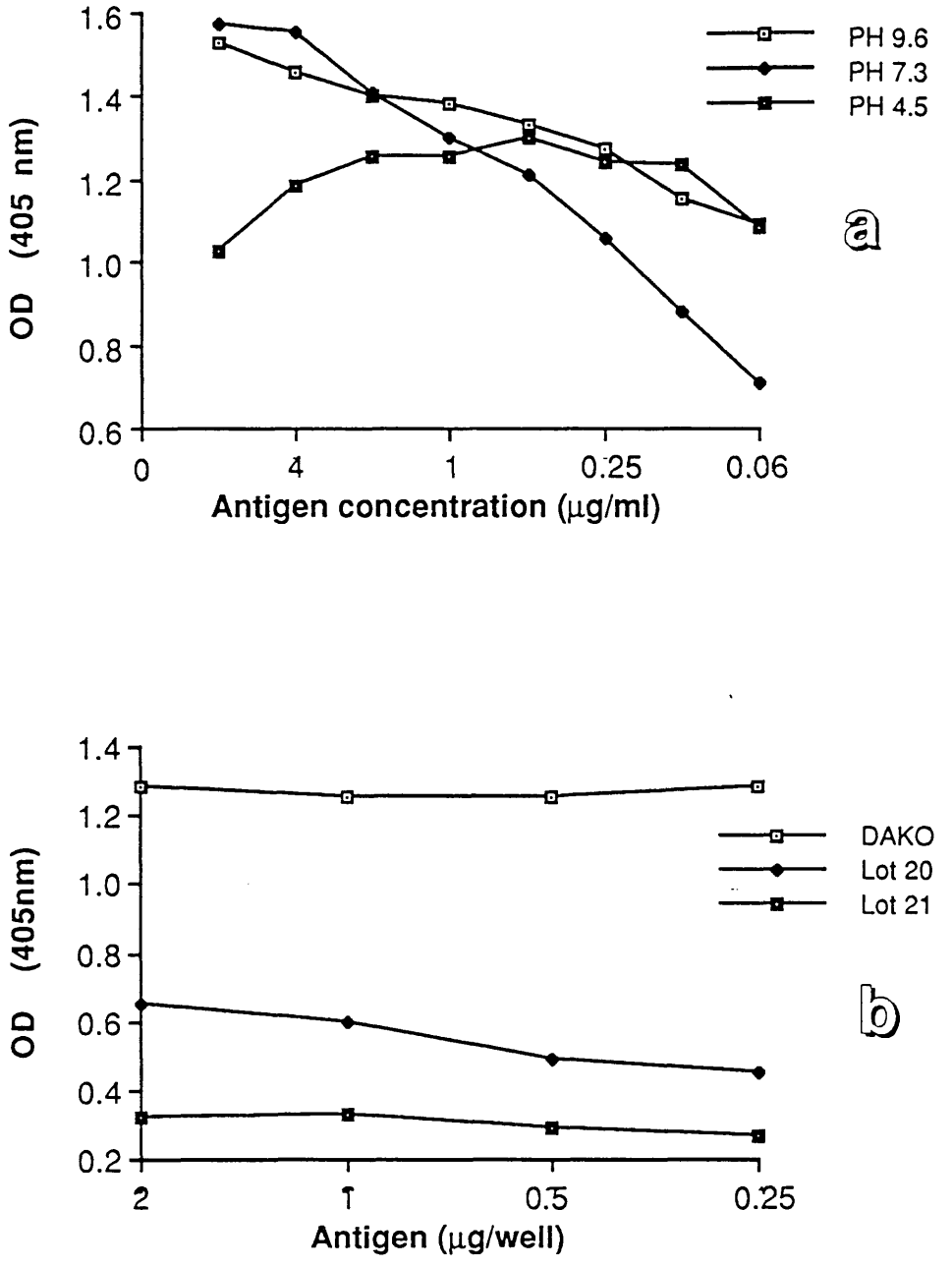


Figure 2.6: Determination of optimal pH and concentration of immunoglobulin (antigen) for coating ELISA plates, when a) immunoglobulin is coated onto the plate at pH 9.6, 7.4, 4.5 and at various concentrations, and b) immunoglobulin is coated onto the plate at pH 9.6 at various concentrations, using three different anti-Ig reagents (directly conjugated to HRP) for its detection. On the vertical axis is the absorbance (OD) measured at 405 nm.

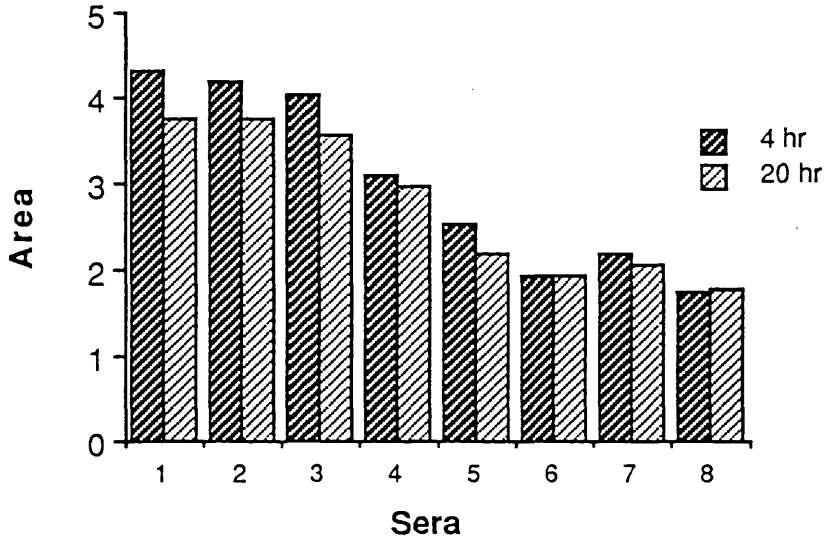


Figure 2.7: Effect of incubation time when immunoglobulin (antigen) is coated onto ELISA plate at 0.5 μg/ml in pH 9.6, after 4 hr and 20 hr incubation at 37°C. Eight different human anti-Ig sera were used. On the vertical axis the results are expressed as area under the sigmoidal curve derived from the titration of the sera (see text for details).

indicating that this is not a critical factor in our ELISA. The 8 antisera belonged to patients with ovarian cancer, who had previously undergone monoclonal antibody therapy with HMFG1. The results are expressed as areas under the serum titration curves as will be discussed later (section 2.5).

2.4 Determination of antibody concentration. The effect of affinity

In the previous paragraph one important factor for each immunoassay has been discussed: adsorption of proteins to the solid phase and quantitation of Ag coated. Nevertheless, there are many other aspects that characterize a solid phase ELISA, antibody affinity being one of the most crucial physical parameters.

Affinity, expressed as a binding constant, is determined at an equilibrium state by the equation :

$$Ab + Ag \underset{K_d}{\overset{K_a}{\rightleftharpoons}} AbAg$$

where AbAg represents the antibody-antigen complex and K_a, K_d the association and dissociation constants respectively (Steward and Lew, 1985). A mathematical theory to characterize the equilibrium composition of a multicomponent cross-reactive ligand-binding system (like the ELISA), was developed by Feldman in 1972 (Feldman, 1972) by solving the mass action equations:

$$\frac{K_a}{K_d} = K = \frac{[AbAg]}{[Ab] \times [Ag]}$$

where K is the equilibrium constant of affinity of the reaction. Antibody affinity is therefore a thermodynamic expression of the primary binding of an

antibody to its epitope, and must not be confused with avidity, which, although depends in part on affinity, also involves other parameters such as Ab valency, Ag valency and other factors associated with binding. When a monoclonal antibody is used, its affinity is known and remains (under standard conditions) always the same. Nevertheless, within a polyclonal antiserum, there is a heterogeneity of antibody affinities. It is not entirely correct to assume that all different affinities will be distributed in a normal Gaussian manner (Steward and Lew, 1985). Affinity heterogeneity can be skewed or even bimodal (Werblin and Siskind, 1972; Steensgaard et al., 1980), and it could pose a problem for accurate Ab quantitation, because even small quantities of high affinity antibodies dramatically decrease the ability to detect those of low affinity (Stanley et al., 1983).

In solid phase assays, like the one that we are using, when the antigen concentration is kept constant and the antibody is titrated out, there is a so called "linear region" on the titration curve, where all Ab binds onto the Ag (Fig. 2.8). In reality the binding in this region is less than 100%, because it is influenced by affinity, avidity and heterogeneity. Thus, it has been reported that primary antibody would bind in a linear fashion over a 3 log range (Koertge and Butler, 1985), but in the typical sigmoidal curve observed in ELISAs (Fig. 2.8), the linear portion only extends over approximately 1 log (Koertge and Butler, 1985).

As measured by ELISA, the detection of primary Ab when it is present at very high concentrations, markedly deviates from its actual concentration. This so called "prozone" or "hook effect" (Fig. 2.8) may be caused by various processes: steric hindrance that prevents the secondary Ab (enzyme conjugated) from binding onto the primary Ab (Koertge and Butler, 1985), a shift from bivalent to monovalent binding (Vos et al., 1987), or simply competition between the

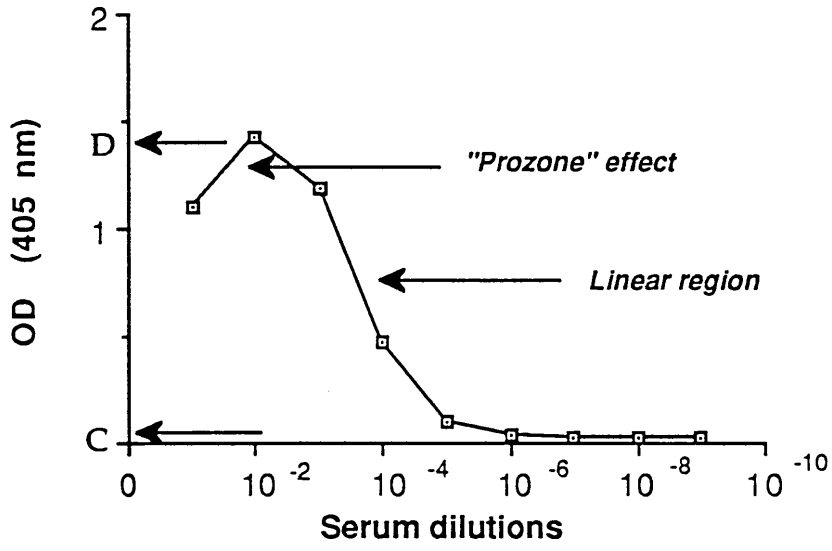


Figure 2.8: A typical sigmoidal curve obtained by ELISA when the antigen concentration is kept constant, and the antibody is titrated out (horizontal axis). On the vertical axis the absorbance is measured at 405 nm.
D and C are the values of the higher and lower (asymptote) points respectively.

binding of the primary Ab to the Ag on the plate (via its binding sites) and to the secondary Ab (via its antigenic determinants) (Vos et al., 1987).

Initially, we determined the optimal concentration of the secondary Ab in our system. Sterilin microtitre plates were coated with 500 ng/well of a mouse monoclonal antibody (EGFR1). The plate was washed with PBS/0.05% tween 20, pH 7.4 (washing buffer) and three different anti-mouse Ig-HRP reagents were applied at dilutions 1:250-1:2000: two batches of sheep anti-mouse Ig-HRP (Lot 20 and 21, Amersham) and a rabbit anti-mouse Ig-HRP (DAKO). The plate was incubated for 1hr at 37°C, washed, and the development of the colour measured after addition of the ABTS substrate. We found that the optimal dilution of secondary antibody is 1:500 (Fig. 2.9). Similar results were obtained when polyclonal human IgG was coated onto the plates and two batches of sheep anti-human Ig-HRP (Amersham) were applied. It was not necessary to stop the enzymatic reaction, since the plate reader takes less than 15 seconds to read a plate; moreover, we had the advantage of obtaining readings at various time points. The increase of enzymatic activity (signal) with time is shown in Figures 2.10a and b for two of the above used secondary antibodies (Lot 20 and 21, Amersham), when the plate was coated with the same mouse monoclonal antibody (EGFR1) as in the previous experiment.

Nevertheless not all commercially available anti-Ig secondary reagents were suitable. Interspecies Igs share common epitopes, and as predicted, most of the anti-Ig reagents cross reacted with Igs from other species. The abolition of cross reactivity was essential for our type of ELISA and only the species-specific anti-Ig-HRP antibodies from Amersham showed no interspecific reactivity in our system. These reagents were therefore used (at 1:500 dilution) in all subsequent experiments.

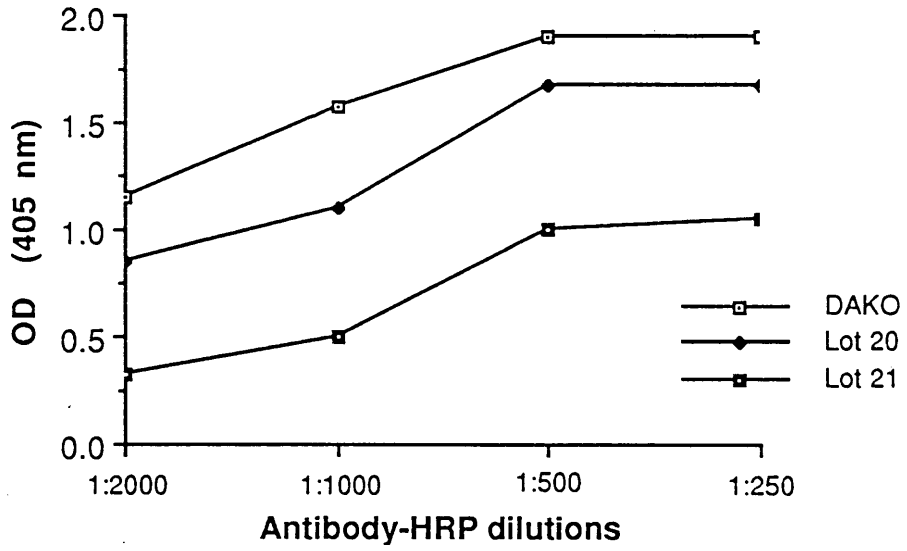


Figure 2.9: Determination of the optimal concentration of the secondary antibody (HRP conjugated), when an ELISA plate was coated with a mouse monoclonal antibody.

rabbit anti-mouse Ig-HRP (DAKO)

sheep anti-mouse Ig-HRP (LOT 20, Amersham)

sheep anti-mouse Ig-HRP (LOT 21, Amersham)

The reagents' dilutions are shown on the horizontal axis. The vertical axis the results are given as absorbance at 405 nm.

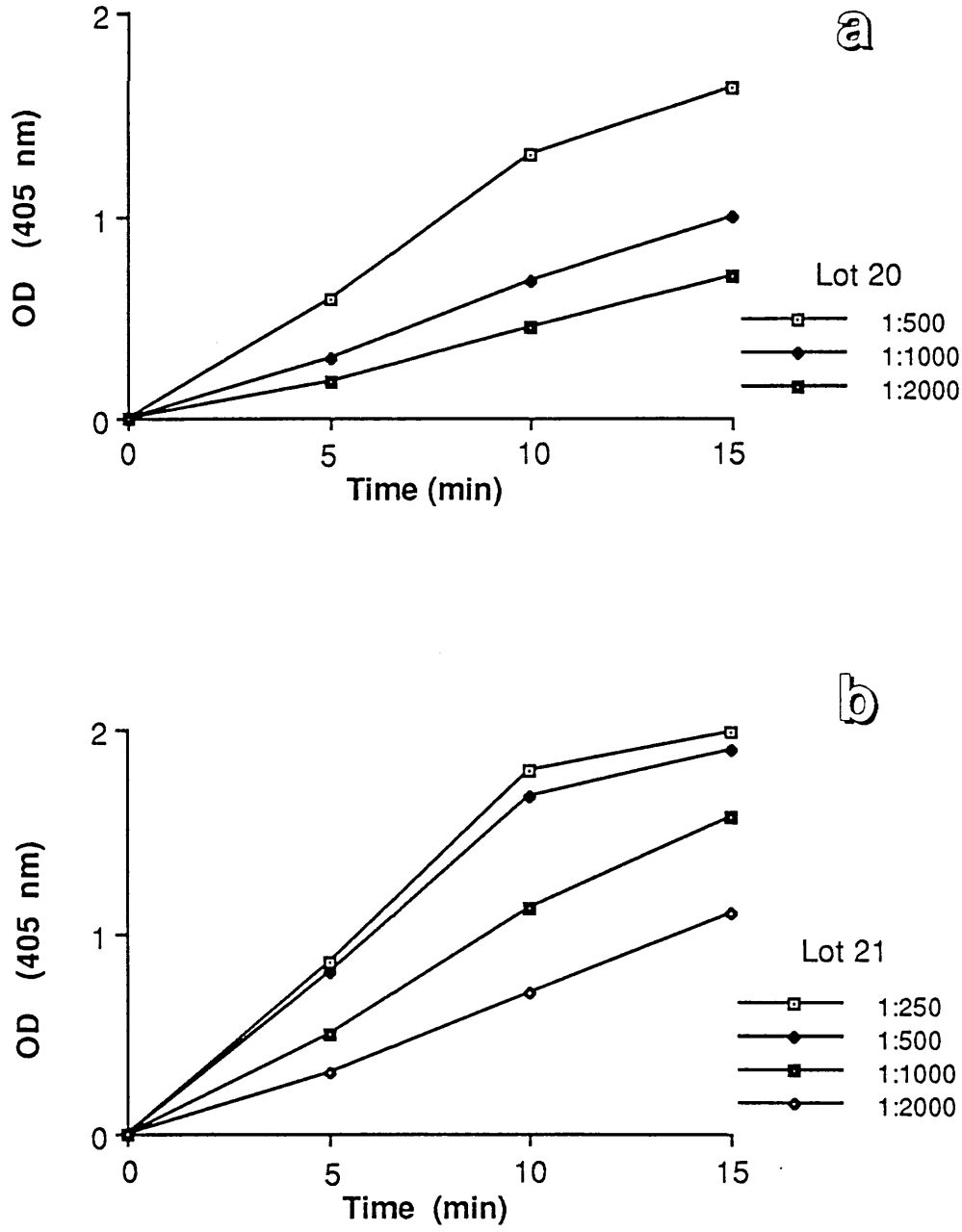


Figure 2.10: Increase of enzymatic activity with time (horizontal axis), when two batches of commercially available HRP-conjugated antisera were used against their respective antigens at three different dilutions.
a) sheep anti-mouse Ig-HRP, LOT 20, Amersham
b) sheep anti-mouse Ig-HRP, LOT 21, Amersham
On the vertical axis the values are given as absorbance measured at 405 nm.

In order to determine the concentration of the primary antibody (unknown serum), under the conditions to be used, a chequer-board ELISA was performed as follows: three microtitre plates (Sterilin) were coated with polyclonal human IgG (Sigma) at various concentrations (2000-15 ng/well) across the plate, starting from column 4 (Fig. 2.11). Three different mouse sera were applied in each plate at dilutions 10^{-1} to 10^{-8} downwards, starting from the first row (A3 and A5-A12). All mice were previously immunized with human IgG. The plate was incubated for 2hr at 37° C, washed, and a species-specific anti-mouse Ig-HRP antibody (Amersham) was added. After 1hr incubation at 37°C the substrate ABTS was applied, and the absorbance was measured as previously described.

Column 1 showed the background absorbance due to the plastic and the non oxidized substrate; these values were automatically subtracted from all wells by the multiscan spectrophotometer. In column 2, where there was no antigen or serum but only second layer antibody, we observed no non specific binding of the HRP-conjugated reagent, as used at 1:500 dilution. In column 3, where there was no Ag, but only serum and second layer reagent, we observed very low non specific binding of the test serum. In column 4, where there was only antigen and the secondary layer, we observed no cross reactivity of the HRP-conjugate. All the above controls became standard procedure for subsequent ELISAs. A starting serum dilution 10^{-1} (followed by 1:10 titration) gave a good signal with the minimum background. We confirmed an Ag concentration of 0.5 μg/ml as optimal (Fig. 2.12). The results shown in Figure 2.12 are from one of the sera at dilution 10^{-1} and 10^{-2} (i.e. rows A5-A12 and B5-B12 from Fig. 2.11); these are representative of the results obtained from all the other serum concentrations as well as the two other mouse sera. In addition, the same results were obtained when mouse Ig (monoclonal antibody HMFG1) was immobilized onto the plate and rabbit anti-mouse Ig antiserum followed by species-specific anti-rabbit Ig-HRP (Amersham) was used.

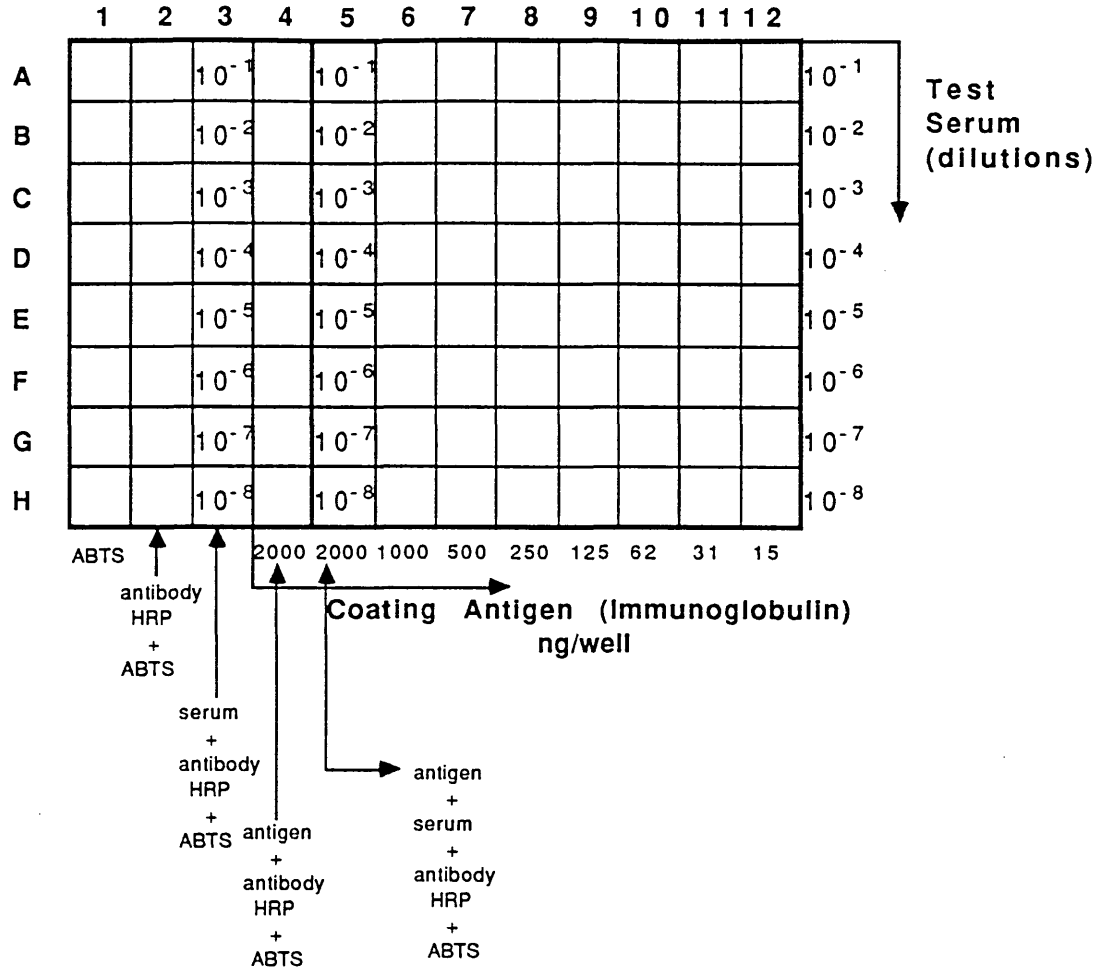


Figure 2.11: Diagram of the chequer-board ELISA used to determine the optimal antigen (immunoglobulin) and test serum concentrations. The antigen was titrated across the 96-well plate (Columns 5-12, 2000-15 ng/well), and the serum was titrated downwards (Rows A-H, 10^{-1}-10^{-8}).
Column 1 contained only substrate ABTS; column 2 contained second layer (HRP conjugated) and ABTS; column 3 contained serum, second layer and ABTS; column 4 contained antigen, second layer and ABTS.

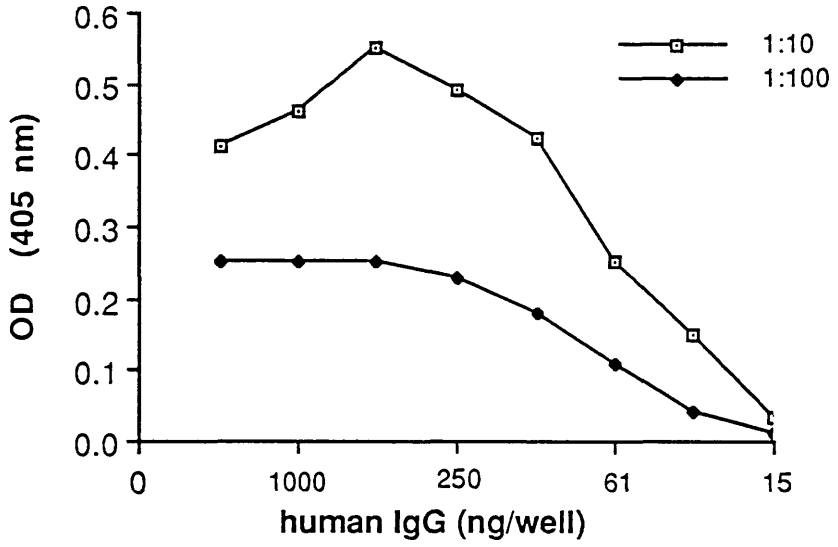


Figure 2.12: Part of the results obtained from the chequer-board ELISA shown in Fig. 2.11, where 96-well microtitre plates were coated with various concentrations of immunoglobulin across the plate, and anti-immunoglobulin antiserum was added at various dilutions downwards. The results shown are for serum dilutions 1:10 and 1:100. On the vertical axis the values are given as absorbance (OD) at 405 nm.

2.5 Data analysis and result evaluation

The final stage in the development of a reliable ELISA is the satisfactory expression and correct interpretation of the results. The immediate signal obtained from an ELISA is absorbance; many investigators have expressed the anti-Ig response as the difference in the OD (or cpm in the case of RIA; Sears et al., 1987) observed using a serum sample before and after administration of the xenogeneic Ig (Courtenay-Luck et al., 1986; Murray et al., 1987; Rosen et al., 1987; Courtenay-Luck et al., 1987). Similarly the results could be also shown as the ratio OD_t/OD_{-ve} where OD_{-ve} and OD_t are the optical densities obtained from the negative and the test serum respectively (Schulz et al., 1988).

However, the two main ways to express ELISA results are: the use of the end point titre, or a standard curve. The end point titre is the maximum serum dilution that gives a specific optical density set well above the control value (background or normal levels) (Primus et al., 1988). On the other hand, the standard curve is plotted using a reference reagent of known antibody concentration. It is essential that the latter is as close to the test sample as possible in terms of constitution (i.e. if the unknown sample is human serum, the reference should also be in human serum). The absorbance reading of a single serum dilution can be directly related to the reference sample, and thus translated to concentration (mg/ml or Units/ml) (Miller et al., 1983; Schroff et al., 1985; Shawler et al., 1985; Hafler et al., 1986; Lowder et al., 1987; Ledermann et al., 1988a; Ledermann et al., 1988b).

ELISA results are usually given as OD measurements vs. serum dilution. Based on this relationship, investigators use a variety of functional forms such as OD as a linear function of log[serum dilution] (De Savigny and Voller, 1980), log[OD] as a linear function of log[serum dilution] (Butler et al., 1978), OD as a quadratic or quartic polynomial function of log[serum dilution] (Platt et al.,

1981; Vos et al., 1982), in order to present their results. We have chosen to do a weighted non linear least squares analysis of 4 parameter logit (De Savigny and Voller, 1980; Karpinski et al., 1987), which is given by the equation:

$$OD = C + \frac{D - C}{1 + e^{-2(a+bx)}}$$

Since a sigmoidal curve is obtained when the results are plotted (Fig. 2.8), the above equation represents the OD as function of x that starts at an upper asymptote and decreases smoothly to a lower asymptote as x increases. C and D are the OD values at the lower and upper asymptotes respectively, a and b are indications of location and steepness of the curve (Karpinski et al., 1987).

However, calculation of Ig concentration for ELISA data is time consuming and can be simplified by specifically designed computer programmes. An increasing number of commercial multiscan spectrophotometers are capable of being interfaced to computers and allow more efficient analysis of the data (Richardson et al., 1983; Slezak et al., 1983; Franco et al., 1984; Schwarz, 1985; Thibaut et al., 1987).

With the help of Dr. R. Edwards (Department of Clinical Pharmacology, Royal Postgraduate Medical School, London, UK) and Dr. E. Sidiropoulos (Department of Physics, Polytechnic of Thessaloniki, Thessaloniki, Greece), we have developed a simple software for the Apple Macintosh computer, written in MS Basic. The programme works as follows: absorbance from each serum dilution is entered and the C and D points of the above equation are estimated (essential in case of a prozone effect). The computer will then automatically plot a sigmoidal curve - based on non linear least squares analysis on the log scale - print all the serum dilution points with their corresponding ODs, estimate any intermediate points that may be missing, give the true C, D, a and b values, find the midrange points for x (serum dilution) and y (OD) and

calculate the area under the curve. A representative example is given in Figure 2.13. With the help of this programme, a standard curve could be accurately plotted. For the quantitative analysis of an unknown sample, the procedure was repeated, the new curve was plotted, and only points at or very close to the linear portion of the standard and test curves were related. It must be emphasized here however, that such analysis using standard curves is often meaningless, due to the high test-sera heterogeneity (Steensgaard et al., 1980; Nimmo et al., 1984). Thus, an ELISA that uses a variety of polyclonal antisera can at best only be semi-quantitative. Therefore, in the majority of our experiments the results were expressed as areas under the serum titration curves. Thus, different sera could be easily compared, and variations analyzed using the Student's t test.

However, the programme was unable to plot a curve for negative or weakly positive sera. Given only one or two OD points above the background, a proper sigmoidal curve could not be plotted. In this case the computer calculated the area under the curve, by connecting the points with a straight line. Since these values were very low, there was no discrepancy with the theoretically correct ones.

2.6 Improving the sensitivity of the ELISA

After careful consideration of all parameters that characterize ELISAs, and with the help of our computer programme, we developed a reproducible and sensitive system for detecting anti-Ig antibodies in the serum. In many cases we were able to detect as little as 5 ng/ml of anti-Ig antibodies.

However, we considered ways of increasing the sensitivity of our assay. ELISA signals are usually amplified by the use of a more suitable enzyme or substrate

Figure 2.13: A typical computer print out after the ELISA values were entered into our programme.
The sigmoidal curve (absorbance vs. antibody dilution) is plotted; the actual OD values for each antibody concentration, the area under the curve, the midrange points as well as the a (alpha), b (beta), C and D values are also given (see text for details).

PATIENT MGB

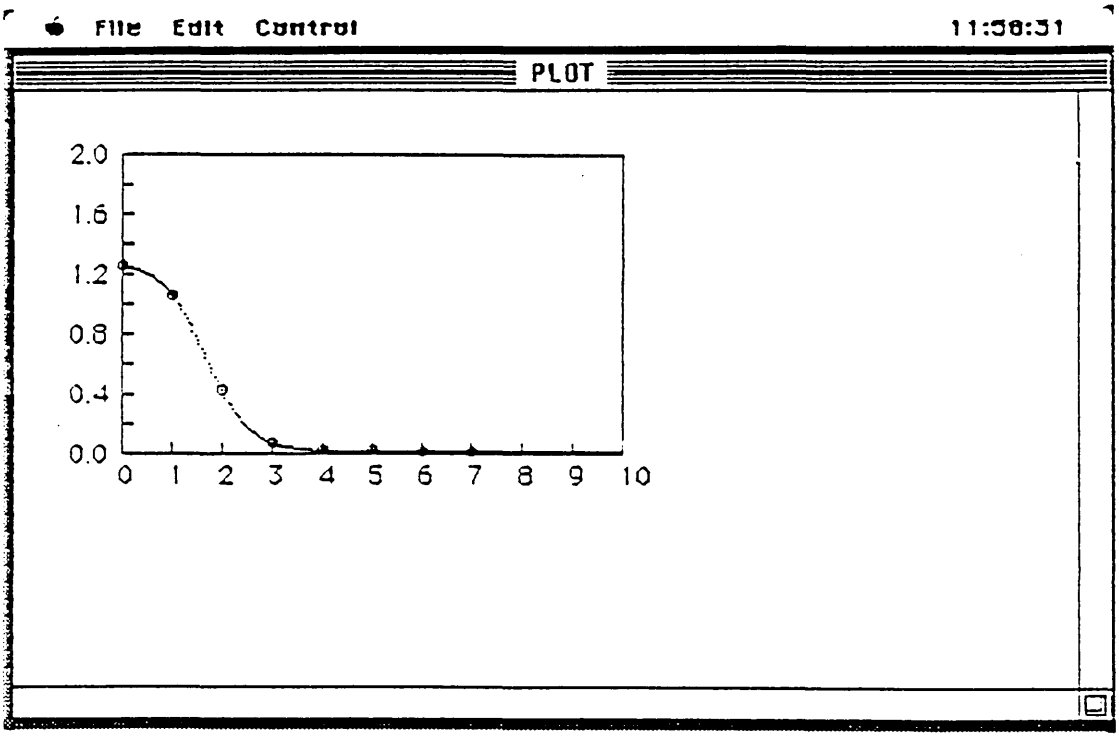
1	0	1.26
2	1	1.07
3	2	.43
4	3	.08
5	4	.044
6	5	.033
7	6	.025
8	7	.02

ALPHA= 1.9274795940926
BETA=-1.1458222704092
C= .019
D= 1.2875841739926

AREA= 2.1455864057974

MIDRANGE POINT COORDINATES

XM= 1.6821802506983 YM= .6532920869963



(Self, 1985; Johansson et al., 1986; Carr et al., 1987; Lee et al., 1988; Macy et al., 1988), a third layer antibody (Taylor et al., 1983; Linsemayer et al., 1988) or the avidin-biotin system (Wilchek and Bayer, 1984; Ziai et al., 1985). We have attempted the last two approaches.

A microtitre plate (Sterilin) was coated with polyclonal human IgG, and mouse antisera were applied at 1:10 dilutions. The mice were previously immunized with human IgG. After 2hr incubation at 37°C, the plate was washed, and a species-specific sheep anti-mouse Ig-HRP antibody (Amersham, 1:500 dilution) was added and incubated for 1hr at 37°C. Subsequently the plate was washed again, and a species-specific donkey anti-sheep/goat-HRP reagent (Amersham, 1:1000 dilution) was added for 1hr at 37°C. The colour was developed using the ABTS substrate. Unfortunately the background was so high, that any evaluation of data was impossible. This may partly be due to amplification of both specific signal and background noise, but additionally could be due to the third layer antibody, which may have not been entirely sheep Ig specific.

The avidin-biotin complex is used in many scientific applications. The unique property of the glycoprotein avidin with 4 high affinity binding sites for the vitamin biotin, makes the complex attractive for many solid phase immunoassays (Wilchek and Bayer, 1984). In an attempt to increase the sensitivity of our ELISA, we have used biotinylated antisera and HRP conjugated to streptavidin (Amersham), because the latter forms more stable complexes with biotin, and shows lower backgrounds in immunochemical techniques, than does avidin. Thus, when the above experiment was repeated, replacing the secondary layer with a species-specific biotinylated anti-mouse Ig (Amersham, 1:500 dilution) followed by streptavidin-HRP (Amersham, 1:1000 dilution), the signal was significantly amplified. Although the background was also enhanced, after subtraction the signal still remained higher than that

obtained with the directly HRP conjugated anti-mouse Ig (Fig. 2.14). We conclude therefore, that the streptavidin-biotin system offers a potentially useful amplification method.

2.7 Discussion

In this thesis we examine various ways of reducing the immunogenicity of Igs, and attempt to induce specific immunosuppression in the recipient against the administered foreign immunoglobulin. Regular, consistent monitoring of the host's response to the xenogeneic antibody is therefore of paramount importance. Thus, it was necessary to develop an assay to measure anti-Ig antibodies that would be sensitive, accurate, reproducible, easy to operate, and requiring the minimum amount of reagents, time and cost. Although a variety of techniques are available and in use (Lukic´ et al., 1975a; Carpenter et al., 1983; Chatenoud et al., 1986; Sears et al., 1987; Ledermann et al., 1988b), we chose the ELISA system for the following reasons: it can be quantitative as well as qualitative, it does not need special skills or protection facilities, it is relatively cost effective considering the number of sera that can be screened daily, and can be easily standardized. In addition, this assay is extremely sensitive in detecting antibodies, with capabilities that reach into pg range, requiring only a few μl of test serum.

The ELISA's closest "rival", Radioimmunoassay, although quite sensitive, has several disadvantages. Radioactive materials pose safety and waste disposal problems. The reagents used have a limited shelf half life due to the physical decay of the radioisotope. RIAs require expensive apparatus (β- and γ-counters), relatively longer signal-counting times, and in addition the specific activity of the reagent may be inadequate to achieve the maximum sensitivity (Witherspoon, 1983). In contrast, ELISA reagents are safer and more user-friendly (with the exception of some substrates that are treated as suspected

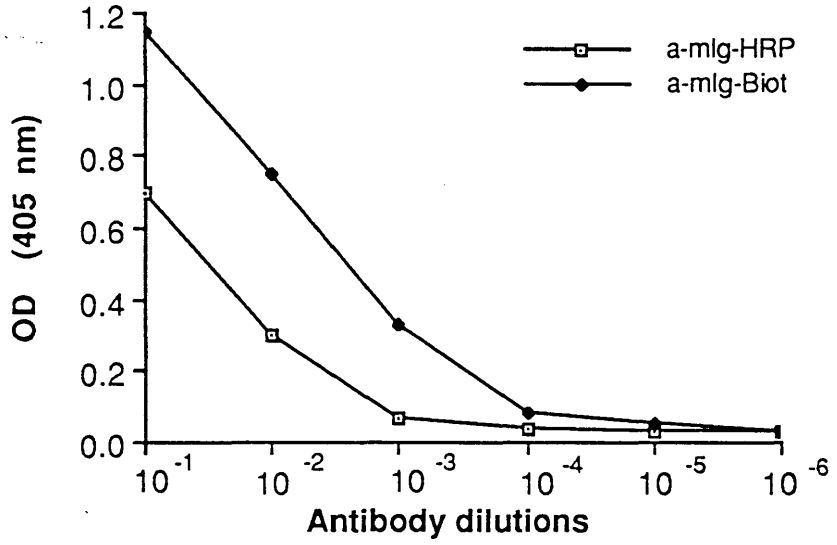


Figure 2.14: Amplification of the ELISA signal when the directly conjugated to HRP second layer was replaced by a biotinylated one, which was then followed by streptavidin-HRP.

second layer-HRP

second layer-biotin + Streptavidin-HRP

carcinogens), and there is no waste problem (Hill and Matsen, 1983). Enzyme conjugated antibodies are stable for a long time, and can be stored at 4°C for years (Avrameas and Ternynck, 1971; Hashida et al., 1984; Tsang et al., 1984; Montoya and Castell, 1987). They can be lyophilized (Montoya and Castell, 1987) or kept frozen at -20°C (Nygren, 1982) without loss of either enzymatic or antibody activity. Nevertheless some care should be taken for the selection of the appropriate reagent (Shields and Turner, 1986; Snoijink, 1987). The sensitivity of RIAs compared to ELISA is debatable; comparative studies from a number of laboratories indicate that ELISA can be more sensitive (Di Felice et al., 1984; Brock et al, 1984; Liebert et al., 1985).

ELISA is a technique that is based either on the simple adsorption of antigen onto a solid phase or on covalent linkage to agarose beads, cellulose (Fuchs and Sela, 1978), nylon (Hendry and Hermann, 1980; Djurup et al., 1983), polyester film (Sarkar and Mandal, 1985), porous glass (Line and Becker, 1975) or various non porous materials (Puttonen and Maasch, 1981; Tyson and Coulter, 1981). For this study we chose to adsorb our Ag onto polystyrene 96-well plates, and experienced no problem with reproducibility in this system. However, in our initial studies we did observe great variability among microtitre plates (Kenny and Dunsmoor, 1987), and finally selected the Sterilin ones, since they gave the lowest levels of non specific binding.

Our washing buffer contained 0.05% of the non ionic detergent tween 20, in order to keep the non specific binding at low levels. tween 20 "sticks" on the plastic surface with its hydrophobic tail, creating therefore a hydrophilic layer of detergent head-groups that prevent electrostatic and hydrophobic non specific binding of test serum Igs or enzyme conjugated second layer (Attwood and Florence, 1983; Graves, 1988). In every ELISA performed, control wells, where there was no Ag, but only serum and/or the enzyme conjugated second layer were always included. We did not observe any non specific binding at

serum dilution 1:10 (or higher), when a species-specific anti-Ig-HRP from Amersham was applied. However, when low background values were observed due to prolonged incubation time of the substrate, they were easily subtracted from the test samples (Kaspar et al., 1988). We believe that this lack of background reflects the correct choice of plate, since it has been reported that Igs can displace detergents on plates with high binding capacity (Kenny and Dunsmoor, 1987). In such cases, casein (alone or with thimerosal, Kenna et al., 1985), a matrix coat based on BSA-tween 20 (Graves, 1988), gelatin (Voller et al., 1979), irrelevant proteins (eg. BSA) (Voller et al., 1979; Kenny and Dunsmoor, 1987), even human tears (Boonstra et al., 1985) may be used in order to reduce the noise.

Generally, there is no rule for the choice of the right microtitre plate and blocking reagent. Every assay is different and has to be optimized independently. Multistep blocking procedures may be essential when moieties are used that bind onto plastic surfaces even in the presence of detergents (Gardas and Lewartowska, 1988), such as immunoglobulin aggregates that bind onto microtitre plates irrespective of the blocking reagent used (Cafruny et al., 1986; Brown et al., 1989; O'Hara et al., 1989), even anti-polystyrene antibodies that have occasionally been detected in tissue culture supernatant (Conger et al., 1988). In contrast, there are reports that in some cases there is no need for sophisticated buffers or blocking steps, and simple washing with distilled or tap water will not affect the outcome of the ELISA (Vos et al., 1980; Salonen, 1982; Ashorn and Krohn, 1986).

Antigen concentration is a crucial factor determining the sensitivity of the ELISA, since sensitivity is dramatically reduced when small amounts of antigen are used. In addition, low affinity antibodies remain undetected (Nimmo et al., 1984). Exceedingly high Ag amounts on the other hand, produce weak protein-protein interactions that generate a "prozone"-like effect (Pesce et al., 1981). Optimal Ag concentration ensures both sensitivity and

reliability of the assay. There are many methods to determine this concentration: binding of peroxidase (Munoz et al., 1986), protein determination using a kit from Pierce (Sorensen and Brodbeck, 1986), or chequer-board ELISA (Munoz et al., 1986; Vos et al., 1987). Using the latter we found that 500 ng/well of Ig is sufficient to saturate each well. In order to determine this concentration, we tested various antisera, since differences in the affinity can shift the optimal concentration to lower (high affinity antisera) or higher (low affinity antisera) values (Munoz et al., 1986).

Since our Ag is non covalently adsorbed on the plates, addition of serum and washings may promote a low level desorption (Butler et al., 1987). However, this did not seem to affect our ELISA, as also reported by others in similar studies (Nieto et al., 1986). The choice of the coating buffer is important, but it is generally believed that pH around 9.6 is best (Brock et al., 1984; Di Felice et al., 1984; Sorensen and Brodbeck, 1986; Geerligs et al., 1988), something that we have also confirmed. Temperature could also affect binding, and it seems that higher temperatures help adsorption (Butler et al., 1987). All our incubations were performed at 37°C.

Presentation of the Ag is also important. Adsorption can result in substantial loss of antigenic activity. We tried to avoid harsh methods of coating, such as fixation or air drying (Schroff et al., 1985; Ledermann et al., 1988a), fearing that certain Ag determinants might be altered. Although the Ag is better presented when covalently conjugated to a linker (Kavai et al., 1985; Brennand et al., 1986; Smith and Wilson, 1986; Karpinski et al., 1987; Jitsukawa et al., 1989), we felt that at pH 9.6 the Ig molecule is adequately presented, with its Fc portion adhering on the solid support (Sorensen and Brodbeck, 1986). Antigen density is another factor that affects immunoabsorbent methods (Nimmo et al., 1984). However, immunoglobulins are large molecules that would present a large number and variety of epitopes to anti-antibodies. In addition, we ensured that the binding capacity of the microwells was saturated. Naturally, this could be

increased by covalent link of the antigen (Ig) to the plastic, but this application for ELISAs became commercially available only very recently.

Antibody-antigen interactions are characterized by molecular association and dissociation rate constants and by their ratio. Binding affinity therefore is very important in ELISAs. Bivalent binding is more stable than monovalent (Kemp and Morgan, 1986). Bivalent binding depends on Ag density, and both Ag and Ab concentration. If the Ab is present in high amounts, there is an increase in monovalent interactions, which results in lower avidity of Ab-Ag binding (Vos et al., 1987). In our assays we have sometimes observed a "prozone" effect even at serum dilutions of 1:100.

The concentration of first and second layer antisera was titrated to find the concentration at which they would give the highest positive binding with the lowest background. Having immunoglobulins as antigens, there was considerable cross reactivity with many commercially available second layers. We chose the species-specific anti-Ig-HRP reagents from Amersham, because these were the only ones that consistently gave very low levels of cross reactivity; in addition horseradish peroxidase is a suitable enzyme (Beyzavi et al., 1987). We chose ABTS as substrate, since it consistently gave good results. A particular advantage is its self-limiting property of enzyme reaction. In addition, it keeps at 4°C ready to be used for months (Portsmann et al., 1981). Reduced ABTS is rather colourless, absorbing at 340 nm. When it is oxidized, it absorbs maximally at 414 and slightly less at 405 and 650 nm. When overdeveloped, the OD can be measured at 492, 570 or 630 nm (Liebert et al., 1985). We have measured the absorbance at 405 nm (Gallati, 1979).

A notable feature of the titration plot of an Ag specific ELISA is the sigmoidal nature of the titration curve, when absorbance is plotted against serum dilution. The shape of this curve is influenced by the amount of test serum and their relative affinity. It is generally believed that measurement of

antibody concentration at high absorbance values, close to the plateau of the binding curve, is greatly affected by antibody affinity, whereas low OD points are more concentration-dependent (Péterfy et al., 1983). Nevertheless, it has been suggested that low epitope density could influence measurements even at minimal absorbances (Nimmo et al., 1984). Thus, although evaluation of the results using low endpoint titres to high density antigens may be advantageous over other methods, we felt that by expressing our data as areas under the test-serum titration curves, would take into account all relevant variables. Using the simple computer programme that we developed, an accurate curve was easily plotted and the area automatically given. This also enabled us to proceed to quantitation of the response when a reference serum was available. However, since standard sera were polyclonal, it would be wrong to assume that they would all have similar affinities (Mäkelä and Péterfy, 1983; Nimmo et al., 1984). We have therefore used a pool of standard sera, in order to obtain values close to the real one (Mäkelä and Péterfy, 1983).

The interplate variation was very small. All serum samples were tested in either duplicate or triplicate (Kenna et al., 1985) to achieve more correct measurements and identify possible errors. In our laboratory, performance of duplicates was standard practice, despite the fact that its cost-effectiveness is sometimes questioned (Rodgers, 1987; Lester and Corns, 1988). Finally, by obtaining areas under the ELISA sigmoidal curves, with the help of our computer programme, or actual concentrations (when standard serum was available), the results could be statistically evaluated using the Student's t test.

In summary, we have developed an ELISA system to detect anti-xenogeneic Ig antibodies in serum. This was simple, reliable, sensitive (detecting <5 ng/ml of antibody without the need of any amplification), and required only a small amount of test serum (20 μl). Non specific binding and cross reactivity were minimized, and sera (tested in duplicates) revealed very little intraplate and interplate variations.

Chapter 3

Kinetics of mouse monoclonal antibodies in man

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3.1 Introduction

Antibody mediated diagnosis and therapy of malignancies primarily depends on two factors; tumour accessibility and the properties of the antibody. Over the past 15 years, many obstacles have been overcome in the endeavour to utilize passively administered antiserum in cancer: antibody-specificity has been largely understood and improved, and promising attempts were made towards increasing tumour vascular permeability in order to achieve higher antibody uptake (Msirikale et al., 1987; Stickney et al., 1987). We have the opportunity now to choose among monoclonal antibodies (mAbs) derived from various species, that are of different classes, subclasses and affinities for tumour associated/specific antigens. Moreover, the toxic properties of these reagents have been reinforced by their covalent or non-covalent association with "lethal" compounds, such as radioisotopes (Bucholz, 1987; Epenetos et al., 1987a; 1987b; Larson, 1987; Hnatowich et al., 1988; Sharkey et al., 1988; Ward et al., 1988; Zalutsky et al., 1989), cytostatic drugs (Takashi et al., 1988; Pietersz et al., 1988a; 1988b; Endo et al., 1988b) and natural toxins (Blackey et al., 1988a; Pirker, 1988; Fauser et al., 1988; Hertler et al., 1988).

However, despite much experience in the construction of "magic bullets" for immunotherapy, the interrelationships between catabolism, kinetics, immunogenicity and tumour localization of these reagents are less well understood. Reduction of antibody immunogenicity will certainly affect its kinetics, and hopefully enhance tumour localization. It is essential therefore, to begin a study on immunosuppression by first examining the natural processing of the antigen (immunoglobulin) by the host.

There are many reports in the literature on the kinetics of passively administered mAbs (Hnatowich et al., 1985; Rosenblum et al., 1985; Pimm et al., 1985; Koizumi et al., 1986; Rosen et al., 1987; Eger et al., 1987; Kirkwood et al., 1987). Although all studies show that Fab fragments are generally cleared faster

than $F(ab')_2$ or whole immunoglobulins (Igs), when administered systemically, there is disagreement as to the actual catabolic rate. This is not surprising, considering the variety of reagents and recipient species used. Many of the initial experiments were performed in animals such as mice, rats, dogs and non-human primates. Variations in their defense mechanisms, and qualitative differences in their metabolic pathways, make any direct correlation with humans almost impossible. In addition, discrepancies in the antibody kinetics in Phase I and II clinical studies can also be explained by the nature of the antibody used, the compound to which it was conjugated and even the way in which blood clearance measurements were made. Moreover, polymorphism and variation in the outbred human population presents an additional obstacle for any attempted standardization.

We therefore decided to perform our own studies on the stability and kinetics of the panel of radiolabelled tumour-associated mouse mAbs (whole and fragments), used at the Hammersmith Hospital for radioimaging and therapy of neoplastic lesions. The most common route of administration was i.v.. However, since intracavity administration is also routinely used (Myers and Collins, 1983; Epenetos, 1986b; Ward et al., 1986; Lawson et al., 1986; Ward et al., 1987; Rowlinson et al., 1987; Colcher et al., 1987), we examined additionally the kinetics of mAbs administered i.p. for therapy to patients with ovarian cancer, and discuss the relative advantages of these different routes of administration. Knowledge of the *in vivo* behaviour of these murine mAbs should facilitate planning strategies as to how to reduce their immunogenicity, thus improving cancer immunotherapy. Our approaches will be discussed in the subsequent chapters.

Finally, although not within the original scope of this thesis, we considered the possibility that small, non-immunogenic molecules with high affinity and specificity for malignant cells might provide an effective alternative to mAb-mediated cancer therapy.

Biotin (Vitamin B_8 or Vitamin H) is a very small molecule with high affinity for avidin or streptavidin (approximately 10^{15}/mole). In tumour-bearing animals it has been shown that radioactive biotin will bind specifically onto neoplastic lesions, when administered a few days after an anti-tumour mAb-avidin (or streptavidin) conjugate (Hnatowich et al., 1987; Paganelli et al., 1988; Rowlinson et al., 1988b). The theoretical advantage of this approach for radioimaging (and therapy) is that biotin either binds with very high affinity onto tumour cells (via the mAb-streptavidin conjugate), or disappears rapidly from the circulation, thus reducing the chance of non-specific damage to normal tissue. A further advantage that we foresee, is that, since avidin/streptavidin can bind up to 4 biotin molecules, one could use lower, subimmunogenic, amounts of mAb to obtain satisfactory imaging and therapy. In a limited number of patients, we have tested this system: a small amount of an anti-tumour mAb-streptavidin conjugate was administered, followed by ^{111}Indium-labelled biotin a few days later. We examined the kinetics of antibody-conjugate, biotin, and the patients' anti-mouse Ig response, and we discuss the feasibility of this two step approach for immunodiagnosis and immunotherapy.

However, biotin's specificity depends upon the correct deposition of mAb-streptavidin (or avidin) on the tumour cell. In an attempt to seek alternatives to mAbs, we have also used radiolabelled human epidermal growth factor (EGF) in animal studies. The choice of human EGF as a potential candidate for cancer therapy was made on the basis of its small size (6KD), absence of immunogenicity and high affinity to EGF receptors, found to be expressed at elevated levels on the surface of many cancer cells (Fabbro et al., 1986; Gullick et al., 1986; Kamata et al., 1986; Scott Goustin et al., 1986). The kinetics and biodistribution of human EGF in monkeys are described, and the possibility of its future use in clinical trials will be discussed.

I would like to thank again Drs. H. Kalofonos, S. Stewart and V. Hird (ICRF, Oncology Laboratories) for injecting the radiolabelled reagents to the patients, and for collecting the blood samples that I have analyzed.

3.2 Materials and Methods

3.2.1 Patients: The kinetics of the mouse monoclonal antibodies, were studied in patients with cancer, admitted to the Department of Clinical Oncology, Hammersmith Hospital, for immunoscintigraphy and immunotherapy. Patients with primary non-small cell lung cancer (NSCLC) and primary or metastatic breast cancer received radiolabelled mAbs for diagnostic purposes, whereas patients with residual ovarian cancer received the radiolabelled mAbs for therapy, after conventional treatment (surgery, followed by chemotherapy/radiotherapy) had failed. All patients gave their written consent before entering the study.

3.2.2 Monoclonal antibodies - administration: The following murine monoclonal antibodies, directed against cell-surface tumour-associated antigens, were used throughout this study : a) HMFG1 and HMFG2, both IgG1, that recognise different epitopes of a large mucin, normally expressed on the milk fat globule membrane of lactating breast, and abnormally found on the majority of ovarian, breast and other carcinomas (Taylor-Papadimitriou et al., 1981); b) HMFG1 $F(ab')_2$ fragments, prepared by Dr. N. Courtenay-Luck in our Department of Immunology (Courtenay-Luck, 1989); c) AUA1, an IgG1 that recognises a 40KD glycoprotein expressed by a wide range of adenocarcinomas (Spurr et al., 1986); and d) H17E2, an IgG1 raised against placental alkaline phosphatase, an enzyme normally only synthesized by syncytio-trophoblast from the 12th week of pregnancy, but abnormally expressed on many neoplasms, including ovarian cancer, testicular tumours and gliomas (Travers and Bodmer, 1984).

All antibodies were provided by Unipath-Oxoid in purified form, sterile and free of pyrogens. Monoclonal antibodies used for immunoscintigraphy were administered i.v. as a bolus injection, whereas therapeutic mAbs were delivered i.p., via a small catheter inserted into the patient's peritoneal cavity (under local or general anaesthesia) as a slow infusion with 1.5 l physiological saline.

3.2.3 Radiolabelling of the monoclonal antibodies: MAbs were labelled with ^{111}Indium (^{111}In, 0.5-1.5 mCi/patient) for immunoscintigraphic studies, and ^{90}Yttrium (^{90}Y, up to 30 mCi/patient) or ^{131}Iodine (^{131}I, up to 200 mCi/patient) for therapy. The specific activity never exceeded 10 mCi/mg, to ensure that the antibodies retained their immunoreactivity. The final radiolabelled preparations ready for *in vivo* use, were diluted to 0.25% human serum albumin (HSA, Blood Products Laboratory, Elstree, UK).

3.2.3.a Indium-111: The radiometal ^{111}In was chelated onto the monoclonal antibodies, via diethylene triamine pentaacetic acid (DTPA), using a modification of the method described by D. Hnatowitch et al. (Hnatowitch et al., 1983). The antibody was concentrated to approximately 10 mg/ml, and the pH raised to 8.5-9.0, using 0.1M carbonate/bicarbonate buffer, pH 9.0. The appropriate amount of DTPA (cyclic anhydride, Sigma, UK) was added, either as dry powder or as a solution in anhydrous dimethyl sulphoxide (DMSO, Aldrich, UK). The starting molar ratio (MR) was 20-30 DTPA molecules/antibody molecule. After 5 min incubation at room temperature (RT), bound and unbound DTPA were separated by Sephadex G-50 (Pharmacia, Sweden) size exclusion chromatography. The column was washed and eluted with PBS, pH 7.4. One ml fractions were collected, and the absorbance (OD) was measured at 280 nm (SP6-500 Spectrophotometer, Pye Unicam UK). Since DTPA does not absorb at this wavelength, only one peak was detected, corresponding to antibody-DTPA. The optical density was converted to

concentration using the formula:

$$\text{Antibody concentration (mg/ml)} = \frac{OD_{\text{antibody}}}{OD^{1\%}_{1cm}} \times 10$$

The extinction coefficient at 280 nm for mouse IgG is approximately 13.6. Throughout the process only metal-free buffers, glassware and pipettes were used, to avoid saturation of the DTPA's chelating capacity with metals other than ^{111}Indium.

The radionuclide was supplied by Amersham, UK as carrier free, in 0.04M HCL. The pH was raised to 6.0-6.5, using 0.1M sodium citrate buffer; at higher pH, ^{111}In forms colloids with water and is thus not incorporated onto the antibody. Antibody-DTPA was incubated with ^{111}In for 15 min at RT; bound and free isotope were then separated by gel filtration (Sephadex G-50), as described above. The radioactivity of the fractions was measured in a well-counter (SIEL isotope calibrator, UK), and of the 2 peaks observed, the first represented the antibody-DTPA-^{111}In and the second the free ^{111}In.

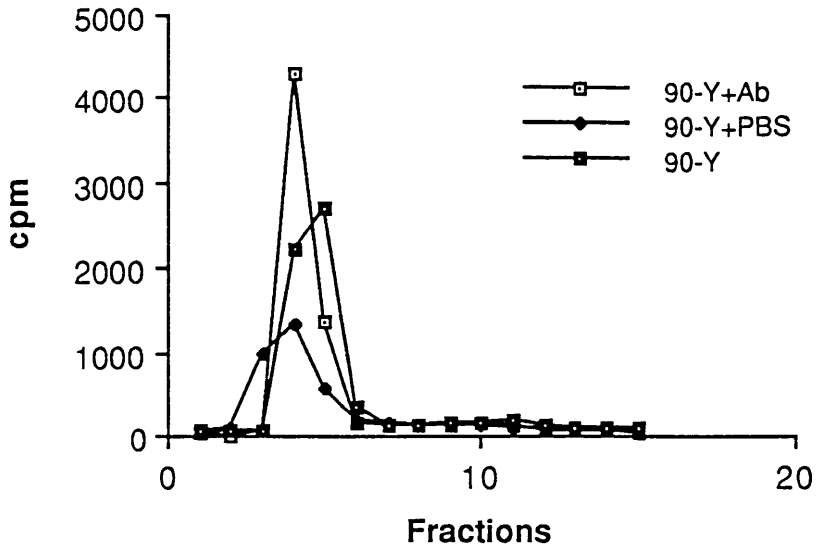
The actual number of DTPA molecules linked to the antibody was estimated as follows. After DTPA coupling and before the first gel filtration, a small aliquot of antibody-DTPA and free DTPA mixture was incubated with a trace amount of ^{111}In (under conditions were DTPA was in excess). Size exclusion chromatography (Sephadex G-50) revealed 2 peaks: the first one was antibody-DTPA-^{111}In and the second one DTPA-^{111}In. By calculating the areas under the peaks, and the percentage of the first peak over the total, we were able to estimate the antibody-bound DTPA number over the total. We found that starting with a MR of 20-30 DTPA/antibody the final, coupled ratio was 1.5-2.0 DTPA/antibody.

3.2.3.b Yttrium-90: The radiometal ^{90}Y (Oak Ridge, USA, or AERE Harwell, UK) was chelated onto the antibodies with a radiolabelling technique similar to that described for ^{111}In. In preliminary experiments using dry ^{90}Y from AERE Harwell, we found that under identical labelling conditions as above, ^{90}Y forms colloids. As shown in Figure 3.1a, the gel filtration profile of antibody-DTPA (in PBS) and ^{90}Y (in citrate buffer, pH 6.5) is the same as that with only PBS and ^{90}Y or ^{90}Y alone, indicating the formation of high molecular weight colloids under the above pH conditions. The results are given as counts per minute (cpm); the differences in counts being due to different amounts of isotope used. The "labelling efficiency" of antibody-DTPA-^{90}Y was 84.6%, of PBS-^{90}Y was 68.3% and of ^{90}Y alone 79.7%.

After testing a variety of conditions, the following protocol was found to be the most successful for ^{90}Yttrium labelling of our antibodies. The pH of the isotope (in 0.04M HCL, supplied by Oak Ridge) was raised to 5.5 with 0.5M citrate buffer. The antibody-DTPA conjugate (in 0.1M citrate buffer, pH 6.0) was then incubated with this ^{90}Y for 1-2 hr at RT. Lastly, the reaction mixture was purified by gel filtration (Sephadex G-50), and the column washed and eluted with PBS.

3.2.3.c Iodine-131: The monoclonal antibodies were labelled with ^{131}Iodine (Amersham, UK) using iodogen (Fraker and Speck, 1978) or n-bromo succinamide (NBS, Reay, 1982) as oxidizing reagents.

i) Iodogen method: the antibodies were concentrated to approximately 10 mg/ml, and the pH raised to 7.5-8.0, using 0.1M phosphate buffer, pH 8.5. The preparation was incubated with ^{131}I in iodogen-coated plastic vials for 15 min at RT; free and bound isotope were then separated by size exclusion chromatography, as described above. The iodogen-coated vials were prepared as follows: a solution of 500 µg/ml iodogen (Pierce, UK) was made in chloroform,



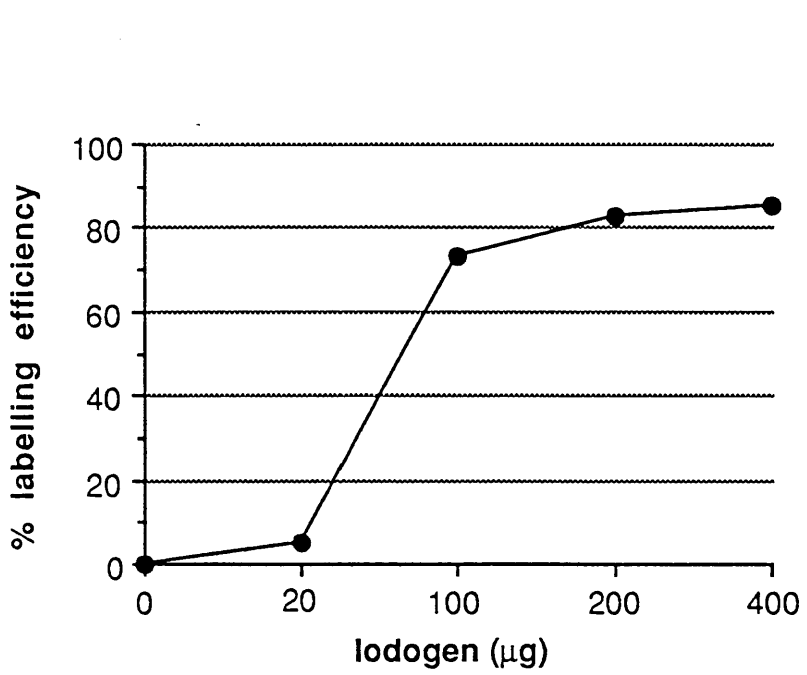


Figure 3.1: a) Size exclusion chromatography profile (Sephadex G25) of 100 µl monoclonal antibody-DTPA conjugate (in PBS) incubated with ^{90}Yttrium, 100 µl PBS incubated with ^{90}Yttrium, or ^{90}Yttrium alone. The presence of one high molecular weight peak is indicative of colloid formation under the pH conditions used. The results are expressed as counts per minute (cpm).
b) Labelling efficiency of a mouse monoclonal antibody when radiolabelled with ^{131}Iodine using iodogen in solution as the oxidizing reagent. The labelling efficiency (vertical axis) was measured by 10% TCA paper chromatography, after incubation of up to 400 µg of iodogen (horizontal axis).

the appropriate amount was then transferred into a plastic vial (100-400 µg/vial) and left to dry overnight in a fume cupboard. The vials were either kept dessicated for short periods at 4°C, or stored at -20°C.

When iodogen-coated vials were not available, a modification of the previously described process was followed. Iodogen (from a freshly prepared solution in acetone) was added to PBS at a final concentration of 2 mg/ml. The solution was sonicated for 2 min, and 100-200 µl (equivalent to 200-400 µg iodogen) were added to the antibody/^{131}I mixture. Incubation time and purification remained the same as described above. Figure 3.1b, shows an example of labelling 100 µg of an antibody (HMFG1) with 1.5 mCi of ^{131}I, using this modified method of iodogen in solution. The maximum labelling efficiency (as measured by 10% trichloroacetic acid precipitation) was reached with iodogen concentrations of 200 µg and above.

ii) N-bromo succinamide method: this method is similar to that using iodogen in solution. NBS was prepared fresh, at a concentration of 1 mg/ml in distilled water, and added dropwise to the concentrated antibody (pH 7.0-7.5). After 15 min incubation, the labelling efficiency was measured by 10% trichloroacetic acid (10% TCA) precipitation paper chromatography. The addition of NBS was continued until the labelling efficiency was over 90%. Separation of free ^{131}I (when needed) was performed by gel filtration (Sephadex G-50), as described above.

3.2.4 Quality control of the monoclonal antibodies: Before labelling, all monoclonal antibodies used were shown to be free of aggregates and other irrelevant proteins. This was assayed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography, using Superose 6 or 12 columns (Pharmacia), connected to a fast protein liquid chromatography (FPLC, Pharmacia), at flow rates 0.3-0.8

ml/min. Antibodies remained aggregate-free after iodination. This was confirmed by autoradiography (after SDS-PAGE) and FPLC analysis. For the analysis of radioactive samples we modified the FPLC apparatus: with the help of Prof. D. Hnatowich. The pen of the chart recorder (usually reserved for the salt gradient in ion exchange chromatography), was connected to a Geiger meter (NE Scaler Ratemeter, SR3, USA) enabling us to follow the protein trace and the radiolabel signal simultaneously. When the radioactivity loaded was not sufficient to be detected by the Geiger meter, fractions were collected, the radioactivity was counted in a γ-counter (Minaxiγ, Packard, USA), and the result was plotted onto the protein trace by hand.

However, SDS-PAGE revealed, that after DTPA-coupling, there was a degree of antibody aggregation. This was expected, since cyclic DTPA is a bifunctional reagent (Fig. 3.2) and cross-links two or more antibody molecules, resulting in the formation of dimers, trimers and polymers. A low DTPA/antibody MR avoided the problem of aggregates but resulted in poor radiolabelling, whereas a high MR led to undesirable aggregate formation. We therefore selected the best compromise conditions for conjugation to give the highest radiolabelling with the lowest polymer formation. Although the degree of aggregation varied between preparations, high molecular weight polymers were always present in both whole antibody-DTPA and, to a lesser degree, $F(ab')_2$ fragment-DTPA conjugates. Figure 3.3, shows Coomassie blue stained 7.5% SDS-polyacrylamide gels of six IgG-DTPA (Figs. 3.3a and b), and three $F(ab')_2$ fragment-DTPA (Fig. 3.3c) conjugates. Dimers and polymers are predominantly visible in the IgG preparations. This observation was also confirmed by FPLC size exclusion chromatography (Superose 6). As shown in Figure 3.4, some antibody-DTPA preparations showed a higher degree of aggregate formation (Figs. 3.4a and b) than others (Figs. 3.4c and d). Monomeric IgG is indicated by the arrow, whereas dimers, trimers and polymers are located to the left of the arrow.

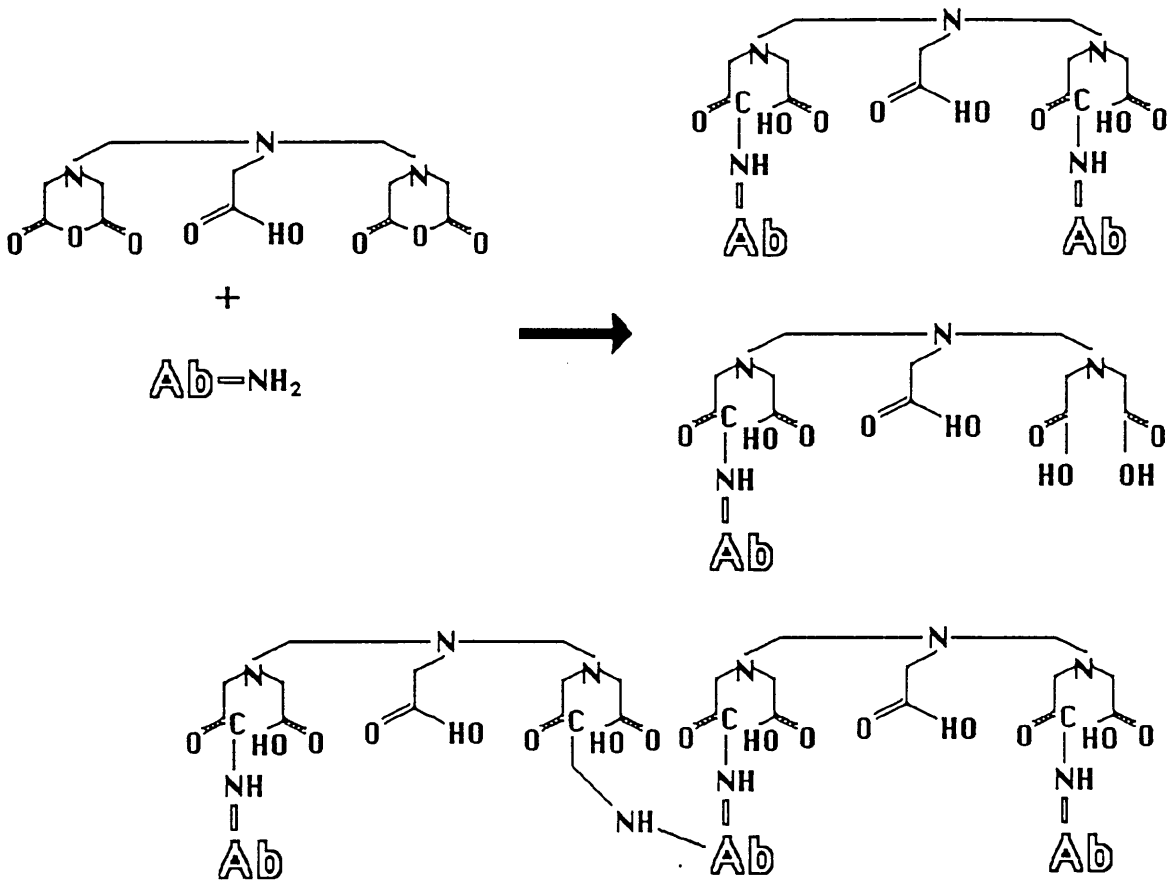


Figure 3.2: Conjugation of antibodies to DTPA, using the cyclic anhydride form, leads to antibody cross-linkage and formation of dimers, trimers and polymers.

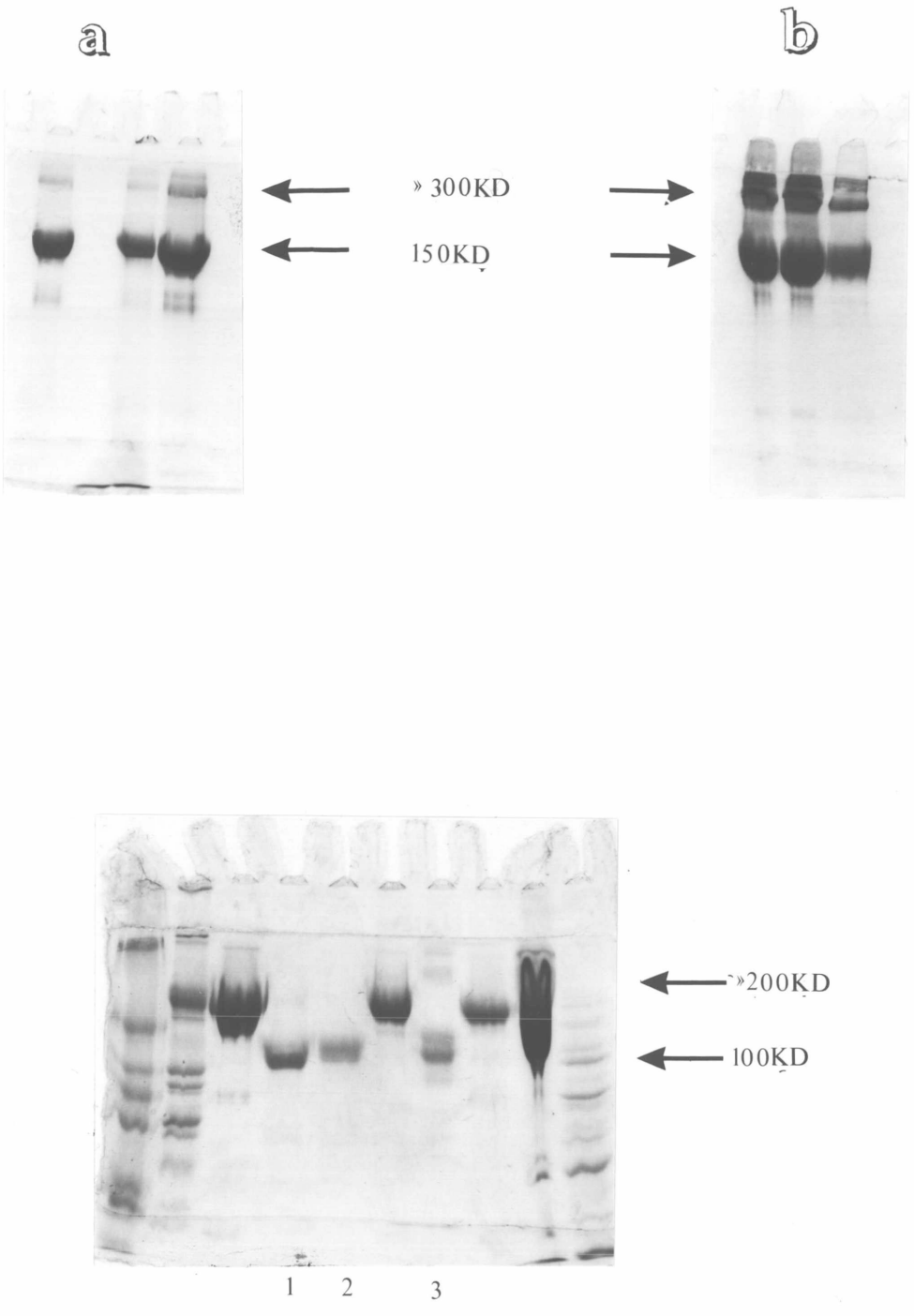


Figure 3.3: a) and b) SDS-PAGE of 6 whole antibody-DTPA conjugates (lanes 1-6). The monomeric (150KD) and aggregated (≥300KD) monoclonal IgGs are indicated by the arrows.
c) SDS-PAGE of 3 monoclonal antibody $F(ab')_2$ fragment-DTPA conjugates (lanes 1-3). The monomeric (100KD) and aggregated antibody fragments (≥200KD) are indicated by the arrows. Coupling of DTPA to antibody fragments did not always result in polymer formation (lanes 1 and 2).

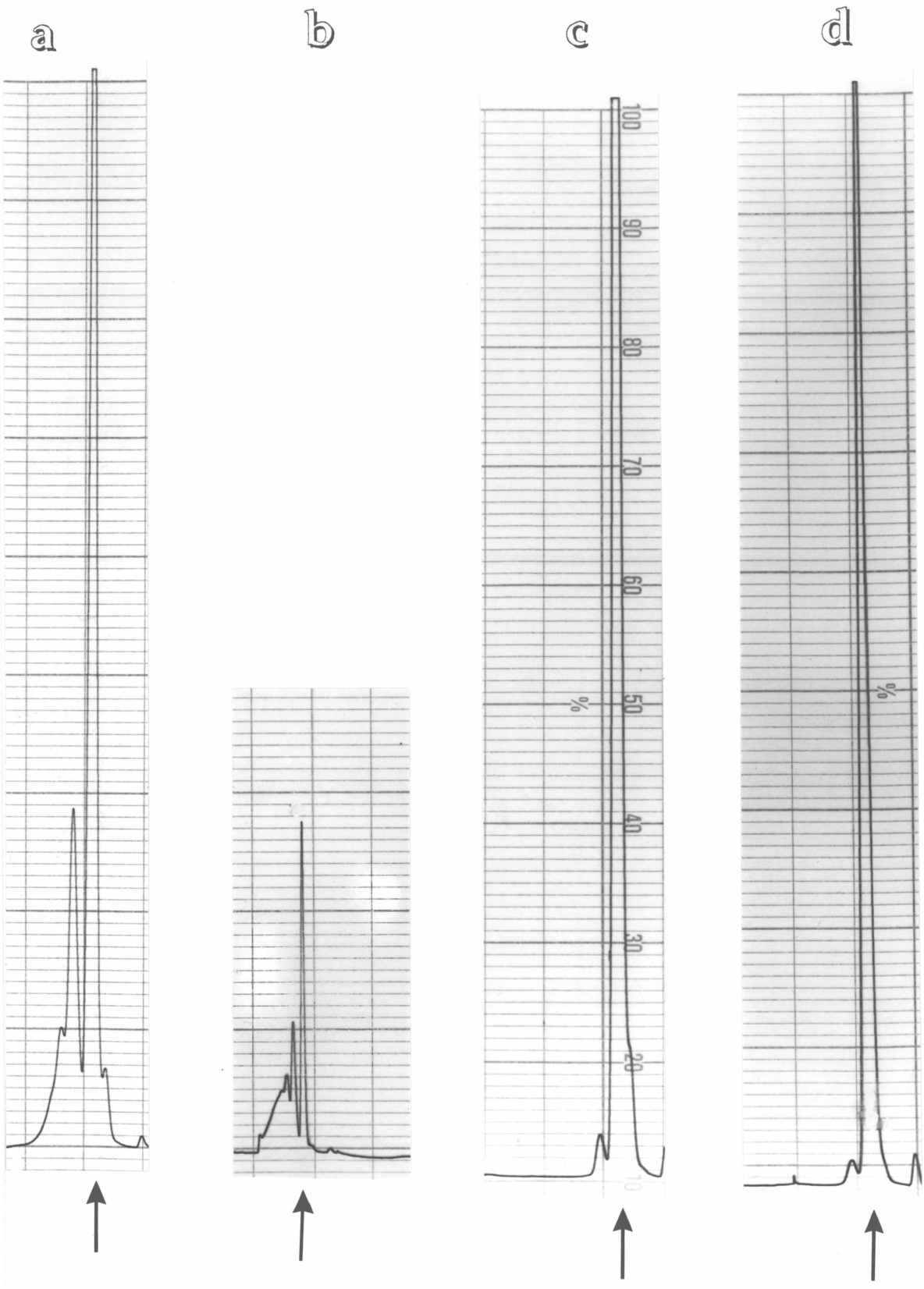


Figure 3.4: FPLC analysis (Superose 6) of 4 whole monoclonal IgG-DTPA preparations: a) HMFG1-DTPA, b) AUA1-DTPA, c) H17E2-DTPA and d) another batch of HMFG1-DTPA. Arrows indicate the monomeric antibody fraction (150KD), whereas all the peaks to the left show the location of immunoglobulin polymers.

This phenomenon became more profound after labelling of the DTPA coupled antibodies with ^{111}In and ^{90}Y. As shown in Figure 3.5, autoradiography revealed considerable aggregate formation in ^{111}In-DTPA-HMFG1 (a), ^{90}Y-DTPA-HMFG1 (b) and ^{90}Y-DTPA-AUA1 (c) preparations (7.5% gel in all case). Arrows indicate the monomeric and aggregated IgG; polymers were radiolabelled as well, if not better, as monomeric antibody. FPLC analysis confirmed the above autoradiography results. Figure 3.6a shows the profile of an ^{111}In-DTPA-HMFG1 conjugate (before separation from free ^{111}In) when a Superose 6 column was used, and Figure 3.6b, the same conjugate (free ^{111}In was removed) when an HR-Sepharose S-200 column (Pharmacia) was used. The blue line represents the protein profile, and the red one the radioactivity. The presence of HSA was expected, since it was added immediately after radiolabelling, as discussed earlier (section 3.2.3). Again Ig aggregates were also radiolabelled, but we found a striking difference in the resolution of these 2 columns. Clearly Superose 6 is superior to HR-Sepharose S-200, but 10 times more expensive. In subsequent experiments, where the test sample was human serum (see section 3.2.5 below), HR-Sepharose S-200 was used, since replacement of the latter column was financially more realistic.

3.2.5 Kinetics of the monoclonal antibodies: The effective half life ($T_{1/2}$) of the radiolabelled mouse mAbs in circulation was measured as follows. The patients were bled at regular time points, and the serum removed after the blood had been left to clot for 3 hr at 37°C. The radioactivity in the serum was measured in a γ-counter, and the counts were corrected for physical decay of the isotope. In addition, 10% TCA paper chromatography or Sephadex G-50 gel filtration revealed the percentage of these counts that corresponded to free isotope. The results were therefore further corrected, and expressed as percentage of injected dose, using an aliquot of the initial injection as standard.

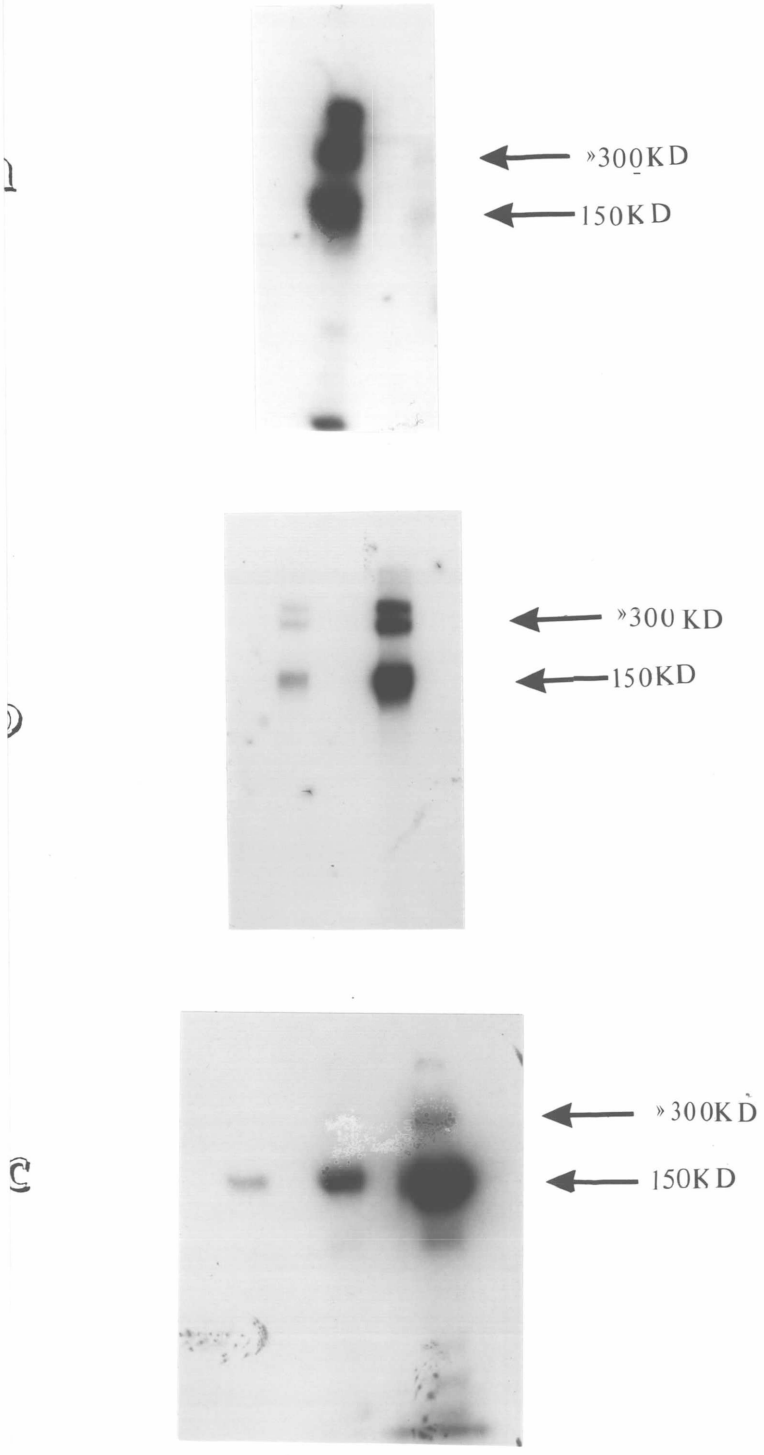


Figure 3.5: Autoradiography of 3 radiolabelled monoclonal antibodies, prior to administration to patients with cancer for imaging or therapy of the neoplastic lesion. a) ^{111}Indium-DTPA-HMFG1, b) ^{90}Yttrium-DTPA-HMFG1, neat (right) and 1:10 dilution (left), and c) ^{90}Yttrium-DTPA-AUA1, neat (right), 1:10 (middle) and 1:100 dilution (left). Monomeric (150KD) and aggregated (≥300KD) antibodies are indicated by the arrows.

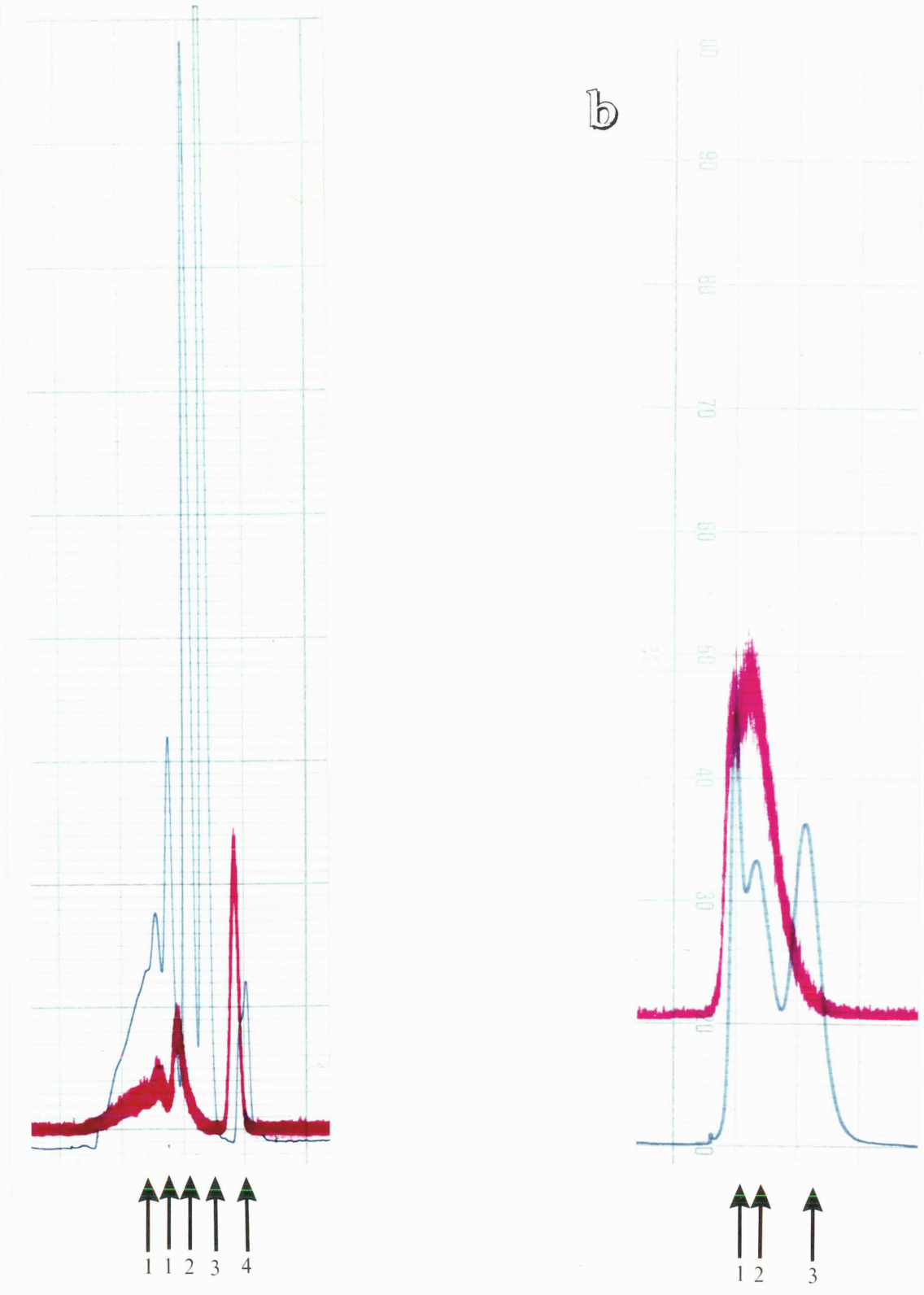


Figure 3.6: a) FPLC profile using Superose 6 column, of ^{111}In-DTPA-HMFG1 monoclonal antibody. The blue line represents the protein and the red line the radioactivity trace. Arrows indicate the location of monomeric (2), aggregated ^{111}In-DTPA-HMFG1 (1), free ^{111}Indium (4), as well as HSA (3), since the radiolabelled preparation was diluted in 0.25% HSA prior to administration. b) FPLC profile using HR-Sepharose S-200 column of the same ^{111}In-DTPA-HMFG1 preparation analyzed in a). The blue line shows the protein trace, whereas the red line corresponds to the radioactivity. Arrows indicate the position of monomeric (2), aggregated ^{111}In-DTPA-HMFG1 (1) and HSA (3).

For further examination of the serum, autoradiography and FPLC size exclusion chromatography (HR Sepharose S-200) were used. This enabled us to determine whether the protein bound radioactivity was still associated with antibody, or whether dehalogenation (for ^{131}I) and transchelation (for ^{111}In and ^{90}Y) had led to the transfer of radioactivity to other serum proteins.

3.2.6 Measurement of the anti-monoclonal antibody response: The patients' response against the murine mAb administered was tested 14 days after administration, using an ELISA, as described in detail in chapter 2. Briefly, the immunogen (mAb) was coated onto 96-well plates, the hosts' sera were applied in serial 1:10 dilutions, and the presence of anti-mouse Ig antibodies was detected by an anti-human Ig-horseradish peroxidase conjugate. The results are expressed as areas under the serum-titration curves (before and 14 days after administration of the mAb), as described in the previous chapter.

3.2.7 The biotin-streptavidin system in vivo: 1 mg of HMFG1-streptavidin conjugate was administered i.v. to 6 patients with metastatic squamous cell carcinoma of the lung. The conjugate was prepared in Prof. D. Hnatowich's laboratory (University of Massachussets, USA) by biotinylation of the HMFG1, followed by mixing with streptavidin. We received the preparation in purified form, at an HMFG1/streptavidin MR of 1:1, sterile and pyrogen free.

Serum samples were collected for a period of 3 days post administration, and the presence of the HMFG1-streptavidin conjugate in the circulation measured using a "sandwich" ELISA method. 96-well plates were coated with sheep species-specific anti-mouse Ig-biotin (Amersham), in carbonate/bicarbonate buffer, pH 9.6, for 3 hr at 37°C. The plate was washed with PBS/0.05% tween 20 three times, and test sera (various time points) applied at 1:2 dilutions. After 2 hr at 37°C, the plates were washed, and a sheep species-specific anti-mouse Ig-horseradish peroxidase reagent (Amersham) was applied, at 1:500 dilution. The plates were washed again (after 1 hr at 37°C), the substrate ABTS added, and the

absorbance was measured at 405 nm. Each plate included known concentrations of HMFG1-streptavidin in serum; from these, a standard curve was plotted, and the experimental results expressed in μg of conjugate/ml of serum.

Approximately 3 days after administration of the HMFG1-streptavidin, the patients received 50 μg biotin, labelled with 2 mCi ^{111}Indium. Biotin-DTPA (Sigma) was reconstituted in metal-free 0.05M bicarbonate buffer, and mixed with the isotope as described above (section 3.2.3a). The labelling efficiency, at 40 mCi/mg, was >99%. This was tested by Dr. H. Kalofonos, who performed the *in vitro* stability tests with ^{111}In-DTPA-biotin-streptavidin, in the presence and absence of serum. In our own experiments, we found that ^{111}In-DTPA-biotin incubated with normal human serum for up to 20 min at 37°C, remained mainly (>90%) non-protein bound. The blood clearance was estimated by measuring the radioactivity of serum samples, in a γ-counter, as described above (section 3.2.5).

The patients' anti-HMFG1 and anti-streptavidin response was measured before and 14 days after administration of the conjugate, using the ELISA method described above (section 3.2.6). For the detection of anti-streptavidin antibodies, the plates were coated with 0.5 μg/well streptavidin (Sigma), a concentration that was determined by a chequer-board ELISA (see chapter 2), using rabbit anti-streptavidin polyclonal antiserum (Sigma). The results were expressed as areas under the serum-titration curves.

3.2.8 Alternatives to antibodies - epidermal growth factor: Human EGF was obtained as lysyl human urogastrone epidermal growth factor (in lyophilized form), from ICI Pharmaceuticals, UK. It was reconstituted in PBS, pH 7.4, and labelled with ^{131}Iodine using the iodogen-coated vial method (200 μg/vial), as described above (section 3.2.3c), at a specific activity of 3.5 mCi/mg, and with a labelling efficiency of >90%. Bound and free isotope were separated by Sephadex G-25 gel filtration. EGF was also labelled with ^{123}Iodine (Amersham) at a

specific activity of 10 mCi/mg. The labelling method was identical to that used for ^{131}I-labelling, except that the pH of the ^{123}I was raised to 7.0-7.5 with citrate buffer, before mixing with the growth factor. Both iodinated preparations fully retained their capacity to bind to EGF-receptors *in vitro*, as shown in a direct cellular radioimmunoassay. Approximately 10^6-$5x10^6$ viable A431 cells (provided by Dr. W. Gullick, ICRF Molecular Oncology laboratories, Hammersmith Hospital) were incubated with unlabelled and labelled EGF, for 1 hr on ice. The concentration of "hot" EGF was kept at 0.8 μg while the "cold" EGF was titrated in at concentrations from 0.01 to 10 μg. The cells were then washed and the radioactivity measured in a γ-counter. As shown in Table 3.1, ^{131}I-labelled EGF competed well with the "cold" EGF.

Approximately 0.7 mCi (200 μg) of ^{131}I-labelled and 3 mCi (300 μg) of ^{123}I-labeled EGF were injected i.v. into primates (two male, adult Cynomolgus monkeys). Blood samples were obtained at regular time points, and the $T_{1/2}$ of EGF was calculated from the serum radioactivity measured in a γ-counter. Again, the counts were corrected for physical isotope decay, and the percentage of free iodine (determined by Sephadex G-25 gel filtration) was subtracted from the values found. In addition, the animals were placed under a γ-camera (Starport, IGE, USA), and dynamic views for the first 20 min, as well as static pictures for a period of 24 hours were taken . The data were transferred to a Starcam system (IGE, USA) for analysis, and the biodistribution of EGF was studied.

cpm	^{131}I-EGF (μg)	EGF (μg)
16,800	0.08	0.01
12,400	0.08	0.10
4,800	0.08	1.00
2,500	0.08	10.00

Table 3.1: Competition of unlabelled human EGF with ^{131}Iodine-labelled EGF, in a direct cellular radioimmunobinding assay using live A431 cells (cell line expressing EGF receptor).

3.3 Results

3.3.1 Kinetics of the i.v. administered monoclonal antibodies: ^{111}In-labelled HMFG1 and HMFG1 F(ab')$_2$ fragments, were administered i.v. to 11 patients with NSCLC and 3 patients with breast cancer, for diagnostic purposes. We found that F(ab')$_2$ fragments disappeared rapidly from the circulation. Five hours after administration of the antibody-fragments, only 45% of the injected dose was found in the blood. From that point the clearance slowed down, with a $T_{1/2}$ of 55 $\pm$ 4 hours. The catabolism of the whole antibody followed a slower rate. At 5 hr post administration, almost 78% of the injected dose was still in the circulation, and cleared out slowly, with a $T_{1/2}$ of 65 $\pm$ 3 hours.

FPLC analysis of the serum (HR Sepharose S-200), showed that the radioactivity was bound to protein with molecular weight 150KD or higher when whole immunoglobulin was administered, and 100KD or higher when F(ab')$_2$ fragments were injected. Due to DTPA cross-linking (see section 3.2.4) of the immunoglobulin, we were unable to distinguish the preformed dimers and polymers from potential *in vivo* formed immune complexes of the mouse mAb and human anti-mouse Ig antibodies. However, as stated earlier (section 3.2.4, Fig 3.3b), aggregate formation was less for the fragment preparation than for the whole antibody. In one patient, therefore, who received ^{111}In-HMFG1-F(ab')$_2$ with no aggregates, we observed two radioactive compounds in the serum, instead of the expected one. Figure 3.7 shows the FPLC profile for protein (a) and radioactivity (b) of a serum sample taken from this patient 24 hr after administration of the radiolabelled antibody fragment. The blue line represents the trace due to the total serum proteins (Fig. 3.7a). When the radioactivity was measured, it corresponded mainly to 2 high molecular weight proteins, 100KD and ≥200KD (Fig. 3.7b), as confirmed by the standard ^{111}In-HMFG1-F(ab')$_2$ sample, which was run through the column

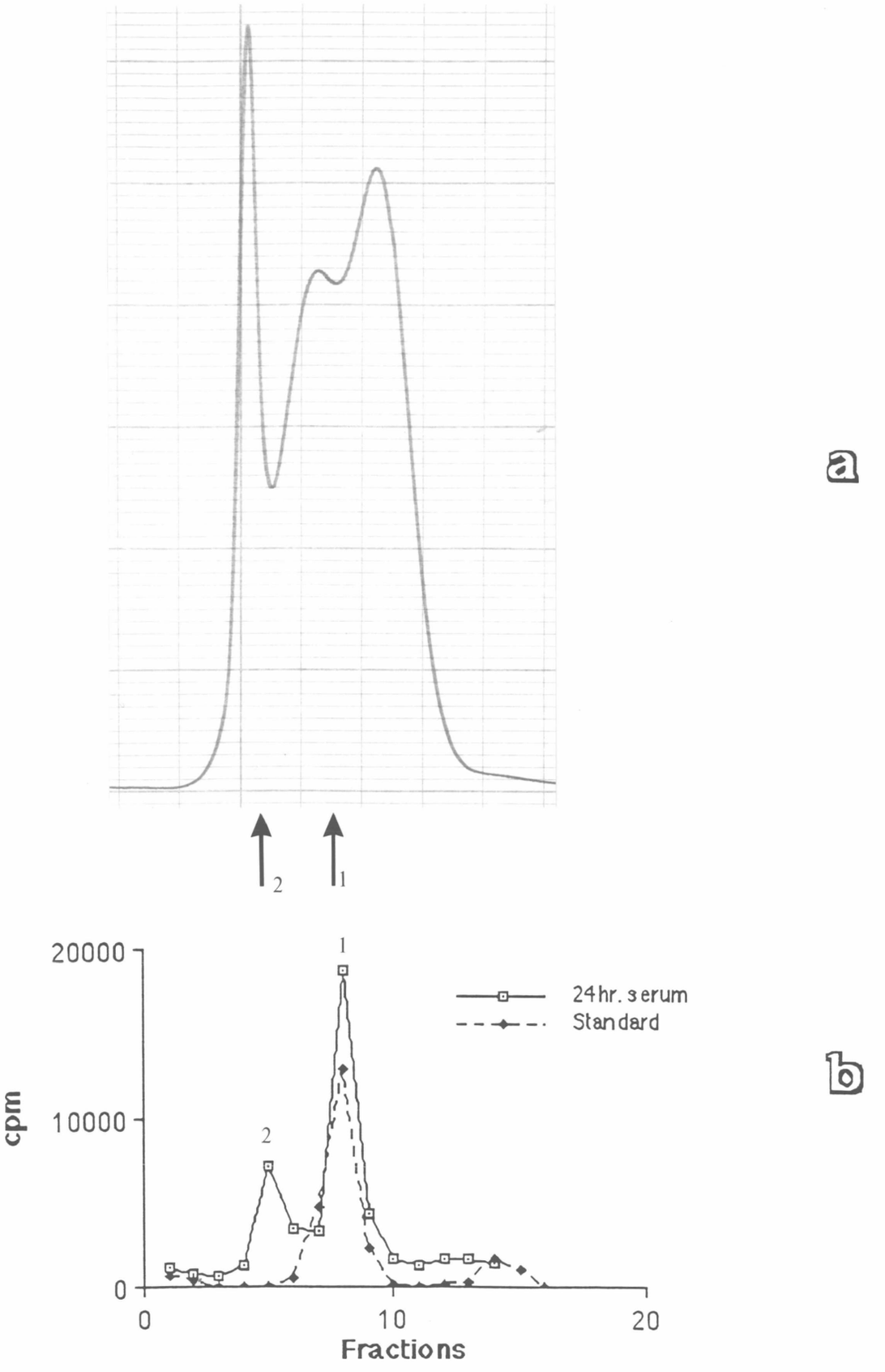


Figure 3.7: FPLC profile (HR-Sepharose S-200) of a patient's serum 24 hr after administration of ^{111}In-DTPA-HMFG1 $F(ab')_2$ fragments. a) the protein trace and b) the radioactive trace of the serum and the standard (^{111}In-DTPA-HMFG1 $F(ab')_2$). Arrows on Figure a indicate the position of the radioactive peaks measured in Figure b. 1: monomeric $F(ab')_2$ fragment (100KD) and 2: aggregated $F(ab')_2$ fragment (≥200KD).

under identical conditions (Fig. 3.7b). The area of the serum-protein profile, in which we located the radioactivity, is shown by the arrows in Figure 3.7a.

Autoradiography of the patients' sera, collected at various time points, confirmed that the radioactivity was associated with the monoclonal antibody (monomeric or aggregated) for up to 3 days. We were unable to detect any radioactivity after that. Figure 3.8a shows a 7.5% gel of two different patients' sera, taken immediately (lanes 2 and 3 respectively) and 24 hr (lanes 4 and 5 respectively) after injection of ^{111}In-labelled HMFG1 F(ab')$_2$ fragments. On the left is the SDS-PAGE with the sera and the 100KD molecular weight marker (lane 1), and on the right the autoradiography film. There was no transchelation of ^{111}In to other serum proteins, and all the activity was associated with molecular weights of 100KD or higher, since the preparations administered were partially aggregated. Figure 3.8b shows a 10% gel of a patient's serum, taken immediately (lane 1), after 1 hr (lane 2) and after 48 hr (lane 3), post ^{111}In-HMFG1-F(ab')$_2$ fragment administration. Again no transchelation was observed. However, radioactivity was only on the monomeric F(ab')$_2$ fragment, since the administered preparation in this case was free of aggregates.

3.3.2 Kinetics of the i.p. administered monoclonal antibodies: ^{131}I-labelled HMFG1, AUA1 and H17E2, were administered i.p. to 18 patients with ovarian cancer. Another group of 12 patients received the mAbs labelled with ^{90}Y. We found that the antibody migrated slowly from the peritoneal cavity into the blood stream, a process that was slightly slower for ^{90}Y-labelled antibodies than for iodinated ones (Fig. 3.9). The radioactivity reached a peak in the blood at approximately 50 hr after i.p. administration of the ^{131}I-mAb (40 hr for ^{90}Y-mAb). From that time point, both preparations followed a slow clearance rate, with a $T_{1/2}$ of approximately 50 hours. The maximum percentage of

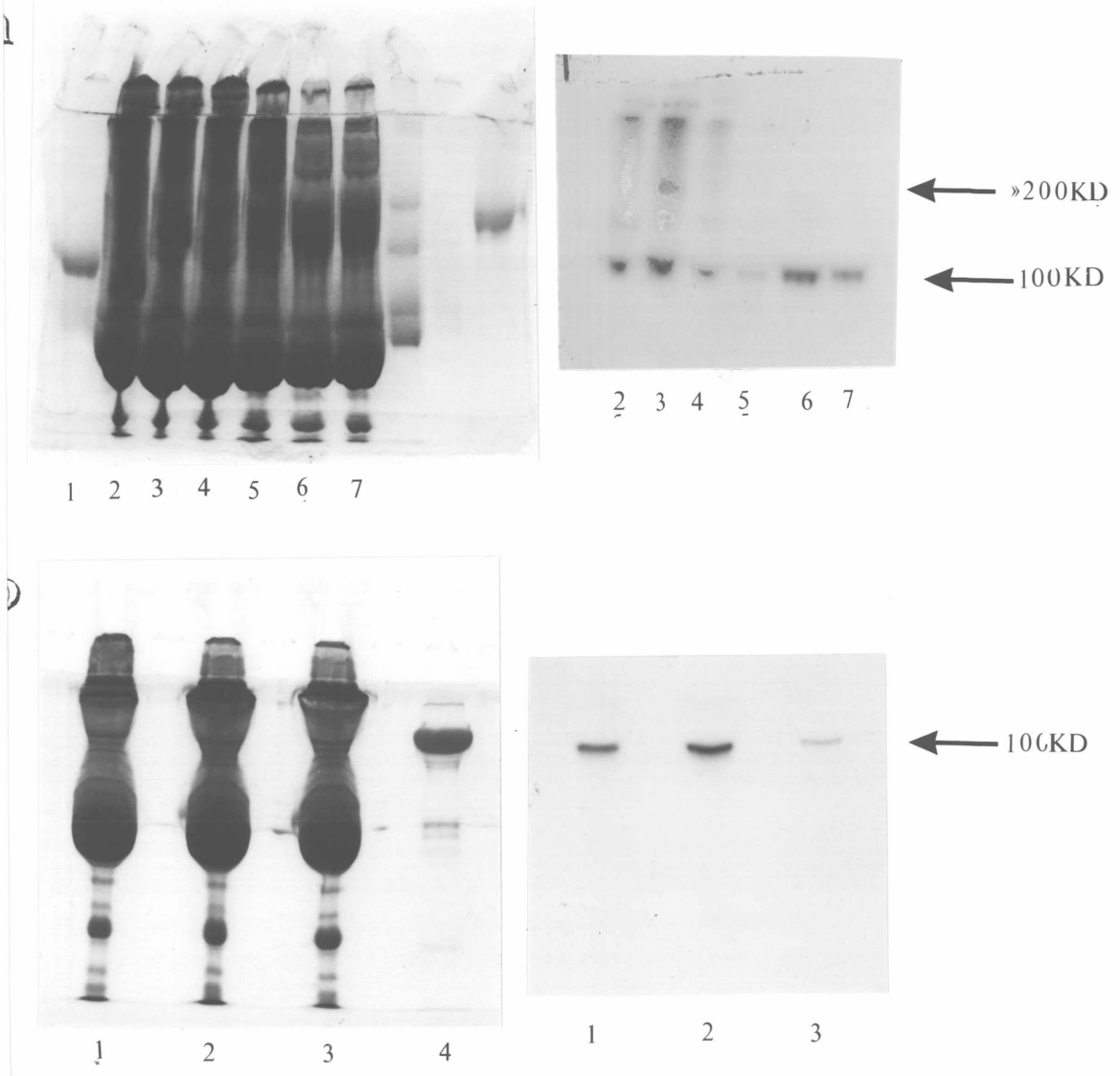


Figure 3.8: a) Left: a 7.5% SDS-PAGE of 2 patients' sera, immediately (lanes 2 and 3 for the two sera respectively) and 24 hr (lanes 4 and 5 for the two sera respectively) after i.v. injection of ^{111}In-DTPA-HMFG1 F(ab')$_2$. Lane 1 contains an HMFG1 F(ab')$_2$ fragment as molecular weight marker and lanes 6 and 7 contain the same serum samples as lanes 2 and 3 but in 1:5 dilution. Right: autoradiography of this gel. Arrows show the monomeric (100KD) and aggregated F(ab')$_2$ fragment (≥200KD).
b) Left: 10% SDS-PAGE of a patient's serum taken immediately (lane 1), after 1 hr (lane 2) and 48 hr (lane 3) post administration of ^{111}In-DTPA-HMFG1 F(ab')$_2$ that was free of aggregates. Lane 4 contains the HMFG1 F(ab')$_2$ fragment as molecular weight marker. Right: autoradiography of this gel. The monomeric (100KD) F(ab')$_2$ fragment is well detected even after 48 hours.

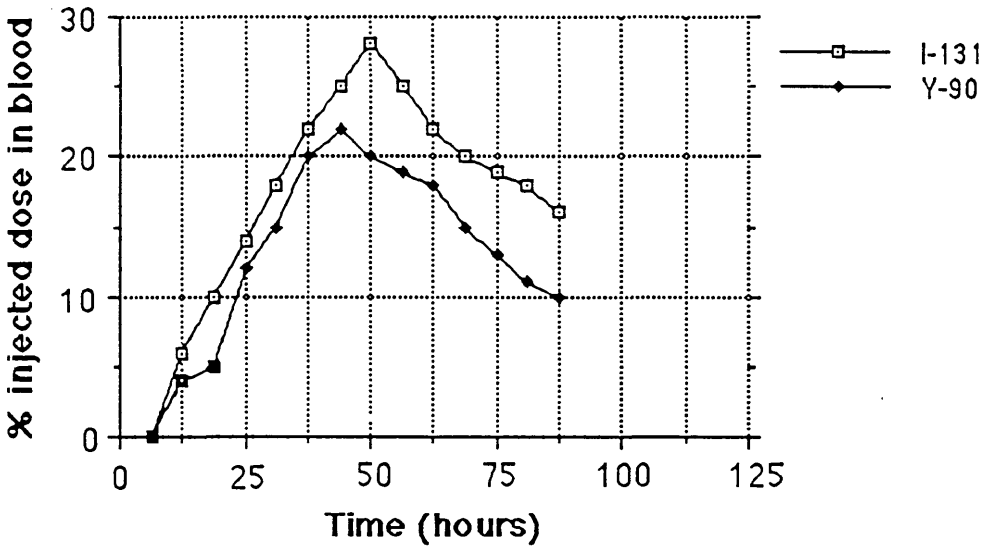
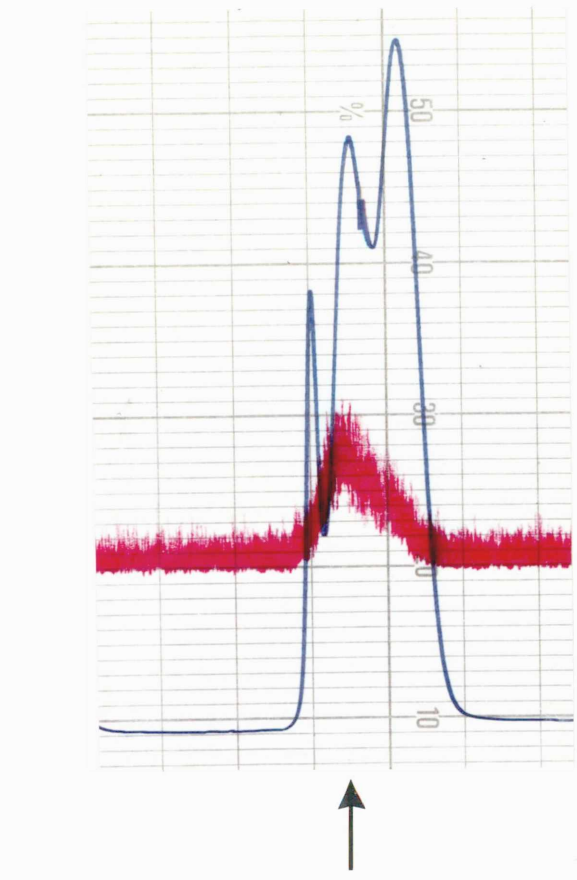


Figure 3.9: Percentage of radiolabelled anti-tumour monoclonal antibody found in the blood after i.p. administration to patients with cancer. Up to 28% (for ^{131}Iodine-antibody) and 22% (for ^{90}Yttrium-antibody) of the injected dose was in the circulation.

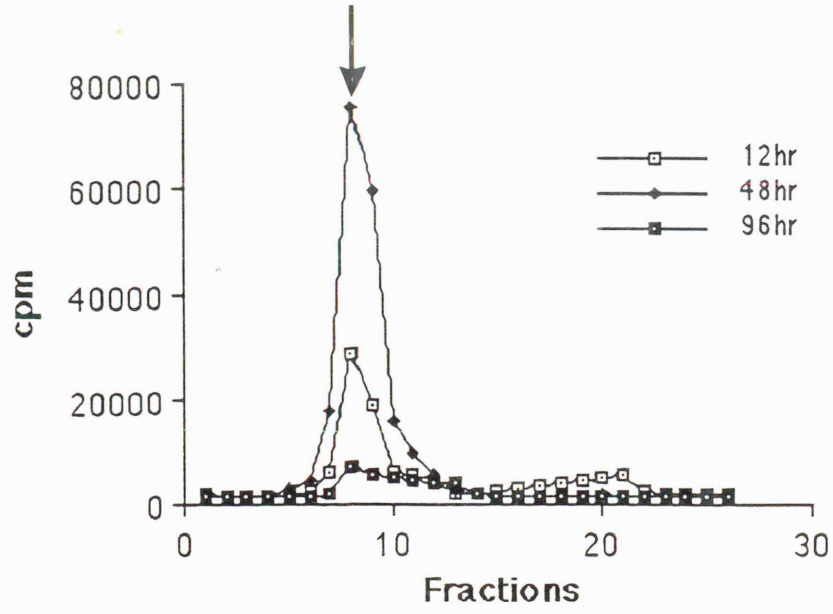
radiolabelled mAb in the circulation was 28% for ^{131}I-labelled, and 22% for ^{90}Y-labelled, of the total injected dose.

FPLC analysis (HR Sepharose S-200) of sera from 10 patients who had received ^{131}I-mAb showed that all the radioactivity was bound to proteins of a size corresponding to IgG (150KD). Figure 3.10a, shows a typical profile of a patient's serum, 48 hr after i.p. administration of ^{131}I-HMFG1. Only one radioactive peak (red pen) was observed and that corresponded to the IgG fraction (arrow). Similarly, the FPLC radioactive profile of the serum of another patient is shown in Figure 3.10b. Again, only one peak was present; this was higher at 48 (2 days) than at either 12 or 96 hours (4 days). After testing a large number of human sera it became clear that there was a big variation in the actual amount of the three main serum-protein compounds, namely: fibrinogen/IgM, IgG and albumin (Davies et al., 1986; Koizumi et al., 1986). However, the monomeric radioactive peak of ^{131}I-mAb always coincided with the IgG fraction. Figure 3.11a shows the radiolabelled antibody present in the blood at 48 hr for three different patients. In Figures 3.11b and c, the FPLC protein profiles of 2 of these are shown. The arrows indicate the area where the radioactive peak was located; as before it corresponded to the IgG fraction of the serum.

Similarly, when we tested the sera from 6 patients who had received ^{90}Y-mAb, we also found one radioactive peak at the 150KD IgG area of the protein FPLC profile. Figure 3.12a shows serum samples from one patient tested 24 and 48 hr post administration of the radiolabelled antibody. There was only a single radioactive peak corresponding to IgG, as indicated by the arrow in Figure 3.12b (FPLC, serum protein profile). However, when a standard (^{90}Y-HMFG1) was run on the same column under identical conditions, we observed both the expected monomeric and polymeric fractions, due to DTPA cross-linking



a



b

Figure 3.10: a) FPLC profile (HR-Sepharose S-200) of a patient's serum 48 hr after i.p. administration of ^{131}Iodine labelled HMFG1. The blue line shows the total serum protein profile, whereas the red line the single radioactive peak corresponding to the IgG fraction of the serum (arrow).
b) Radioactive trace after FPLC analysis (HR-Sepharose S-200) of a patient's serum taken 12, 48 and 96 hours post i.p. administration of ^{131}Iodine-HMFG1. The single peak corresponds to monomeric IgG (arrow).

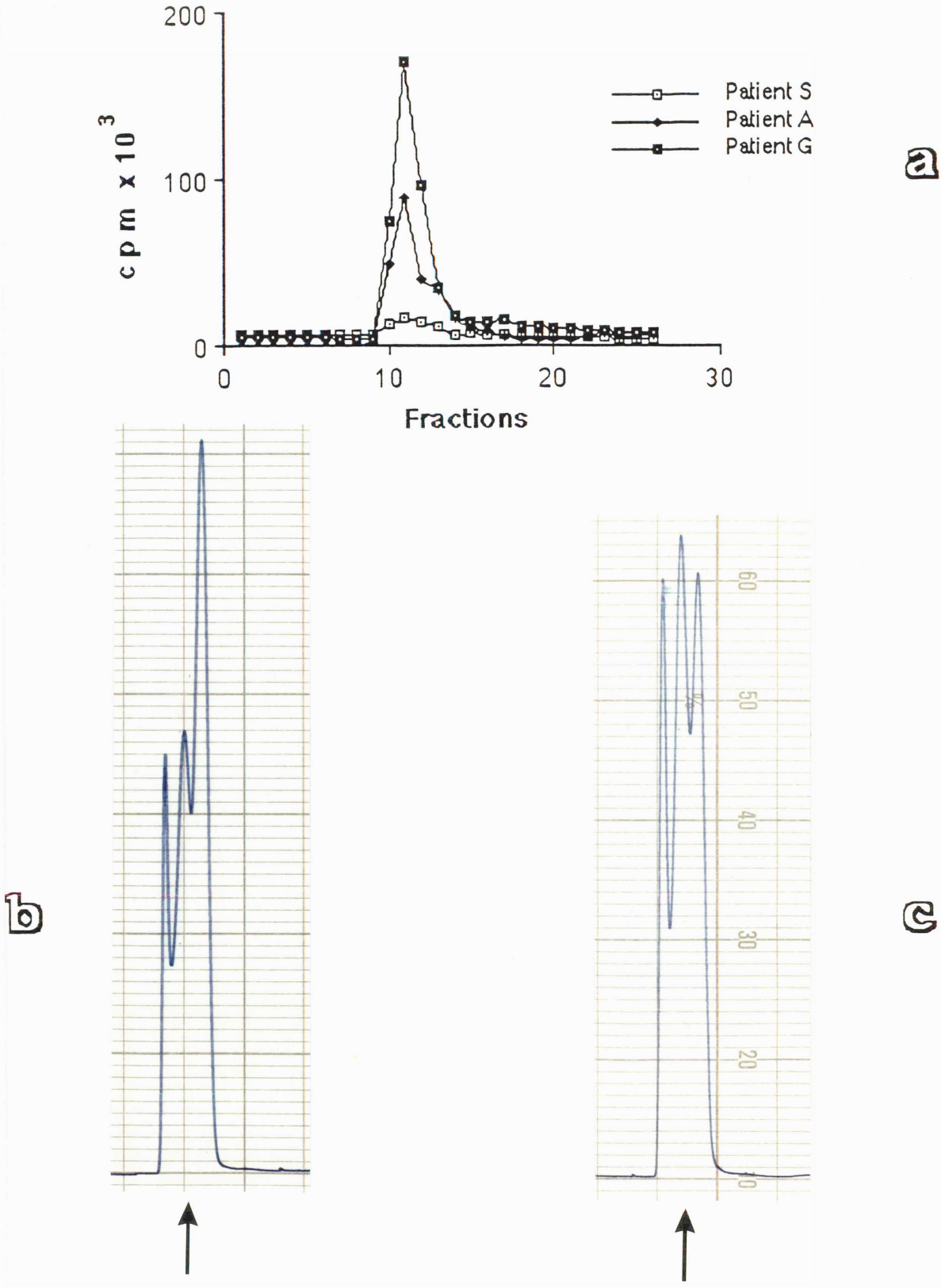
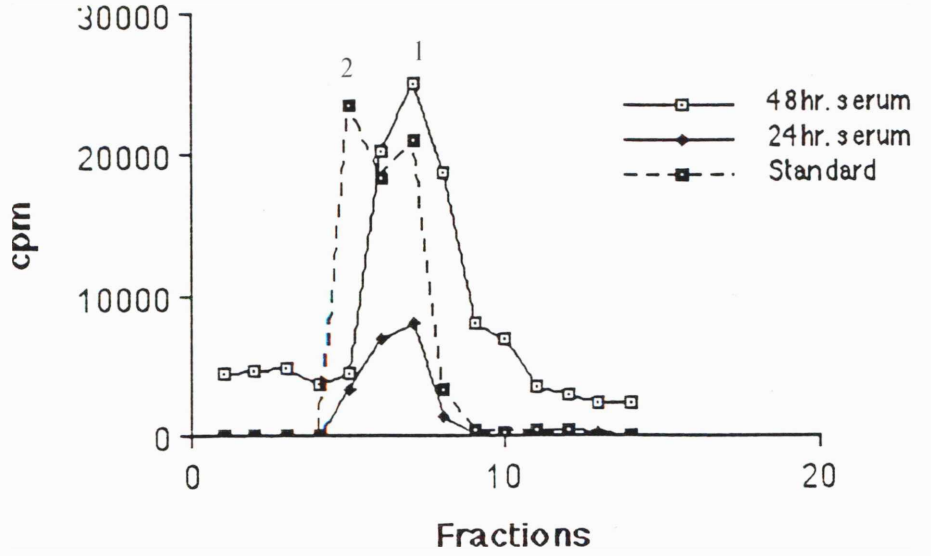


Figure 3.11: FPLC profile (HR-Sepharose S-200) of 3 patients' sera, 48 hr after i.p. administration of ^{131}Iodine-HMFG1. Figure a shows the radioactive traces whereas Figures b and c the total serum protein traces of two of them (patients S and A respectively). The single radioactive peak of Fig. a corresponds to monomeric IgG, and its location is shown by the arrows on Figures b and c.

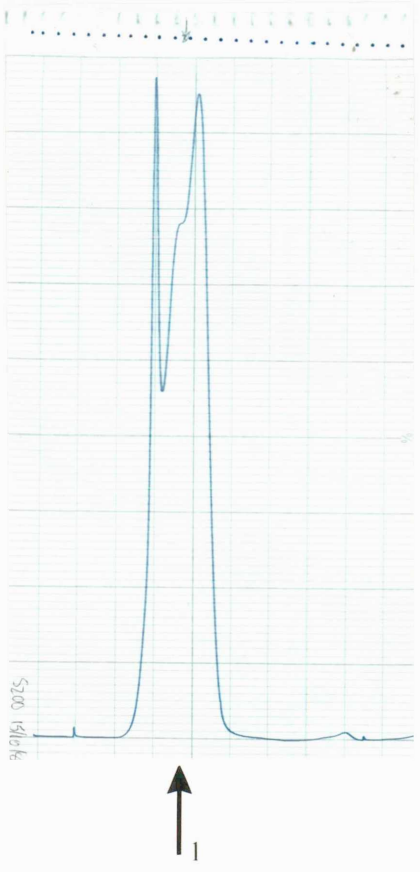
Figure 3.12: a) Radioactive trace (FPLC using HR-Sepharose S-200) of one patient's serum 24 and 48 hr after i.p. administration of ^{90}Yttrium-labelled HMFG1. The single peaks correspond to monomeric IgG, as found when standard ^{90}Yttrium-HMFG1 was tested in the same column under identical conditions. 1: monomeric monoclonal antibody (150KD) and 2: aggregated monoclonal antibody (≥300KD).

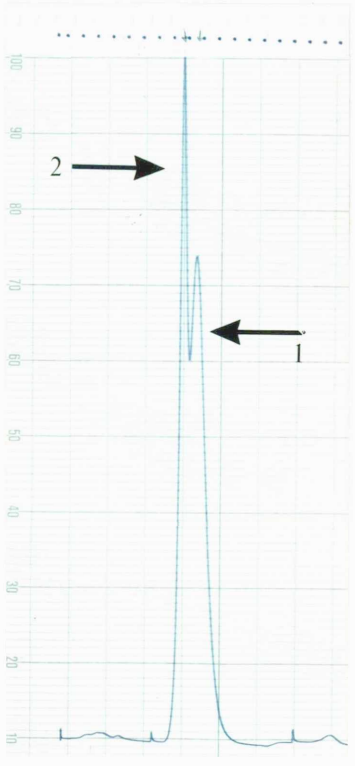
b) FPLC profile (HR-Sepharose S-200) of the total serum proteins, where the arrow (1) indicates the position of radioactive peak measured in the patient's serum in Figure a. This activity corresponds to the IgG fraction.

c) FPLC protein trace (HR-Sepharose S-200) of the standard ^{90}Yttrium-HMFG1 used in Figure a. The two radioactive peaks measured on Figure a correspond to monomeric (150KD) and aggregated (≥300KD) fractions of the antibody as shown by the arrows (1 and 2 respectively).


30000
20000
10000
0
cpm
2
1
48hr. serum
24hr. serum
Standard
0
10
20
Fractions

a


1


2
1

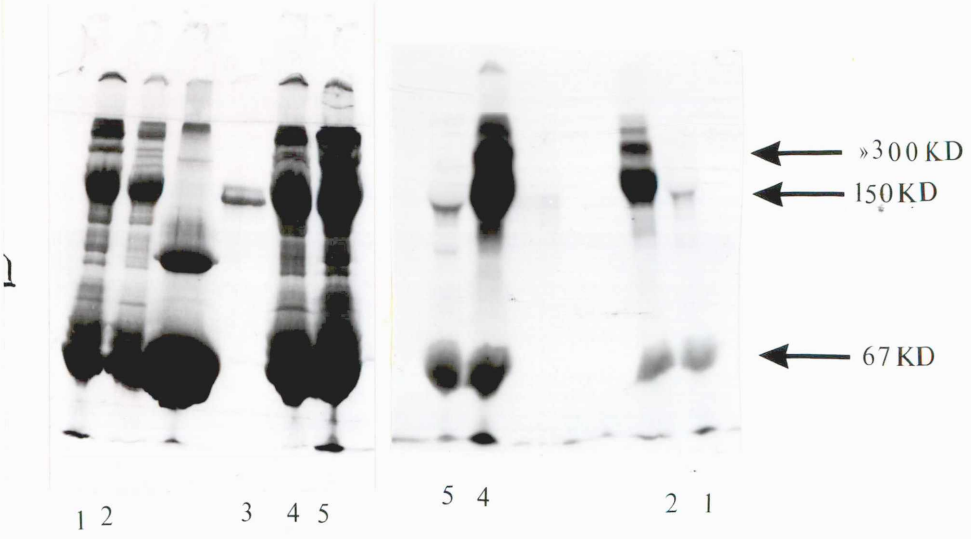
c

(see section 3.2.4 above), for both the protein (second and first peak in Fig. 3.12c) and the radioactive trace (second and first peak in Fig. 3.12a).

The association between radioactivity and administered antibody was also demonstrated by autoradiography. This was tested not only in the patients' sera, but also in patients' ascitic fluid (after the cells had been removed) although for the above kinetics studies patients with ascites were excluded, since the mAb migration into the blood stream follows a different and irregular pattern (S. Stewart, personal communication). Figure 3.13a shows an autoradiograph (7% gel) of a patient's serum (lanes 1 and 5) and ascitic fluid (lanes 2 and 4), at two dilutions, 24 hr after i.p. administration of ^{90}Y-HMFG1. In addition to radioactivity at 150KD, dimers and polymers were also visible in the ascitic fluid but not in the serum. Radioactivity was also associated with proteins of molecular weight close to albumin (67KD). Figure 3.13b, shows an autoradiograph (7.5% gel) of a patient's ascitic fluid 3 hr after administration of ^{131}I-HMFG1. Almost all radioactivity was associated with monomeric mAb. However on this gel, a very small proportion of higher molecular weight proteins were also observed.

Five patients treated i.p. with ^{90}Y-HMFG1, were retreated with ^{131}I-HMFG1. All these patients had developed anti-mouse antibodies, due to their earlier exposure to HMFG1. Measurement of blood levels of the radiolabelled mAb showed that only a very low percentage (approximately 5%) was found in the circulation, which peaked at 15-20 hr post administration, and was then cleared out very slowly (Fig. 3.14).

3.3.3 The biotin-streptavidin system in vivo: We examined the clearance of 1 mg HMFG1-streptavidin in the patient after i.v. administration. One hour after



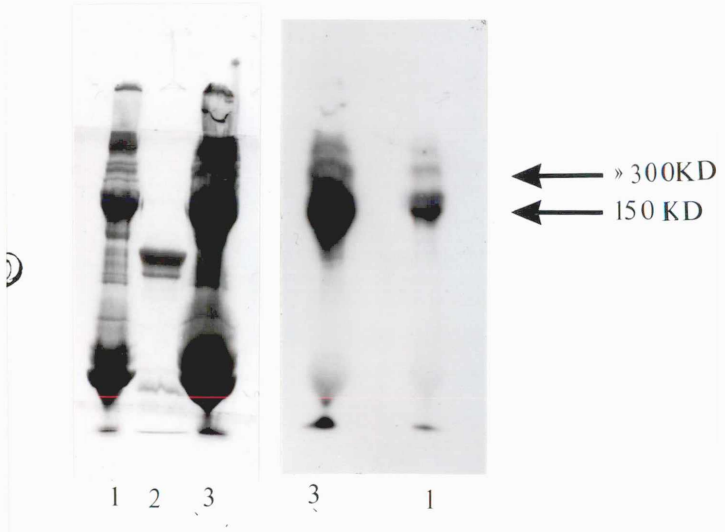


Figure 3.13: a) Left: 7% SDS-PAGE of a patient's serum (lane 1, 1:50 dilution and lane 5, 1:10 dilution) and ascitic fluid (lane 2, 1:50 dilution and lane 4, 1:10 dilution), 24 hr after i.p. administration of ^{90}Yttrium-HMFG1. Lane 3 contains HMFG1 as molecular weight marker. Right: autoradiography of the above gel. Monomeric radiolabelled antibody (150KD) is present in serum and ascites, whereas aggregates (≥300KD) are only present in ascites (arrow). Lower molecular weight proteins are also radiolabelled (~67KD, arrow).
b) Left: 7.5% SDS-PAGE of a patient's ascitic fluid (lane 1, at 1:50 dilution and lane 3 at 1:5 dilution) 3 hr after i.p. administration of ^{131}Iodine-HMFG1. Lane 2 contains HMFG1 F(ab')$_2$ as a molecular weight marker (100KD). Right: autoradiography of this gel showed the presence of the radiolabelled antibody (150KD), as well as a small proportion of antibody dimers and polymers (≥300KD, arrow).

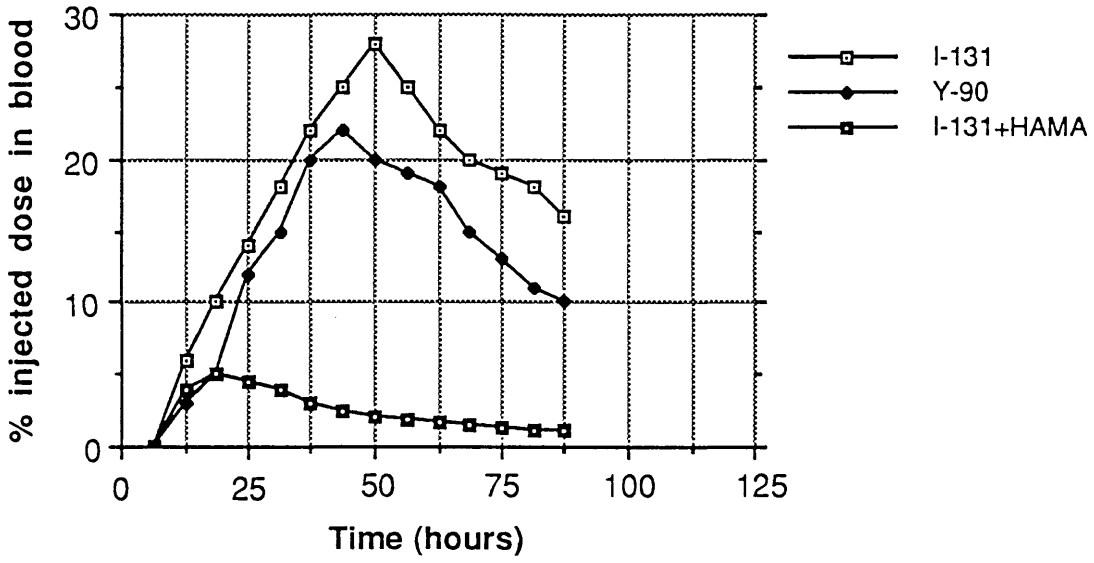


Figure 3.14: Percentage of radiolabelled anti-tumour monoclonal antibody found in the blood after a second i.p. administration to patients with cancer. These patients, who had already developed anti-mouse Ig antibodies, cleared the radiolabelled antibody faster from the circulation (I-131 + HAMA; HAMA = human anti-mouse Ig antibodies) compared with patients treated with monoclonal antibody only once (I-131 and Y-90).

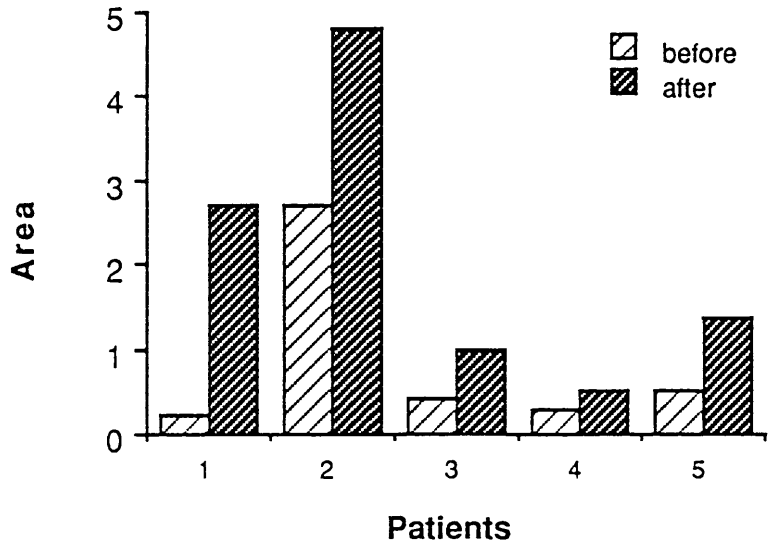
administration of the conjugate, approximately 25% was present in the circulation. At 24 and 48 hr, the serum concentration was 10 and 5% of the injected dose respectively.

Radiolabelled biotin, administered i.v. 3 days after the HMFG1-streptavidin conjugate, disappeared from the circulation very rapidly. 20 hr after administration of ^{111}In-biotin, only a very small percentage of radioactivity was still in circulation (<8%).

As shown in Figure 3.15a, all patients, who received the HMFG1-streptavidin conjugate, developed antibodies against the mouse Ig. In addition, in 3 out of the 5 patients, we detected anti-streptavidin antibodies (Fig. 3.15b).

3.3.4 Alternatives to antibodies - epidermal growth factor: The kinetics of human EGF were studied in two monkeys that received ^{131}I- and ^{123}I-labelled EGF i.v.. This small growth factor was removed from the circulation very quickly; the clearance was biphasic, with a $T_{1/2}\alpha$ of 12 ± 2 min and $T_{1/2}\beta$ of 20.0 $\pm$ 1.5 hr (Fig. 3.16). The scintigraphic scan showed that most of the EGF accumulated in the liver and kidneys within the first 30 min (Fig. 3.17a for ^{123}I and c for ^{131}I). However, 24 hr later all the EGF had been catabolized, with only low activity remaining in the stomach, large bowel (probably free iodine), kidneys and bladder (Fig. 3.17c for ^{123}I and 3.17d for ^{131}I). There was no uptake of iodine in the thyroid, since it had been blocked with KI which had been added to the animals' drinking water 3 days prior to administration of the radiolabelled reagent. Figure 3.17 also shows the striking difference and the superiority in the quality of the signal obtained, when ^{123}I is used in radioscintigraphic studies over ^{131}I.

a



b

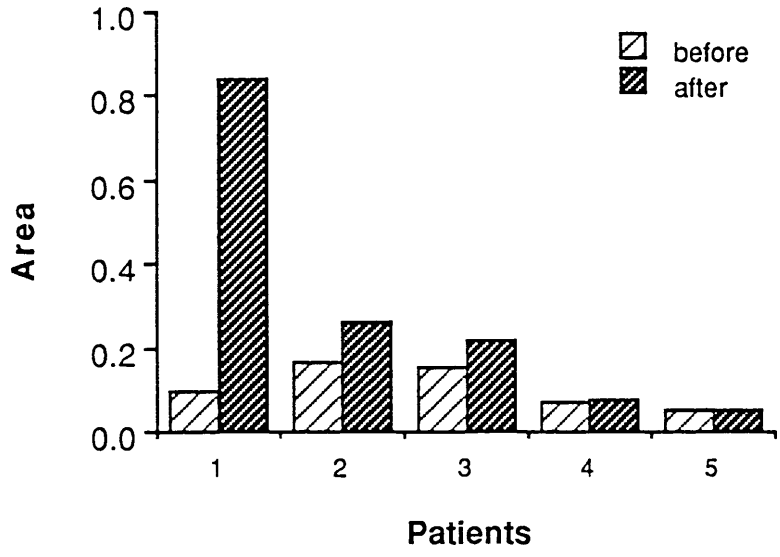


Figure 3.15: a) Anti-mouse Ig response of 5 patients who received 1 mg of HMFG1-streptavidin conjugate i.v., as measured by ELISA. The results are expressed as areas under the serum titration curve, when measured before and 14 days after administration of the antibody conjugate.
b) Anti-streptavidin response of the same patients, as measured by ELISA. The results are expressed as areas under the serum titration curve, when measured before and 14 days after administration of the antibody conjugate.

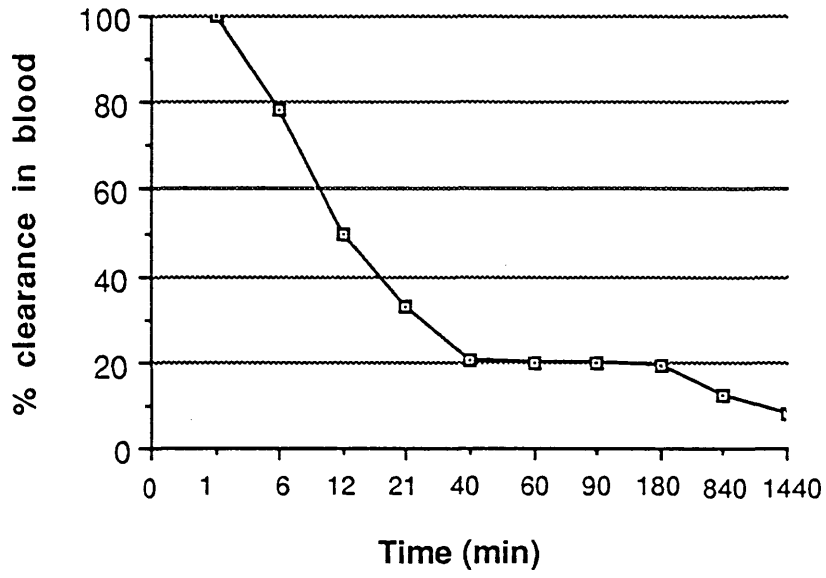


Figure 3.16: Blood clearance of epidermal growth factor after i.v. administration to Cynomolgus monkeys. The kinetics follow a biphasic pattern, with a T1/2α of 12+2 min and T1/2β of 20.0+1.5 hours.

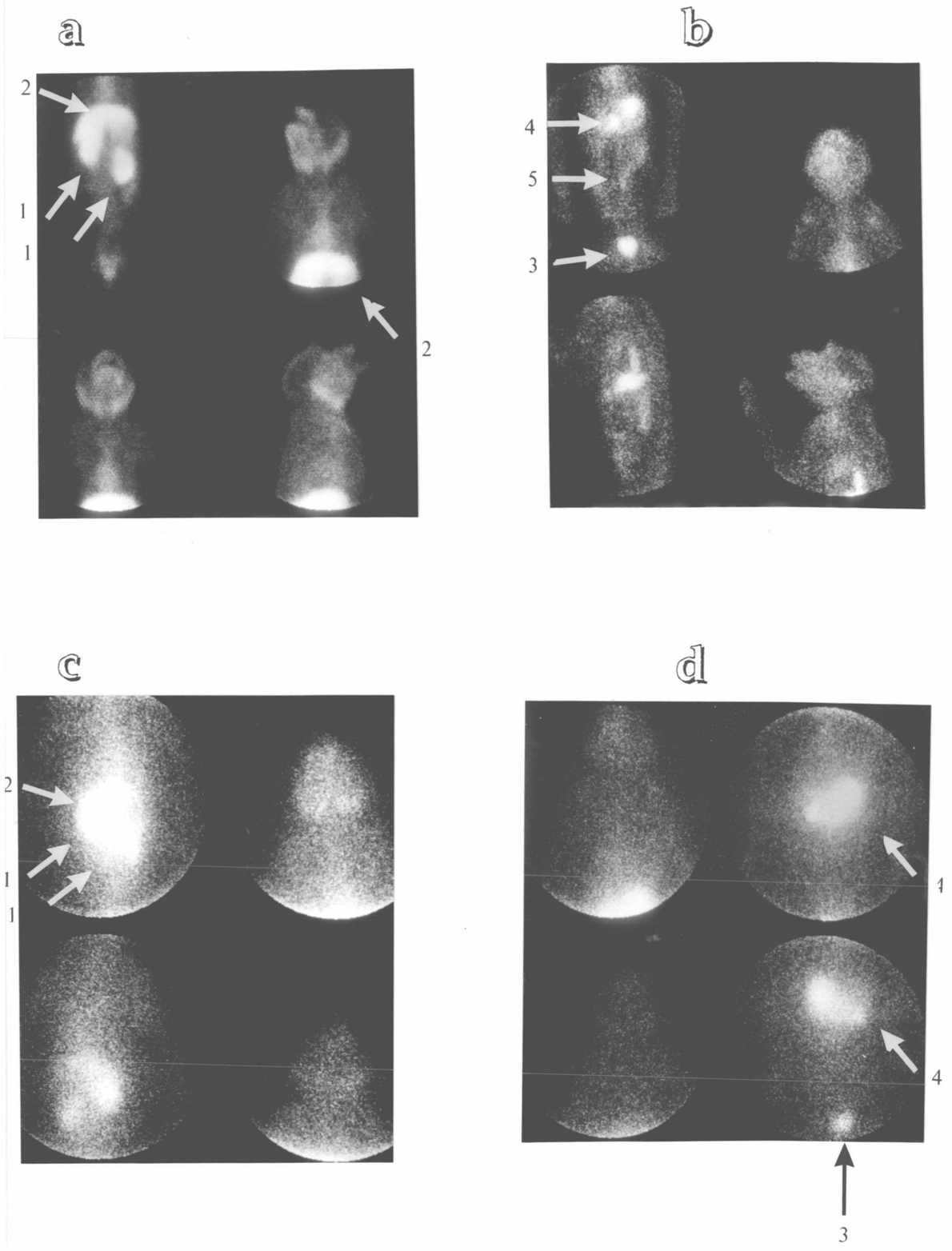


Figure 3.17: Scintigraphic scan of a Cynomolgus monkey 20 min (a and c) and 24 hr (b and d) after i.v. administration of ^{123}Iodine- (a and b) and ^{131}Iodine-labelled (c and d) human EGF. In the anterior views, arrows indicate kidneys (1), liver (2), bladder (3), stomach (4) and bowel (5)

3.4 Discussion

In our studies we confirmed data reported by a number of investigators that $F(ab')_2$ antibody fragments, when administered i.v., are cleared more rapidly from the circulation than whole immunoglobulins (Wahl et al., 1983; Khaw et al., 1984). However, if sufficient amounts of Ig fragments are taken up by the tumour, they may appear advantageous over intact molecules in immunodiagnosis and immunotherapy, since their immunogenicity and non-specific damage to normal tissue is minimized by removal of the Fc region.

Unfortunately, DTPA coupling of the antibodies, which is essential for chelating heavy metals such as ^{111}In and ^{90}Y, results in the formation of antibody aggregates. Since these isotopes have the right characteristics for tumour imaging and therapy respectively, new methods must be developed for controlled chemistry, and site-specific isotope conjugation. Poisson statistics reveal that, if we wish to couple three DTPA moities/antibody, using the existing methodology, only 23% will posses three chelates, 15% will posses five and 5% will not have any DTPA at all (Mears and Goodwin, 1984). Large aggregates have major defects: they cannot deeply penetrate the tumour mass; they are overall less immunoreactive; they trigger an immune response more easily; and they are cleared out more rapidly, hence the kinetics (especially the $T_{1/2}\alpha$ reported for ^{111}In-labelled mAbs by many investigators) may not represent the true values (Mears and Goodwin, 1984). However, one cannot readily reduce aggregate concentration, without sacrificing the labelling efficiency and specific activity.

Using FPLC size exclusion chromatography and autoradiography, we were able to analyze the labelled antibody in the circulation for up to 3 days post i.v. administration. Preformed aggregates in DTPA-coupled IgG preparations prevented us from detecting immune complexes, resulting from human anti-

mouse Ig antibodies that might have formed *in vivo*. However, since $F(ab')_2$ fragments rarely aggregated with DTPA, we were able to look for *in vivo* formed immune complexes in patients receiving $F(ab')_2$ antibodies. Immune complexes were detected in only one patient out of fourteen (Figs. 3.7a and b). Unfortunately, we did not test this serum for anti-mouse Ig antibodies by ELISA.

Intraperitoneally administered IgG migrates into the blood stream where it reaches a peak at about 40-50 hr after administration. The percentage of the injected dose that reaches the circulation is high (28% for ^{131}I- and 22% for ^{90}Y-labelled antibodies), considering that, for the treatment of patients with ovarian cancer, circulating radiolabelled Igs unnecessarily irradiate normal tissue and stimulate the host's immune system (Fig. 3.9). The presence of anti-mouse Ig antibodies in 5 patients considerably lowered the overall amount of circulating mAb (and hence total radioactivity); however it is not clear whether the effectiveness of the therapy was also reduced (Fig. 3.14).

Following i.p. administration, we analyzed the radiolabelled mAbs by FPLC size exclusion chromatography and autoradiography, for up to 4 days. We did not detect any form of immune complexes in the serum of any patient at these time points (Figs. 3.10, 3.11, 3.12). This was surprising, considering that the ^{90}Y-labelled preparations administered already contained Ig aggregates. These polymers, although visible in the ascitic fluid (Fig. 3.13a), seemed not to be present in the serum (Fig. 3.13b), indicating that their size may hinder their entry into the blood stream. It is possible that such aggregates may be catabolized by reticuloendothelial cells in the draining lymphatic system. Autoradiography also revealed that a small amount of ^{90}Yttrium was associated with a low molecular weight protein (around 67KD). This may be explained by the transchelation of the radioisotope to transferrin. Since the transport from the peritoneal cavity into the blood stream seems to be size-restricted it would be interesting to examine the kinetics of i.p. administered

IgM. This may be advantageous for treatment of intraperitoneal malignancies, since the radiolabelled antibody could reside in the peritoneal cavity, irradiating the tumour for a longer time.

Intraveneously injected radiolabelled biotin disappeared from the circulation rapidly. In addition, although the kinetics of the mAb-streptavidin conjugate were studied in only one patient, we found that it was cleared significantly faster than the unconjugated whole Ig. All patients responded to the mouse mAb, whereas in 3 out of the 5 patients we also detected anti-streptavidin antibodies (Figs. 3.15a and b). It is likely, therefore, that this two step approach increases the overall immunogenicity of the therapy. However, as shown in our preliminary clinical studies (Kalofonos et al., 1990), the biotin-streptavidin system may prove to be useful for tumour radioimaging purposes, since radiolabelled biotin would either bind to the tumour (because of the mAb-streptavidin), or be excreted rapidly.

Finally, our preliminary experiments using radiolabelled human EGF in monkeys were very encouraging. Since EGF is a human product, it is highly unlikely to trigger an immune response. In addition, it binds with high affinity to the EGF receptor (found on various malignant cells), it labels with radioiodines without loss of binding reactivity (Table 3.1), its size allows deep tumour penetration, and it is excreted by the kidneys within the first 24hr (Fig. 3.17). EGF should therefore provide a good alternative to antibody-mediated diagnosis and treatment of neoplasms. Growth factors like EGF, whose receptors are upregulated in neoplastic cells may therefore become important therapeutic molecules in the future. Other molecules in this category might be interleukin 4 and the ligand for c-erb-B2, since both the interleukin 4 and c-erb-B2 receptors are thought to be elevated in certain carcinomas (Gullick et al., 1986; Al Jabaari et al., 1989)

In this chapter, we have analyzed the *in vivo* behaviour of mouse monoclonal antibodies used for diagnosis and therapy of patients with cancer. These data provide the background against which strategies for achieving immunosuppression or tolerance to the mAb can be planned. Alternatives to mAbs have also been considered, and our initial data suggest that small site-specific reagents such as growth factors may ultimately be more effective for the treatment of malignancies.

Chapter 4

Immunosuppression by passive administration of anti-immunoglobulin antiserum

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4.1 Introduction

Passive administration of antibodies for the treatment of cancer is not a new idea (Hericourt and Richet, 1895; Pressman and Keighley, 1948). The development of large quantities of antibodies with single specificity (Köhler and Milstein, 1975) paved the way for an increasingly growing number of clinical trials; knowledge of the kinetics and biodistribution of anti-tumour antibodies in man, has put immunodiagnosis and immunotherapy under a new perspective. A large number of monoclonal antibodies (mAb) have been used so far; the fact that they are all of different origin, class, subclass, specificity, affinity, size, administered via various routes at a wide range of doses, and directed against many types of neoplasms, in relatively small numbers of patients, makes the overall evaluation of the results very difficult. It is not surprising therefore, that not only are these discrepancies in the kinetics of mAbs reported (discussed in chapter 3), but also their specificity and efficacy in diagnosis and treatment of cancer is often questioned (Bradwell et al., 1985; Epenetos et al., 1986b; Kemshead et al., 1987; Kalofonos et al., 1988; Freimanis et al., 1988; Kemshead et al., 1989).

However, one universal feature of this therapy is that the actual amount of therapeutic antibody that reaches the target is always less than 0.01% of the injected dose/gramme of tumour (Epenetos et al., 1986b; Richardson et al., 1986; Beatty et al., 1986; Coakham and Kemshead, 1987; Esteban et al., 1987; Zalutsky et al., 1989). Whether this is sufficient, remains to be proven. Nevertheless, the fact is that most of the antibody remains in the blood and extravascular space. This causes additional problems both in immunoscintigraphy, where the background from the blood pool "masks" the signal from the tumour area for at least 2 days, and in immunotherapy, where the "lethal" antibodies that reside mainly in the liver and spleen will non-specifically cause damage to normal tissues.

The rate of antibody clearance can be increased by the use of antibody fragments. These lack the Fc portion ($F(ab')_2$ and Fab), and, being smaller in size than whole immunoglobulins, are more rapidly catabolized (Wahl et al., 1983; Buchegger et al., 1983; Kalofonos et al., 1989b). However, fragmentation is often accompanied by low tumour accumulation and retention (Herlyn et al., 1983; Colcher et al., 1983; Harwood et al., 1985).

Other approaches have involved the administration of a second antibody in order to "chase out" the first one. Studies using anti-antibodies were reported as early as the 1960s, when I. L. Spar et al. showed a decrease in the excess of rabbit anti-fibrinogen antibody in rats, after administration of anti-rabbit immunoglobulin (Ig) antiserum (Spar et al., 1964). Similarly in clinical trials, R. J. McCardle et al. showed a dramatic reduction of polyclonal rabbit anti-human fibrinogen antibody from the circulation, after goat anti-rabbit Ig was administered to two patients (McCardle et al., 1966). Since then, a number of groups have used a second antibody in order to improve radioimaging and the therapeutic tumour/normal tissue ratio. Antiserum was administered alone or entrapped onto liposomes in animal (Sharkey et al., 1984; Begent et al., 1987; Senekowitsch et al., 1987; Klibanov et al., 1988; Pedley et al., 1989; Blumenthal et al., 1989) and clinical studies (Begent et al., 1982; Begent et al., 1987). All evidence suggested that there was a rapid increase of the tumour/blood ratio, without significant influence on the anti-tumour effect. Moreover, formation and transient accumulation of immune complexes mainly in the liver and spleen did not seem to cause serious side effects.

Passive administration of a second antibody, has also been used for the *in vivo* clearance of various antigens (Campbell et al., 1980), and in some cases was shown to prevent sensitization by various immunogens (Wigzell, 1966; Ryder and Schwartz, 1969; Rowley et al., 1969), most notably the rhesus system in humans (Freda et al., 1967; Woodrow, 1970). In related studies, administration of sheep red blood cells (SRBCs) to animals failed to elicit a humoral immune

response when allogeneic or syngeneic anti-SRBC antiserum was injected prior to or simultaneously with the antigen (Murgita and Vas, 1972; Coutinho and Forni, 1981; Heyman and Wigzell, 1984). This suppression of the primary response was specific for the antigen injected (Murgita and Vas, 1972). Moreover, antibodies against a hapten or the carrier prevented the anti-hapten primary humoral response (Takatsu et al., 1974; Collisson et al., 1979; Brüggemann and Rajewsky, 1982). The *in vivo* immunosuppressive effect due to passive immunization seems to be more affinity- rather than isotype-dependent, since in experiments using mAbs, those with the highest affinity for erythrocyte-associated antigens showed the maximum suppressive activity (Heyman and Wigzell, 1984). Similarly, administration of anti-bovine γ globulin (BγG) to rats rendered the animals unresponsive to a further challenge of BγG (Rowley et al., 1969). In more recent experiments, the primary response of monkeys against mouse mAbs was reduced after they had received multiple injections of syngeneic anti-mouse Ig antiserum. Interestingly, in the latter study the immunosuppressive effect of passive immunization was greater than the effect of an immunosuppressive drug (cyclosporin A) (Lowder et al., 1987).

The mechanism of action of this Ig-mediated immunosuppression is not clear. "Masking" of antigenic determinants is one explanation (Uhr and Möller, 1968). However, rapid clearance of antibody-antigen complexes and transfer to catabolic sites of the reticuloendothelial system (RES), or direct disturbance of Fc receptor bearing cells, such as B-, T- and antigen presenting cells, have been postulated as important factors (Ryder et al., 1969; Hoffmann and Kappler, 1978; Kölsch et al., 1980; Hoffmann, 1980; Heyman and Wigzell, 1984).

In this chapter, we describe the effect of human anti-mouse Ig antibodies administered to patients with cancer, who were undergoing radiolabelled (^{131}Iodine) murine mAb treatment. The aim of this study was to examine whether the kinetics of the therapeutic mouse mAb would be altered (thus

reducing the non-specific whole body radiation), and if the expected faster clearance would reduce immunogenicity of the xenogeneic Ig.

As discussed earlier, systemic administration of anti-mAb antiserum, following intravenous injection of the therapeutic mAb, resulted in a dramatic drop of the mAb levels from the circulation. However, the second antibody was given hours (Sharkey et al., 1984; Senekowitsch et al., 1987; Pedley et al., 1989) or days (McCardle et al., 1966; Begent et al., 1982; Begent et al., 1987; Blumenthal et al., 1989) after the therapeutic antibody, so that there was no reduction of the anti-tumour effect. In our experiments, all patients received the therapeutic mAb i.p. and the second antibody i.v.. From previous studies (chapter 3), we found that i.p. administered ^{131}Iodine-labelled mAb gradually migrates into the blood stream, reaching a peak at approximately 2 days post administration that corresponds to at least 25% of the total injected dose. This considerable amount of radioactivity adds to the whole body radiation dose, without adding anything to the tumour radiation effect. Moreover, the unnecessary circulating mAb may also facilitate the immune response against itself. By intravenously administering the second antibody, we minimized any interference with the anti-tumour effect, and hoped that both non-specific irradiation and immunogenicity would be reduced.

Unlike previous studies, we did not use liposomally entrapped anti-mAb antibodies, but chose to employ second antibody alone, since it has been shown that the liposome-Ig complexes accumulate in the liver, spleen and other major organs for a long time (Begent et al., 1982). The anti-mAb antiserum used was affinity purified from patients who had developed anti-mouse Ig antibodies in previous studies. Human (though allotypically different) antibodies were essential for such immunosuppressive studies, since foreign antiserum (Begent et al., 1982) would enhance rather than suppress the anti-xenogeneic Ig response.

4.2 Materials and methods

4.2.1 Monoclonal antibody: The mouse mAb HMFG1 was administered i.p. to patients with ovarian cancer. This is a mouse IgG1 that recognises a large mucin expressed on the cell surface of many carcinomas of epithelial origin (Taylor-Papadimitriou et al., 1981).

4.2.2 Patients: Fourteen patients with advanced ovarian cancer received radio-immunotherapy for the first time, after cytoreductive surgery and post operative chemotherapy had failed. Formaldehyde fixed, parrafin embedded sections of tumour obtained at each patient's original operation, demonstrated positive immunoperoxidase staining with HMFG1. The RPMS, Hammersmith Hospital's Ethics Committee approved a protocol where 5 patients could enter a pilot study, to explore the potential of i.v. administered human anti-mouse immunoglobulin antibodies (HAMA).

Donors for exogenous HAMA were chosen from patients with ovarian cancer who had received i.p. radiolabelled HMFG1 for therapy in other studies, had developed anti-mouse immunoglobulin antibodies, and who showed no evidence of any autoimmune disease. Five of these patients received mAb therapy for a second time, after they had relapsed. Written informed consent was obtained from all patients.

4.2.3 Purification of human anti-mouse immunoglobulin antibodies: A blood sample (approximately 200 ml) was venesected from patients with high anti-mouse Ig titres, and the serum was removed by centrifugation, after the blood had been left to clot at 37°C for 4-5 hours. A small aliquot was tested for the presence of Hepatitis B surface antigen and antibodies to human immunodeficiency virus, by the Dept. of Virology, Hammersmith Hospital, and found to be negative. The sera were stored frozen at -20°C until use.

Anti-HMFG1 antibodies were purified from these sera by affinity chromatography, using HMFG1 coupled onto CN-Br-activated Sepharose 4B beads (Pharmacia, Sweden). Dry beads were washed with 1mM HCL, and incubated with 10 mg/ml HMFG1 in coupling buffer (0.1M $NaHCO_3$/0.5M NaCl, pH 8.5) for 2 hr at RT. The mixture was then transferred to blocking buffer (1M ethanolamine, pH 8.0) and incubated for a further 2 hr at RT. Finally, the gel was washed twice with sterile coupling buffer, followed by sterile 1M NH_3 and PBS, pH 7.4. The HMFG1 column was kept in a cold room (4°C) throughout this study. No preservatives were added at any stage.

The patients' sera were thawed, centrifuged (to remove large aggregates), and filtered (0.22 μm filter, Millipore, France) before purification of the anti-mouse Ig antibodies. The column was washed with sterile PBS, the serum was added, and the bound anti-HMFG1 antibodies were eluted using 1M NH_3 (pH approximately 12.0). Fractions were collected (Frac 100, Pharmacia), and those containing the desired anti-mouse Ig antibodies were pooled together. These fractions were determined by measuring the absorbance at 280 nm (UV-1, Pharmacia). As Figure 4.1 shows, two peaks were recorded when the absorbance profile was followed by a chart recorder (Rec-492, Pharmacia). The first peak represents the unbound serum proteins and the second the bound and eluted anti-HMFG1 antibodies. Since they were eluted in 1M NH_3, they were immediately neutralized (with 0.5M HCL), and dialyzed overnight against sterile PBS at 4°C. After the concentration was determined by measuring the absorbance at 280 nm (as described in chapter 3, section 3.2.3a), the purified HAMA was filter sterilized (0.22 μm filter), and stored at 4°C until further use.

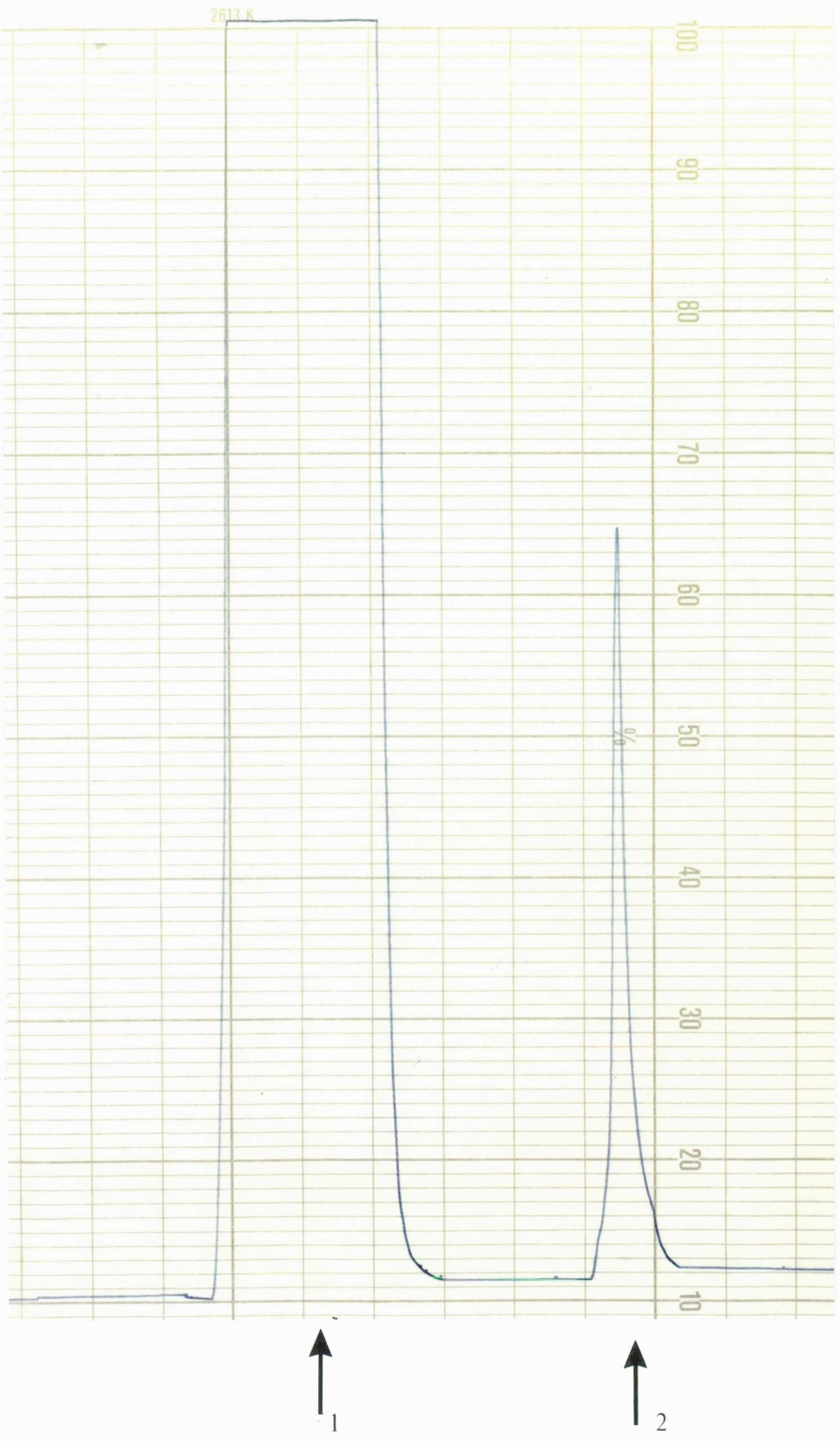
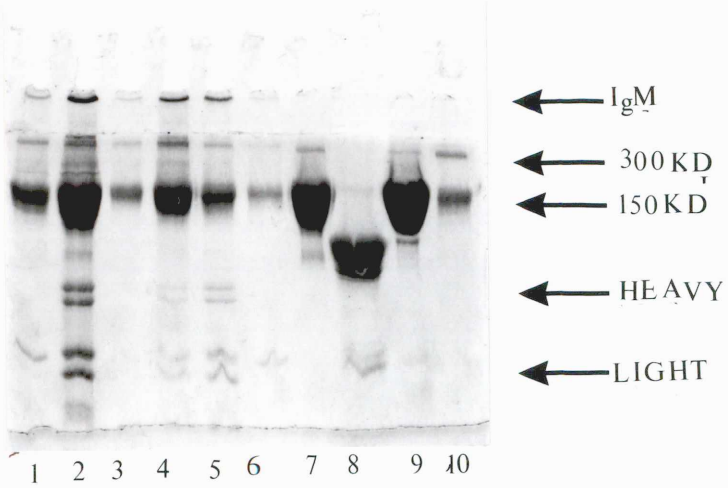


Figure 4.1: Purification of HAMA by affinity chromatography. The first peak (1) corresponds to unbound serum proteins, and the second peak (2) to purified HAMA (as measured by absorbance at 280 nm).

4.2.4 Quality control of the purified human anti-mouse immunoglobulin antibodies: Purified HAMA was tested for the presence of non-Ig proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). All preparations were found to contain only Ig. An example is shown in Figure 4.2a: this is a 8% non-reduced, Coomassie blue stained SDS-PAGE, where HAMA from 6 different patients is shown in lanes 1-6, lanes 7 and 9 contain HMFG1, lane 8 HMFG1 $F(ab')_2$, and lane 10 polyclonal human IgG (Sigma). In addition to monomeric IgG, all HAMA samples also contained free heavy and light chains, IgG dimers, and possibly IgM.

The presence of IgG (and IgG aggregates) was confirmed by Western blotting, where the proteins from the SDS-PAGE gel were transferred onto nitrocellulose paper (Biorad, UK), using a blotting apparatus (Biorad). After transfer was completed, the paper was incubated in 2.5% milk powder in PBS (Marvel, Premier Brands Ltd., Birmingham, UK) for 30 min, in order to block non-specific binding sites. It was then rinsed in washing buffer (0.5% milk powder in PBS), and incubated with goat anti-human IgG (human γ-chain specific) conjugated to horseradish peroxidase (HRP) (Sigma), for 1 hour. The paper was washed, and incubated for 10 min with the substrate diaminobenzidine (DAB, Sigma). A representative example of 5 HAMA preparations is shown in Figure 4.2b, where they contained IgG, and in some cases IgG dimers (lane 4) and IgG breakdown products (lanes 2 and 3).

FPLC size exclusion chromatography of the HAMA (using Superose 6, Pharmacia), revealed that the percentage of IgM and IgG aggregates was very low (<10%) compared to the IgG fraction. In the example shown in Figure 4.3, the arrows indicate the location of IgM, IgG dimers and IgG.



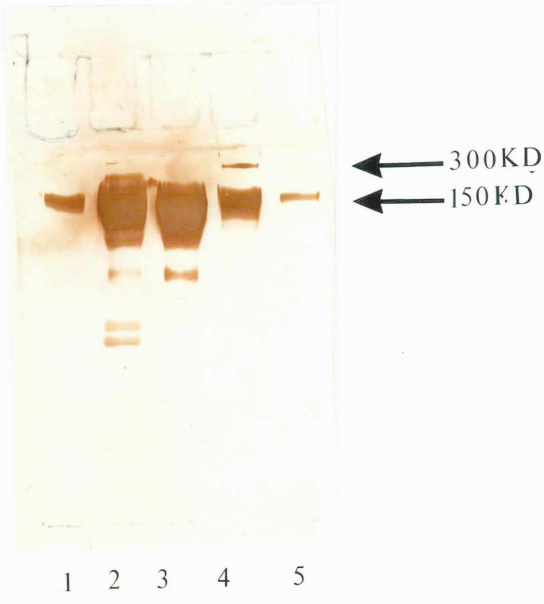


Figure 4.2: a) SDS-PAGE of 6 HAMA preparations (lanes 1-6). Lane 7 and 9 contain HMFG1 mAb (IgG1), lane 8 HMFG1 $F(ab')_2$ fragment and lane 10 human IgG (polyclonal). Arrows indicate IgM, IgG, IgG dimers, heavy and light chains.
b) Western blot of 5 HAMA preparations. IgG (150KD) and IgG dimers (300KD) are indicated by the arrows.

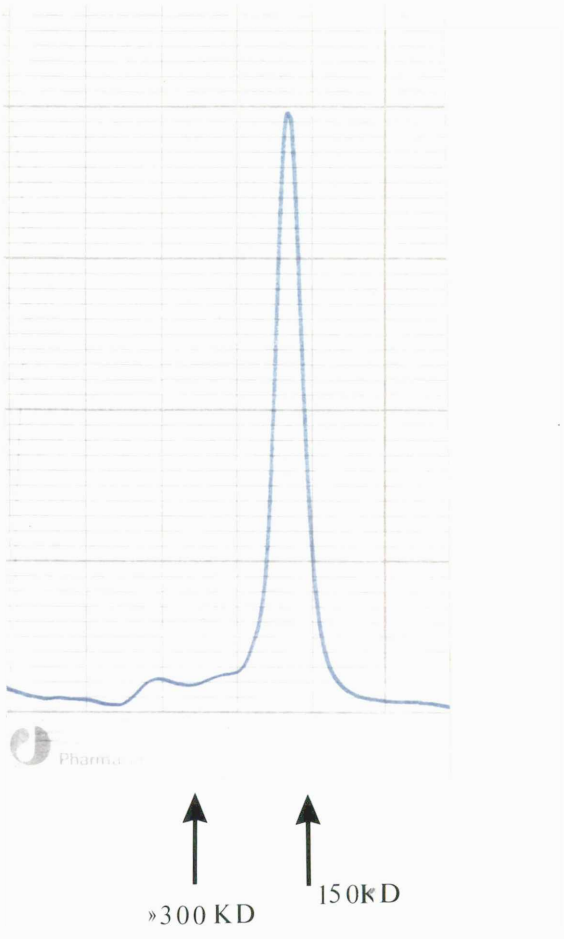


Figure 4.3: FPLC profile (Superose 6 column) of purified HAMA. Monomeric IgG as well as higher molecular weight immunoglobulins (IgG dimers, IgM) are shown by the arrows.

The immunoreactivity of the HAMA preparations was tested by an ELISA, as described in chapter 2. Briefly, 96-well plates were coated with 0.5 μg/ml HMFG1, and the HAMA was applied (1:10 serial dilution), followed by a species-specific sheep anti-human Ig-HRP conjugate. The substrate used was ABTS, and the results, expressed as absorbance at 405 nm, were obtained by use of a multiscan spectrophotometer. All preparations were found to retain their capacity to bind HMFG1. They were then pooled, their concentration estimated spectrophotometrically as described above, and stored under sterile conditions at 4°C. Prior to *in vivo* use, the sterility and presence of pyrogens was further tested by an independent laboratory (Blood Products Laboratories, Elstree, UK).

In order to determine to what extent the pooled anti-mouse Ig antibodies were recognizing the Fc portion of the murine mAb, HMFG1 and HMFG1 $F(ab')_2$ fragments were coated on the same plate, and an ELISA was performed as described above. We found that a large proportion (46% as estimated by the area under the curves) was directed against the Fc part of the mouse IgG (open squares, Fig. 4.4). Preincubation of the HAMA with polyclonal human IgG (Sigma) removed some of the anti-HMFG1 response, whereas the anti-HMFG1 $F(ab')_2$ reactivity remained unaffected (Fig. 4.4). There was therefore a small percentage of our HAMA preparation (12% as estimated by the area under the curves), that recognised both human and mouse Fc.

Using a rabbit anti-human IgM-HRP conjugate (human μ-chain specific, DAKO, Denmark), as second layer in the above ELISA, we confirmed the SDS-PAGE (Fig. 4.2a) and FPLC (Fig. 4.3) results, showing that approximately 13% (as estimated by the areas under the curve) of the HAMA was IgM (Fig. 4.4). These IgMs were mostly rheumatoid factors, since a large proportion of their reactivity (60%) could be absorbed out by preincubation with polyclonal human IgG (Sigma) (Fig. 4.4) and since they did not bind to HMFG1 $F(ab')_2$ fragment (Fig. 4.4).

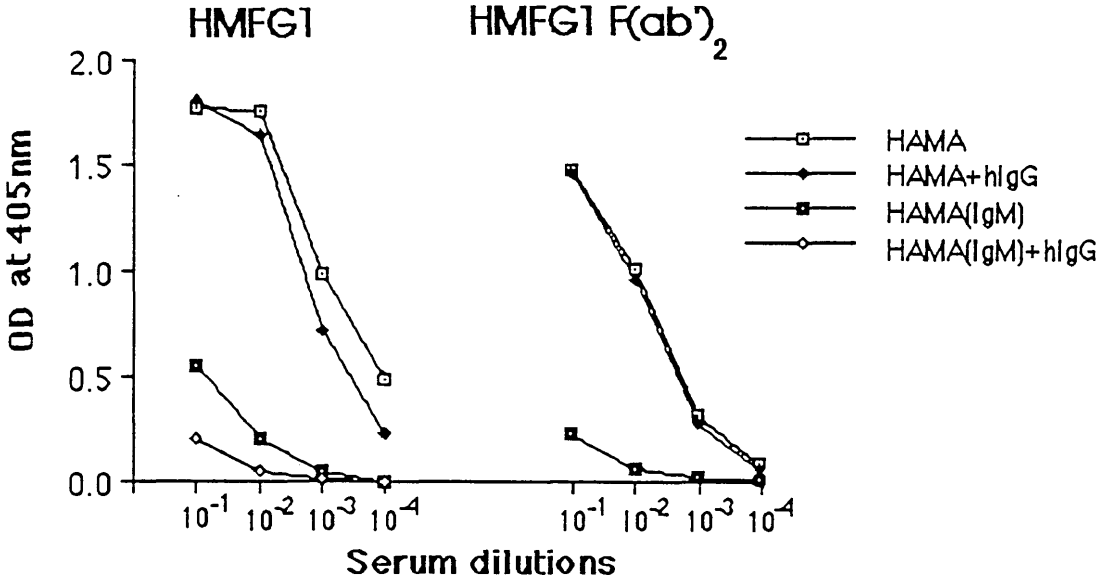


Figure 4.4: Reactivity of purified HAMA against whole HMFG1 and HMFG1 F(ab')2 fragment, as measured by ELISA.
On the vertical axis is the absorbance (measured at 405 nm); on the horizontal axis are the HAMA serial dilutions.

—□— Reactivity of total HAMA

—◆— Reactivity of total HAMA after incubation with polyclonal human IgG

—■— Reactivity of the IgM fraction of HAMA

—○— Reactivity of HAMA IgM after incubation with polyclonal human IgG

4.2.5 Radiolabelling: The mouse mAb HMFG1 was supplied by Unipath Ltd. (Bedford, UK) and Compagnie Oris Industrie (Saclay, Paris, France), labelled with ^{131}Iodine, at a specific activity of approximately 10 mCi/mg. Sterility, labelling efficiency and immunoreactivity were tested by the suppliers. However, we always performed a 10% trichloroacetic acid precipitation paper chromatography, prior to administration, in order to confirm that the activity incorporated onto the protein was >90%.

HAMA was radiolabelled with ^{125}Iodine, in order to measure its kinetics after i.v. administration. This was done using the iodogen-coated vial method, as described in chapter 3 (section 3.2.3c). The specific activity was 0.02-0.05 mCi/mg.

4.2.6 Treatment schedule: 10-15 mg of the therapeutic ^{131}I-labelled HMFG1 (113-130 mCi) was administered i.p., by Dr. S. Stewart. This was performed via a small catheter inserted into the peritoneal cavity. The labelled mAb was slowly infused with 1.5-2.0 l of 0.9% saline. All patients were given KI for 2 days prior to and 28 days following therapy, in order to minimize the iodine uptake by the thyroid.

HAMA, 30-36 mg, was administered i.v. (by Drs. S. Stewart and V. Hird), as a bolus injection, in either one or two doses, at various time points after the i.p. infusion of the ^{131}I-HMFG1. The treatment schedule is shown in Table 4.1.

4.2.7 Pharmacokinetics: Blood samples were taken at regular time points (Drs. S. Stewart and V. Hird). After clotting, the serum was removed and the radioactivity was measured in a γ-counter (Minaxiγ, Packard, USA). The kinetics of the i.p. administered ^{131}I-HMFG1, in 5 patients who received exogenous HAMA intravenously, were compared with the kinetics of the i.p. injected mAb in the presence and absence of endogenous anti-mouse Igs, as

	^{131}I-HMFG1 i.p.		HAMA i.v.	
	Amount (mg.)	*Radioactivity (mCi.)*	*Amount (mg.)*	*Time (hr.)*
Patient 1	10	117	30	17
Patient 2	15	130	18	0
			18	36
Patient 3	10	115	18 *	17
			18 *	39
Patient 4	10	113	15 *	0
			15 *	39
Patient 5	10	113	15 *	18
			18 *	41

Table 4.1: Five patients with ovarian cancer were treated with ^{131}Iodine labelled HMFG1 monoclonal antibody intraperitoneally. Purified HAMA was administered i.v. at various time points (last column) after the injection of the therapeutic mAb.
* ^{125}Iodine-labelled HAMA for kinetic studies.

already described in the previous chapter (chapter 3, section 3.3.2, Fig. 3.9). The blood clearance of the ^{125}I-HAMA was also measured in 3/5 patients.

In addition, urine was collected for 5 days after ^{131}I-HMFG1 administration, in order to measure the ^{131}Iodine excreted. Urine ^{131}Iodine activity was measured in a γ-counter, by extrapolating the counts for 2 ml samples to the total volume. Urine sampling and counting were performed by Drs. S. Stewart and V. Hird.

4.2.8 Radiation to liver and bone marrow: The radiation dose to the haemopoietic bone marrow and liver ($D_{[organ]}$) was calculated by Dr. S,. Stewart, using the MIRD formulation (Snyder et al., 1978):

$$D_{[organ]} = \% \times A \times S_{[organ\text{-}organ]} + A \times S_{[whole\ body\text{-}organ]}$$

as described in detail in our recent publication (Stewart et al., 1989). A is the integrated blood isotope activity in μCi-hours for the 100 hr after treatment, S is the factor S of the MIRD formulation (Snyder et al., 1978), and % is the percentage of total blood present in the liver or bone marrow, at every time point. In addition, Dr. M. Myers (Department of Medical Physics, Hammersmith Hospital) monitored the radiation activity over the liver in 3 patients, using a specially shielded scintillation counter. Measurements were taken from these areas every 20 min after i.v. injection of HAMA, for up to 5 days.

4.2.9 Immune complex formation: This work was done by Dr. K. Davies, and is described in detail in our recent publication (Davies et al., 1990). The precipitation of *in vivo* formed immune complexes was performed by addition of 4% polyethylene glycol (PEG, BDH. UK) to plasma and serum samples, followed by centrifugation at 1,000g. The complex size was determined by a 10-50% sucrose gradient and overnight centrifugation at 25,000g (Johns and Stanworth, 1975). The presence of C3d on the immune complexes, as well as

their capacity to bind C1q, was assayed by a direct solid phase radioimmunoassay, when anti-C3d or C1q was coated onto microtitre plates (Immulon II Removawells, Dynatech, UK), followed by the addition of the serum.

4.2.10 Complement assays: This section was also performed by Dr. K. Davies. All assays are discussed in detail in our recent publication (Davies et al., 1990). Briefly, plasma C3, C4 levels were measured using a single radial immunodiffusion, in 1.2% CFD-agarose gel containing anti-C3 and anti-C4 antibodies; CH50 was estimated by a standard haemolytic assay (Lachmann et al., 1973); CR1, C3q and C4d on erythrocytes were measured using a radioligand binding assay (Walport et al., 1985); C3 conversion was demonstrated by crossed immunoelectrophoresis of plasma samples in 1% agarose (Johnstone and Thorpe, 1987); finally, C3a and C5a levels were measured by a solid phase radioimmunoassay (Jose et al., 1983).

4.2.11 Measurement of the anti-mouse immunoglobulin response: The patients' humoral anti-mouse Ig response was assayed 14 days after the i.p. administration of the ^{131}I-HMFG1, using an ELISA, as described in chapter 2 and section 4.2.4 of this chapter. Briefly, after HMFG1 was coated onto microtitre plates, the test serum was added, followed by a species-specific anti-human Ig-HRP linked reagent. The substrate ABTS was used, and the absorbance at 405 nm measured in a multiscan spectrophotometer.

Using our highly purified HAMA preparation, we were able to plot a standard curve and quantitate the patients' response: the test serum-titration curve was plotted onto a semi-logarithmic paper together with the standard curve. As shown in the example of Figure 4.5, we chose an absorbance value which coincided with the linear section of the sigmoidal curve for both test and standard serum. The known concentration of the standard curve (at that

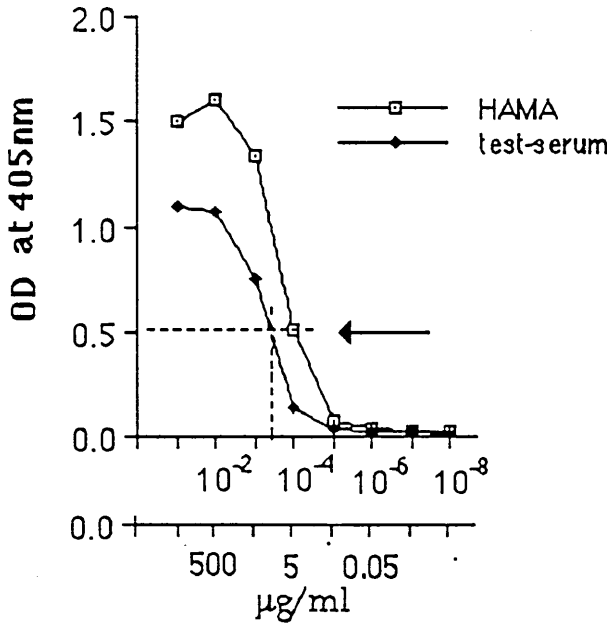


Figure 4.5: Quantitation of the patients' anti-mouse Ig antibodies by ELISA. An unknown concentration of the test serum (rhombus) is directly correlated with a known concentration of the standard purified HAMA (open squares). Both values chosen belong to the linear section of the titration sigmoidal curve. On the vertical axis the results are given as absorbance at 405 nm, whereas the horizontal axis shows serum dilution (for the test serum) and actual concentration in µg/ml (for the standard).

absorbance value), was directly referred to the corresponding dilution of the test serum.

However, as described in chapter 2, accurate quantitation of the anti-Ig response is almost impossible, due to differences in affinity among different samples. We have examined the accuracy of our results, by performing the following two tests. Firstly, on the same ELISA plate, we compared the standard HAMA used in the above quantitative measurements, with the same preparation in the presence of 3 different human serum samples (previously shown to contain no anti-mouse Ig antibodies). As shown in Figures 4.6a, b and c, the values obtained from our reference purified HAMA were only slightly higher (3.6 $\pm$ 1.3%, according to area under the sigmoidal curve), than when it was tested in the presence of normal human serum. Secondly, 20 ml of blood was venesected from 2 patients, who had developed anti-HMFG1 antibodies due to previous immunotherapy. The serum was removed, and the anti-HMFG1 antibodies were quantitated in our ELISA system. The HAMA was found to be 0.33 and 0.21 mg/ml serum respectively. Subsequently, the HAMA from these 2 sera was purified by affinity chromatography, as described previously (section 4.2.3), and the anti-HMFG1 levels, as measured spectrophotometrically, were found to be 0.37 and 0.24 mg/ml serum respectively. The latter was assumed to be the total true value of anti-HMFG1 antibodies in these 2 sera, since all anti-mAb antibodies were bound and eluted from the column, as tested by ELISA. The 10.8-12.5% difference observed, was thought to be a good approximation, considering the wide variability among the different test sera.

4.3 Results

4.3.1 Pharmacokinetics: Figure 4.7a shows the blood levels of ^{131}I-HMFG1 in the 5 patients who received i.v. HAMA. The values are expressed as percentage

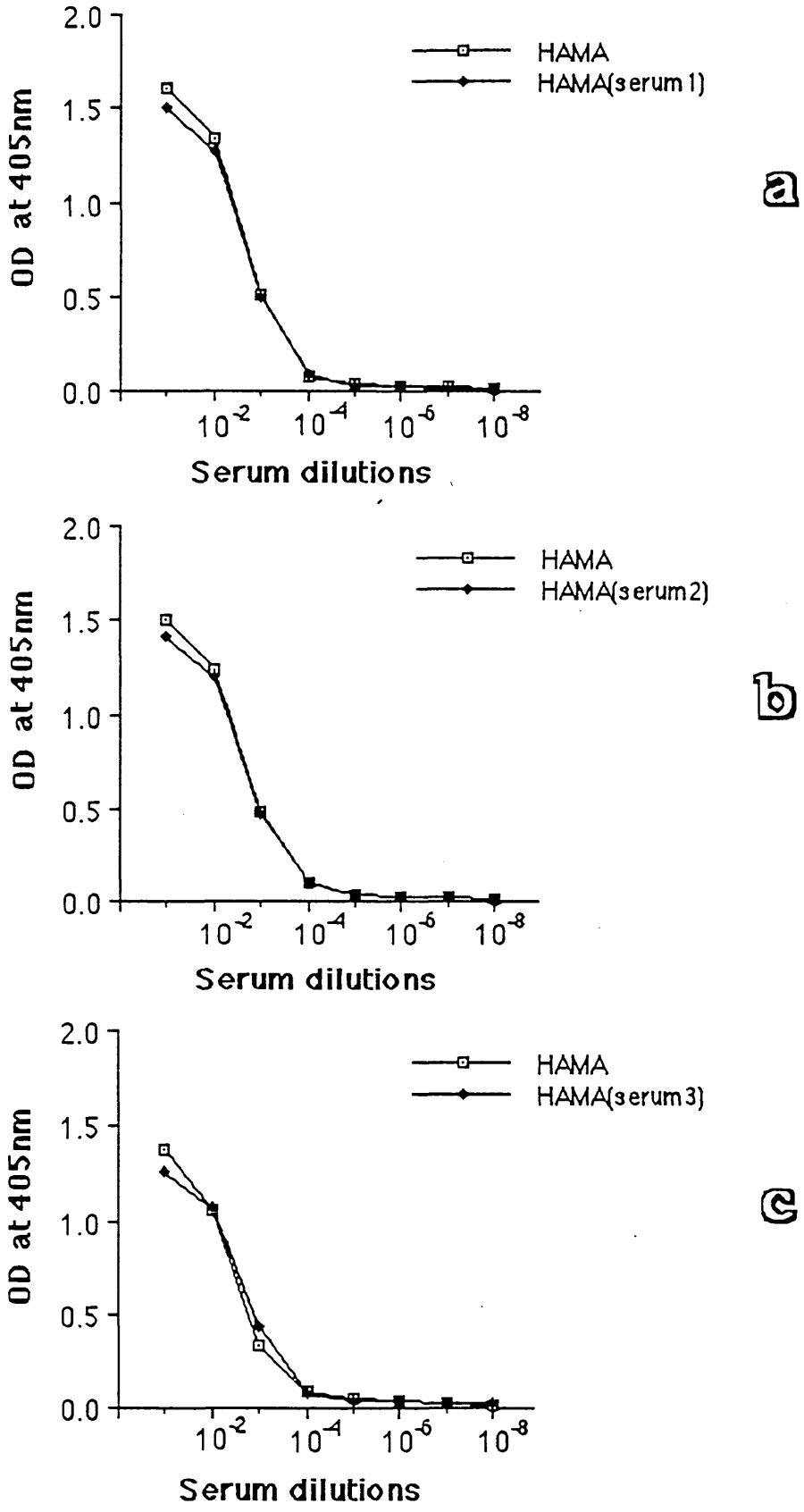


Figure 4.6: Reactivity of HAMA against HMFG1 as measured by ELISA. Comparison between HAMA alone (open squares) and HAMA mixed in 3 different normal human sera (rhombus in a, b and c respectively).

of injected radioisotope activity, measured over a period of 5 days (120 hr) after i.p. injection of the mAb. The activity in the blood is compared with the mean activity measured in 18 patients, who received the ^{131}I-HMFG1 without i.v. exogenous HAMA (Fig. 4.7a), and in 5 patients, who had developed HAMA due to previous therapies (Fig. 4.7b), as previously described (chapter 3, section 3.3.2). We found that in all patients the amount of ^{131}I-HMFG1 fell sharply 10-20 min after the i.v. administration of exogenous HAMA. However, as soon as the concentration of injected HAMA became less than 20% of the initial amount injected, ^{131}I-HMFG1 accumulated again in the blood. We estimated that the total activity present in the circulation in the 5 patients who received exogenous HAMA was 11.4% (mean), compared to 28% in those who did not receive i.v. HAMA, and 5% in those who had developed their own anti-mouse Ig antibodies (chapter 3, section 3.3.2).

The kinetics of HAMA (labelled with ^{125}Iodine) in 3 patients is shown in Figure 4.8. The time of injections is indicated by the arrows. We found that HAMA is rapidly removed from the circulation, as soon as it becomes complexed with the mouse mAb. Free iodine released from the catabolized antibody was excreted in the urine. 50% of the injected radioactivity was excreted within 45 hr of i.p. administration of the ^{131}I-HMFG1, in the 5 patients who received i.v. HAMA (S. Stewart, personal communication). This was compared with 60 hr for the patients who did not receive exogenous HAMA, and 32 hr for the patients who had developed their own anti-mouse Ig antibodies, due to previous therapies. By day 5, 85 and 92% of the total radioactivity had been excreted by the patients with i.v. injected HAMA and endogenous HAMA respectively, compared with 67% excreted by the patients with no anti-mouse Ig antibodies.

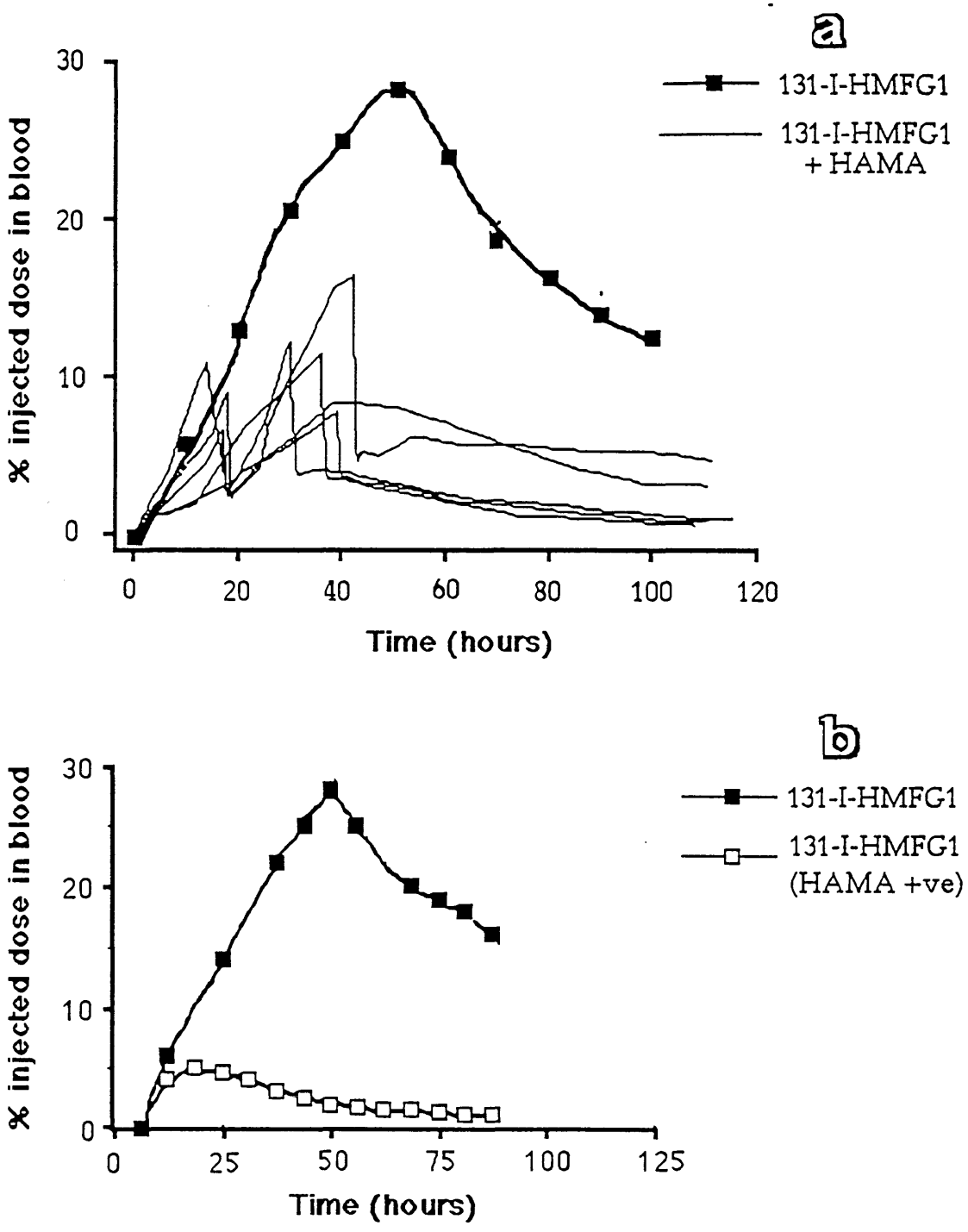


Figure 4.7: Level of radiolabelled mouse monoclonal antibodies in the blood, after i.p. administration in patients who had neither endogenous nor exogenous HAMA (solid squares in Figs. a and b), compared with a) five patients who received i.v exogenous HAMA (plain lines), and b) five patients who had developed anti-mouse Ig antibodies due to previous treatments (open squares).

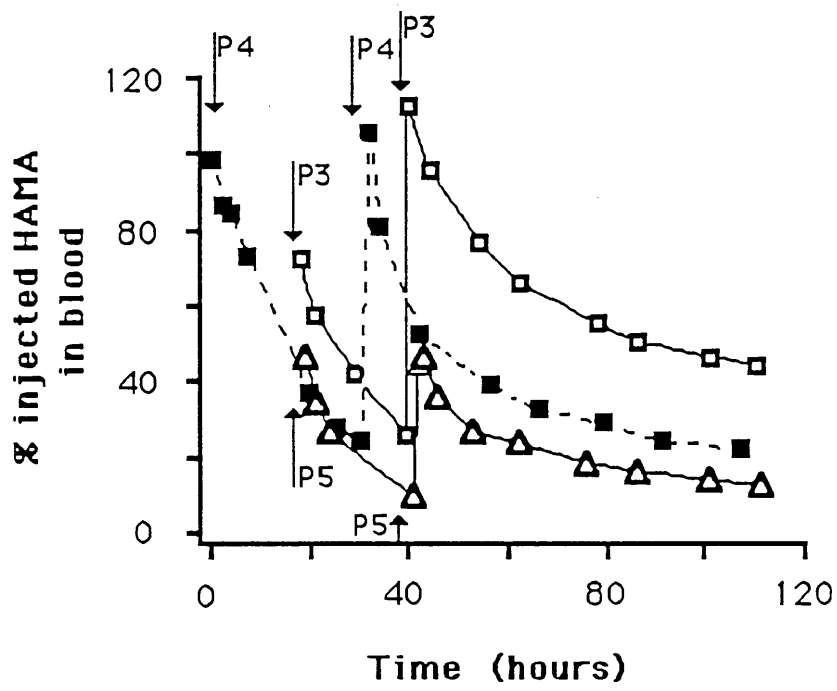


Figure 4.8: Kinetics of i.v. administered HAMA in 3 patients (P3, open squares; P4, closed squares; P5, open triangles). The time of injection is indicated by the arrows.

4.3.2 Immune complexes: The *in vivo* formation of immune complexes between the i.p. administered ^{131}I-HMFG1 and the i.v. administered ^{125}I-HAMA in the circulation, was studied in 3 patients (Patients 3, 4, 5, Table 4.1) by Dr. K. Davies, and described in detail in our recent publication (Davies et al., 1990). It was found that at the time of the HAMA injection, the molar ratio of HAMA/mAb ranged between 15:1 and 38:1. PEG precipitation revealed that the radioactive material precipitated in the blood rose after commencement of the HAMA injection. Sucrose density gradient centrifugation showed that prior to HAMA administration, only a 7S ^{131}Iodine peak was observed in the plasma. However, 5 min after administration of ^{125}I-HAMA, a 7S ^{125}Iodine peak was also observed. Gradually, both ^{131}Iodine and ^{125}Iodine 7S peaks were reduced, giving rise to large immune complexes with a sedimentation coefficient of 19-25S. In addition, only a small percentage of complexes were able to bind to erythrocytes. Finally, peak complex binding to both C1q and C3d was observed 15 min after HAMA i.v. administration.

4.3.3 Complement activation and CR1 levels: The above observations indicated that complement activation resulting from immune complex formation was occurring in all 3 cases. In addition, C3, C4 and CH50 levels fell (30-50%) within 1 hr after HAMA administration. Although C5a was not detectable in plasma, C3a levels rose 10-fold. Fixation of C3dg and C4d on peripheral blood erythrocytes was observed in all patients during the first 2 hr of the HAMA injection. A fall in CR1 numbers was observed in all cases.

4.3.4 Radiation to liver and bone marrow: The radiation dose to liver and bone marrow was calculated by Dr. S. Stewart, applying the MIRD formulation. In the 5 patients who received exogenous HAMA the mean dose to the liver/mCi of ^{131}I-HMFG1 in the circulation was 0.11 cGy, compared to a mean of 0.44 cGy in the 9 patients who did not receive HAMA. Similarly, the dose to the bone marrow was 0.21 cGy and 0.97 cGy for the above two groups

respectively. The ^{131}Iodine radioactivity measured over the liver, rapidly increased 15 min after administration of HAMA. However, this increase did not last longer than 10 hours, when the radioactivity levels fell back to expected levels (i.e. comparable to those seen in the absence of HAMA) (M. Myers, personal communication).

4.3.5 Anti-mouse immunoglobulin response: The anti-mouse Ig response was monitored in the 5 patients who received ^{131}I-HMFG1 i.p. and exogenous HAMA i.v. This was compared with the response of 9 patients who received only ^{131}I-HMFG1, i.p.

The results are shown in Table 4.2, and are expressed in μg endogenous HAMA/ml serum. We found that 9 of the 10 patients tested for pre-existing anti-mouse Ig antibodies, had low levels of anti-HMFG1 antibodies prior to administration. The remaining patient (No 1) had relatively high levels. By day 14 we found that all 14 patients tested had developed an immune response against the HMFG1. There was no statistically significant difference between the group that received i.p. HMFG1 and i.v. HAMA and the group that received i.p. HMFG1 alone (Table 4.2).

Exogenous (i.v. administered) HAMA did not interfere with the quantitation of the endogenous HAMA measured on day 14. As shown in Figure 4.8, in the 3 patients measured, by day 5 (120 hr) the amount of ^{125}I-HAMA was 50, 30 and 15% of the total injected dose respectively. On the assumption that the total serum volume is 2,500 ml, the above percentages translated to 6.4, 3.8 and 1.9 μg/ml (serum), that is, a concentration that is already very low, and which by day 14 would be negligible.

i.p. HMFG1 and i.v. HAMA

Patients	*HAMA (day 0) (μg/ml)*	*HAMA (day 14) (μg/ml)*
1	166	1,250
2	7	53
3	19	480
4	5	50
5	3	26

i.p. HMFG1

Patients	*HAMA (day 0) (μg/ml)*	*HAMA (day 14) (μg/ml)*
6	41	620
7	12	67
8	N.T.	32
9	N.T.	25
10	37	714
11	28	881
12	N.T.	40
13	N.T.	1,590
14	14	324

Table 4.2: Humoral responses against the HMFG1 mAb, administered i.p. to patients for therapy. This was measured by ELISA, before and 14 days after therapy, and is expressed as μg of anti-mouse Ig antibodies/ml of serum. Patients 1-5 also received i.v. exogenous HAMA, whereas patients 6-14 received i.p. HMFG1 alone.
N.T. = Not tested.

4.3.6 Toxicity: We did not observe any serious toxicity related to the administration of exogenous HAMA. Two patients complained of hot flushes associated with tachycardia immediately after injection, and two patients developed a rigor 20 min after HAMA injection. Both side effects were self-limited, and did not need any medical intervention. Four of the 5 patients demonstrated a degree of myelosuppression (S. Stewart et al., 1990).

4.4 Discussion

Polyclonal human anti-mouse Ig antibodies were administered i.v. to patients with ovarian cancer who were undergoing therapy with intraperitoneal radiolabelled murine anti-tumour mAbs (Table 4.1). HAMA were affinity purified and found to be mostly IgG (approximately 87%) (Fig. 4.3). The majority (60%) of the remainder consisted of IgM rheumatoid factors that recognised the Fc portion of both mouse and human IgG (Fig. 4.4).

The kinetics of i.p. administered ^{131}I-HMFG1 were significantly altered in the 5 patients who received i.v. HAMA. The overall mean concentration of radiolabelled mAb found in the blood was 11.4% of the injected dose, compared to 28% measured in 18 patients who did not receive exogenous HAMA, and 5% in 5 patients who had endogenous anti-mouse Ig antibodies from previous treatments (Figs. 4.7a and b, and chapter 3 for details of the two latter groups). Rapid catabolism of the radiolabelled antibody resulted in increased excretion of ^{131}I from the kidneys. By day 5, over 85% of the total dose was excreted from the patients who received exogenous HAMA, compared to 67% in patients without HAMA.

Large immune complexes were formed between HAMA and the mAb, which were rapidly removed from the circulation (Fig. 4.8) and accumulated mainly in the liver. The transient increase in hepatic radiation did not last more than

10 hr, and the overall radiation to bone marrow and liver - as estimated by the MIRD formulation - was reduced to approximately 25% of the dose received by those tissues in patients who did not have HAMA.

Immune complex formation led to complement activation, resulting in a rapid fall of C3, C4 and CH50 levels, as well as CR1 numbers on erythrocytes. Although C3a levels rose 10-fold, C5a was not detectable. Considering the drop in white blood cell counts, immediately after immune complex formation, it is very likely that C5a binds to their cell surface, resulted in rapid removal and catabolism by the RES and lungs (Lewis et al., 1986).

All patients responded to the mouse mAb administered (Table 4.2). Unfortunately, the small number of patients in this study, together with genetic variations and different levels of pre-existing anti-mouse Ig antibodies, made it difficult to make any direct comparison between the group that received exogenous HAMA and that which did not. The fact that all patients had pre-existing antibodies against the mAb prior to immunotherapy generally reduces the immunosuppressive effect of passive immunization, since it is more effective for primary, IgM, responses (Wigzell, 1966; Heyman and Wigzell, 1984). Although the majority of these pre-existing anti-mouse Ig antibodies were cross-reactive IgM rheumatoid factors (Courtenay-Luck et al., 1987), we have not examined whether our 5 patients had IgG anti-mouse Ig antibodies as well, and to what extent. In addition, the timing of the i.v. injections of HAMA appears to be a crucial parameter (Wigzell, 1966; Murgita and Vas, 1972). We were therefore unable to fully assess the immunosuppressive effects of passively administered anti-serum against the administered xenogeneic immunoglobulin.

The amount of HAMA administered is another important factor. In our experiments, the molar ratio of HAMA/^{131}I-HMFG1 was kept to approximately 4/1, which was equal to (Begent et al., 1987) or lower (Goldenberg et al., 1987)

than other similar studies. It may be possible that the amount of HAMA was not sufficient, given that when HAMA levels were below 20% of the initial, ^{131}I-mAb reaccumulated in the blood.

Toxicity that accompanied the i.v. administration of HAMA included rigors, warm sensation, flushing, tachycardia and mild myelosuppression, all phenomena associated with immune complex formation and complement activation (Clarkson et al., 1986). Higher doses of HAMA may worsen the side effects, leading to formation of large insoluble immune complexes that would saturate the degradation capacity of RES and cause tissue injury as well as further reduction in complement and erythrocyte CR1 number.

Our data suggest that slow i.v. infusion of HAMA, starting before the i.p. administration of the radiolabelled mAb, may reduce the above side effects, since immune complex formation will be controlled and their size and number will be kept to the minimum possible. In the future, it would be interesting to examine whether F(ab')$_2$ fragments of HAMA are advantageous over whole Igs, since they lack the Fc portion. In addition, the development of monoclonal human anti-mouse Ig antibodies may offer a new dimension to the existing data. The fact that they have specificity for a certain epitope on the murine mAb, can be selected for their inability to activate complement (Hughes-Jones, 1986; Stewart et al., 1988), and the absence of alloreaction for some patients, may prove important. Polyclonal or monoclonal HAMA preparations must bind to the mouse immunoglobulin with the highest affinity possible, in order to be cleared more efficiently. This accelerated clearance and rapid immune complex formation must be carefully controlled, since tissue damage may occur due to the immune complex deposition (Steward, 1979a; 1981; Lew and Steward, 1984).

In conclusion, exogenously i.v. administered HAMA dramatically reduced the radiation levels in the blood after i.p. administration of ^{131}I-labelled mAb without affecting tumour targeting, since crossing of Ig from the circulation to

the peritoneal cavity is thought to be very low (Young et al., 1987). Side effects due to immune complexes may be reduced by the use of $F(ab')_2$ fragments or slow infusion of small amounts of high affinity HAMA. Theoretically, removal of approximately 1/4 of the injected antibody, would not abolish but should certainly reduce the immune response against the mouse mAb. However, in this limited number of patients we were unable to assess the immunosuppressive effects of HAMA.

Intraperitoneal administration of mAb, followed by its direct penetration to the lymphatics, may make passive immunosuppression extremely difficult to achieve. In addition, binding of specific antiserum against the mAb and its removal to catabolic sites, may increase the antigen's (mAb) presentation to immunocompetent cells, rather than decrease its immunogenicity (Möller and Wigzell, 1965; Henry and Jerne, 1968; Murgita and Vas, 1972; Collisson et al., 1979; Coutinho and Forni, 1981; Heyman and Wigzell, 1984). However, the observed reduction of total body radiation together with the removal of a large proportion of the immunogen must be regarded as a positive step towards better immunotherapy of intraperitoneal tumours.

Chapter 5

Immunosuppression by immunoglobulin deaggregation

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5.1 Introduction

Immunoglobulins (Igs) are unique molecules, characterized by their multiple interactions with cells or soluble compounds. Their significance for immune responses against potential pathogens is mainly based on the huge diversity of their hypervariable regions. However, in addition to the antigen binding site, each Ig domain is responsible for a specific function: for example, binding to Fc receptors on macrophages (mϕs), monocytes, neutrophils and K-cells; binding staphylococcal protein A, streptococcal protein G; fixation of complement (C1q) and complement fragments (C4b), are properties that are controlled by the specific class and subclass of the antibody (Roitt et al., 1985). Thus each individual antibody, produced by a B-cell clone, is unique in terms of its idiotype, allotype and isotype.

Immunoglobulins are large molecules that can elicit an immune response when administered to a foreign host. However, being such pleiotropically functional molecules, their immunogenicity is highly variable, thus, as discussed in the previous chapter, antibodies may suppress as well as induce immune responses to themselves and other antigens. Both C and N terminals are responsible for these variations: the constant region controls the catabolic rate; certain parts of it are less immunogenic than others (Walker et al., 1988), and Fc-derived peptides often influence immune responses (Baxevanis et al., 1986; Dagan et al., 1988). Immune complexes on the other hand, between the antibody's antigen binding site and antigen (or other Ig), also crucially affect immunogenicity, as discussed in the previous chapter. Under these circumstances therefore, variability in anti-Ig responses is the rule rather the exception, even within the same species.

The first report that Ig could be immunosuppressive was made by Dresser in 1962 when he described experiments to show that aggregate-free γ-globulin (bovine) was not only less immunogenic, but was actually tolerogenic when

administered to mice (Dresser, 1962). These initial data were supported and extended by various other investigators, who found that deaggregated human, rabbit, sheep or bovine Ig appeared to induce a state of specific Ig tolerance in mice (Golub and Weigle, 1969; Pepys and Taussig, 1974; Lukic´ et al., 1975a; Hosono and Fujiwara, 1979a; 1979b; Weigle et al., 1987; 1988). In addition, Ig-hapten conjugates failed to elicit an immune response to either the hapten or the antibody (Erard et al., 1976).

However, it soon became apparent that the induction of tolerance depended upon: a) the host (mouse) strain, b) the method of deaggregation, and c) the amount injected. Thus, mouse strains such as C57BL/6, A/J, DBA/2, CBA and C3H/HE, have been shown to be "susceptible" to the induction of Ig-specific unresponsiveness (Golub and Weigle, 1969; Staples et al., 1970; Fujiwara and Cinader, 1974; Lukic´ et al., 1975a), whereas Balb/c, SJL, NZB and DDD mice were particularly "resistant" (Golub and Weigle, 1969; Staples et al., 1970; Playfair, 1971; Lukic´ et al., 1975a; Hosono et al., 1977; Hosono and Fujiwara, 1979a; 1979b). The degree of deaggregation appeared to be crucial in obtaining pure, tolerogenic, Ig monomers. Although deaggregation by ultracentrifugation was the first to be used (Dresser, 1962; Das and Leskowitz, 1970; Pepys and Taussig, 1974; Lukic´et al., 1975b), improved methods such as Na_2SO_4 fractionation or biological filtration were found to be more effective, and able to induce tolerance even in "resistant" strains (Golub and Weigle, 1969; Lukic´et al., 1975a). The route of administration did not seem to play an important role, since tolerogenic Ig was administered i.p (Golub and Weigle, 1969; Pepys and Taussig, 1974; Lukic´and Leskowitz, 1974; Lukic´ et al., 1975a, Hosono and Fujiwara, 1979a, Garnier et al., 1988), i.v. (Erard et al., 1976; Weigle et al., 1987), and even orally (Komatsu et al., 1988), with similar effects. The dose of Ig however, does seem to influence the outcome, although the effects reported for amounts of 5 μg to 10 mg are so varied that it is difficult to draw any conclusions as to their significance (Golub and Weigle, 1969; Lukic´ et al., 1975b;

Hosono and Fujiwara, 1979b; Weigle et al., 1988; Garnier et al., 1988; Komatsu et al., 1988).

The exact mechanism of the observed specific unresponsiveness to deaggregated Ig is still not clear. However, it appears to be complement independent (Pepys and Taussig, 1974; Lukic´ et al., 1975a) and is probably controlled at the level of antigen processing and presentation by mϕs (Lukic´ and Leskowitz, 1974; Lukic´ et al., 1975a; 1975b; Phipps and Scott, 1983; Garnier et al., 1988; Phipps et al., 1988). Thus, deaggregated Ig may be less efficiently processed than aggregated Ig.

The overall success of these experimental studies suggested that deaggregation might provide an effective means of inducing tolerance to mouse Ig in patients receiving murine monoclonal antibodies (mAbs) for the clinical therapy of cancer. We therefore analyzed the effects of Ig aggregation on the anti-mouse Ig response of patients. In addition, we set up a genetically "inverted" experimental model in which human Ig (hIgG) was given to mice, in order to study the immunogenicity/tolerogenicity of different preparations of Ig in more detail.

In our experiments we analyzed the effects of "antigen deaggregation" in 3 different strains of mice given polyclonal human IgG. Since the tolerogenic effect is genetically controlled, we chose mice with different major histocompatibility loci, Balb/c (H-2^d), CBA (H-2^k) and C57BL/6 (H-2^b), belonging to tolerance "resistant" (Balb/c) as well as tolerance "susceptible" (CBA, C57BL/6) groups, and studied whether more stringent deaggregation could render IgG tolerogenic even to "resistant" strains. We used fast protein liquid chromatography (FPLC) size exclusion chromatography, which separates Ig monomers from aggregates under physiological conditions, quickly and most efficiently. Human IgG was administered i.p. since this was the regional administration route used to optimize the tumour localization of mAbs in our

patients with cancer (Epenetos et al., 1987a); this route was, in addition, the most favourable for the induction of tolerance, as reported in previous experiments (Golub and Weigle, 1969; Lukic´ et al., 1975b; Hosono and Fujiwara, 1979a; Garnier et al., 1988; Komatsu et al., 1988). The amount of immunogen used was the minimum producing a detectable response in our anti-Ig assay, and was within the range used in other similar studies.

Finally, in parallel clinical studies, we monitored the anti-mouse Ig response of patients with ovarian cancer, who received non-aggregated and partially aggregated radiolabelled murine mAb i.p. for therapeutic purposes. These patients received ^{131}Iodine-labelled antibodies that remained free of aggregates even after radiolabelling, and ^{90}Yttrium-labelled antibodies, that due to diethylene triamine pentaacetic acid (DTPA) cross-linking, were often aggregated to a high degree (as extensively discussed in chapters 3 and 4). The purpose of this study was mainly to investigate whether deaggregated preparations would be at least less immunogenic and at best tolerogenic, thus rendering them more favourable for clinical application.

5.2 Materials and Methods

5.2.1 Animals: Female Balb/c, CBA and C57BL/6 mice, 8-10 weeks old, were purchased from Olac, UK. Groups of at least 3 mice were used for each experimental situation (section 5.2.5 below).

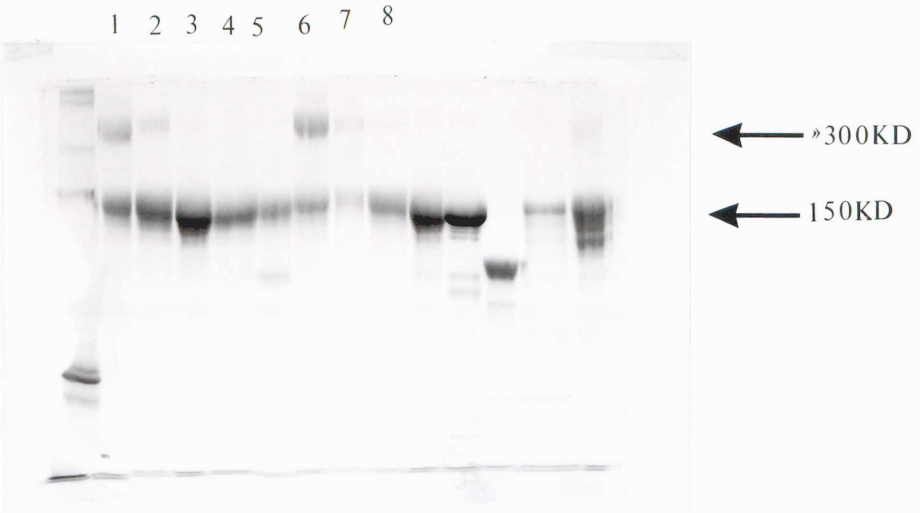
5.2.2 Human immunoglobulin G: Polyclonal human IgG (Sigma, UK) was used as an immunogen for the mice. Each mouse received 70-100 μg of hIgG i.p., in 0.3 ml sterile PBS, pH 7.4. No adjuvants were used.

5.2.3 Deaggregation of human IgG: Human IgG was purchased in lyophilized form. After reconstitution in PBS, at 1 mg/ml, it was found to be

partially aggregated, as determined by sodium SDS-PAGE (Fig. 5.1a). These aggregates were pelleted by centrifugation at 150,000 g for 150 min (L8-70M, Beckman Instr., Palo Alto, USA), and the contents of the top third of the centrifuge tube was then carefully removed for use as the immunogen. Figure 5.1b shows a silver stained 8% SDS-PAGE of various hIgG samples before and after ultracentrifugation. Within the sensitivity limits of the technique, we confirmed that the above deaggregation method yielded monomeric IgGs (Fig. 5.1b).

In further experiments, monomeric IgG was isolated by size exclusion chromatography, using Superose 6 or 12 columns (Pharmacia, Sweden) connected to an FPLC (Pharmacia). Figure 5.2a shows a typical FPLC profile (absorbance measured at 280 nm) of the partially aggregated hIgG. Since the apparatus will automatically measure the areas under the two peaks, we could calculate that our preparation contained 15-20% aggregates (mainly dimers). The dark area on Figure 5.2a, which corresponds to monomeric IgG, was removed for use as an immunogen immediately after determination of concentration by spectrophotometry (as discussed in chapter 4, extinction coefficient approximately 15.0) in order to avoid reaggregation. The whole procedure, from the beginning of gel filtration through to immunization, usually lasted approximately 20-30 minutes. Fresh deaggregated material was used for each immunization.

5.2.4 Aggregation of human IgG: The initial, partially aggregated human IgG was heated for 2 hr at 59°C, and left overnight on ice (Weigle, 1973). Additional aggregate formation was confirmed by FPLC analysis (Superose 6, Fig. 5.2b). After spectrophotometrical determination of the protein concentration, the samples were stored at -20°C.



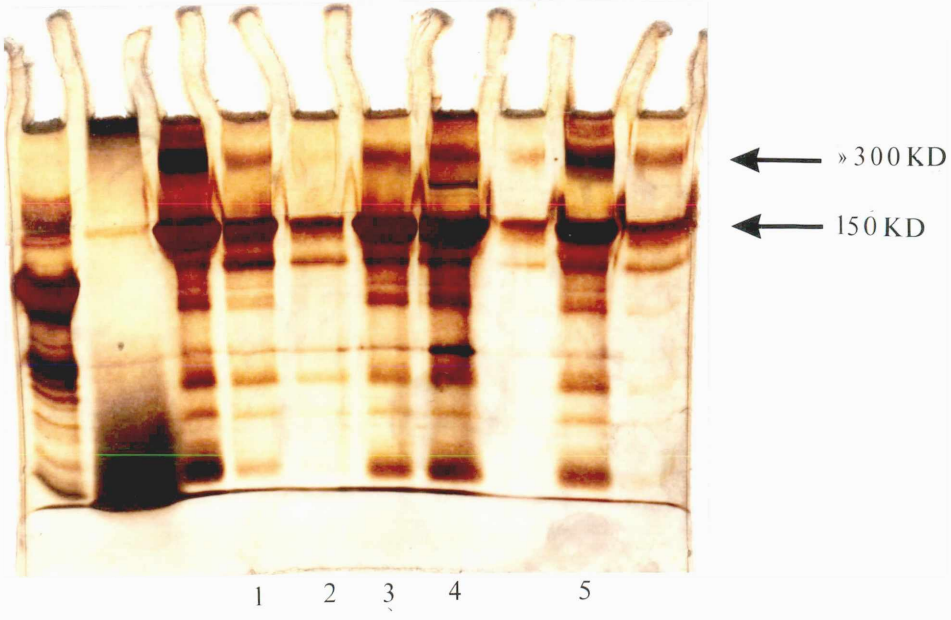


Figure 5.1: a) Coomassie blue stained 7.5% SDS-PAGE of polyclonal human IgG preparations (lanes 1-8), and b) silver stained 8% SDS-PAGE of polyclonal human IgG preparations before (lanes 1, 3, 4 and 5) and after ultracentrifugation (lane 2). Arrows indicate the relative molecular weight of monomeric (150KD) and partially aggregated hIgG (≥300KD).

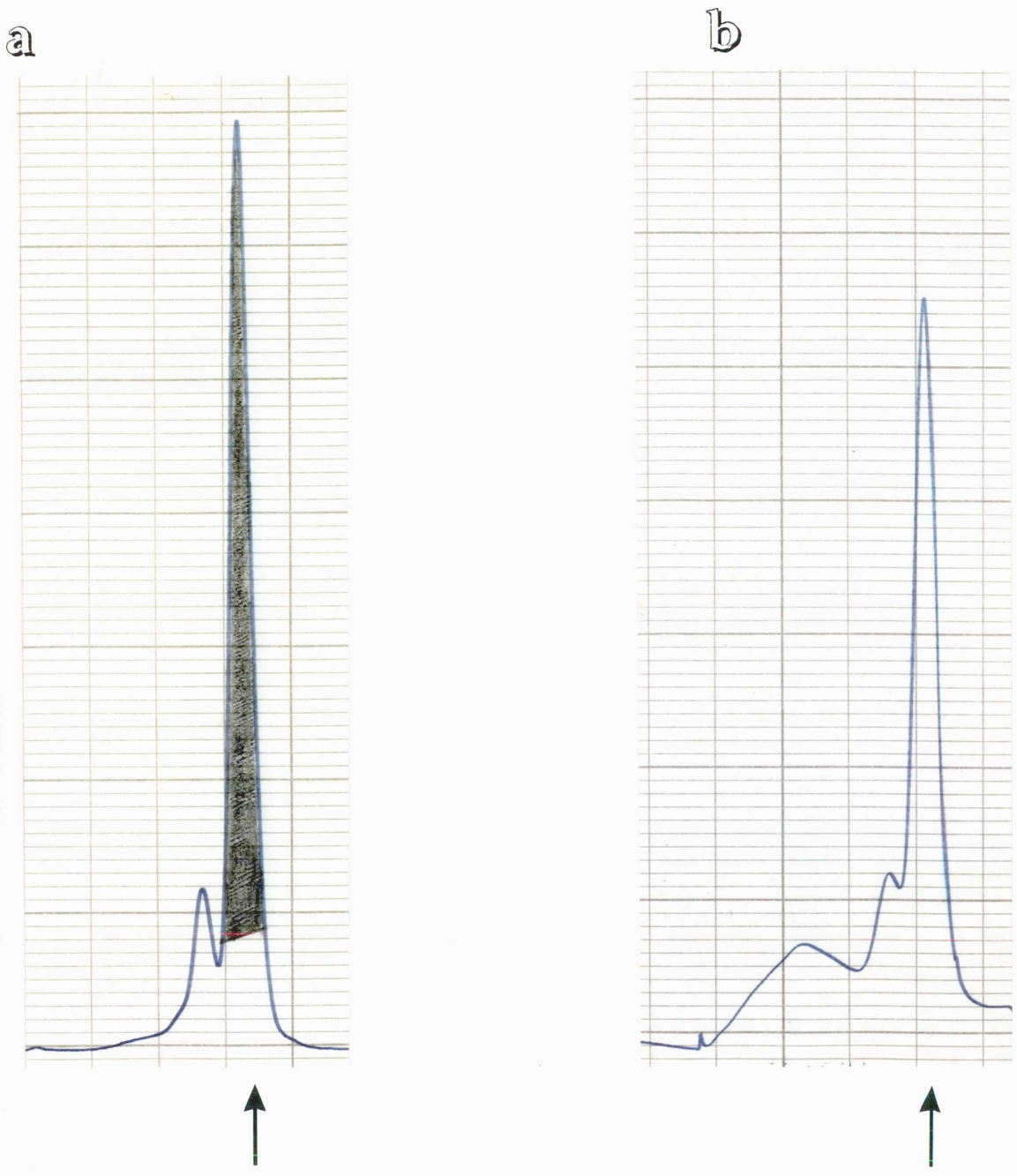


Figure 5.2: FPLC profile (absorbance at 280 nm) of partially aggregated (a) and heat-aggregated (b) polyclonal human IgG. Arrows indicate the monomeric fraction. The dark area in Figure (a) was separated and used as immunogen.

5.2.5 Immunization schedule: Mice received an i.p. injection of the: a) untreated, partially aggregated human IgG (A-hIgG), b) monomeric, deaggregated hIgG (D-hIgG), and c) polymeric, heat-aggregated hIgG (HA-hIgG). Ten days later, half of the mice were killed and bled (cardiac puncture). The serum was removed after centrifugation, and stored at -20°C in aliquots, in order to assess the primary immune response. The remaining animals were challenged for a second time. Seven days later, all mice were killed, bled, and the secondary response against hIgG was measured.

5.2.6 Patients: Twenty-two patients with carcinoma of the ovary were given radiolabelled monoclonal antibody HMFG1 as part of a phase I/II clinical study. Patients received approximately 15 mg of radiolabelled antibody i.p., via a small catheter inserted into the abdomen, under local or general anaesthesia. All patients received monoclonal antibody treatment for the first time. A blood sample was taken before and 14 days after treatment, the serum was removed, and the anti-mAb response measured.

5.2.7 Monoclonal antibody: The mAb used was HMFG1, a mouse IgG1, that recognises a large mucin, normally produced by the lactating breast, but also expressed by the majority of ovarian, breast and other cancers of epithelial origin (Taylor-Papadimitriou et al., 1981). HMFG1 was provided by Unipath-Oxoid (Bedford, UK) in purified, sterile and pyrogen free form. It was radiolabelled with ^{131}Iodine (80-150 mCi, Amersham, UK) using the n-bromo-succinamide method (Reay, 1982), or with ^{90}Yttrium (5-20 mCi, AERE, Harwell, UK) using the cyclic anhydride DTPA (Sigma) as chelating agent (Hnatowitch et al., 1983). The radiolabelling methods are discussed in detail in chapter 3, where it is also shown that native HMFG1 is aggregate free (for at least 6 months, stored in sterile PBS, pH 7.4, at 4°C), and that it remains monomeric after iodination. This was confirmed by FPLC gel filtration as shown in Figures 5.3a and b. In contrast, DTPA, which is necessary for radiolabelling with radiometals

such as yttrium, cross-links Igs, resulting in dimer and polymer formation as shown in Figures 5.3c and d.

5.2.8 Measurement of the anti-immunoglobulin response: We have used an ELISA to measure the anti-Ig response in both experimental and clinical studies. The method is described in detail in chapter 2, but briefly, 5 μg/ml of hIgG or HMFG1 mAb was coated onto 96-well plates. Mouse or human serum was applied using 1:10 serial dilutions. The absorbance in each well was measured in a multiscan spectrophotometer, after a horseradish peroxidase linked species-specific anti-mouse Ig, or anti-human Ig as appropriate, was added, followed by the substrate ABTS. The results are given as the mean value of the areas under the serum titration curves, as described earlier (chapter 2). Since groups of at least 3 animals were used in each experiment, statistical analysis was performed using the Student's t test.

It should be mentioned here, however, that when we first published the results of this study, the data were expressed as absorbance at 405 nm for test serum dilution of 1:10, or 1:100 where there was a prozone effect (Sivolapenko et al., 1989). It was then that we appreciated the need for a better and more reliable method of evaluating the results. The computer programme described in chapter 2, was therefore developed. In this chapter, we have re-evaluated the data using this programme. However, we were still limited by the fact that the same standard serum could not be used for all experiments. Comparisons should therefore be made within, but not between, experiments. Each histogram/figure (with the exception of Figures 5.7a and b that are directly comparable) represents a single experiment. In general, our conclusions here do not contradict those in our previous publication, although the statistical p values were altered (higher values but still ≤ 0.05), and in some cases differences were not as great as initially thought.

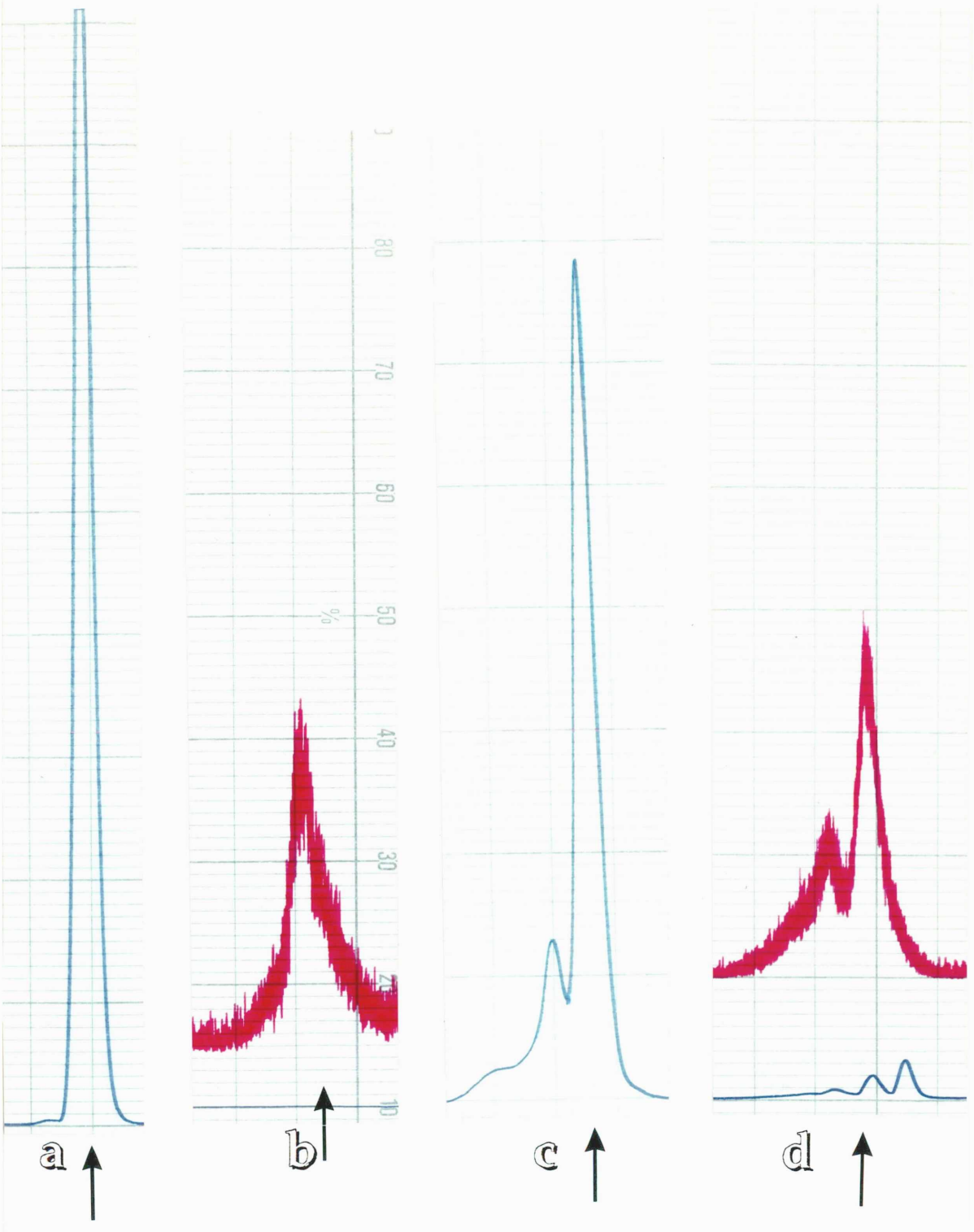


Figure 5.3: FPLC profile (absorbance at 280 nm) of HMFG1 (a), ^{131}Iodine-labelled HMFG1 (b), HMFG1-DTPA (c) and ^{90}Yttrium-labelled HMFG1-DTPA (d). The blue line is the protein trace, whereas the red one is the radioactivity trace (for b and d). Arrows indicate the location of monomeric IgG.

5.3 Results

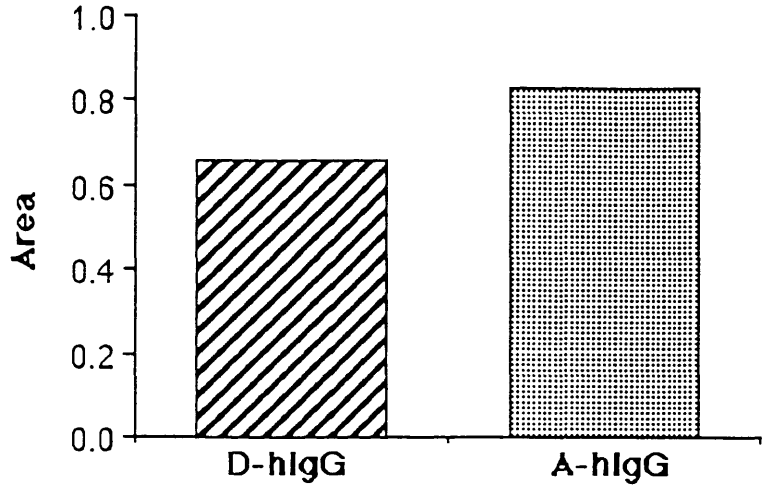
5.3.1 Immune response to human IgG of Balb/c mice: Unprimed Balb/c mice were given i.p. 70 μg of polyclonal human IgG, that was either untreated (A-hIgG) or deaggregated (D-hIgG) by ultracentrifugation. As shown in Figure 5.4a, the mean response against D-hIgG was 0.655±0.245 and against A-hIgG 0.825±0.248. There was no statistically significant difference of the anti-Ig response in these two groups of mice.

Nevertheless, when mice were primed with 100 μg hIgG deaggregated by FPLC gel filtration (D-hIgG), they responded 25.1% (mean area 1.06±0.05) less than those that received the partially aggregated preparation (A-hIgG) (mean area 1.34±0.15, p=0.048), and 26.2% less than mice injected with heat-aggregated hIgG (HA-hIgG) (mean area 1.42±0.19, p=0.045) (Fig. 5.4b). There was no difference in the anti-Ig response between the animals primed with A-hIgG or HA-hIgG.

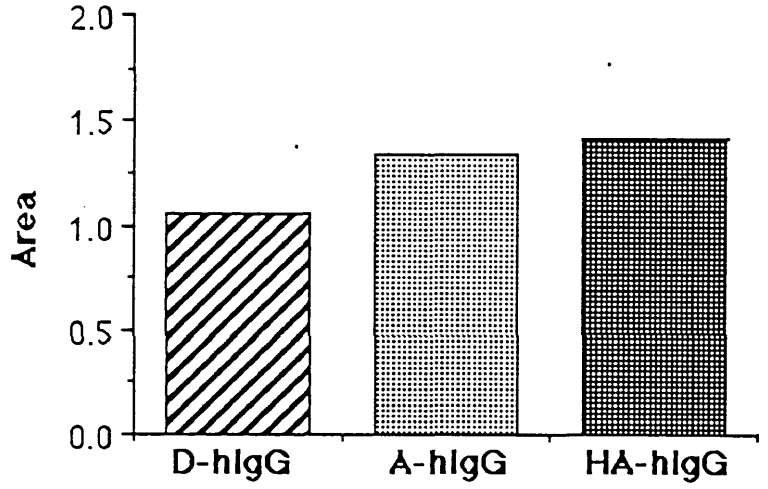
Half of the partially suppressed animals received a further i.p. challenge of 100 μg of D-hIgG, whereas the rest were given A-hIgG. Similarly, half of the mice primed with A-hIgG were injected again with A-hIgG, and the rest received the same amount of D-hIgG. The secondary response against hIgG is shown in Figure 5.4c. We found that all animals that received at least one injection of D-hIgG responded less to the immunogen, than did controls that were immunized with A-hIgG twice (mean area 3.178±0.651). The reduction was 66.2% for the animals primed with A-hIgG and challenged with D-hIgG (mean area 1.369±0.260, p=0.024), 34.7% for mice primed with D-hIgG and challenged with A-hIgG (mean area 2.072±0.295, p=0.045), and 53.4% for those primed and boosted with D-hIgG (mean area 1.48±0.31, p=0.030).

Figure 5.4: a) Primary response of Balb/c mice against ultracentrifuge-deaggregated (D-hIgG) and (partially) aggregated (A-hIgG) polyclonal human IgG.
b) Primary response of Balb/c mice against chromatographically deaggregated (D-hIgG), (partially) aggregated (A-hIgG) and heat-aggregated (HA-hIgG) polyclonal human IgG.
c) Secondary response of Balb/c mice when:
primed and boosted with (partially) aggregated polyclonal human IgG (A-hIgGxA-hIgG);
primed with (partially) aggregated and boosted with chromatographically deaggregated polyclonal human IgG (A-hIgGxD-hIgG);
primed with chromatographically deaggregated and boosted with (partially) aggregated polyclonal human IgG (D-hIgGxA-hIgG);
primed and boosted with chromatographically deaggregated polyclonal human IgG (D-hIgGxD-hIgG).
The results are expressed as areas under the ELISA serum-titration curves, and are the mean value of groups of at least 3 mice.

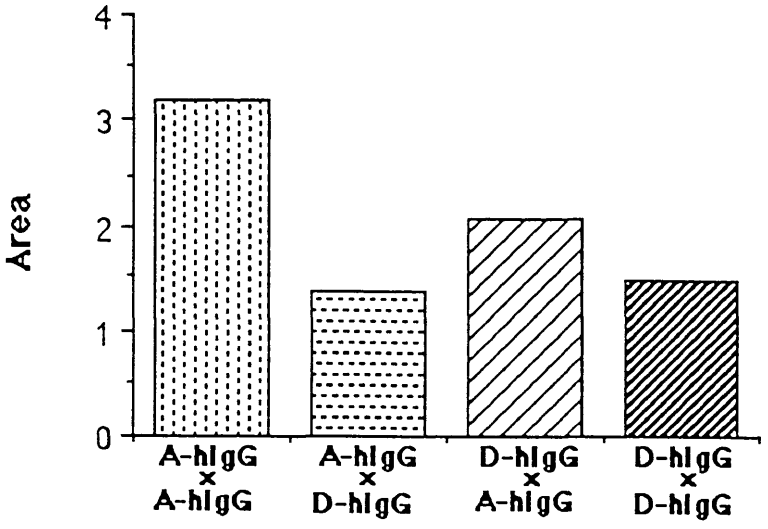
Balb/c


1.0
0.8
0.6
0.4
0.2
0.0
Area
D-hIgG
A-hIgG

a


2.0
1.5
1.0
0.5
0.0
Area
D-hIgG
A-hIgG
HA-hIgG

b


4
3
2
1
0
Area
A-hIgG x A-hIgG
A-hIgG x D-hIgG
D-hIgG x A-hIgG
D-hIgG x D-hIgG

c

In addition, mice primed with D-hIgG and boosted with A-hIgG responded 33.3% and 28.6% more than the ones that were primed with A-hIgG and boosted with D-hIgG, or those that received D-hIgG twice. However, the p values were 0.060 and 0.067 respectively. No other statistically significant differences were observed.

5.3.2 Immune response to human IgG of CBA mice: Unprimed CBA mice received i.p. 100 μg of chromatographically deaggregated (D-hIgG), partially aggregated (A-hIgG) and heat-aggregated (HA-hIgG) human IgG. Figure 5.5a shows their primary anti-hIgG response, where we found that animals primed with D-hIgG (mean area 0.164±0.117) responded 86.8% less than animals that received A-hIgG (mean area 1.245±0.288, p=0.005), and 91.8% less than those given HA-hIgG (mean area 1.996±0.163, p=0.004). In addition, CBA mice appeared to have responded 37.7% more against the HA-hIgG material than against A-hIgG (p=0.050).

After a second i.p. injection of hIgG, we found that animals that were primed and boosted with D-hIgG (mean area 0.579±0.089) responded significantly less than the other groups. As shown in Figure 5.5b, the reduction in the anti-hIgG response was 66.1% compared with animals primed with D-hIgG but boosted with A-hIgG (mean area 1.705±0.821, p=0.045), 78.3% compared with those primed with A-hIgG and boosted with D-hIgG (mean area 2.666±0.619, p=0.011), and 79.5% compared with mice that received A-hIgG twice (mean area 2.870±0.767, p=0.015). Moreover, mice primed with D-hIgG and boosted with A-hIgG responded 39.5% (p=0.005) and 36.0% (p=0.009) less than those injected twice with A-hIgG, or those primed with A-hIgG and boosted with D-hIgG, respectively. No other statistically significant differences were observed.

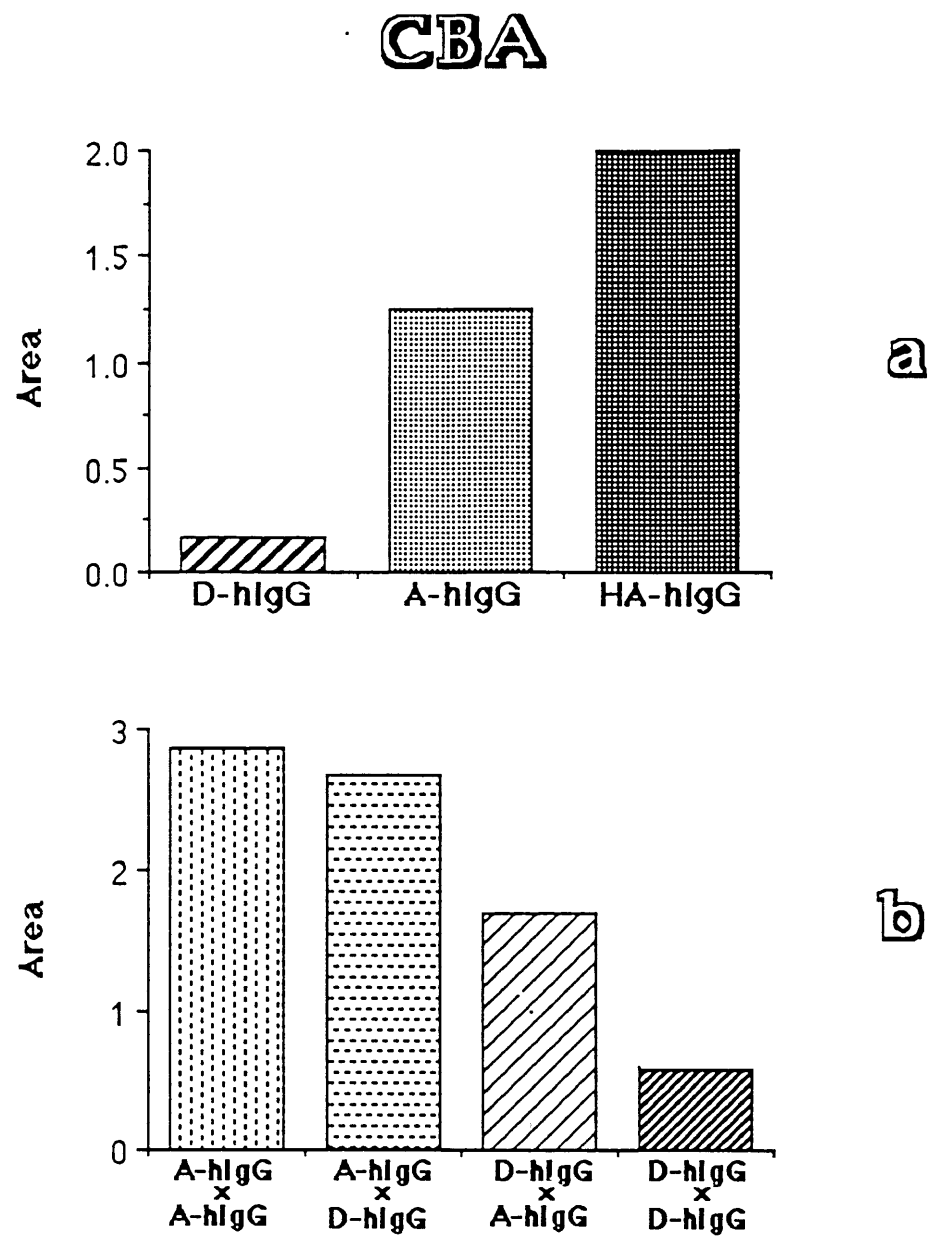


Figure 5.5: a) Primary response of CBA mice against chromatographically deaggregated (D-hIgG), (partially) aggregated (A-hIgG) and heat-aggregated (HA-hIgG) polyclonal human IgG.
b) Secondary response of CBA mice when:
primed and boosted with (partially) aggregated polyclonal human IgG (A-hIgGxA-hIgG);
primed with (partially) aggregated and boosted with chromatographically deaggregated polyclonal human IgG (A-hIgGxD-hIgG);
primed with chromatographically deaggregated and boosted with (partially) aggregated polyclonal human IgG (D-hIgGxA-hIgG);
primed and boosted with chromatographically deaggregated polyclonal human IgG (D-hIgGxD-hIgG).
The results are expressed as areas under the ELISA serum-titration curves, and are the mean value of groups of at least 3 mice.

5.3.3 Immune response to human IgG of C57BL/6 mice: C57BL/6 mice received an i.p. injection of 100 μg chromatographically deaggregated (D-hIgG), partially aggregated (A-hIgG) and heat-aggregated (HA-hIgG) human IgG. Figure 5.6a shows their primary response. Mice responded 63.0% less against D-hIgG (mean area 0.236±0.049) than against A-hIgG (mean area 0.638±0.240, p=0.023) and 87.8% less than against HA-hIgG (mean area 1.947±0.601, p=0.020). Interestingly, C57BL/6 mice responded 67.2% more to HA-hIgG than to A-hIgG (p=0.024).

When we assessed the secondary response, after a second i.p. challenge with 100 μg of the two hIgG preparations, we found that those all animals that received at least once D-hIgG, responded significantly less than those that had two immunizations with A-hIgG (mean area 0.829±0.249, Fig. 5.6b). The reduction in the anti-hIgG response was 44.4% for mice primed with A-hIgG and challenged with D-hIgG (mean area 0.461±0.053, p=0.033), 49.8% for D-hIgG primed and A-hIgG boosted animals (mean area 0.416±0.087, p=0.025) and 51.9% for those that received D-hIgG twice (mean area 0.399±0.086, p=0.024). No other statistically significant differences were observed among the other 3 groups (Fig. 5.6b).

5.3.4 Human anti-mouse immunoglobulin response: Eleven patients with ovarian cancer received i.p. approximately 15 mg ^{131}Iodine-labelled HMFG1 (non-aggregated) for therapeutic purposes, while another 11 patients were given the same amount of ^{90}Yttrium-labelled HMFG1 (aggregated). The patients' anti-mouse Ig response was measured before and 14 days after the mAb administration. As shown in Figures 5.7a and b, we found no statistically significant difference between the anti-HMFG1 response of the group that received the non-aggregated iodinated mAb and the group that received yttrium-labelled mAb.

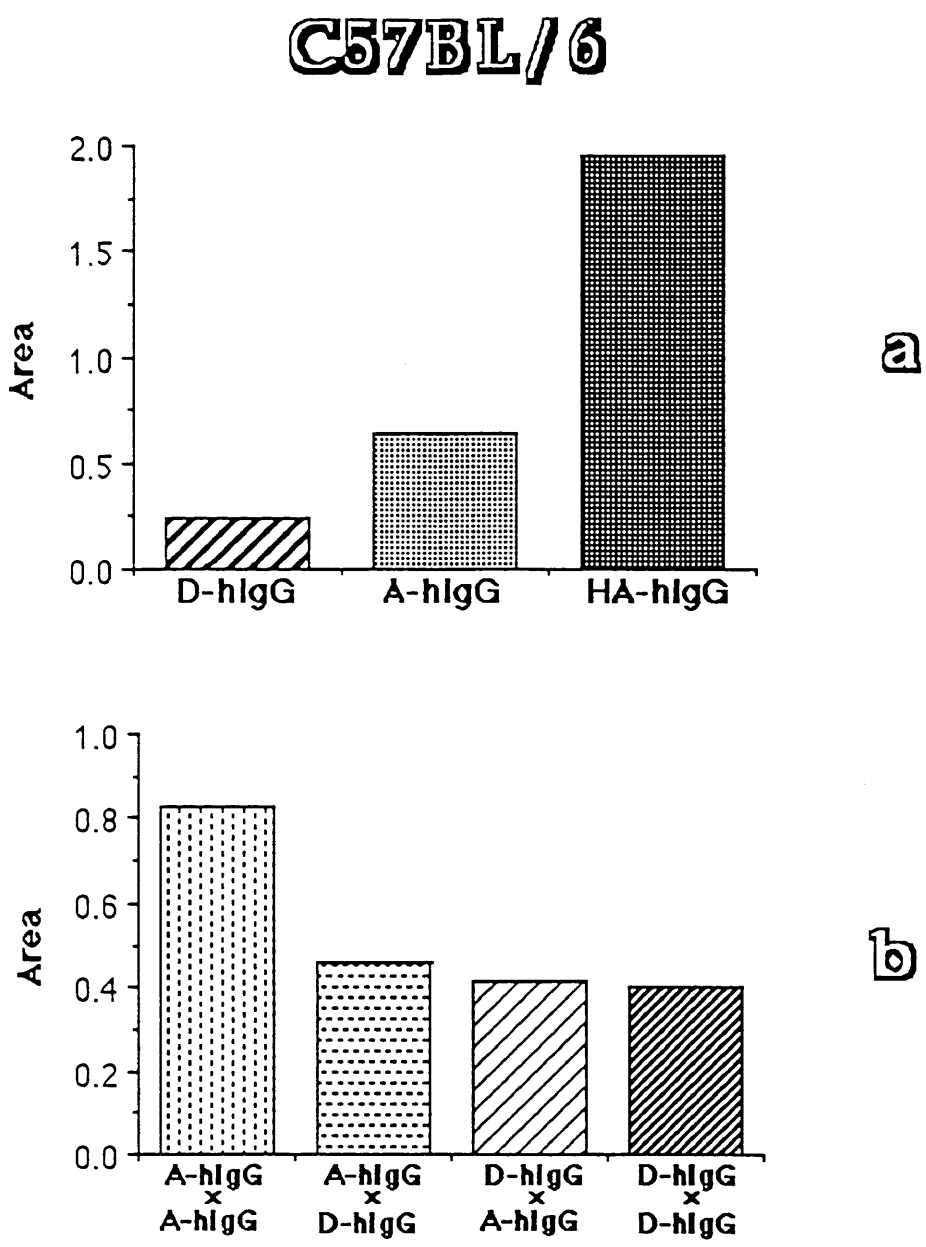


Figure 5.6: a) Primary response of C57BL/6 mice against chromatographically deaggregated (D-hIgG), (partially) aggregated (A-hIgG) and heat-aggregated (HA-hIgG) polyclonal human IgG.
b) Secondary response of C57BL/6 mice when:
primed and boosted with (partially) aggregated polyclonal human IgG (A-hIgGxA-hIgG);
primed with (partially) aggregated and boosted with chromatographically deaggregated polyclonal human IgG (A-hIgGxD-hIgG);
primed with chromatographically deaggregated and boosted with (partially) aggregated polyclonal human IgG (D-hIgGxA-hIgG);
primed and boosted with chromatographically deaggregated polyclonal human IgG (D-hIgGxD-hIgG).
The results are expressed as areas under the ELISA serum-titration curves, and are the mean value of groups of at least 3 mice.

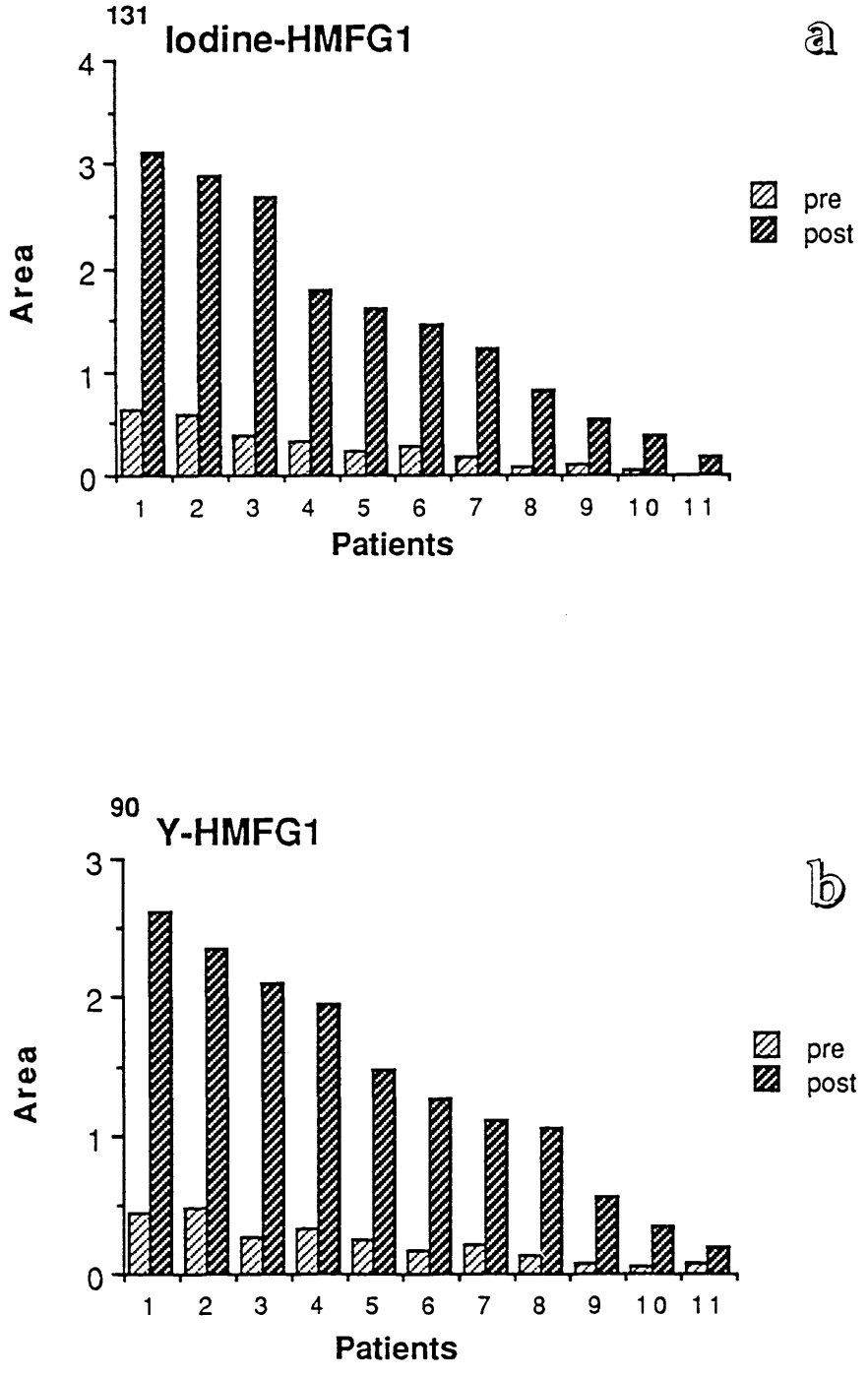


Figure 5.7: Humoral anti-mouse Ig response of 11 patients who received ^{131}I-labelled (a) and ^{90}Yttrium-labelled (b) HMFG1 monoclonal antibody for therapy, as measured before (pre) and 14 days after (post) administration. The results are expressed as areas under the ELISA serum titration curves.

5.4 Discussion

One of the major problems that has to be overcome before mAb therapy for cancer reaches its full potential, is the immunogenicity of the administered immunoglobulin. Since 1962, when Dresser first reported the induction of tolerance to foreign Ig by deaggregation (Dresser, 1962), many investigators have supported and extended the view that monomeric IgG can be tolerogenic in some mouse strains, and that the effect greatly depends upon the degree of deaggregation (Golub and Weigle, 1969; Lukic´ and Leskowitz, 1974; Lukic´ et al., 1975a).

For this study we used 3 different MHC class I haplotype strains (Balb/c, CBA and C57BL/6), and chose to produce monomeric human IgG using FPLC gel filtration, since this provides a rapid and highly efficient method of deaggregation. The superiority of this approach over ultracentrifugation (the technique most often used by others) is shown in Figures 5.4a and b, where tolerance "resistant" Balb/c mice responded 25.1% less to chromatographically deaggregated than to untreated hIgG (Fig. 5.4b), whereas the response against the ultracentrifuged material remained unchanged (Fig. 5.4a).

Generally, Balb/c mice showed a significant reduction in their primary (25.1%) and secondary (mean 51.4%) anti-hIgG response when monomeric IgG was the only (primary response, Fig. 5.4b) or one of the antigen challenges (secondary response, Fig. 5.4c). Similarly, CBA mice primed with deaggregated hIgG responded 86.8% less than when primed with partially aggregated, and 91.8% less than when primed with heat-aggregated hIgG (Fig. 5.5a). After a second challenge, the anti-Ig response was lower (mean reduction 74.6%) in those animals that received deaggregated material twice (Fig. 5.5b). In addition, mice primed with D-hIgG and boosted with aggregated material responded less (mean 37.7%) than those that received A-hIgG twice, or were primed with A-

hIgG and boosted with deaggregated Ig (Fig. 5.5b). In C57BL/6 mice we found that monomeric IgG resulted in 63% suppression of the anti-hIgG response compared with those that received partially aggregated hIgG, and 87.8% compared with animals given heat-aggregated hIgG (Fig. 5.6a). The secondary response followed the pattern observed with Balb/c mice, where the anti-Ig response was reduced (mean 48.7%) in all cases where at least one of the immunization (primary or secondary) was with deaggregated hIgG (Fig. 5.6b).

Overall, the immune response generated by chromatographically prepared monomers was lower than that generated by aggregated hIgG, in both "high responder" (Balb/c) and "low responder" (CBA, C57BL/6) mice. Our data suggest therefore that Ig deaggregation certainly leads to reduction of immunogenicity; this was also supported by the fact that both CBA and C57BL/6 mice responded to heat-aggregated hIgG significantly more than to partially aggregated Ig (37.6% Fig. 5.5a, and 67.2% Fig 5.6a, respectively). In contrast with other investigators, however, we found no evidence of tolerance. This is not the first time that conflicting results have been reported for experiments on the induction of tolerance by Ig deaggregation. For example, Pepys and Taussig were the only investigators that showed the tolerogenic effect of monomeric IgG in the tolerance "resistant" Balb/c strain (Pepys and Taussig, 1974), while Das et al. reported that C57BL/6 mice remained unresponsive even to completely aggregated material (Das et al., 1973). Moreover, some investigators stressed the importance of the Fc region for tolerance induction (Borel, 1976; Kettman et al., 1979; Phipps and Scott, 1988), whereas others reported no relationship (Pepys and Taussig, 1974; Schrader, 1975). Many of the above discrepancies, and the lack of reproducibility of the initial exciting results may be attributed to various factors, such as the source, amount and route of administration of the xenogeneic Ig, the method of deaggregation, the assay system used to measure the anti-Ig response and perhaps the sex and age of the animals.

The method by which the *in vivo* anti-Ig response was detected may also explain discrepancies between our data and previous results. The main system used by previous investigators was the rate of biological clearance of ^{125}Iodine-labelled γ-globulin (Golub and Weigle, 1969; Pepys and Taussig, 1974; Fujiwara and Cinader, 1974; Lukic´ et al., 1975a). This method only detects high affinity antibodies and does not represent the true status of the immune response. As previously reported, biological clearance does not quite correspond to the presence and quantity of anti-Ig antibodies (Rossen et al., 1971; Steward, 1979). For the experiments presented here, we had developed a highly sensitive ELISA in order to assess the response against the foreign Ig; its advantages over biological clearance or other detection systems were extensively discussed in chapter 2. With our system we measured a reduced anti-Ig response which by no means reflects tolerance. This is in accordance with recent publications where using an ELISA, a low anti-Ig response was measured in animals previously reported as tolerance "susceptible" (Phipps et al., 1988; Garnier et al., 1988).

In addition, in all previous *in vivo* studies the response against monomeric Ig was compared with controls that received heavily aggregated IgG (Golub and Weigle, 1969; Lukic´ et al., 1975a; Hosono and Fujiwara, 1979a). The large difference that we also observed in the response to HA-hIgG and D-hIgG in tolerance "susceptible" mice (Figs. 5.5a and 5.6a), combined with of rather insensitive detection techniques, may well have given the appearance of unresponsiveness.

However, despite variability in the reported data, it is clear that deaggregated is less immunogenic than aggregated Ig, and that the magnitude of this effect varies according to the strain of mouse used. As discussed earlier, induction of tolerance by deaggregated Ig is complement independent, since complement depletion by cobra venom proved ineffective in either restoring (Pepys and Taussig, 1974) or inducing Ig-specific immunosuppression (Lukic´et al., 1975a).

It soon became apparent that "key" cells responsible for the ease with which tolerance could be induced were the APCs, and in particular mϕs. Initial experiments showed that administration of carrageenin, known to be toxic for mϕs but not for B- and T-cells, rendered "responder" Balb/c mice unresponsive to a subsequent challenge of Ig. Spleen cells from Balb/c mice (tolerance "resistant") transferred to irradiated DBA/2 mice (tolerance "susceptible", both strains H-2^d) induced responsiveness to Ig after the transferred cells populated important sites and reached an appropriate number (Lukic´and Leskowitz, 1974). Further experiments with F1 and backcross generations of the above strains, revealed that there was a single gene locus affecting tolerance induction that differs from the major histocompatibility complex (MHC), and is associated with the ability of mϕs to present sufficient antigen in association with MHC class II on their cell surface (Lukic´et al., 1975b).

Simple binding of a native antigen onto the mϕ membrane *in vivo* is inefficient for antigen presentation (Buus et al., 1987). As it is generally accepted, the antigen must be endocytosed and processed in a lysozome/endosome compartment, before it reappears on the cell surface, fragmented, and in association to MHC class II (Babbit et al., 1985; Buus et al., 1986). Only then it is able to stimulate cellular (and subsequently humoral) immune responses. It is at the level of antigen endocytosis and processing that the differences in immunogenicity among mouse strains are observed.

In previous *in vitro* experiments, it has been reported that Ig dimers were less effectively internalized and degraded by mouse mϕs than were the larger oligomers or polymers. Whereas Ig aggregates were processed as normal in the endosomes, dimers never entered lysosomes (Finbloom, 1986). In addition, Ig polymers were found to activate the release of interleukin 1 (IL-1), resulting in upregulation of positive antigen presentation by mϕs *in vitro* (Weigle et al., 1987) and *in vivo* (Staruch and Wood, 1983), thus inhibiting induction of tolerance. Immunoglobulin monomers did not have this effect. Moreover,

substances with known ability to stimulate IL-1 production, such as bacterial lipopolysaccharide (LPS), 8-bromoguanosine (8BruGuo) and bacillus of Calmette-Guérin (BCG), were also found to interfere with the induction of unresponsiveness, and facilitated immunity (Louis et al., 1973; Scheuer et al., 1985; Lukic´et al., 1975a). Prostaglandin E (PGE) may also be involved in the anti-Ig suppression, since it has been shown that high levels of PGE_2 were associated with tolerance induction (Goldings, 1986; Phipps et al., 1988), whereas indomethacin (IM) and acetyl salicyclic acid promoted monomeric-Ig immunogenicity, presumably via their well known PGE-antagonistic action (Scheuer et al., 1987).

When an antigen is administered *in vivo*, it accumulates in specific sites within the reticuloendothelial system (RES), where it is catabolized. Accessory cells at these sites are responsible for potentiating the immune response against this antigen. Variations in the efficiency of antigen processing by different APC populations has been shown *in vivo* for T-independent antigens (Humphrey, 1981; Humphrey and Grennan, 1981), and *in vitro* for monomeric Ig (Phipps and Scott, 1983; Phipps et al., 1988; Sadegh-Nasseri and Phipps, 1988), as well as for other T-dependent antigens even using APCs that originated from the same clone (Michalek et al., 1989). Antigen degradation therefore is a crucial factor that determines the degree of stimulation of antigen-specific T-cells. Impaired T-helper cell function observed during deaggregated Ig-mediated unresponsiveness (Hosono and Fujiwara, 1979a; 1979b), without induction of T-suppressor cells (Weigle et al., 1987), is consistent with inadequate antigen presentation by mϕs.

Interestingly, it has been reported that the mouse strains generally regarded as tolerance "susceptible", were also characterized by their inability to produce high affinity antibodies (Alpers et al., 1972; Steward et al., 1973a). Although there was no relation between the actual amount of antibodies produced and their affinity, the latter seemed to interfere with immune clearance of a given

antigen, and also facilitated induction of tolerance (Steward et al., 1973a; 1974). It is highly likely that differences in antigen presentation by mϕs, directly or indirectly regulate affinity. Nevertheless, this can only partially explain the persistent low-affinity antibody production in some mouse strains (or in some immunological disorders), indicating that affinity is influenced by a variety of other factors (Steward et al., 1974; Steward, 1979b).

Enhanced immunogenicity to Ig aggregates (or monomers) however, has some further implications. It has been reported that resistance to tolerance induction by deaggregated Ig in NZB mice increased with age prior to the appearance of autoantibodies (Hosono and Fujiwara, 1979b). In addition, male BxSB/MpJ mice, that spontaneously develop an autoimmune disease resembling systemic lupus erythematosus (SLE), were particularly good responders to deaggregated Ig, whereas female mice did not respond to monomeric Ig, and do not show any incidence of autoimmune disorders. (Garnier et al., 1988). Recently it has been suggested that accessory cells continuously process *in vivo* self proteins as efficiently as foreign antigens (Lorenz and Allen, 1988). Nevertheless, immune responses are not triggered, since self-reactive T-cells have been deleted, as discussed in the introduction of this thesis. All the above, together with the fact that some APCs present antigen in a more immunogenic form than others (Phipps and Scott, 1983; Phipps et al., 1988; Sadegh-Nasseri and Phipps, 1988; Michalek et al., 1989), and that Ig aggregates potentate this presentation (Finbloom, 1986), which is accompanied by increased IL-1 production (Weigle et al., 1987), make one wonder whether there is a link between enhancement of anti-Ig response (aggregated or xenogeneic) and appearance of rheumatoid factors and other autoimmune diseases.

The partially aggregated polyclonal human IgG employed in our animal experiments contained up to 20% aggregates, the majority of which were Ig dimers (Fig. 5.2a). We decided to use this preparation since it was closer to the partially aggregated mouse mAbs that patients were most likely to receive (Fig.

5.3a), although in the later case, complexes were covalently linked, a difference that may be significant. The origin of the hIgG oligomers is not exactly known, but lyophilization certainly played an important role. Recently, it has been shown that the dimers that form in pooled donors' sera are due to idiotype-anti-idiotype interactions (Gronski et al., 1988a; Gronski et al., 1988b). Whereas polyclonal human IgG from a single donor usually contains only a few oligomers, pooling of IgG from hundreds of donors facilitates specific Fab'-Fab' interactions. However, their number is relatively low, resulting almost exclusively in the formation of dimers, with two distinct average functional equilibrium association constants characterized by $K_{app.}$ values of approximately 2.5-3.0 x 10^{10}/mole and 1.0x10^{12}/mole (Gronski et al., 1988a). Dimer formation of human IgG is therefore an ongoing process that may not only occur within the time lapse between deaggregation and injection, but also *in vivo*. This problem can be overcome when monoclonal antibodies are used. With the rare exception of limited dimer formation in tissue culture supernatant or spontaneous disulfide exchange, natural mAb aggregation does not occur (Gronski et al, 1988a). In the future, it would be interesting to examine the effect of deaggregated monoclonal hIgG in mice, since monospecificity would ensure "cleaner" and more controlled antigen preparations. Ig deaggregation by FPLC size exclusion chromatography therefore, despite its advantages over other methods, may be totally ineffective if the Ig preparation has the potential to reaggregate *in vivo*. This occurs not only when an antibody has binding affinity for a cell membrane antigen (Benjamin et al., 1986), but also after idiotype-anti-idiotype interactions, as discussed above. Other deaggregation methods, such as biological filtration, have proved to be more efficient in inducing specific Ig unresponsiveness (Golub and Weigle, 1969; Lukic´et al., 1975a), although in some other cases it was found to be ineffective (Hosono and Fujiwara, 1979b).

Finally, in our clinical trials, we found that there was no difference in the human anti-mouse Ig response between the two groups of patients that

received partially aggregated (yttrium labelled) and non-aggregated (iodinated) mAb (Figs. 5.7a and b). We feel that the effect is not solely dose- or route-dependent, since a wide range of concentrations have been administered so far, via different routes, with similar immunogenicity (Miller et al., 1983; Schroff et al., 1985; Shawler et al., 1985; Courtenay-Luck et al., 1986; Murray et al., 1987; Rosen et al., 1987). The fact that all these mAbs had specificity for a cell membrane antigen, and thus increased antigenicity due to *in vivo* aggregation/immune complex formation (Benjamin et al., 1986), made the conditions worse for the induction of tolerance. Theoretically, one could use monoclonal antibodies with no specificity for human tissue antigens, in order to induce specific unresponsiveness, before administering the specific anti-tumour antibody. However, following the above considerations on the genetic control of antigen processing and presentation, it seems unlikely that Ig deaggregation could be effective in inducing specific suppression in the outbred human population. Moreover, the presence of pre-existing anti-mouse Ig antibodies/rheumatoid factors (Figs. 5.7a and b) in most patients (Courtenay-Luck et al., 1987), further reduces the chances of inducing Ig-mediated specific unresponsiveness. Based on our animal experiments, we feel that deaggregated Igs are essential in passive immunotherapy, in order to decrease immunogenicity and implications with autoimmune disorders. The fact that we did not observe the expected (and undesirable) enhanced immunogenicity with the partially aggregated ^{90}Yttrium-labelled mAb, may be due to a general immunosuppression, since ^{90}Yttrium is a bone-seeking radiometal, that stays and irradiates the bone marrow for long time (S. Stewart et al., 1990). Thus, we may have underestimated the immune response.

In summary, we found that Ig aggregation was associated with enhanced immunogenicity. Although we could reduce the response of mice to deaggregated human IgG, in no case did we succeed in inducting tolerance. The particular polyclonal hIgG preparation used may explain our failure to induce permanent immune suppression, and our sensitive anti-Ig assay may account

for the disagreement with previous studies. The use of mAbs without specificity for mouse tissue may improve the partial unresponsiveness we observed. However, the key factor for this reduced immunogenicity appears to be antigen processing. Since this is under genetic control, it is impossible to predict how the equivalent system may operate in man. The outbred human population may "mask" such "responder" from "non-responder" phenotypes. However, given our experimental data, it would seem wise to use monomeric/deaggregated mAbs for clinical therapy wherever possible.

Chapter 6

Pharmacological immunosuppression: cyclosporin A

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6.1 Introduction

Advances in pharmacology in the last 30 years may not be characterized so much by the discovery of new, revolutionary, drugs, as by our better understanding of the mechanism of action, at the cellular and molecular level, of the existing pharmaceutical compounds. This, together with detailed *in vivo* kinetics and biodistribution studies, resulted in improved treatment protocols, and led to the synthesis of active derivatives with more favourable chemical and physical properties. Since the early 1960s, when successful drug-induced impairment of immune responses was demonstrated (Schwartz and Dameshek, 1959; McLaren, 1961; Schwartz and Dameshek, 1963), immunosuppressive drugs have been used with increasing frequency in a variety of clinical situations, such as transplantation, autoimmune disorders and lymphoproliferative diseases. Therefore, another approach to reducing the host's response to the xenogeneic immunoglobulin would be to use an immunosuppressive reagent. The complexity of an immune response together with the multiple action of most chemical immunosuppressants, often narrows the margins between desirable activity and unwanted side effects. Optimization of the conditions presupposes an exact knowledge of the mode of action and the level at which a drug interferes with immunity. Correct timing of drug administration, relative to antigenic challenge, determines the degree, duration and specificity of immune suppression. In addition, since animal studies precede clinical trials, inter-species variations in the drug kinetics, activity and side effects should also be taken into consideration prior to application.

Corticosteroids are one of the therapeutic agents most widely used, since they are very effective in modulating immune responses. They are normally synthesized from cholesterol in the adrenal cortex, and are of two classes: mineralcorticoids and glucocorticoids. Generally, members of the latter group are thought to interfere more with the immune system (Dougherty et al.,

1964). However, a large number of synthetic analogues have been produced with improved physical properties and increased immunosuppressive activity (Reviewed by Steinberg and Williams, 1978). Glucocorticoids bind to steroid receptors located in the cytoplasm of the target cells. The exact mechanism of drug internalization is still unknown. However, the receptor-hormone complex is soon found associated with chromatin, controlling mRNA synthesis (Baxter and Funder, 1979). The *in vivo* effects of corticosteroids on white blood cells vary: lymphocytes, monocytes, eosinophils and basophils are dramatically reduced (Fauci and Dale, 1974; Fauci, 1975; Dunsley et al., 1979; Thomson et al., 1980), whereas the number of neutrophils rises (Bishop et al., 1968; Dale et al., 1975). The effect of steroids on macrophages (mϕs) is of great importance for immune suppression, since mϕ activation is prevented or inhibited (Wang et al., 1972; Gemsa et al., 1973), a phenomenon often accompanied by reduction of Fc-receptor function (Hunninghake and Fauci, 1977). Thus, the T- and B-cell cooperation against thymus dependent (TD) antigens is impaired; this is also reflected by reduced primary (mainly) antibody responses (Gabrielsen and Good, 1967). Glucocorticoid-induced humoral unresponsiveness is further enhanced in experimental animals, since the T-helper (T_h) cell activity is also inhibited (Lee et al., 1975; Markham et al., 1978). In man however, steroids act against T-suppressor (T_s) cells, often resulting to augmentation of antibody production rather than suppression (Tuchinda et al., 1972; Waldmann et al., 1976).

Since the early 1950s a variety of purine analogues have been synthesized, but azathioprine and 6-mercaptopurine (6-MP) are those most often used for immunosuppression. Both substances are inactive in their native form. However, *in vivo* they are metabolized to thioinosinic acid which competively inhibits the enzymes responsible for the chain conversion: inosinic acid to xanthylic acid and adenylosuccinic acid to adenylic acid. This exerts feedback inhibition on the formation of 5-phosphoribosylamine, thus inhibiting DNA synthesis (reviewed by Rees and Lockwood, 1982). In addition,

another purine analogue, 6-thioguanine, may be directly incorporated into DNA as a false base and lead to lethal errors in transcription and/or duplication (Scannell and Hitchings, 1966). Purines, when administered *in vivo*, reduce lymphocyte and monocyte number, and also impair Killer-cell function (Hersch et al., 1966a; Campbell et al., 1976; Thomas et al., 1977). Humoral responses are readily suppressed following high doses of the drug given simultaneously or soon after antigenic challenge, since the effect on immunocompetent cells is exerted when they undergo proliferation after antigen stimulation (Levin et al., 1964; Hersch et al., 1966b; Santos, 1967).

Several alkylating agents, such as busulfan, chlorambucil, cyclophosphamide, ifosfamide, melphalan or thiotepa, are successfully used in cancer therapy. Their toxicity for rapidly dividing cells suggested their use as immunosuppressants. Cyclophosphamide (Cy) is one of the most commonly used. Like purine analogues, Cy is not toxic itself, but its two active metabolites, phosphoramide and nor-nitrogen mustards, are characterized by high alkylating (and consequently immunosuppressive) activity (Jardine et al., 1978; Shand and Howard, 1979). Apart from alkylation of purine bases in DNA, Cy has the additional property of covaiently cross-linking DNA strands, thus preventing their separation during mitosis (Roberts et al., 1971). Its presence *in vivo* is associated with leucopenia (Bach and Dardenne, 1972; Winkelstein, 1979) and reduced humoral responses. The immunosuppressive effect is more profound in experimental animals, where it is caused by relatively low doses, often leading to antigen-specific tolerance particularly against thymus independent (TI) antigens (Santos and Owens, 1966; Aisenberg and Davis, 1968; Howard and Shand, 1979; Shand and Howard, 1979). The mechanism of Cy-induced tolerance is not yet clear, but it has been suggested that the drug inhibits re-expression of surface immunoglobulins (Igs) on B-cells, and the tolerogenic effect is enhanced with increasing doses of antigen (Howard and Shand, 1979; Shand and Howard, 1979). Nevertheless, in man higher (thus more toxic) doses must be given simultaneously with the antigen for

induction of unresponsiveness, and no evidence of tolerance has been observed (Santos et al.,1969; Mackay et al., 1973).

Cyclosporin A (CSA), a naturally occurring fungal metabolite, is a relatively new agent found to possess immunosuppressive activity (Borel, 1976b; Borel et al., 1977). Thus, it has been extensively and successfully used in transplantation (reviewed by Shevach, 1985) and other, mainly autoimmune, disorders (reviewed by James, 1988). CSA acts primarily on T-lymphocytes (T_h-cells) not by direct killing, but indirectly by inhibiting their proliferation (Shevach, 1985; Klaus and Chisholm, 1986). It has been shown to block production of many cytokines including interferon-γ (IFN-γ), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 3 (IL-3) which are important for T- and B-cell as well as mϕ and dendritic cell activation and proliferation (Shevach, 1985; Granelli-Piperno et al., 1986). Recently it has been proposed that CSA may be responsible for excessive extracellular accumulation of calcium, resulting to Ca^{2+}/Na^{+} imbalance that restricts the cell to Go phase and prohibits cell cycle progression (Kimball and Sell, 1989). Granulocyte-macrophage colony stimulating factor (GM-CSF) production does not seem to be affected by CSA (Bickel et al., 1987), but there is evidence that other lymphokines (such as interleukin 4, interleukin 6) may be also directly or indirectly inhibited.

Chronic administration of CSA results in a dramatic reduction of all cells that usually reside in the thymus medulla (Kanariou et al., 1989). This has major implications for T-cell "education" of "self", which occurs in the thymus. Thus, intensive CSA treatment may lead to autoimmune diseases, especially at an early age (Sorokin et al., 1986; Sakaguchi and Sakaguchi, 1988). CSA has little or no bone marrow toxicity but in man it appears to possess nephrotoxic activity when given at high doses (James, 1988).

Corticosteroids, purine analogues, Cy and CSA are the most commonly used immunosuppressants, although several anti-mitotic drugs (pyrimidine

analogues, folic acid antagonists, antibiotics, alkaloids etc.) have also proved useful in many clinical situations (reviewed by Steinberg and Williams, 1978). In the previous chapter we showed that Balb/c mice were particularly good responders when challenged with aggregated and deaggregated polyclonal human IgG (hIgG). Although the deaggregated material was less immunogenic, Balb/c mice responded better overall to hIgG than CBA and C57BL/6. Their primary response was only marginally reduced when the immunogen was deaggregated hIgG, and their secondary response was lowered only when one of the challenge was with highly monomeric Ig (chapter 5, section 5.3.1).

In this chapter we combine immunization with aggregated and deaggregated hIgG in Balb/c mice, with chemical immunosuppression. We chose to use CSA because of the absence of myelosuppression. Cyclosporin A was administered orally at a low concentration throughout the experimental period. If successful, similar low doses in man would minimize nephrotoxic side effects. The multiple inhibitory action of CSA on the production of lymphokines that facilitate immune responses, further justify its use. In addition, its action was never associated with abrogation or inhibition of T_S-cells, resulting in immune response enhancement instead of suppression, as reported with corticosteroids, 6-MP and Cy treatment (Chanmougan and Schwartz, 1966; Tuchinda et al., 1972; Waldmann et al., 1976; Duclos et al., 1977; Berd and Mastrangelo, 1987).

The immunogen was deaggregated as well as aggregated hIgG, because of their different effects on mϕs (IL-1 production) as suggested in the previous chapter. Although the immunosuppressive effects of CSA are more profound in primary humoral responses (Homan et al., 1980; Lindsey et al., 1980), in some cases the secondary response was also reduced (Borel et al., 1977). In this work we focused mainly on whether CSA could suppress secondary responses to

IgG, and also whether there is any additive effect or synergism of action between CSA and deaggregation of the immunogen.

6.2 Materials and Methods

6.2.1 Animals: Groups of at least 3 female Balb/c mice (Olac, UK), 6-8 weeks old, were used throughout these experiments.

6.2.2 Human IgG: Polyclonal human IgG (Sigma, UK) was used as the immunogen for the mice. As described in chapter 5 (section 5.2.2), we found that, after reconstitution with PBS, pH 7.4, the lyophilyzed hIgG material contained up to 20% aggregates (mainly dimers). For each immunization animals received 100 μg of (partially) aggregated (A-hIgG) or deaggregated (see below) human IgG (D-hIgG) intraperitoneally.

6.2.3 Deaggregation of human IgG: The partially aggregated human IgG preparation was deaggregated by fast protein liquid chromatography (FPLC) size exclusion chromatography, using Superose 6 column, as described in chapter 5 (section 5.2.3). The deaggregated material was prepared only shortly before each immunization, in order to avoid reaggregation.

6.2.4 Cyclosporin A: Sandimmun, containing 100 mg/ml cyclosporin A in oil, was obtained from Sandoz Pharmaceuticals (Switzerland). Cyclosporin A was administered to mice orally, throughout the immunization period which was 10 days for the primary and 7 days for the secondary (see below). In two separate experiments, mice were given either 7 or 15 mg/kg/day of CSA. Control mice received oil alone.

6.2.5 Immunization schedule: Mice were primed with untreated, partially aggregated human IgG (A-hIgG), deaggregated human IgG (D-hIgG), or the

same preparations in combination with 15 mg/kg/day CSA. Ten days later, all the CSA treated animals were killed and bled (cardiac puncture), together with 3 mice that had received A-hIgG and D-hIgG and oil alone. The serum was removed by centrifugation, and stored until assessment of the primary anti-Ig response. From the remaining animals, mice primed with aggregated hIgG were divided in 4 groups and received A-hIgG, D-hIgG or the same preparations in combination with 15 mg/kg/day CSA. Similarly, mice primed with deaggregated hIgG were challenged with A-hIgG, D-hIgG or the same immunogens combined with 15 mg/kg/day CSA. Seven days later all animals were killed, bled by cardiac puncture, and the secondary anti-hIgG response was measured. This series of experiments were repeated with CSA at 7 mg/kg/day.

6.2.6 Measurement of the anti-immunoglobulin response: The mouse anti-hIgG humoral response was measured by an ELISA as described in the previous chapter (chapter 5, section 5.2.8). The results are expressed as mean area under the serum-titration curves. Since at least 3 mice were used in each experimental situation, statistical analysis was performed using the Student's t test. In our data, results obtained from less than 3 samples are excluded, and are shown on the histograms as not tested (N.T.).

As mentioned in the previous chapter (section 5.2.8), originally the data was expressed as absorbance vs. serum dilution of 1:10, or 1:100 when there was a prozone effect (Sivolapenko et al., 1989). However, these have now been re-evaluated using our computer programme (chapter 2). As before, since we could not use the same standard serum for all the experiments, each histogram should be seen as a single experiment. Generally, the data presented here do not differ from those of our publication. The p values are higher, but still within the statistically accepted levels.

6.3 Results

6.3.1 Immune response to hIgG after treatment with 15 mg/kg cyclosporin A: As shown in Figure 6.1a, cyclosporin A given to mice primed with hIgG, at a dose of 15 mg/kg/day, reduced their primary anti-Ig response. Mice treated with CSA, showed similar low anti-Ig levels, irrespective of whether they were primed with deaggregated (mean area 1.045±0.112) or aggregated hIgG (mean area 0.955±0.170). The reduction observed in A-hIgG primed and cyclosporin treated versus untreated mice, was 45.7% for those primed with A-hIgG (mean area 1.76±0.290) and 38.8% for those primed with D-hIgG (mean area 1.560±0.310) alone. Similarly, mice administered with D-hIgG together with CSA responded 40.6% and 33.0% less than the ones that received only A-hIgG or D-hIgG respectively. In all cases the p values were <0.04. In addition, although the response to D-hIgG was 11.3% lower than to A-hIgG, this was not statistically significant.

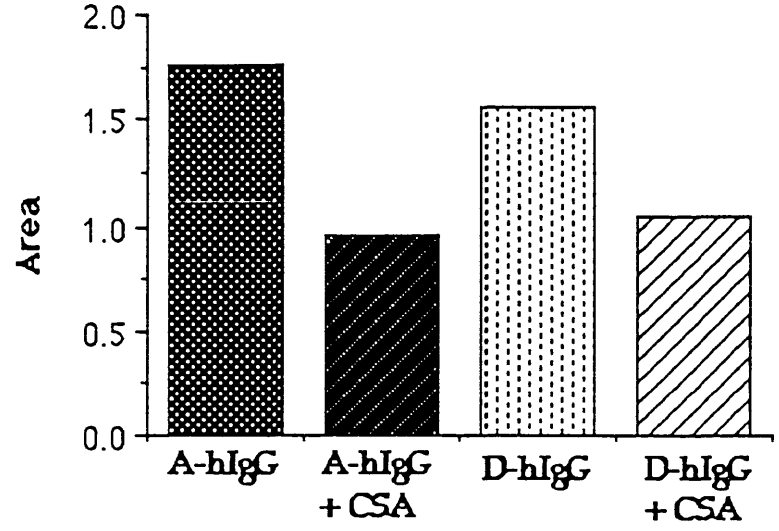
Animals primed with deaggregated hIgG showed a reduced secondary anti-Ig response when they were challenged with A-hIgG or D-hIgG in combination with 15 mg/kg/day CSA (Fig. 6.1b). Mice boosted with A-hIgG in combination with CSA (mean area 1.540±0.092), responded 11.8% less compared with those that received a second immunization of only A-hIgG (mean area 1.747±0.086, p=0.05), but there was no difference in the anti-Ig response when the second immunogen was D-hIgG alone (mean area 1.450±0.160). Nevertheless, in the latter case the response was further reduced when combined with CSA (mean area 0.869±0.105). The reduction was 41.5% and 50.3% compared with the response observed in mice injected for a second time with only D-hIgG or A-hIgG respectively (Fig. 6.1b). In all cases the lower anti-Ig responses were statistically significant, with p<0.04.

Figure 6.1: a) Primary response of Balb/c mice against (partially) aggregated human IgG (A-hIgG), (partially) aggregated human IgG combined with 15 mg/kg/day cyclosporin A (A-hIgG+CSA), chromatographically deaggregated human IgG (D-hIgG) and chromatographically deaggregated human IgG combined with 15 mg/kg/day cyclosporin A (D-hIgG+CSA).

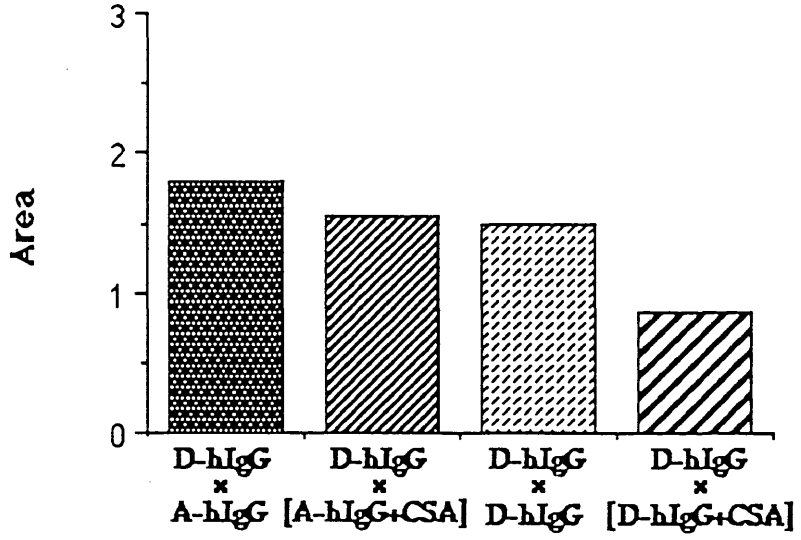
b) Secondary response of Balb/c mice when primed with chromatographically deaggregated polyclonal human IgG and boosted with:
(partially) aggregated polyclonal human IgG (D-hIgGxA-hIgG),
(partially) aggregated polyclonal human IgG combined with 15 mg/kg/day cyclosporin A (D-hIgGx[A-hIgG+CSA]),
chromatographically deaggregated polyclonal human IgG (D-hIgGxD-hIgG),
chromatographically deaggregated polyclonal human IgG combined with 15 mg/kg/day cyclosporin A (D-hIgGx[D-hIgG+CSA]).

c) Secondary response of Balb/c mice when primed with (partially) aggregated polyclonal human IgG and boosted with:
(partially) aggregated polyclonal human IgG (A-hIgGxA-hIgG),
(partially) aggregated polyclonal human IgG combined with 15 mg/kg/day cyclosporin A (A-hIgGx[A-hIgG+CSA]),
chromatographically deaggregated polyclonal human IgG (A-hIgGxD-hIgG),
chromatographically deaggregated polyclonal human IgG combined with 15 mg/kg/day cyclosporin A (A-hIgGx[D-hIgG+CSA]).

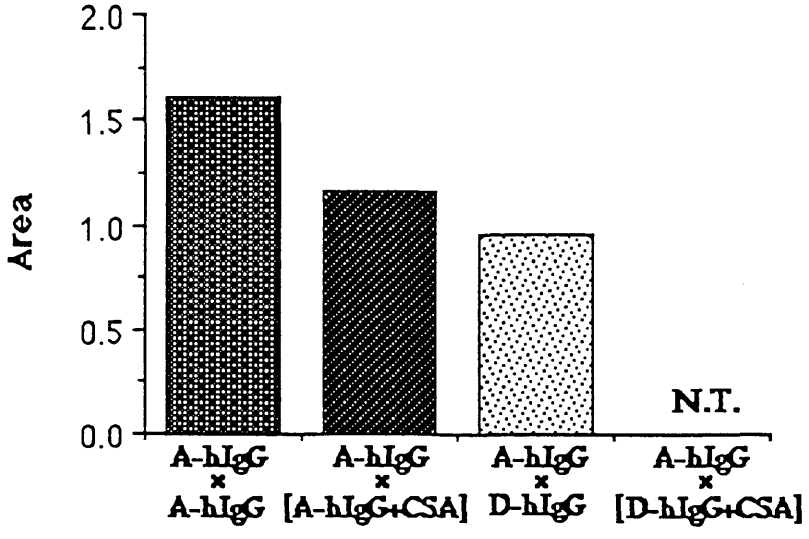
All results are expressed as areas under the ELISA serum-titration curves, and are the mean value of groups of at least 3 mice. N.T. = not tested.


Area
2.0
1.5
1.0
0.5
0.0
A-hIgG
A-hIgG + CSA
D-hIgG
D-hIgG + CSA

a


Area
3
2
1
0
D-hIgG × A-hIgG
D-hIgG × [A-hIgG+CSA]
D-hIgG × D-hIgG
D-hIgG × [D-hIgG+CSA]

b


Area
2.0
1.5
1.0
0.5
0.0
N.T.
A-hIgG × A-hIgG
A-hIgG × [A-hIgG+CSA]
A-hIgG × D-hIgG
A-hIgG × [D-hIgG+CSA]

c

Similarly, in A-hIgG primed mice, CSA reduced the secondary anti-Ig response after they were challenged with A-hIgG (mean area 1.165±0.190) (Fig. 6.1c). The reduction was 27.1% compared with those that received a second immunization of A-hIgG alone (mean area 1.602±0.211, p=0.04). However, the response was even lower (40%) when the animals were boosted with monomeric IgG (mean area 0.961±0.105, p=0.035) (Fig. 6.1c). Unfortunately we were unable to assess whether a combination of CSA and deaggregated Ig would further reduce the secondary response, due to inadequate number of samples.

6.3.2 Immune response to hIgG after treatment with 7 mg/kg cyclosporin A: Cyclosporin A given at a dose of 7 mg/kg/day reduced the primary anti-Ig response in mice injected with either aggregated or deaggregated hIgG (Fig. 6.2a). For the animals primed with A-hIgG (mean area 0.153±0.055), the reduction was 50.0% and 45.7% compared with the response when only A-hIgG (mean area 0.315±0.045) or D-hIgG (mean area 0.290±0.039) respectively was administered. Similarly, the combination of CSA with deaggregated IgG (mean area 0.189±0.021) resulted in 40.1% and 34.8% reduction of the primary anti-Ig response, compared with animals primed with only A-hIgG or D-hIgG respectively (Fig. 6.2a). All the p values were <0.035. The low (8%) difference observed between mice injected with A-hIgG and D-hIgG alone, is not statistically significant.

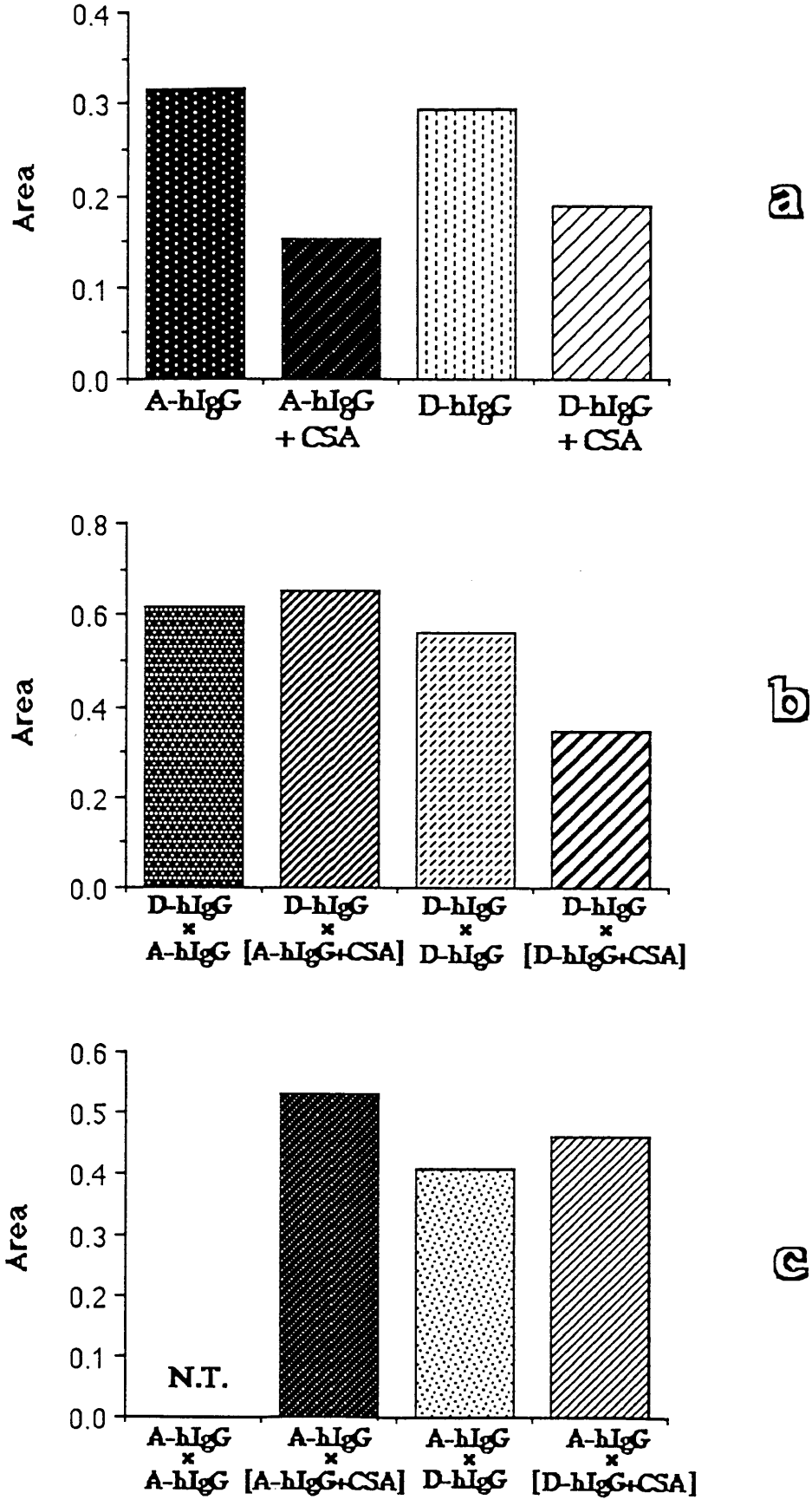
Cyclosporin A, given at 7 mg/kg/day, did not have any effect in the secondary anti-Ig response of mice primed with deaggregated hIgG and boosted with A-hIgG (Fig. 6.2b). However it appeared to be effective when the D-hIgG was used for the second immunization (mean area 0.348±0.086, Fig. 6.2b). The reduction of the immune response was 43.7% and 38.2% compared with animals boosted with only A-hIgG (mean area 0.618+0.123, p=0.03) or D-hIgG (mean area 0.563+0.990, p=0.03) respectively.

Figure 6.2: a) Primary response of Balb/c mice against (partially) aggregated human IgG (A-hIgG), (partially) aggregated human IgG combined with 7 mg/kg/day cyclosporin A (A-hIgG+CSA), chromatographically deaggregated human IgG (D-hIgG) and chromatographically deaggregated human IgG combined with 7 mg/kg/day cyclosporin A (D-hIgG+CSA).

b) Secondary response of Balb/c mice when primed with chromatographically deaggregated polyclonal human IgG and boosted with:
(partially) aggregated polyclonal human IgG (D-hIgGxA-hIgG),
(partially) aggregated polyclonal human IgG combined with 7 mg/kg/day cyclosporin A (D-hIgGx[A-hIgG+CSA]),
chromatographically deaggregated polyclonal human IgG (D-hIgGxD-hIgG),
chromatographically deaggregated polyclonal human IgG combined with 7 mg/kg/day cyclosporin A (D-hIgGx[D-hIgG+CSA]).

c) Secondary response of Balb/c mice when primed with (partially) aggregated polyclonal human IgG and boosted with:
(partially) aggregated polyclonal human IgG (A-hIgGxA-hIgG),
(partially) aggregated polyclonal human IgG combined with 7 mg/kg/day cyclosporin A (A-hIgGx[A-hIgG+CSA]),
chromatographically deaggregated polyclonal human IgG (A-hIgGxD-hIgG),
chromatographically deaggregated polyclonal human IgG combined with 7 mg/kg/day cyclosporin A (A-hIgGx[D-hIgG+CSA]).

All results are expressed as areas under the ELISA serum-titration curves, and are the mean value of groups of at least 3 mice. N.T. = not tested.


Area
0.4
0.3
0.2
0.1
0.0
A-hIgG
A-hIgG + CSA
D-hIgG
D-hIgG + CSA
a
Area
0.8
0.6
0.4
0.2
0.0
D-hIgG × A-hIgG
D-hIgG × [A-hIgG+CSA]
D-hIgG × D-hIgG
D-hIgG × [D-hIgG+CSA]
b
Area
0.6
0.5
0.4
0.3
0.2
0.1
0.0
N.T.
A-hIgG × A-hIgG
A-hIgG × [A-hIgG+CSA]
A-hIgG × D-hIgG
A-hIgG × [D-hIgG+CSA]
c

In addition, as shown in Figure 6.2c, mice primed with aggregated hIgG responded equally well when they were challenged with A-hIgG combined with 7 mg/kg/day CSA, D-hIgG alone, or D-hIgG and CSA.

6.4 Discussion

Corticosteroids, purine analogues and alkylating agents have been extensively used *in vivo* because of their immunosuppressive activity (reviewed by Rees and Lockwood, 1982), and partial or total reduction of humoral responses against various antigens were often observed (Hersh et al., 1966b; Santos, 1967; Rossen et al., 1971; Mackay et al., 1973; Markham et al., 1978). In addition, cyclophosphamide was shown to reduce antibody affinity (Steward et al., 1973b) and even induce a state of permanent antigen-specific unresponsiveness, which was more profound for TI antigens (Howard and Shands, 1979), than TD ones (Many and Schwartz, 1970b; Miller and Mitchell, 1970; Ramshaw et al., 1977). However, exact timing of drug and immunogen administration was crucial, not only because most of the above drugs act on proliferating cells, but also because they inhibit or eliminate T_s-cells, resulting to enhanced instead of lower immune responses (Chanmougan and Schwartz, 1966; Tuchinda et al., 1972; Waldmann et al., 1976; Duclos et al., 1977; Berd and Mastrangelo, 1987). This, together with the high myelosuppression associated with their immunoregulatory action, suggested the use of an alternative drug: cyclosporin A. CSA blocks production of vital lymphokines such as IFN-γ, IL-1, IL-2, IL-3, thus preventing humoral responses (Borel et al., 1977; Homan et al., 1980; Lindsey et al., 1980). No damage to bone marrow occurs, although chronic administration of CSA at high doses leads to thymus-microenvironment changes (Kanariou et al., 1989) and nephrotoxicity in man (James, 1988). In recent studies, CSA induced and maintained immunosuppression against a mouse monoclonal antibody in rabbits

(Ledermann et al., 1988a). Moreover, in recent clinical trials it has been reported to prevent the development of anti-murine Ig antibodies in patients undergoing monoclonal antibody therapy (Ledermann et al., 1988b).

As discussed in the previous chapter, Balb/c mice are particularly good responders against human IgG, and our deaggregated material reduced but did not abolish the response. Here we combined immunization with deaggregated or aggregated hIgG with two different low doses of CSA, given orally for the whole period of the primary or the secondary response.

We found that CSA reduced the primary anti-Ig response at both 15 and 7 mg/kg doses (Figs. 6.1a and 6.2a). Although the two experiments are not directly comparable, from the reduction observed compared to the controls, we see that 7 mg/kg is as effective as 15 mg/kg. In addition, there was no difference when the primary immunogen was aggregated or deaggregated hIgG, as shown by both experiments (Figs. 6.1a and 6.2a). However, what is important to emphasize is that we did not observe any statistically significant difference in the response of control mice that received A-hIgG and D-hIgG alone (Figs. 6.1a and 6.2a). This contrasts with our previous data, where deaggregated Ig was approximately 25% less immunogenic than partially aggregated Ig (chapter 5, section 5.3.1). Nevertheless, the difference observed then, was at the border line of statistical significance ($p=0.05$), indicating that such results should be treated with more scepticism than excitement.

Similarly, the reduction of the secondary response in mice primed with D-hIgG and boosted with A-hIgG combined with 15 mg/kg CSA (Fig. 6.1b) was so low and variable (11.8%, $p=0.05$), that, although within statistically acceptable levels, we would not like to over emphasize their importance. However, mice challenged with D-hIgG combined with CSA, showed a significant reduction in the anti-Ig response, when both 15 mg/kg (Fig. 6.1b, mean reduction 45.9%) and 7 mg/kg (Fig. 6.2b, mean reduction 40.9%) of CSA were used ($0.3<p<0.4$).

This was unexpected, since assuming that a small proportion of deaggregated material was processed by antigen presenting cells and presented to T_h-cells, whose activation was further inhibited by CSA, we should expect this phenomenon to be the same if not enhanced when aggregated material was used with CSA. But as explained above, this may have not occurred (Figs. 6.1b and 6.2b). At the moment we are unable to explain this additive effect between deaggregated Ig and CSA in secondary responses. Further experiments are required to determine a possible direct influence of monomeric Ig on immunocompetent cells, and the way in which this enhances CSA action.

Cyclosporin-mediated reduction of the secondary response was observed when mice were injected twice with aggregated hIgG (Fig. 6.1c). As shown in Figure 6.1c, aggregated hIgG was significantly more immunogenic than D-hIgG, something that is also confirmed by our experiments in the previous chapter (chapter 5, section 5.3.1, Fig. 5.4c). The reduction of the anti-Ig response observed to a second challenge with A-hIgG combined with 15 mg/kg CSA, could be attributed more to the drug's activity against IL-1 production (that aggregated hIgG facilitates, see chapter 5, section 5.4: discussion), than to inhibition of IL-2. This is supported by the fact that when the second immunogen was D-hIgG alone (does not trigger IL-1 production), the response was equally low (Fig. 6.1c).

Unfortunately, lack of sufficient serum samples made it impossible to assess the anti-Ig response of mice primed with A-hIgG and boosted with D-hIgG in combination with CSA (N.T., Fig. 6.1c). It would have been of great interest to see whether the same degree of additive effect between CSA and deaggregation is observed, as described above (Figs. 6.1b and 6.2b). As shown in Fig 6.2c, the same problem was encountered when less than 3 serum samples were obtained from the control mice that received A-hIgG twice. However, since there appears to be a a consistent difference of 40-66% in the response compared to mice primed with A-hIgG and boosted with D-hIgG (section 6.3.1,

Fig. 6.1c, and chapter 5, section 5.3.1, Fig 5.4c), we may assume that 7 mg/kg of CSA also reduced the secondary response against A-hIgG, presumably via its IL-1-production inhibition, as described above. In addition, no additive effect was observed between deaggregation and CSA, at least at this concentration (7 mg/kg).

In conclusion, cyclosporin A reduced equally the primary response against aggregated and deaggregated hIgG in Balb/c mice, when used at both 15 and 7 mg/kg doses. The secondary response was also reduced when both immunogens were aggregated hIgG, which, as suggested in the previous chapter, are potent inducers of IL-1. This indicates that CSA may "break" a pathway of IL-1 production. When one of the injections was deaggregated hIgG, CSA appeared not to affect the immune response. However, administration of monomeric material twice, in combination with CSA, further reduced the secondary anti-Ig response, in a way that at the moment we cannot explain.

In the future, new immunosuppressive drugs (eg. 15-deoxyspergualin, FK-506) may further optimize desirable activity and toxic side effects, becoming therefore more useful for drug-induced unresponsiveness (Dickneite et al., 1988; Woo et al., 1988; Leonard et al., 1989). Nevertheless, the absence of specificity of this type of immunosuppression, makes this approach less attractive for future application in patients with cancer receiving monoclonal antibody therapy, since they are already "overloaded" with chemotherapy and radiotherapy. Unless immunosuppressive drug treatment renders the host permanently unresponsive to a given antigen, our goal still remains to induce antigen-specific tolerance, leaving the rest of the immune system intact.

Chapter 7

Antigen "suicide"

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7.1 Overview

The binding of antigen (Ag) on the surface immunoglobulin (Ig) of B-cells is very important, since it is the determining factor which decides the fate of both antigen and B-cell. B-cells recognise Ags in their native conformation, either free in solution or bound on cell surface membranes. For immature B-cells, antigen cross-linking of the surface Ig results in their inhibition and effective elimination (reviewed by Nossal, 1983; Rudich et al., 1988), whereas mature B-cells are stimulated and expand clonally to become antibody secreting or memory cells. Nevertheless, for thymus-dependent Ags, simple binding is not sufficient for B-cell activation. B-cells need T-helper cell co-operation in order to receive the full signaling that promotes their growth and differentiation from small resting cells to those that secrete antibody (reviewed by Lanzavecchia, 1985).

It is generally believed that B-cells capture their Ag, process it, and present it to T-helper cells, like macrophages do (Fauci, 1982; Chesnut and Grey, 1986; Hayglass et al., 1986). The only difference being that antigen recognition of B-cells is specific, via their surface immunoglobulin receptor, whereas other antigen presenting cells (APC) take up the antigen non-specifically. This is the reason why B-cells can bind, and efficiently present to T-cells, antigen at a concentration 1000 times lower than that required by conventional APCs (Lanzavecchia, 1985; Tony and Parker, 1985).

B-cell stimulation and differentiation is outlined in Figures 7.1a and b: antigen binds specifically onto the surface receptor Igs of B-cells. Thus the cell, with the help of several lymphokines, is activated and proliferates. During this clonal expansion, somatic mutation in the variable (V) region genes allows a wider range of antibody binding sites to be present in this B-cell population. Those cells with receptors with the highest affinity for their antigen, are likely to multiply preferentially (Tonegawa, 1983). B-cells will then either become

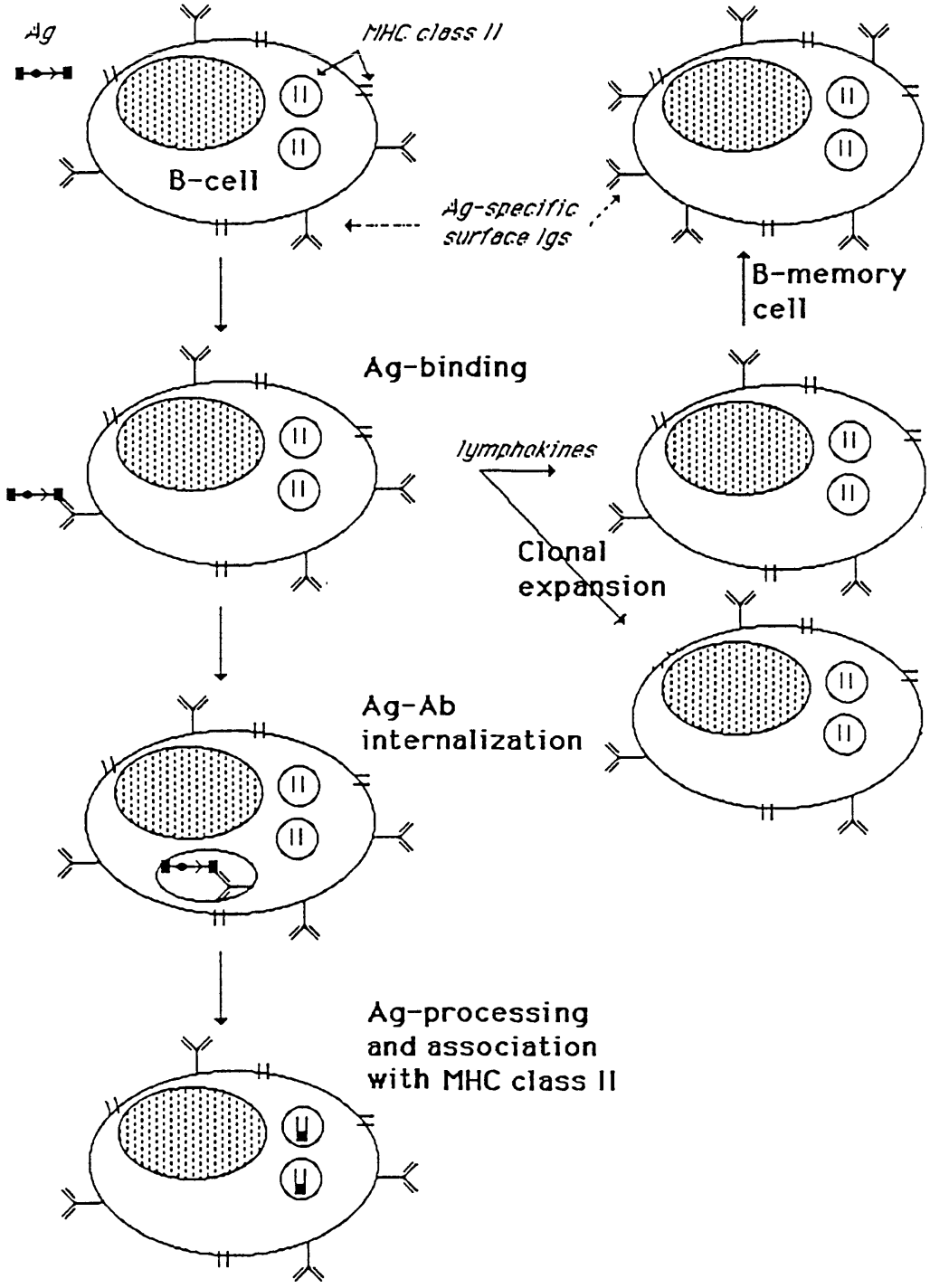


Figure 7.1.a: B- and T-cell interactions in immune responses. B-cells bind the antigen via their specific surface-immunoglobulins, and undergo proliferation. Some cells become memory cells, whereas others internalize the antigen and process it in association with MHC class II.
Ag: Antigen.
Igs: B-cell surface specific immunoglobulins.

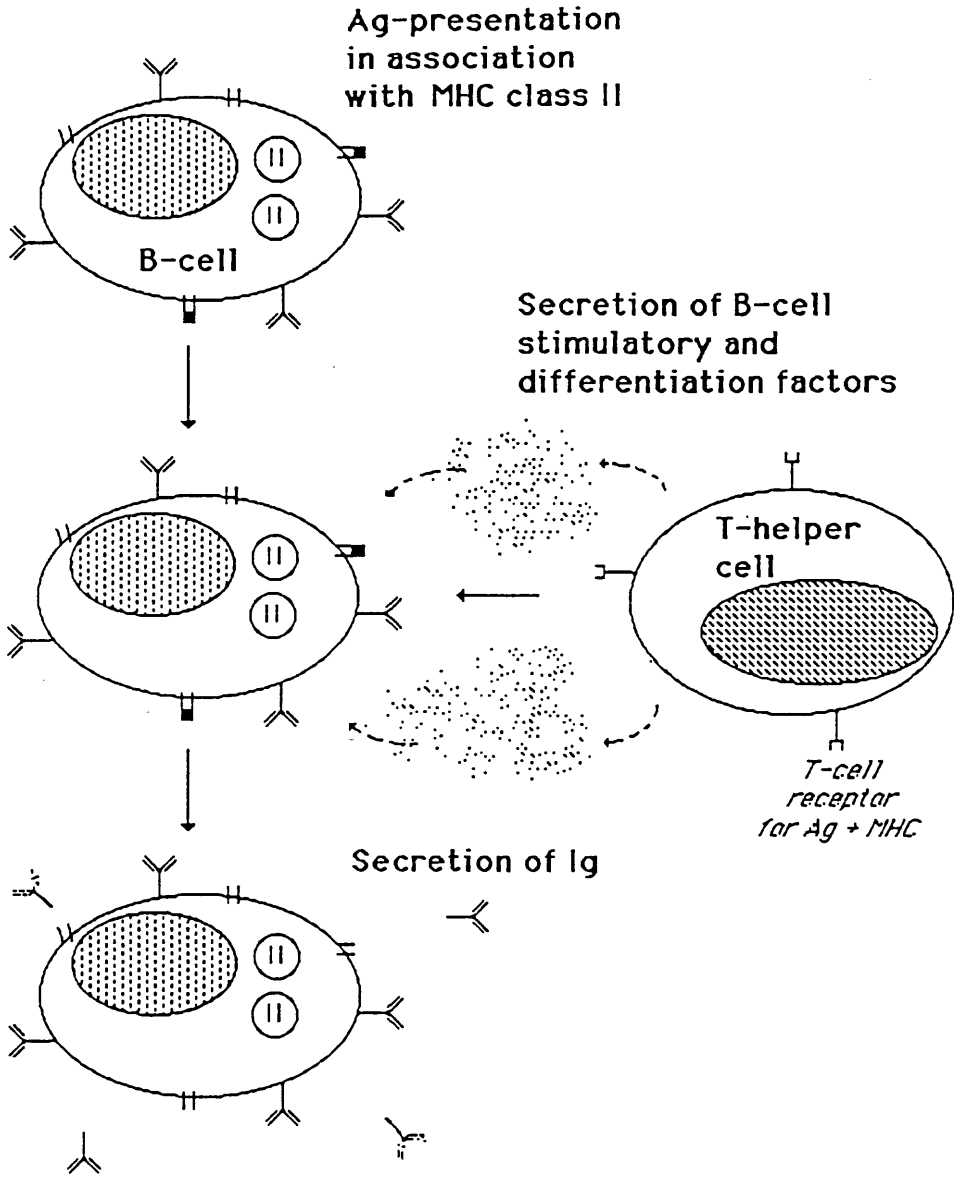


Figure 7.1.b: B- and T-cell interactions in immune responses. The antigen is presented on the B-cell surface in conjunction with MHC class II. This complex is recognized by T-helper cells that, by releasing certain factors, stimulate the B-cell to secrete its specific Immunoglobulins.
Ag: Antigen.
Ig: B-cell surface specific immunoglobulins.

memory cells or continue their differentiation. B-memory cells are relatively long-lived small resting lymphocytes with an increased density of surface Ig, which they do not secrete, unless they are restimulated by Ag with T-cell co-operation. B-cells destined to fully differentiate, internalize the antigen and process it in association with the Major Histocompatibility Complex (MHC) class II, before it appears on the cell surface. The MHC class II/antigen-fragment complex is now recognizable by the T-cell receptor of the T-helper cells (Fig. 1b), that - having been already stimulated by APCs - bind to the B-cells. This encounter restimulates the T-cells to produce stimulatory factors in the B-cell's microenvironment, which in turn trigger the latter to secrete their antibody (Fauci, 1982).

Theoretically therefore, if an antigen is somehow conjugated to a toxic compound, it should kill rather than drive the B-cells to differentiation and antibody secretion. This is the concept of antigen "suicide" (Humphrey and Keller, 1969). Thus, only the B-cell population that bears Ig receptors specifically for the "lethal" antigen will be deleted. Specific immunosuppression, and perhaps even tolerance, may be achieved under conditions where the rest of the immune system remains intact and fully potent to react against potential pathogens.

The overall aim of this study is to identify ways by which the host's immune response to xenogeneic Ig, administered for therapeutic purposes, can be reduced or eliminated. In this chapter we describe two approaches to the construction of Igs that have lethal properties to lymphocytes *in vivo*. The first approach was to heavily radiolabel the antibody molecule with various radioisotopes, while the second was to use an antibody-drug conjugate. We examined whether immunocompetent cells that recognise the Ig molecule as foreign would be impaired by either the radioactivity or the cytostatic drug, when the "lethal" immunoglobulin was administered *in vivo* to mice and

rabbits. The results, if successful, could be of importance not only to immunotherapy, but also to other clinical situations such as autoimmunity, allergy or transplantation.

7.2.1 Radiolabelled Antibody - Introduction

The theory of B-cell clonal selection in immune responses was first proposed by M. Burnet in 1959. As originally envisaged, immunological recognition was effected through antibody-like sites present on antigen-binding cells, that were apparently lymphoid cells (Burnet, 1959). The exact nature of the cells involved in antigen recognition was unclear at the time. M. Fishman et al. proposed that macrophages were the important cells for an immune response (Fishman and Adler, 1963; Fishman et al., 1963), whereas J. L. Gowans and J. W. Uhr suggested that other lymphocytes are responsible (Gowans and Uhr, 1966). It was essential to identify the type of cells involved in antigen binding and the specificity of this encounter.

Initial experiments by many investigators (Nossal et al., 1966; Han and Johnson, 1966; Naor and Sulitzeanu, 1967) showed that when an antigen is injected into animals, it is found on lymphocytes mainly in the spleen. In addition, D. Naor and D. Sulitzeanu reported that only very few splenic lymphocytes could recognise a given antigen (bovine serum albumin, BSA, in his case), and that there were cells capable of binding more BSA than others (Naor and Sulitzeanu, 1967). It is important to note here, that all the above experiments were performed using radiolabelled Ag (mainly with ^{125}Iodine, ^{125}I), followed by autoradiography, since this was the most sensitive and readily available detection method at the time.

Following on from these studies, G. L. Ada and P. Byrt reported an unexpected phenomenon: irradiated mice reconstituted with syngeneic spleen cells that

had been previously exposed to ^{125}I-labelled antigen (flagellin) failed to respond to a subsequent challenge with flagellin, whereas they responded normally to other antigens (Ada and Byrt, 1969). Later the same year J. H. Humphrey and H. U. Keller reported that mice failed to exhibit a primary and secondary response against a synthetic antigen (TIGAL), when it was heavily radiolabelled with ^{125}I (Humphrey and Keller, 1969). Two years later the same group confirmed and extended Ada's initial experiments, reporting that the same degree of specific unresponsiveness was observed in irradiated mice, even when the spleen cells - *ex vivo* incubated with ^{125}I-labelled antigen before transfer - were primed (Humphrey et al., 1971). The most likely explanation was that only those cells that could specifically bind the antigen (*in vitro* and *in vivo*) were lethally irradiated and killed. Although initiated to explore the nature of the B-cell repertoire, these experiments established the concept of antigen "suicide". Thus, an antigen can be made "lethal" by labelling with the appropriate β-emitter; it will then kill rather than stimulate immunocompetent cells to proliferation, maturation and eventually secretion of antibodies (Fig. 7.1a and b).

Following the first reports on B- and T-cell co-operation for antibody production (Mitchell and Miller, 1968), A. Basten et al. showed that specific unresponsiveness to ^{125}I-labelled chicken IgG, could only be achieved if both thymus derived T-cells and spleen derived B-cells were together incubated with the radioactive antigen (Basten et al., 1971). E. Unanue extended the above results, when he found that bone marrow cells exposed to highly radioactive keyhole limpet haemocyanin (KLH), failed to adoptively transfer immunity to irradiated syngeneic mice (Unanue, 1971). In addition, it has been shown that in a hapten-carrier situation, no anti-hapten response was observed after specific killing of lymphocytes that recognised the carrier (using ^{125}I-labelled carrier) (Roelants and Askonas, 1971; Golan and Borel, 1972). Antibody affinity was not very important for this antigen "suicide"; avidity and antigen structure and valency seemed to be the crucial factors (Willcox et al., 1974).

Finally, it has recently been shown that allergy to birch pollen could be prevented in rabbits when highly radiolabelled birch pollen was administered *in vivo* (Filipp et al., 1981). Similarly, ^{125}I-labelled egg albumin (OVA) and human serum albumin (HSA) failed to sensitize guinea pigs, and the expected anaphylaxis did not occur even after subsequent challenges with unlabelled OVA or HSA (Csaba et al., 1985).

We therefore chose to test the efficacy of this "lethal" radiolabelled antigen approach in our xenogeneic *in vivo* serotherapy system. We selected two experimental systems: a) human IgG administered to mice, as the reciprocal of murine Igs given to humans, thus keeping the "genetic difference" between donor and recipient constant, and b) murine monoclonal antibodies (mAbs) to rabbits, since this enabled us to use the same reagents as those used clinically. In our experiments with mice, polyclonal human IgG (hIgG) was radiolabelled with ^{111}Indium (^{111}In), since this isotope has been shown to be particularly toxic to lymphocytes (Chisholm et al., 1979; Ten Berge et al., 1983). Even when no effect on viability or cell surface phenotype is evident, there may be a significant reduction in antibody dependent cellular cytotoxicity (Signore et al., 1983), and significant functional abnormality (Segal et al., 1978; Chisholm et al., 1979; Kraal and Geldof, 1979). Generally, labelling of lymphocytes with ^{111}In, even at very low levels, is associated with alteration of the proliferative capacity (Chisholm et al., 1979; Kraal and Geldof, 1979) and chromosomal abnormality of the cells (Ten Berge et al., 1983). In addition, since the effects of ^{125}I-labelled antigen were demonstrated in various experiments mentioned above, the *in vivo* effect of a highly radiolabelled (with ^{125}I) mouse monoclonal antibody was studied, when administered to rabbits.

7.2.2 Materials and Methods

7.2.2.1 Animals - Immunization schedule: Three 6-8 week old unprimed female Balb/c mice (Olac, UK) were injected i.p. with ^{111}In-labelled polyclonal human IgG (^{111}In-hIgG), and "cold" HSA as an irrelevant antigen. Another group of three mice received the same amount of unlabelled hIgG and HSA. Ten days later they were killed, bled by cardiac puncture, and their primary anti-hIgG response measured. Similarly, the secondary response was examined: six mice were primed with hIgG and HSA i.p.; after 10 days 3 of them were boosted i.p. with ^{111}In-hIgG / "cold" HSA, and the other 3 with unlabelled hIgG / HSA. They were bled on day 17 as above.

For the second experiment, a group of 3 unprimed "1/2 lop" male rabbits (Froxfield Farm, UK), 3-4 kg, were injected i.v. (ear vein) with a mouse monoclonal antibody labelled with ^{125}Iodine. Another three rabbits received the same amount of unlabelled antibody. Seven days later they were bled and boosted with the same "cold" mouse monoclonal antibody, i.p. The rabbits' anti-mouse Ig response was assayed on day 7, 10, 14, and 21.

7.2.2.2 Antigen - Radiolabelling: Polyclonal human IgG, for use as an antigen in mice, was purchased from Sigma, UK. Its purity was assayed and described in detail in Chapter 5. For each immunization 50 μg of hIgG and 100 μg of HSA (Blood Products Laboratory, Herts, UK) were injected in complete Freund's adjuvant (CFA, Difco, USA). Prior to labelling with ^{111}Indium, hIgG was conjugated to the chelating agent diethylenetriamine pentaacetic acid (DTPA) as previously described (Chapter 3). We tried to keep the DTPA/hIgG ratio as high as possible in order to achieve the maximum radiolabelling. The starting ratio was 50-80 DTPA/Ab, and the final estimated bound DTPA molecules per Ab were 8-10. Free ^{111}Indium was separated from that bound to Ab using Sephadex G-50 (Pharmacia, Sweden) size exclusion chromatography.

The mouse monoclonal antibody used for the rabbit experiments was the 225-28S, produced by Sorin Biomedica, Italy, and kindly provided in purified form by Dr. G. Paganelli (Dept. of Nuclear Medicine, St. Rafael Hospital, Milan, Italy). This antibody recognizes a high molecular weight glycoprotein expressed on human melanomas and some skin carcinomas (Wilson et al., 1981; Siccardi et al., 1986). Labelling with ^{125}Iodine was performed using either n-bromo succinamide (as described in chapter 3 for ^{131}Iodine, section 3.2.3c) or iodogen (as described in chapter 3, section 3.2.3c). For the first and second immunization, 4 μg and 1 mg of mouse antibody was administered respectively. Prior to the initial injection with the ^{125}I-225-28S, Sephadex G-50 gel filtration and 10% trichloroacetic acid (10% TCA) precipitation showed that the labelling efficiency was over 90%.

7.2.2.3 Immunoreactivity: We examined whether the mouse monoclonal antibody 225-28S reacted with any antigen on normal rabbit tissue, by indirect immunoperoxidase. Frozen sections (5 μm) of rabbit liver, kidneys, lung and skin, were fixed in acetone for 10 min and incubated with 1-2 μg (100 μl) of the antibody for 45 min (room temperature). As negative control we have used the 4D513/2118 mouse monoclonal IgG1, which recognises the idiotope of the immunoglobulin expressed on a human B-cell lymphoma. The slides were washed (phosphate buffered saline, pH 7.4) and incubated for a further 45 min with a rabbit anti-mouse Ig conjugated to horseradish peroxidase (DAKO, Denmark). For the development of the colour, the substrate diaminobenzidine (Sigma) was used. We found that the 225-28S reacted with rabbit kidney (glomeruli) and weakly with liver.

7.2.2.4 Kinetics: The *in vivo* kinetics of the mouse monoclonal antibody in rabbits was measured as follows: immediately after injection of the radiolabelled 225-28S, all 3 rabbits were placed under a γ-camera (IGE 400A, USA), and whole body counts were recorded. At the same time a blood sample was taken, in order to examine the percentage of free ^{125}Iodine in the

circulation. After the blood was left to clot at 37°C for 4 hr, the serum was removed; an aliquot was loaded on a Sephadex G-50 column, and 0.5 ml fractions were collected after elution with PBS, PH 7.4 (as described in Chapter 3). The same procedure was repeated once daily for 7 days.

7.2.2.5 Anti-immunoglobulin response: An ELISA, as described in Chapter 2, was used for the measurement of the anti-Ig response. Briefly, 96-well plates were coated with hIgG (to test mouse sera) or 225-28S (to test rabbit sera). Mouse and rabbit blood samples were left to clot overnight at 4°C, and the serum removed. It was applied to the corresponding antigen at 1:10 serial dilutions, in duplicate, followed by a species-specific sheep anti-mouse Ig-HRP or species-specific donkey anti-rabbit Ig-HRP (Amersham) as appropriate. The absorbance in each well was measured after addition of the substrate ABTS. The results are given as area under the sigmoidal curve obtained from the titration of the sera.

Prior to administration, all rabbits were tested for pre-existing or cross-reactive anti-mouse Ig antibodies, using the above ELISA. Only animals with no anti-mouse Ig reactivity were chosen for the experiment. This was not possible with the mice, since it was difficult to bleed them without sacrificing them. Therefore out of a large cage of this inbred population, three mice were randomly chosen before injection, and used as negative controls.

7.2.2.6 Anti-albumin response: An ELISA similar to that above was developed for the detection of anti-HSA antibodies, where Sterilin microtitre plates were coated with HSA, in carbonate/bicarbonate buffer pH 9.6. The optimal concentration of HSA was determined by a chequer-board ELISA as described in detail in chapter 2 (section 2.3): human serum albumin was coated onto 96-well plate at different dilutions, from 1,600 - 12.5 ng/well; rabbit anti-HSA-HRP (DAKO) was applied and the saturating concentration found to be 200 ng/well. After determination of the Ag concentration, the test sera, second layer,

substrate, development and evaluation of the results was the same as for the anti-Ig response described above.

7.2.3 Results

7.2.3.1 Radiolabelling: Under the particular conditions of DTPA coupling and ^{111}In labelling, the highest specific activity achieved for the ^{111}In-hIgG was 15 mCi/mg for the first and 7 mCi/mg for the second immunization.

Several mouse mAbs were labelled with ^{125}I using the n-bromo succinamide method, but the 225-28S consistently labelled to the highest specific activity (80 mCi/mg). This was also true when iodogen was used, although with this method we succeeded in incorporating more ^{125}I into the 225-28S Ab molecule: the specific activity achieved was 180 mCi/mg. The latter preparation was used as an immunogen in rabbits. There was no evidence of radiolysis; the antibody was administered immediately after Sephadex G-50 gel filtration, which showed that all radioactivity was associated with the protein. In addition, the kinetics of the antibody (see section 7.2.3.2 below) followed the pattern usually reported for mouse antibodies in rabbits.

No side effects were observed due to the administration of the radiolabelled protein. Only those mice that received the first injection of ^{111}In-hIgG showed a mild diarrhoea one day after administration, lasting for approximately 24 hours.

7.2.3.2 Kinetics: The clearance of the ^{125}I-225-28S in rabbits is shown in Figure 7.2. The curves are corrected for isotope decay and the percentage of free ^{125}Iodine co-measured was subtracted, since it was known after size exclusion chromatography. The half-life of the Ab was found to be 49 $\pm$ 4 hours.

7.2.3.3 Anti-immunoglobulin and anti-albumin response: The mouse anti-hIgG primary response is shown in Figure 7.3a. Mice that were primed with ^{111}In-hIgG (15 mCi/mg) responded 13% less than those that received the "cold" hIgG ($Mean_{^{111}In\text{-}hIgG}$ = 2.035±0.038, $Mean_{hIgG}$ = 2.342±0.16, p = 0.0485). Mice primed with hIgG and boosted with ^{111}In-hIgG (7 mCi/mg), also responded 10% less than those that had been injected twice with hIgG (Fig. 7.3b), although this difference was not statistically significant (p = 0.09). As seen in Figures 7.4a and b, the response against HSA was similar in all groups of mice (for both primary - Fig. 7.4a -, and secondary - Fig. 7.4b - responses), indicating that the slight reduction of the primary immune response was specific for the hIgG molecule.

The rabbit anti-225-28S response is shown in Figure 7.5. On day 7 no primary response was measurable, possibly due to the low amount of Ab injected (4 μg), We were unable to administer more Ab due to the extremely high specific activity; a higher Ab dose would have exceeded the permitted radiation levels, and considerable side effects (including generalized immunosuppression) would be expected. On day 10 both groups exhibited similar anti-mouse Ig levels, that reached a peak on day 14 and leveled off on day 21. As shown in Figure 7.5, no difference was observed in the anti-225-28S response of the rabbits irrespective of whether they had been primed with radiolabelled or "cold" 225-28S.

7.2.4 Discussion

In the last two decades, various investigators have exploited the use of radiolabelled antigens as potential immunosuppressive reagents. It has been shown that when an ^{125}I-labelled antigen is incubated with spleen or isolated B/T-cells *in vitro*, antigen-specific lymphocytes are eliminated. This becomes evident when the spleen cells repopulate syngeneic irradiated mice, that are

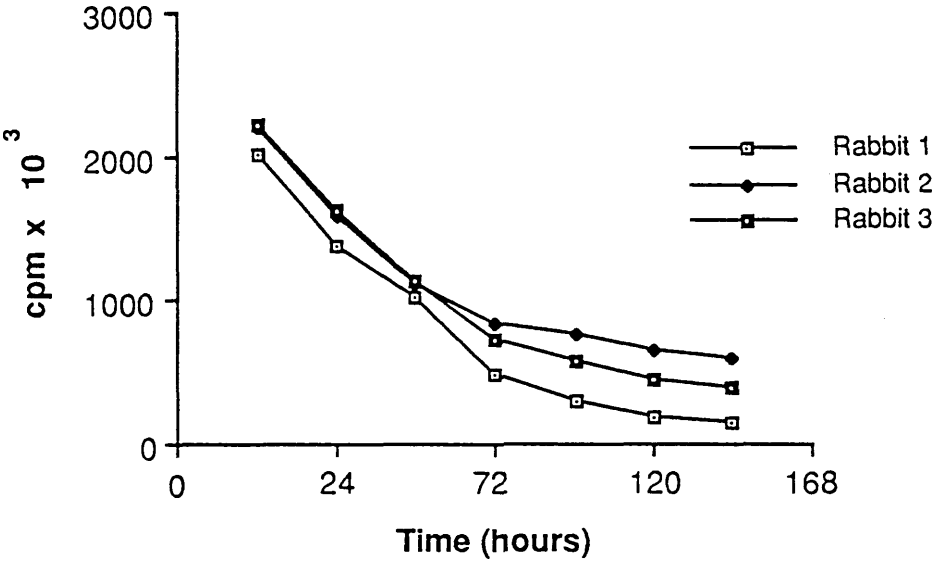


Figure 7.2: The kinetics of the 225-28S mouse monoclonal antibody, when labelled with ^{125}Iodine and administered i.v. in rabbits. On the vertical axis are the whole body counts per minute, when the animals were placed under a γ-camera, and counted over a period of 7 days (168 hours) (horizontal axis).

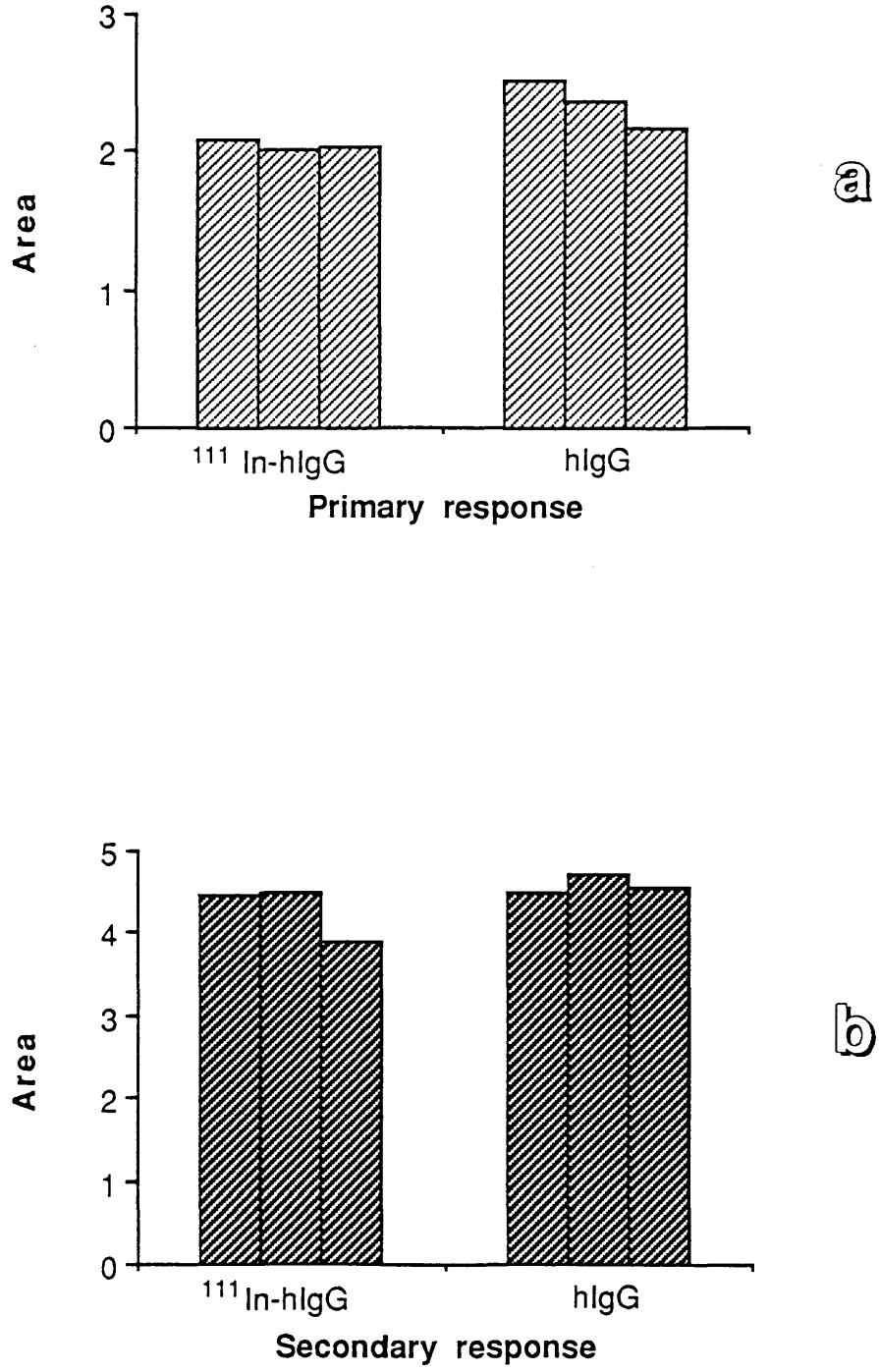


Figure 7.3: a) primary and b) secondary humoral responses of mice to "cold" and ^{111}Indium-labelled polyclonal human IgG, as measured by ELISA. The results are expressed as areas under the test-sera titration curves (vertical axis) (see text for details).
hIgG: polyclonal human IgG.
^{111}In-hIgG: ^{111}Indium-labelled polyclonal human IgG.

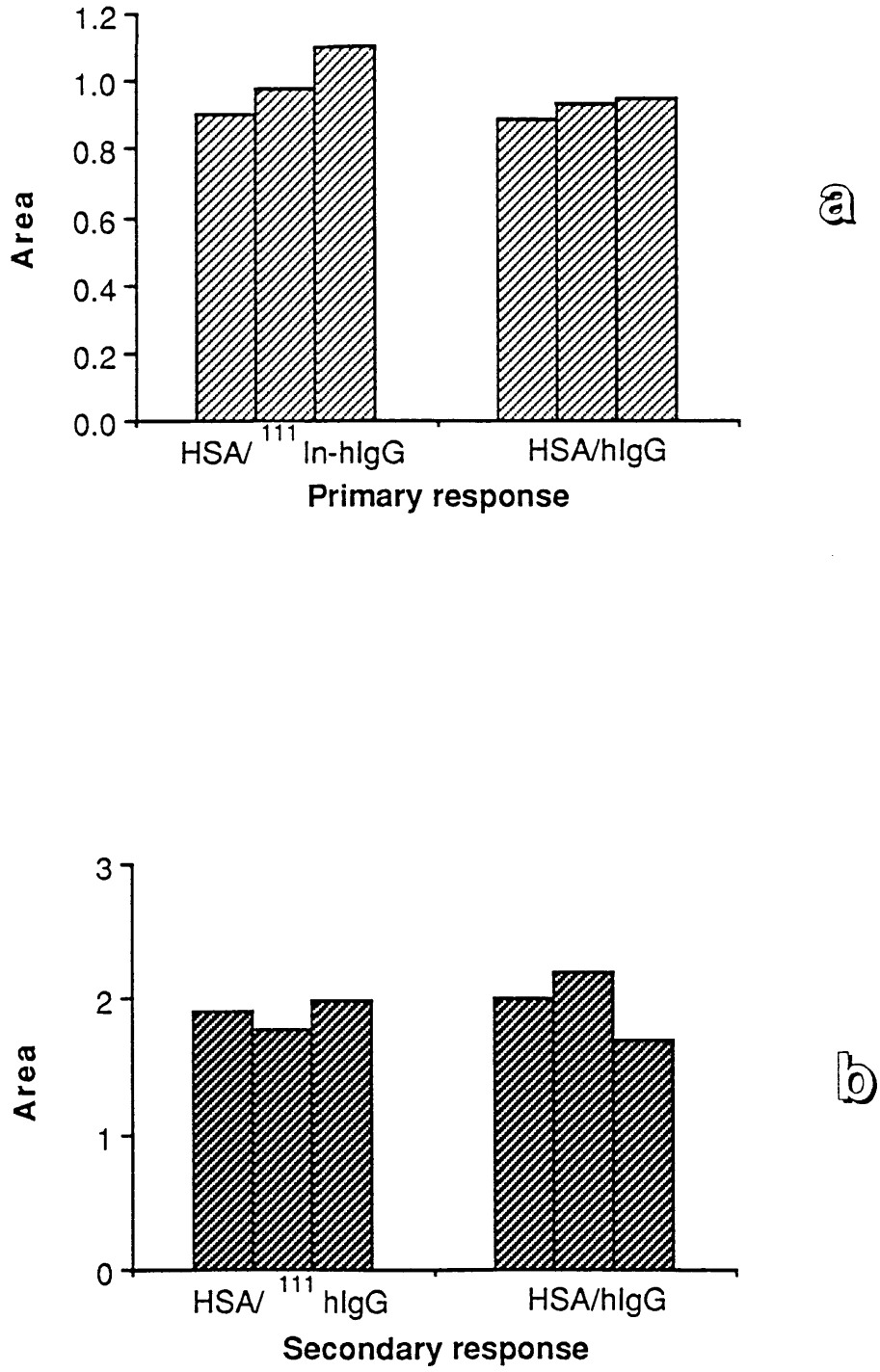


Figure 7.4: a) primary and b) secondary humoral responses of mice to human serum albumin, co-administered with ^{111}Indium-labelled or "cold" polyclonal human IgG, as measured by ELISA. The results are expressed as areas under the test-sera titration curves (vertical axis) (see text for details).
HSA: human serum albumin.
hIgG: polyclonal human IgG.
^{111}In-hIgG: ^{111}Indium-labelled polyclonal human IgG.

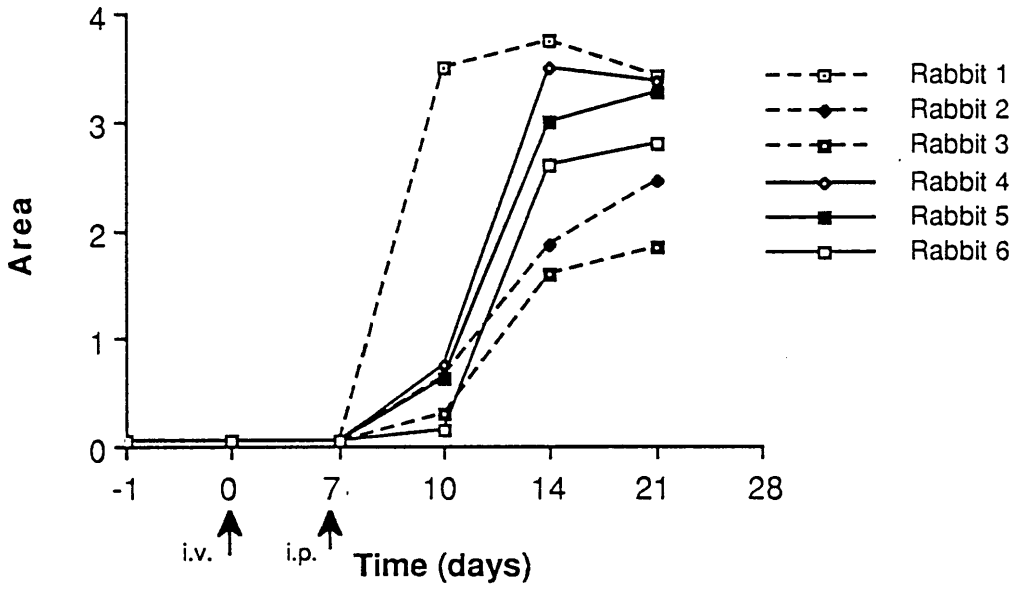


Figure 7.5: Rabbit humoral responses to a mouse monoclonal antibody (225-28S), when they were primed i.v. with ^{125}Iodine-labelled antibody (rabbits 1,2,3) or "cold" antibody (rabbits 4,5,6), and boosted with "cold" antibody 7 days later (all rabbits), as measured by ELISA over a period of 21 days (horizontal axis). The values are given as areas under the test-sera titration curves (vertical axis) (see text for details).

subsequently challenged with the same "cold" antigen. The animals either do not respond, or respond significantly less, to antigen challenge (Ada and Byrt, 1969; Basten et al., 1971; Roelants and Askonas, 1971; Unanue, 1971; Golen and Borel, 1972), even if the cells have been previously primed (Roelants and Askonas, 1971, Humphrey, 1971). In addition, in a limited number of *in vivo* studies, radiolabelled antigens administered to animals were found to be less immunogenic than the equivalent unlabelled ones (Humphrey and Keller, 1969; Filipp et al., 1981; Csaba et al., 1985). From these studies two points become apparent: a) an antigen that carries a suitable β-emitter has the potential of lethally irradiating antigen-specific lymphocytes, and b) the immunosuppression observed is quite distinct from tolerance; immunocompetent cells that have escaped, and newly recruited cells, will proliferate and differentiate in order to inactivate the foreign antigen.

Nevertheless, even short term and transient unresponsiveness would be of considerable value in immunotherapy using xenogeneic antibodies. We therefore studied the *in vivo* effects of radiolabelled Ig on the immune system. We found that ^{111}In-labelled hIgG is marginally less immunogenic than "cold" hIgG, when used for priming mice (Fig. 7.3a), although the secondary response does not seem to be affected by the use of radiolabelled hIgG (Fig. 7.3b). The immunosuppression of the primary response observed was antigen-specific, since the animals responded normally to an irrelevant antigen (HSA). To our knowledge, this is the first report using ^{111}In-labelled antigen, for antigen "suicide" experiments. Unfortunately, ^{125}I-labelled mouse monoclonal antibody injected to rabbits, failed to induce even the low suppression observed in mice (Fig. 7.5).

The toxicity of ^{111}Indium and ^{125}Iodine to lymphocytes (that are very radiosensitive anyway) is well documented (Kraal and Geldof, 1979; Ten Berge et al., 1983; Signore et al., 1983), the failure to induce specific

immunosuppression in our experiments may be due to inadequate radiolabelling. The specific activities used by others were considerably higher than ours. With the exception of the results reported by B. Csaba (Csaba et al., 1985), all other investigators who have shown immunosuppressive effects of radiolabelled antigen *in vivo*, used specific activities of up to 900 mCi/mg (Filipp et al., 1981) and 2,500 mCi/mg (Humphrey and Keller, 1969). Even in the *ex vivo* treatment of spleen cells - where specific killing is more likely to be successful due to closer contact of antigen and antibody, and where the toxic effects increase by increasing the incubation time (Golan and Borel, 1972) - the antigens were labelled at specific activities of 150-400 mCi/mg (Basten et al., 1971; Roelants and Askonas, 1971; Unanue, 1971; Golan and Borel, 1972). It is possible therefore, that the specific activities of 7-15 mCi/mg for ^{111}Indium or 180 mCi/mg for ^{125}Iodine, that we have achieved, were not high enough. Within the time limits of this study we were unable to label the corresponding antigens with higher specific activity. We feel that there may be space for improvement with ^{111}Indium-labelling, and recently we have succeeded in radiolabelling mouse monoclonal antibodies up to 30 mCi/mg. For ^{125}Iodine, the use of alternative spacers and linkers might make it possible to label Ig to a higher specific activity (Bolton and Hunter, 1973). However, the ^{125}Iodine activity achieved with the 225-28S monoclonal antibody (180 mCi/mg), was one of the highest ever achieved not only by us, after testing several mouse monoclonal antibodies, but by other investigators as well. Finally, the degree of labelling depends on the nature of proteins. Immunoglobulins will have an upper limit, above which they cannot incorporate more ^{125}Iodine or ^{111}Indium.

Another crucial factor is the recipient animal. Although rabbits (Filipp et al., 1981) and guinea pigs (Csaba et al., 1985) have been used, in most cases the animal model was the mouse. As discussed in Chapter 5, there is such variability in antigen processing among various strains, that the results obtained may significantly differ from experiment to experiment. In previous

antigen "suicide" immunosuppression approaches, CBA/C57 (Ada and Byrt, 1969; Humphrey and Keller, 1969; Humphrey et al., 1971), CBA/DBA (Golan and Borel, 1972), CBA/H (Willcox et al., 1974), C3H (Basten et al., 1971; Roelants and Askonas, 1971) and A/J (Unanue, 1971) were used, strains generally regarded as "tolerance susceptible" (Golub and Weigle, 1969; Staples et al., 1970; Fujiwara and Cinader, 1974; Lukic´ et al., 1975b). In contrast, the Balb/c that we have used are believed to be "tolerance resistant" (Golub and Weigle, 1969; Lukic´ et al., 1975a), especially when the immunogen is aggregated immunoglobulin, as is our preparation. On the other hand, since radioactivity inactivates proliferating cells, would it be more likely that immunocompetent cells would preferentially be inhibited when the radioisotope is carried on a potent stimulant rather than a less immunogenic molecule? If this explains the partial unresponsiveness we observed with our ^{111}In-labelled aggregated IgG, it is possible that in future experiments immunosuppression may be induced when higher specific activity is used.

The dose of immunogen and the route of administration may also be of importance. The antigen concentrations reported in the literature ranged from a few μg (Humphrey and Keller, 1969) to 1 mg in mice (Golan and Borel, 1972) and up to 10 mg in rabbits (Filipp et al., 1981). We feel therefore, that the amounts that we have used (50 μg in mice - with CFA -, 4 μg and 1 mg in rabbits), are comparable; in addition, a type of adjuvant (Alum precipitate, *B. Pertussis*, CFA) was also often used (Humphrey and Keller, 1969; Golan and Borel, 1972; Willcox et al., 1974; Csaba et al., 1985). The routes of administration that we have chosen (i.p. for mice, i.v. for rabbits) are consistent with previous studies. It therefore seems unlikely that antigen concentration and route of injection were key factors that determined the lack of unresponsiveness in our experiments. On the other hand, every antigen is unique; hence, our Ig preparation may need a different optimal concentration and a favourable route of administration in order to induce suppression.

Finally, the antibody detection system should be considered. Even in the most recent reports (Filipp et al., 1981; Csaba et al., 1985), the assays used were haemmagglutination tests or plaque forming cell assays (Basten et al., 1971; Golan and Borel, 1972). As explained in detail in Chapter 2, we have reason to believe that our ELISA technique is more sensitive, reliable and reproducible than many assays used so far. However, the differences in the immune response among animals that received radiolabelled and unlabelled Ig are so small, that it is doubtful whether less sensitive assays would have produced different results.

In summary, in our experiments we could not induce significant specific suppression to immunoglobulin in mice and rabbits when radiolabelled Ig was administered. The amount injected, the route of administration, and the animal strain, are important parameters for the induction of unresponsiveness. The fact that the mouse mAb administered to rabbits had affinity for a cell-surface antigen further enhanced its immunogenicity (Benjamin et al., 1986), rendering the attempted induction of immunosuppression even more difficult. However, it is most likely that the specific activity was too low for the conditions, and if increased, the desirable unresponsiveness might be achieved.

7.3.1 Drug complexed monoclonal antibody - Introduction

The continuously expanding use of monoclonal antibodies in the last decade, and the great progress in biochemistry, has enabled the production of novel immunoconjugates. MAbs have therefore been conjugated to toxins (of plant or bacterial origin) and drugs, in order to increase their potency. Initial results were very promising: toxin/drug immunoconjugates appear to localize specifically onto cells, and to kill their targets via the toxic compound that they

carry (Vitetta et al., 1987; Baldwin and Byers, 1987; Blakey et al., 1988a; Byers and Baldwin, 1988; Ahmad, 1988; Pirker, 1988). Would it also be possible to use such immunoconjugates to eliminate immunocompetent cells *in vivo* ? Could such immunoconjugates play the role of "lethal" antigen ?

The first report in this area came from D. J. Volkman et al., who showed that antibody production against tetanus toxoid antigen was abrogated when peripheral blood mononuclear cells, from recently immunized people, were preincubated with a tetanus toxoid-ricin conjugate (Volkman et al., 1982). The unresponsiveness was antigen-specific, and due to elimination of the tetanus toxoid-specific B-cell population. Similarly, J. A. Killen and J. M. Lindstrom reported that acetylcholine receptor (AchR)-ricin conjugates were able to suppress specifically the *in vitro* response to AchR in lymph node cell cultures derived from rats with experimental autoimmune Myasthenia gravis (EAMG) (Killen and Lindstrom, 1984).

Ricin is a toxin derived from the plant *Ricinus communis*, and is composed of two subunits: the A- and B-chain. The A-subunit is responsible for blocking the cell's protein synthesis, whereas the B-chain functions as a lectin by binding to sugar residues on the surface of any cell (Benson et al., 1975; Fodstad and Olsnes, 1977; Olsnes and Refsnes, 1978; Villafranca and Robertus, 1981). Thus, excessive amounts of lactose had to be used in the previous *in vitro* experiments, in order to block the toxin's non-specific lethal potential.

This problem was overcome by the use of the A-chain alone. E. S. Vitetta et al. reported that irradiated mice failed to respond to dinitrophenol (DNP), when they were reconstituted with syngeneic spleen cells preincubated with DNP-HSA-ricin A-chain (Vitetta et al., 1983). In another experiment, C. Morimoto et al. specifically inhibited *in vitro* anti-nucleoside antibody production by eliminating specific B-cells derived from patients with Systemic Lupus

Erythematosus (SLE), using a nucleoside-ricin A-chain conjugate (Morimoto et al., 1983).

Despite the encouraging initial reports on the immunosuppressive effects of ricin conjugates, further *in vivo* experiments were limited by the fact that ricin A-chain was less toxic than the native toxin (Lappi et al., 1978; Youle and Neville, 1982; Vallera et al., 1984), and that the whole molecule had undesirable non-specific binding properties of the B-chain. Inevitably, ricin was replaced by other toxins. S. Brust et al. showed that horseradish peroxidase-gelonin complexes (a toxin derived from the plant *Gelonium multiflorum),* could inhibit specific T-cell proliferation *in vitro,* when mixed with lymph node cells derived from rats previously immunized with horseradish peroxidase (Brust et al., 1987). In addition, AchR-gelonin could inhibit the *in vivo* production of anti-AchR antibodies, and improve the disease status of rats with EAMG (Brust et al., 1987).

Although these reports on specific immunosuppression using toxin-conjugated antigens remained limited, other workers in the area turned to drug conjugates. Initial encouraging *in vitro* experiments, such as the specific inhibition of B-cells (derived from SLE patients) producing anti-DNA antibodies, using DNA-daunorubicin complexes (Takeuchi et al., 1986), were followed by a series of *in vivo* antigen "suicide" reports: P. N. Shek et al. showed that liposome-entrapped methotrexate (MTX) was responsible for reducing the response to BSA in mice, when BSA was conjugated onto the surface of the vesicles (Shek et al., 1986); and E. Diener et al. observed similar specific anti-trinitrophenol (TNP) suppression, when TNP-OVA-daunomycin was injected into mice (Diener et al., 1986). More recently it has been shown that daunomycin-conjugated AchR inhibited the *in vivo* anti-AchR response in rats (Shelton et al., 1988), and daunomycin-mouse monoclonal antibody conjugates abolished the primary rat anti-mouse Ig response (Durrant et al., 1989).

For our antigen "suicide" experiments, we have used a bispecific mouse monoclonal antibody. This antibody recognises a tumour-associated antigen (carcinoembryonic antigen, CEA) with one binding site, and Vinca alkaloids with the other (Corvalan and Smith, 1987). We examined whether this antibody, when "loaded" with the cytostatic drug vinblastine (VLB), could specifically suppress the *in vivo* anti-mouse Ig response in rabbits.

7.3.2 Materials and Methods

7.3.2.1 Animals: Male 1/2 "lop" rabbits (Froxfield Farm, UK), 3-4 Kg, were used throughout this study.

7.3.2.2 Antigen - Immunization schedule: The bispecific mouse monoclonal antibody 28.19.8. was used as immunogen for rabbits. This antibody was produced at the Lilly Research Centre, Surrey, UK, and kindly provided to us by Dr. J. Corvalan. It is a mouse IgG1-IgG2a hybrid, derived from the fusion of a hybridoma cell line producing anti-CEA monoclonal antibodies (designated as 11.285.14), and spleen cells from a mouse previously immunized with vindesine-BSA (VDS-BSA). This hybrid-hybrid antibody (designated as 28.19.8) has the capacity of binding CEA with one binding site and Vinca alkaloids with the other (Corvalan and Smith, 1987) (Fig. 7.6). It has been shown, that when "loaded" with vindesine (Corvalan et al., 1987a) or vinblastine (Corvalan et al., 1987b), it is toxic to CEA expressing cells, not only *in vitro* but also *in vivo* against human tumour xenografts in nude mice (Corvalan et al., 1988).

The immunization schedule of the first experiment is shown in Figure 7.7a. Four groups, of 3 rabbits each, were injected i.v. (ear vein) with 1 mg of the following antigens: a) the bispecific, hybrid-hybrid, mouse monoclonal antibody 28.19.8 mixed with VLB (as VLB sulphate, Eli-Lilly, USA) (HH/VLB) at

a molar ratio (MR) of 1:1 (equivalent to 5.45 μg VLB/rabbit), b) the parental anti-CEA antibody 11.285.14 mixed with 5.45 μg VLB (anti-CEA/VLB), c) the bispecific monoclonal antibody 28.19.8 alone (HH) and lastly d) the bispecific monoclonal antibody 28.19.8 covalently linked to VLB (HH-c-VLB), on the Vinca binding site, at MR 1:1 (Fig. 7.8). The conjugation method is described later in this chapter (section 7.3.2.4). All rabbits were bled on day 6, and boosted i.p. with 1 mg of the parental anti-CEA antibody on day 7. They were bled again on days 10, 14, 21 and 28. On day 29 the 4 groups received i.v. 1 mg of the initial immunogen as on day 0. A blood sample was taken seven days later (day 35), and on day 36, all rabbits were boosted again i.p. with 1 mg of the anti-CEA (as on day 7). They were bled on days 39, 43, and from then on, once weekly for a period of 3 weeks (up to day 64). In addition, 4 mg of ovalbumin (OVA) (Sigma, UK) was simultaneously administered with each injection of the antigen.

Figure 7.7b shows the immunization schedule for the second experiment. A group of 6 rabbits was injected i.v. with 1 mg of the hybrid-hybrid antibody 28.19.8 (HH) mixed with VLB at a MR 1:1 (5.45 μg/rabbit/injection), for 5 consecutive days. A group of 6 rabbits served as controls; these received the same injections as the above group except that the HH antibody was replaced by 1 mg of the anti-CEA. On day 11 blood samples were taken, and all animals were boosted i.p. with 1 mg of anti-CEA. The rabbit anti-mouse Ig response was assessed on days 14, 18 and 25.

The third experiment consisted of 2 groups of 6 rabbits each. In all animals, we implanted an osmotic mini-pump (Alzet, USA), filled with 1 mg of VLB in distilled water. These pumps released the drug at a constant flow rate of 10 μl/hour for a period of 7 days (detailed description of the pump in 7.3.2.5 below). Five days after implantation, the two groups received i.v. 1 mg of either the bispecific antibody (HH) or the parental anti-CEA. The animals were bled, and the primary anti-mouse Ig response assessed on days 7, 12 and 60 after administration of the immunogen.

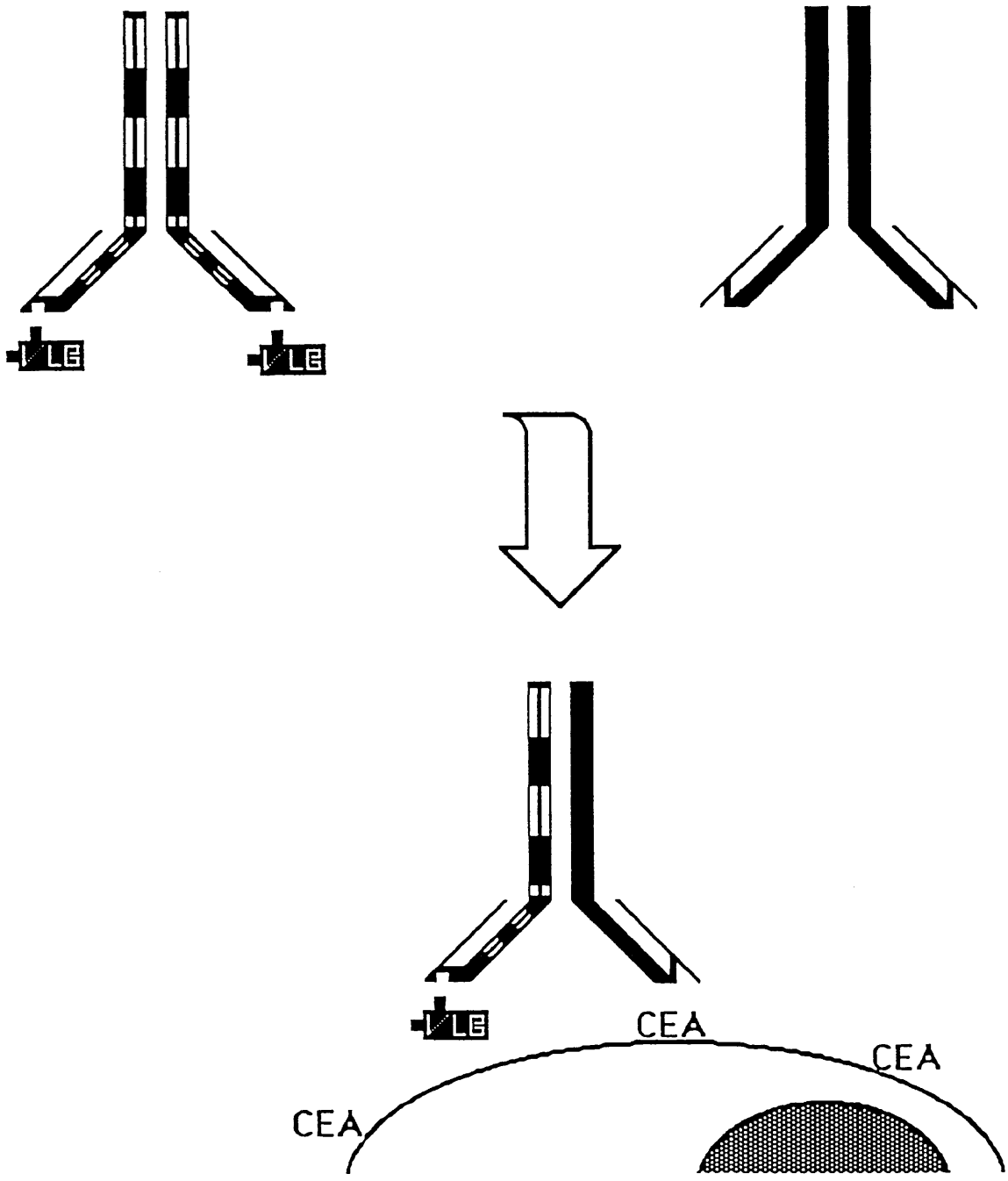


Figure 7.6: The fusion of a hybridoma cell line with specificity for CEA, and spleen cells from mice immunized with vindesine-BSA, yielded to a monoclonal antibody with dual specificity: for CEA and Vinca alkaloids (in our case vinblastine, VLB).

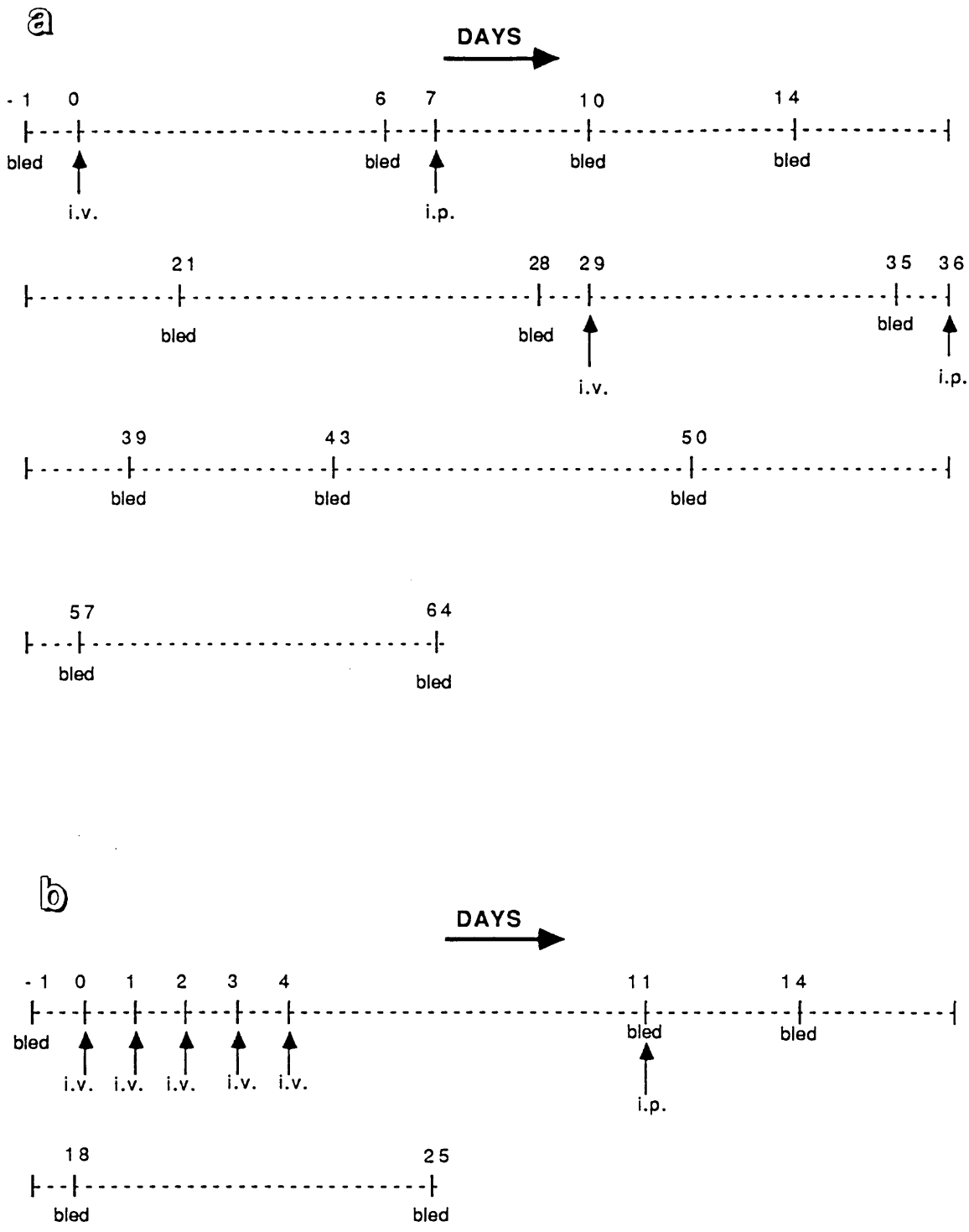


Figure 7.7: Immunization schedules for:
a) the first experiment. Four groups of 3 rabbits received i.v.: the anti-CEA antibody with vinblastine (VLB), the hybrid-hybrid antibody (HH) with VLB, the HH with VLB covalently linked and the HH alone (days 0 and 29). All the i.p. injections were with anti-CEA (days 7 and 36), and
b) for the second experiment. Two groups of six rabbits received i.v. either HH with VLB or anti-CEA with VLB, for 5 consecutive days (Days 0, 1,2, 3, 4). On day 11, they were all boosted with anti-CEA.

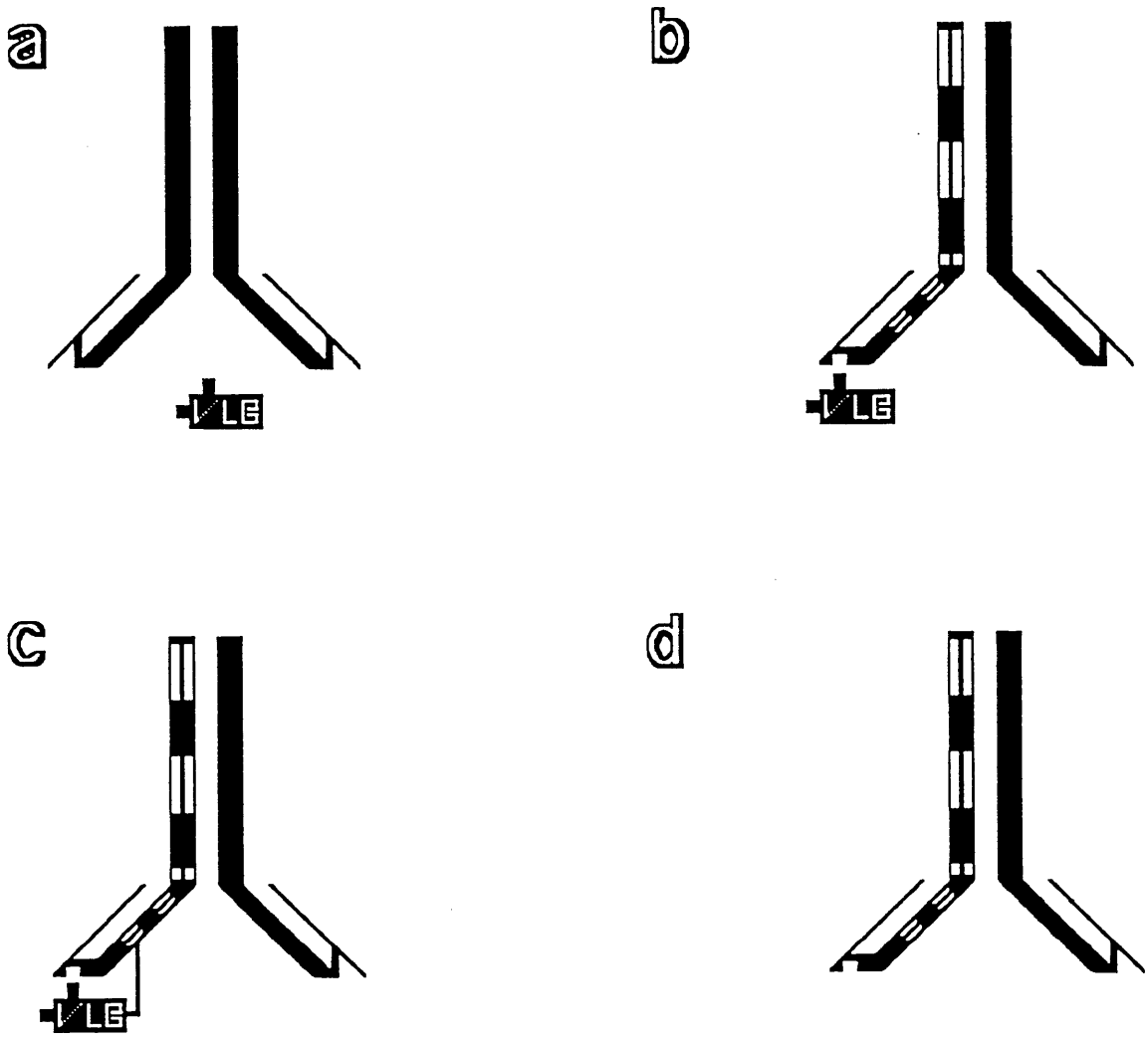


Figure 7.8: The immunogens used in rabbits for the first experiment: a) the anti-CEA antibody mixed with vinblastine, b) the hybrid-hybrid antibody mixed with vinblastine, c) the hybrid-hybrid antibody covalently linked to vinblastine and d) the hybrid-hybrid antibody alone.

7.3.2.3 Immunoreactivity: Prior to each administration, the ability of the antibody to bind VLB was checked. This was done by either ELISA or gel filtration. For the ELISA, 96-well plates (Sterilin) were coated with 200 ng/well VLB-BSA (conjugation method described in 7.3.2.4 below); the test antibody was applied, followed by an anti-mouse Ig-HRP (Amersham) at 1:500 dilution. For the gel filtration, the test antibody was incubated with ^{3}H-labelled VLB (^{3}H-VLB, Amersham), and passed through Sephadex G-25 or G-50 columns (Pharmacia). 1 ml fractions were collected, and the radioactivity was measured in a β-counter (LKB-Wallac, Sweden). In both experiments, the anti-CEA antibody served as negative control.

The immunoreactivity of the parental anti-CEA antibody was tested by ELISA: Nunc (UK) 96-well plates were coated with 1 μg/well purified CEA (kindly provided by W. Smith, Lilly Research Centre, Surrey, UK) at room temperature (RT), overnight. The test antibody was added, followed by a rabbit anti-mouse Ig-alkaline phosphatase (Prepared by W. Smith, at Lilly Research Centre). The substrate used was para-nitro phenol phosphate (p-NPP, Sigma), and the absorbance was measured at 410 nm, using a Titertek spectrophotometer (Flow Laboratories).

In addition, both immunogens, HH and anti-CEA, were tested for binding to normal rabbit tissue, by indirect immunoperoxidase do determine whether these also recognise antigens in the rabbit. 5 μm frozen sections of rabbit liver, kidney, lung and skin, were fixed in acetone for 10 min. 100 μl of test antibody (20 μg/ml) was added, and incubated for 45 min at RT As negative control, the mouse monoclonal antibody 4D513/2118 was used. This is an anti-idiotypic antibody (Skarlos et al., 1989), that was kindly provided to us by Unipath-Oxoid, UK. The slides were then washed with PBS, pH 7.4, and rabbit anti-mouse Ig-HRP (DAKO) added at 1:50 dilution, for 45 min. After further washing, a brown peroxidase colour was developed using diaminobenzidine (DAB, Sigma) as

substrate. We found that both HH and anti-CEA antibodies were positive on normal rabbit kidney (glomeruli) and liver.

7.3.2.4 Preparation of vinblastine-conjugates: The hybrid-hybrid/VLB mixture was prepared by simply mixing the antibody with VLB sulphate (Eli-Lilly, USA) at RT Prior to each administration all antibody and antibody-VLB mixtures were tested and found to be free of aggregates. This was done by size exclusion chromatography using a Superose 6 column connected to an FPLC system (Pharmacia)

Vinblastine was covalently linked to the bispecific antibody as follows: n-hydroxy succinamide ester of 4-succinoyl desacetyl vinblastine (Eli-Lilly, USA) was allowed to bind to the bispecific antibody as antigen, whilst stirred for 1 min in PBS, pH 6.2, at 4°C. The starting MR was 5 vinblastine esters per antibody molecule. The antibody/vinblastine ester complex was immediately separated from free ester by size exclusion chromatography (P-6 column, Biorad, UK) in borate buffer, pH 8.6, at 4°C. The complex was warmed to RT, and stirred for 1 hour. After overnight dialysis in PBS, pH 7.4, the conjugation ratio was estimated by dual wavelength spectroscopy at 270 and 280 nm, and found to be 1.03 VLB/antibody. As tested by ELISA, the immunoreactivity of the conjugate for CEA remained intact (assay performed as described in section 7.3.2.3 above), whereas it was reduced to 2.7% for VLB, when compared with the unconjugated antibody (assay performed as described in section 7.3.2.9 below).

Similarly, VLB was conjugated to BSA using the same succinamide ester as above. The mixture was incubated in borate buffer, pH 8.6, for 4 hr at RT, and BSA-VLB was purified from unbound VLB ester by gel filtration (P-6 column, Biorad) in PBS, pH 7.4. The MR was estimated as above, and found to be 3.75:1 (starting MR 15:1).

7.3.2.5 Stability - Kinetics: The *in vitro* stability of the HH/VLB complex was tested as follows: 1 mg of the HH (or anti-CEA that served as negative control) was incubated in PBS or normal rabbit serum, with 5.6-6 μg of VLB sulphate (MR slightly greater than 1:1), and 2.5 μCi of ^{3}H-VLB (Amersham). The specific activity of the tracer ^{3}H-VLB was so high (up to 30 Ci/mg), that the actual increase of total VLB was regarded as negligible. A sample was taken immediately, after 20 min, 1 , 6 and 24 hours, and gel filtration (Sephadex G-25, Pharmacia) used to determine the percentage of VLB bound onto the antibody. Size exclusion chromatography was performed as follows: the column was loaded with 100 μl of the antibody/VLB mixture (from each time point). The column was then washed and eluted with PBS, pH 7.4, and 0.5 or 1 ml fractions collected. 300 μl of each fraction was subsequently mixed with 3 ml of scintillation fluid (BDH, UK), and the radioactivity measured in a β-counter (LKB-Wallac) over a period of 5 min. The results are expressed as counts per minute (cpm).

The stability of the HH/VLB complex was also tested *in vivo*: 1 mg of the HH antibody (or anti-CEA that served as negative control), was mixed with 5.45 μg (MR 1:1) or 100 μg (MR 18:1) of VLB, and 150 μCi of ^{3}H-VLB. The mixture was administered i.v. to unprimed rabbits. Blood samples were taken immediately, and after 5, 15 and 30 min, 1, 2 and 5 hours. The blood was then left to clot for 4 hr at 37°C, and the serum removed. 300 μl of this serum was mixed with 3 ml of scintillation fluid (BDH), and the radioactivity measured in a β-counter (LKB-Wallac), as above. The results (cpm) were expressed as percentage of the injected dose, by using an aliquot of the injected radioactivity as standard, and calculating the rabbit's total serum volume from the body weight (70 ml blood/kg, 60% haemmatocrit). The percentage was subsequently translated to absolute total amount of VLB in the blood (expressed in μg).

However, we observed a big difference in the counts among the various blood samples, due to differences in quenching. This was corrected by using an

internal standard: a known amount of ^{3}H-VLB was added to each test blood sample (approximately 10 nCi) and the total radioactivity measured. From this number the previously measured counts were subtracted; the remaining radioactivity was that added, and should have been the same in every sample. In fact this number was different in all samples (due to different quenching), but it enabled us to check the counting efficiency, to do the corrections, and finally to obtain the true counts. An example of this procedure is given in Table 7.1.

The kinetics of VLB were also measured when the drug was delivered to unprimed rabbits by constant infusion, using an osmotic mini-pump (Alzet, USA). This was a miniature self-powered pump, that when implanted subcutaneously to rabbits, released VLB at a controlled rate of 10 µl/hr for 1 week. The pump consists of a collapsible reservoir of flexible, impermeable material, surrounded by an osmotic agent contained within a semipermeable membrane (Fig. 7.9). When the filled pump is put into an aqueous environment (subcutaneously), the osmotic agent imbibes water (tissue fluid) at rate that is controlled by the semipermeable membrane. The imbibed water generates hydrostatic pressure on the flexible lining of the reservoir, gradually compressing it, producing a constant flow of VLB through the delivery portal. Only the drug leaves the pump, whereas the osmotic agent cannot pass into the reservoir or out through the semipermeable membrane. The pump's delivery rate is fixed and predetermined at manufacture. The actual amount of drug delivered is set by the concentration of VLB placed in the vehicle.

Rabbits were therefore implanted with mini-pumps containing 4 or 1 mg VLB mixed with a trace amount of ^{3}H-VLB (approximately 150 µCi). Blood samples were taken immediately, and then daily for a period of 3 weeks. The radioactivity was measured using a β-counter, as described above. The results

Sample	Time (min)	Activity counted (cpm)	Activity + Standard (cpm)	Difference (cpm)	% Standard	Corrected activity (cpm)
1	2	1286	9744	8458	33.22	3871
2	5	1255	14070	12815	50.33	2494
3	11	1212	16706	15494	60.85	1992
4	35	1111	16502	15391	60.44	1838
5	57	481	12491	12010	47.17	1020
6	180	183	4946	4763	18.71	978
7	300	515	18366	17851	70.11	735
Standard	-	-	25463	-	100.00	-

Table 7.1: Example of counting ^{3}H-vinblastine in different blood samples. The differences in quenching were corrected for using an internal standard, and the true counts obtained.

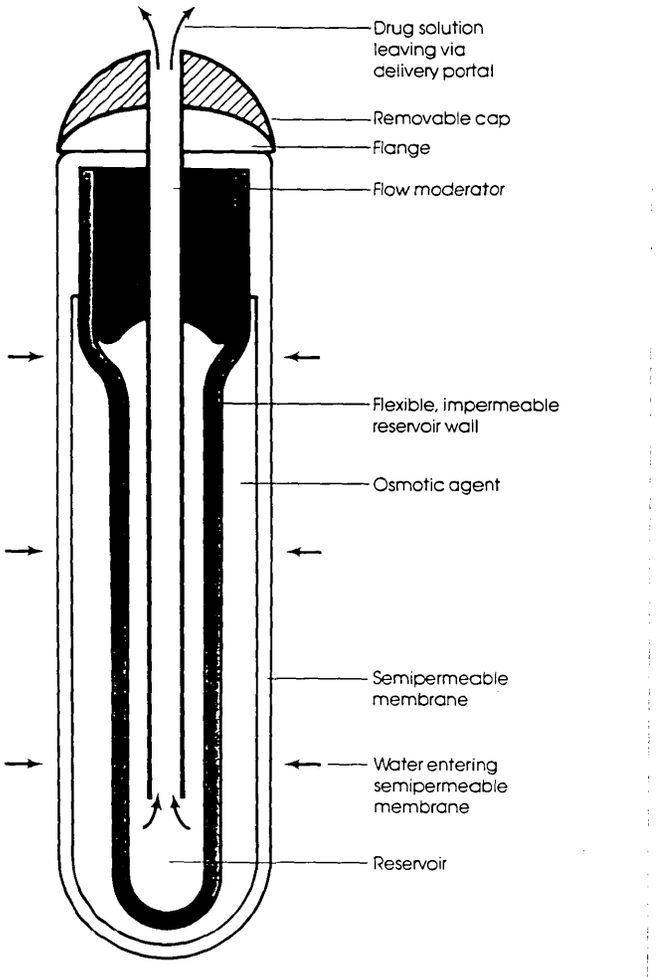


Figure 7.9: The osmotic mini-pump used subcutaneously for constant infusion of vinblastine in rabbits (photographed from the manufacturer's brochure).

(cpm) were expressed as a percentage of the injected dose, and were subsequently translated into the actual total concentration in the blood (in μg) at each time point.

The kinetics of the bispecific monoclonal antibody (HH), when administered i.v. to rabbits, were measured as follows: 1 mg of the HH antibody was injected i.v., and blood samples were taken at various intervals for 5 days. The blood was left to clot at 37°C for 4 hr, the serum was then removed and incubated in 96-well plates (Sterilin) coated with 200 ng/well VLB-BSA. After 2 hr the plate was washed with PBS/0.05% tween 20, pH 7.4, and incubated with an anti-mouse Ig-HRP (Amersham) at 1:500 dilution, for 1 hour. The plate was washed, and the absorbance (using ABTS substrate) was measured at 405 nm. The results are given as areas under the sigmoidal curve, obtained from the serial dilutions (1:10) of the test sera, as described in chapter 2. The value obtained from the serum sample taken immediately after administration, was regarded as the injected dose (100%).

7.3.2.6 Measurement of circulating immunoglobulin and vinblastine: The concentration of the mouse antibodies, used as immunogens (HH or anti-CEA), in the circulation was measured by ELISA. 96-well microtitre plates (Nunc, UK) were coated with 1 μg/well purified CEA. Test serum was added, and the mouse antibodies were detected by a rabbit anti-mouse Ig-alkaline phosphatase (prepared by W, Smith at Lilly Research Centre, UK) and p-NPP substrate. The absorbance was measured at 410 nm, by a multiscan spectrophotometer, a standard curve plotted, using known concentrations of the corresponding antibody (mixed with rabbit serum), and the circulating immunogen quantitated.

Similarly the concentration of circulating VLB was estimated as follows: ELISA plates were coated with CEA (as above), and a saturating amount of the

bispecific antibody was incubated for 1 hr at RT The plates were washed with PBS/0.05% tween 20, pH 7.4, and the test serum was added. Circulating VLB was detected, by direct competition with VLB-alkaline phosphatase (prepared by W. Smith at Lilly Research Centre, UK). The substrate used was p-NPP, and the absorbance was measured at 410 nm as above. This was performed after we established that there was no HH antibody in circulation, to interfere with our assay.

7.3.2.7 Measurement of antigen-specific B-cells: Immunized rabbits were killed, and bled by cardiac puncture. Peripheral blood lymphocytes (PBLs) were isolated as follows: blood was gently added at the top of a gradient (Lymphoprep, Nyegaard, Norway), and centrifuged at 1,200 rpm for 10 min. The layer containing the white cells was carefully removed and the PBLs were washed twice with RPMI medium (Flow Laboratories). The spleen was also removed, teased with forceps and 23G needles, and the cell suspension washed twice with RPMI medium. Approximately $5x10^5$ - 10^6 PBLs or spleen cells were incubated in LP3 plastic tubes, with 100 µl (100 µg/ml) fluorescein isothiocyanate (FITC) conjugated HH or anti-CEA antibody. After 1 hr incubation on ice, the cells were washed twice in RPMI, and the B-cells having Ig-receptors for the mouse monoclonal antibodies were detected using a cell sorter (Epics, Coulter, USA).

The conjugation of the monoclonal antibodies with FITC was performed as follows: antibodies were dialyzed in carbonate/bicarbonate buffer, pH 9.0, and concentrated down to 10 mg/ml. FITC (Sigma) solution of 20 mg/ml in dimethyl sulphoxide (DMSO anhydrous, Aldrich, UK) was prepared freshly and added to the antibody solution at the equivalent of 250 µg/10 mg of antibody. After 2 hr incubation at RT, under constant stirring, free FITC was separated from bound by gel filtration (Sephadex G-25, Pharmacia). The column was washed and eluted with PBS, pH 7.4, and 1 ml fractions were collected. The absorbance (O.D.) at 280 nm (for the antibody) and 495 nm (for FITC) was

measured, and the antibody concentration estimated using the equation:

$OD_{antibody} = OD_{280\ nm} - 35\%\ OD_{495\ nm}$

The molar ratio FITC/antibody molecule was estimated as follows:

$$M.R. = \frac{2.87 \times OD_{495}}{OD_{protein}}$$

and found to be 4.1 FITC/HH and 4.9 FITC/anti-CEA.

7.3.2.8 Anti-immunoglobulin response: The rabbits' responses against the mouse monoclonal antibodies administered, were measured by ELISA, as described earlier (chapter 2). Briefly, 96-well plates were coated with the antibody used as antigen, and the corresponding test sera were added. Rabbit anti-mouse Ig antibodies were detected by a species-specific anti-mouse Ig-HRP second layer and ABTS substrate. The results are expressed as areas under the sigmoidal curve. Before any experiment, we ensured that none of the rabbits used had any pre-existing anti-mouse Ig antibodies. Statistical analysis of the data was performed using the Student's t test on the logs of the areas.

7.3.2.9 Anti-ovalbumin and anti-vinblastine response: The rabbits' response to the administered VLB or OVA was measured with an ELISA similar to the above. The only difference was that the plates were coated with either 200 ng/well VLB-BSA or 500 ng/well OVA. The optimal concentration of VLB and OVA for coating the plates was estimated by doing a chequer-board ELISA (as described in chapter 2), using anti-BSA (DAKO), anti-VLB (our bispecific antibody 28.19.8) and anti-OVA (Sigma) antibodies. In the case of anti-VLB response, all positive sera were also tested against BSA alone, in order to prove the presence of true anti-VLB antibodies. Before each experiment, rabbits were chosen that did not have any pre-existing anti-VLB or anti-OVA antibodies. Statistical analysis of the data was performed using the Student's t test on the logs of the areas.

7.3.3 Results

7.3.3.1 In vitro stability of the monoclonal antibody/vinblastine complex: 1 mg of the bispecific antibody (HH) was incubated with 6 μg of VLB and a trace amount of ^{3}H-VLB (2.5 μCi), in PBS at RT An aliquot was taken at various time points (immediately, after 20 and 60 min, 1 and 24 hr), passed through a size exclusion column, and the radioactivity was measured in each fraction. We found that up to 24 hr, VLB remained associated with the antibody (Figs. 7.10a and b). The radioactivity was shared between the bound and unbound fractions (47.5 ± 5.0 %) (Figs. 10a and b), since "cold" VLB was added in slight excess of MR 1:1. The same experiment was repeated, replacing the bifunctional antibody with the parental anti-CEA one. This antibody has no binding sites for VLB and served as negative control. We confirmed that all VLB remained unbound (Figs. 7.10c and d).

In addition, the ability of the bifunctional antibody to bind VLB in the presence of serum, at 37°C, was tested. One mg of the HH antibody was incubated in normal rabbit serum with 5.6 μg VLB and a tracer of ^{3}H-VLB (2.5 μCi). An aliquot was taken at various time points (immediately, after 3 and 6 hr). Size exclusion chromatography revealed that VLB remained bound onto the antibody for at least the time period tested (6 hr) (Fig. 7.11a). Since "cold" VLB was added again at a slight excess of MR 1:1, the percentage of radioactivity bound onto the antibody was 65.3 ± 2.3 % (Fig. 7.11a). When the control antibody (anti-CEA) was incubated with VLB and ^{3}H-VLB in normal rabbit serum, we found that almost all VLB remains unbound (Fig. 7.11b). There is only a small amount of VLB (7% at 3 hr, 12% at 6 hr) associated with high molecular weight rabbit serum proteins (Fig. 7.11b).

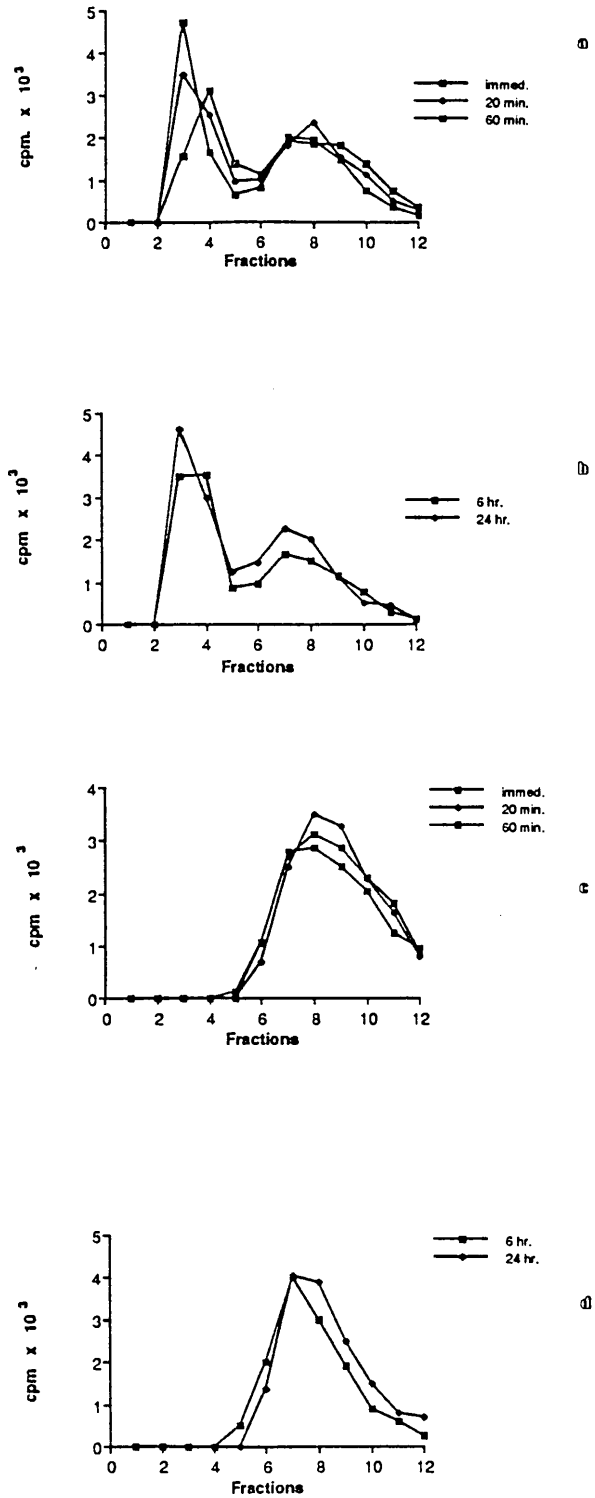


Figure 7.10: a,b) Size exclusion chromatography profile of the bispecific (HH) antibody, immediately, after 20, 60 min (a), 6 and 24 hr (b) incubation with ^{3}H-vinblastine and "cold" vinblastine (MR > 1:1) in PBS, at RT.
c,d) Size exclusion chromatography profile of the anti-CEA antibody, immediately, after 20, 60 min (c), 6 and 24 hr (d) incubation with ^{3}H-vinblastine and "cold" vinblastine (MR > 1:1) in PBS, at RT.
On the vertical axis the data is presented as counts per minute (cpm). On the horizontal axis are the number of fractions collected. The first peak represents vinblastine bound onto the antibody (for a and b), whereas the second peak is free vinblastine (for a, b, c and d).

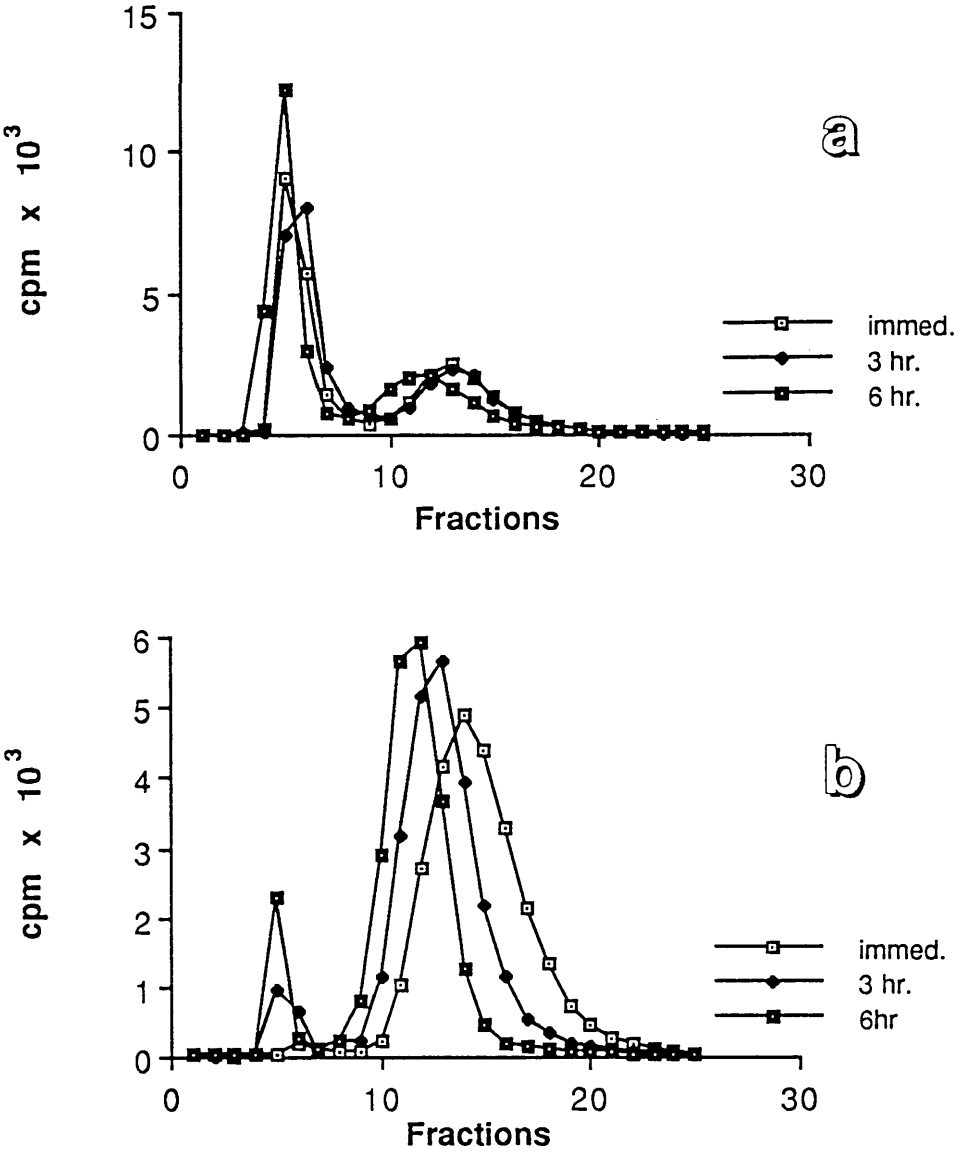


Figure 7.11: a) Size exclusion chromatography profile of the bispecific (HH) antibody, immediately and after 3 and 6 hr incubation with ^{3}H-vinblastine and "cold" vinblastine (MR > 1:1) in normal rabbit serum, at 37°C.
b) Size exclusion chromatography profile of the anti-CEA antibody, immediately and after 3 and 6 hr incubation with ^{3}H-vinblastine and "cold" vinblastine (MR > 1:1) in normal rabbit serum, at 37°C.
On the vertical axis the data are presented as counts per minute (cpm). On the horizontal axis are the number of fractions collected. The first peak represents vinblastine bound onto serum proteins, whereas the second peak is free vinblastine.

7.3.3.2 In vivo kinetics and stability of vinblastine and monoclonal antibody/vinblastine complex: The kinetics of free VLB in rabbits were measured as follows: the control anti-CEA antibody was incubated with VLB (MR 1:1) and a trace amount of ^{3}H-VLB (150 μCi). The mixture was administered to rabbits i.v., and serum samples were taken at various time points up to 15 min. Since we found earlier that the anti-CEA does not recognise the drug, the counted radioactivity counted must represent the clearance of free VLB. We found that the drug is removed from the circulation very rapidly, and that 10 min after administration only approximately 1% of the injected dose remains in the blood (Fig. 7.12a).

In addition, the kinetics of free VLB were studied after infusion from the subcutaneous osmotic mini-pump. A pump was filled with 4 mg of VLB and a trace amount of ^{3}H-VLB (150 μCi). The animal was bled daily for up to 8 days. We found that the drug was released slowly into the circulation, and as shown in Figure 7.12b, its concentration increased with time, from 0.2% (8 μg total) on day 1, to 0.5% (20 μg) on day 3 and 0.82% (33 μg) on day 7. Unfortunately, on day 8 the animal died, probably due to excessive dose of VLB. The same experiment was therefore repeated, using 1 mg of VLB in the pump. Figure 7.12c shows a steady increase in the serum level of VLB over the first 7 days as before (Fig. 7.12b). Although the actual concentration is 4-fold less than in the previous experiment, the percentage of the total is the same since only 1/4 of the initial dose was used. In this second animal we found that the amount of VLB reached a peak 7-9 days after implantation, after which it slowly dropped back to zero (Fig. 7.12c). We estimated that from day 5 to day 12 there is a "window", where the total amount of VLB in the blood is over 5 μg, i.e. a MR of approximately 1:1 when 1 mg of HH is injected. The animal remained alive and well throughout and long after this experiment. Its immunological status was checked, when on day 12 we administered 1 mg of the anti-CEA antibody. We obtained a normal primary response on day 19.

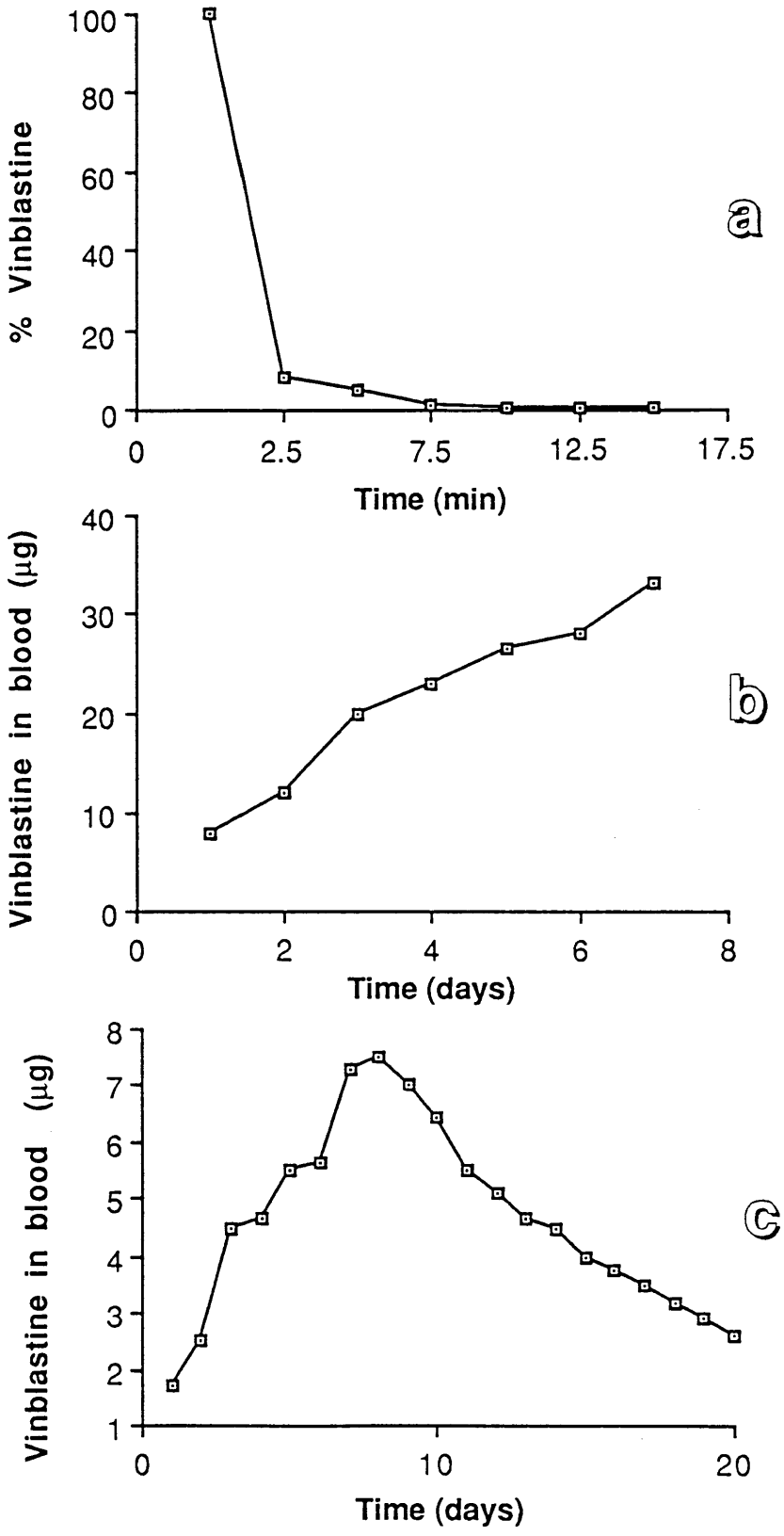
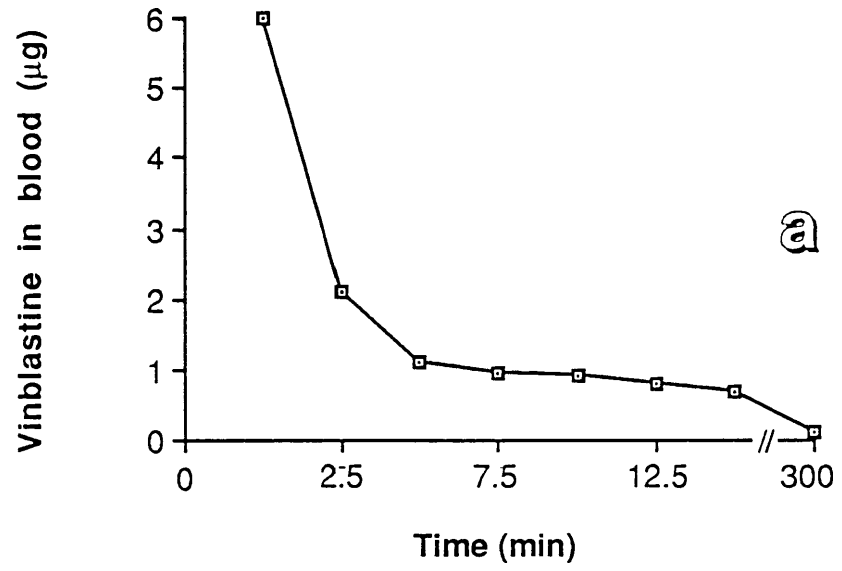


Figure 7.12: a) Clearance of vinblastine in rabbits, after i.v. administration (given as % found in the serum over a period of 15 min).
b, c) Total concentration of vinblastine in the serum of rabbits (given in µg), when 4 mg (b) or 1 mg (c) is constantly infused, by subcutaneously implanted osmotic mini-pumps, over a period of 8 (b) and 20 (c) days.
Each figure shows the data from one rabbit.

The clearance of VLB when simultaneously administered with the hybrid-hybrid antibody was also measured, over a period of 5 hours. When VLB (including the tracer ^{3}H-VLB) was injected i.v. into a rabbit, in the presence of HH at a MR of 1:1, the drug was rapidly removed from the circulation (Fig. 7.13a), though not as rapidly as for free VLB (Fig. 7.12a). As shown in Figure 7.13a, at 10 min after administration approximately 13% (0.8 μg) of the injected amount of VLB is in circulation, associated with the HH antibody. This clearly showed that within the first 10 min, the MR dropped 8-fold. Moreover, at 5 hr, the theoretically calculated molar ratio (assuming again that the concentration of HH remained unchanged), was 0.02 molecules of VLB/antibody molecule.

Nevertheless, if VLB is administered at 18-fold excess (i.e. 100 μg/HH mg), the actual concentration of VLB in the blood was high enough to keep the HH's binding sites occupied for a long time, although the rate of clearance remained the same (Fig. 7.13b). Thus, at 10 min we measured 14 μg, and after 5 hr 6 μg of VLB was still found in the circulation, an amount that theoretically was well above a 1:1 MR, if 1 mg of HH antibody was administered. There was evidence therefore, that by increasing the administered VLB concentration, we also increase the chances of retaining an antibody/VLB complex for a longer period of time in circulation.

7.3.3.3 Kinetics of the bispecific monoclonal antibody: The hybrid-hybrid antibody was injected i.v. into a rabbit, and serum samples were collected at various time points for 5 days. The amount of antibody present in circulation at each time point was determined by its ability to bind VLB immobilized onto an ELISA plate. The amount of HH present in the blood - and also capable of binding to its antigen - remained unchanged (100% of the injected dose) for up to 6 hr after administration. From that point on, it diminished with a $T_{1/2}$ of 68 $\pm$ 5 hours (Fig. 7.14).



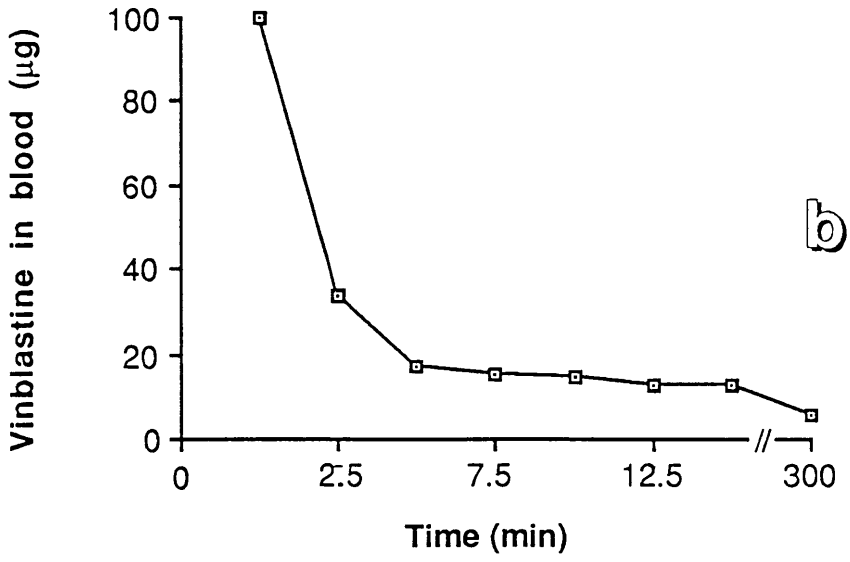


Figure 7.13: Total amount of vinblastine (in µg) found in rabbit serum, over a period of 300 min, when 6 µg (a) or 100 µg (b) of drug is i.v. injected mixed with the bispecific antibody 28.19.8.
Each figure shows the data from one rabbit.

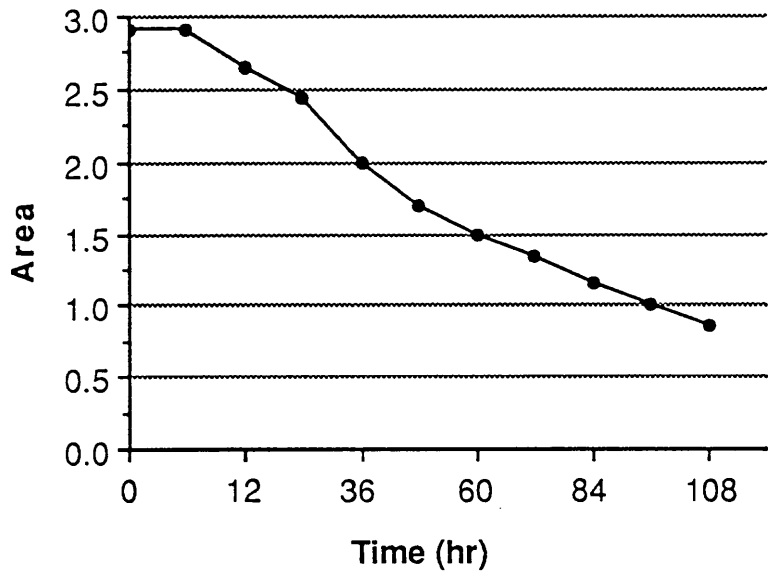


Figure 7.14: Clearance of the bispecific antibody 28.19.8 in rabbits, after i.v. administration. On the vertical axis is the area under the ELISA curve (see text for details) and on the horizontal axis is time (hours).
Data from one rabbit are presented.

7.3.3.4 Anti-immunoglobulin response: In the first experiment (Fig. 7.7a), rabbits were primed i.v. with the HH, HH covalently conjugated with VLB, HH mixed with VLB and anti-CEA mixed with VLB (the last three preparations were used at a MR of 1:1, Fig. 7.8). When we measured the primary anti-mouse Ig response, 7 days later, we found that rabbits receiving the HH/VLB mixture responded significantly less to the mouse Ig. As shown in Figure 7.15, rabbits primed with the VLB-"loaded" HH, developed 53.4% less anti-mouse Ig antibodies, than those that received HH alone ($Mean_{HH/VLB}$ = 1.368±0.370, $Mean_{HH}$ = 2.932±0.304, p=0.004), 52.4% less than those receiving the HH antibody covalently linked to VLB ($Mean_{HH\text{-}c\text{-}VLB}$ = 2.875±0.407, p=0.006) and 59.8% less than the ones that received the parental anti-CEA antibody mixed with VLB ($Mean_{a\text{-}CEA/VLB}$ = 3.401±0.018). Unfortunately one of the animals belonging to the latter group died (for reasons not connected with the experiment), making statistical analysis of this group impossible.

On day 7, all groups were boosted i.p. with the anti-CEA antibody. As shown in Figure 7.16, the anti-mouse Ig secondary response was measured over a period of 3 weeks. We found that initially there was no difference in the response among the 4 groups. Nevertheless, the anti-mouse Ig titres started to drop more rapidly (on day 21) in the group of rabbits that were primed with the HH/VLB mixture. By day 28, the anti-mouse Ig response for this group was 28% less (p=0.035) than in the control groups (Fig. 7.16).

On day 29, the 4 groups received an i.v. injection of their corresponding antigens (Igs) (as on day 0), and 7 days later they were boosted with an i.p. injection of the anti-CEA antibody alone (as on day 7) (Fig. 7.7a). The humoral response was followed up to day 64; we observed no difference in the rabbits' anti-mouse Ig responses among all groups, irrespective of the preparations with which they had been primed and boosted.

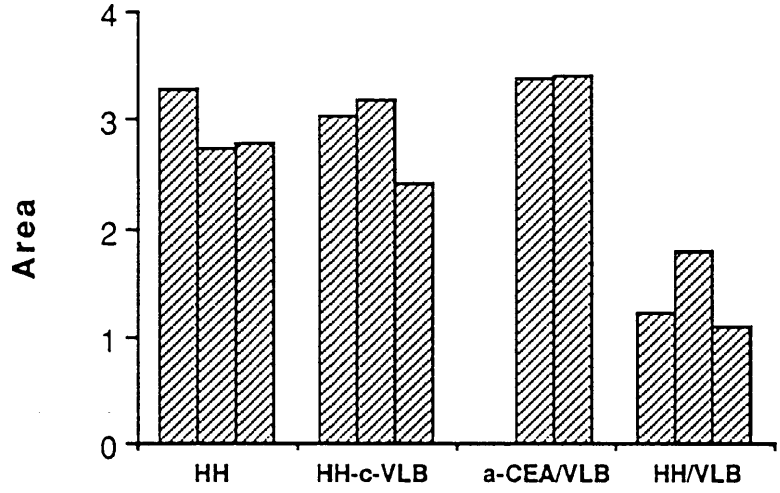


Figure 7.15: Rabbit anti-mouse Ig primary response, when the following immunogens were administered i.v. to groups of 3 rabbits: the hybrid-hybrid antibody (HH), the hybrid-hybrid antibody covalently linked to vinblastine (HH-c-VLB), the anti-CEA antibody mixed with vinblastine (a-CEA/VLB) and the hybrid-hybrid antibody mixed with vinblastine (HH/VLB). The a-CEA/VLB group consisted of only 2 rabbits.
The results are expressed as areas under the ELISA curves (see text for details).

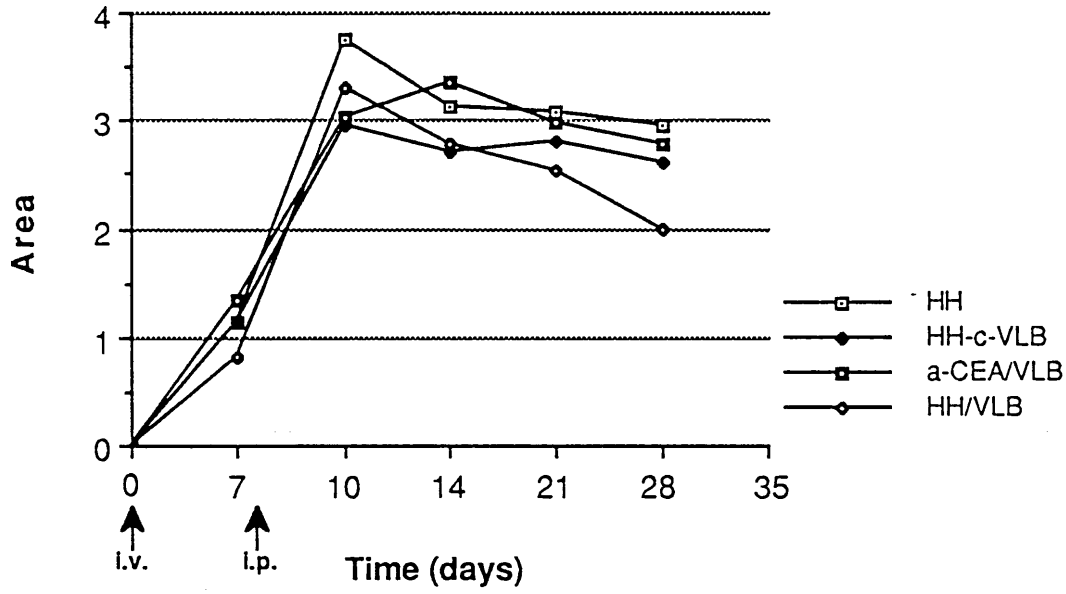


Figure 7.16: Rabbit anti-mouse Ig responses, measured over a period of 4 weeks, when 4 groups of rabbits were primed i.v. (day 0) with: the hybrid-hybrid antibody (HH), the hybrid-hybrid antibody covalently linked to vinblastine (HH-c-VLB), the anti-CEA antibody mixed with vinblastine (a-CEA/VLB) and the hybrid-hybrid antibody mixed with vinblastine, and subsequently boosted i.p. (day 7) with the a -CEA antibody.
On the vertical axis the results are expressed as areas under the ELISA curves (see text for details).

In the second experiment (Fig. 7.7b), two groups of 6 rabbits each, received 5 i.v. injections at daily intervals, of HH/VLB and anti-CEA/VLB mixtures respectively (MR 1:1). On day 11, they were all boosted with anti-CEA antibody alone. The response was assessed on days 11, 14, 18 and 25 post first injection. As shown in Figure 7.17, no difference in the anti-mouse Ig response was observed between the 2 groups.

In the third experiment, 12 rabbits were implanted with osmotic mini-pumps, releasing VLB into the circulation at a constant flow rate. Five days after implantation, 6 rabbits received the HH antibody and the other 6 the parental anti-CEA. The rabbits' primary response was measured on days 7, 12 and 60 post antibody administration. As shown in Figure 7.18a, rabbits injected with the HH, responded 78% less than those that received the control antibody ($Mean_{HH}$ = 0.480$\pm$0.237, $Mean_{a\text{-}CEA}$ = 2.177$\pm$0.802, $p<0.001$). On days 12 and 60, the reduction in the anti-mouse Ig response was found to be 55% ($Mean_{HH}$ = 1.799$\pm$0.614, $Mean_{a\text{-}CEA}$ = 3.587$\pm$0.463, $p=0.001$) and 63% ($Mean_{HH}$ = 1.128$\pm$0.345, $Mean_{a\text{-}CEA}$ = 3.007$\pm$0.941, $p<0.001$) respectively (Figs. 7.18b and c). The overall anti-mouse Ig response, over the period of 60 days, is shown in Figure 7.19. The mean $\pm$ standard deviations (not shown on the graph) for the rabbits that received the HH are: day 7 = 0.750$\pm$0.452, day 12 = 2.193$\pm$0.428, day 60 = 1.330$\pm$0.746, and for those that received the anti-CEA: day 7 = 2.806$\pm$0.841, day 12 = 4.373$\pm$0.564, day 60 = 3.145$\pm$0.874 (Fig. 7.19a). The overall mean reduction in the anti-mouse Ig response for the rabbits that received the bispecific antibody, was 60%, as calculated by the area under the curve (Fig. 7.19b).

7.3.3.5 Measurement of antigen-specific B-cells: In the first experiment of antigen "suicide" (Fig. 7.7a), we found that despite the initial reduction in immunogenicity of the VLB-"loaded" HH antibody, the response (after several immunizations) was the same against all immunogen preparations. On day 64, we measured similar levels of anti-mouse Ig antibodies in all rabbits (see 7.3.3.4

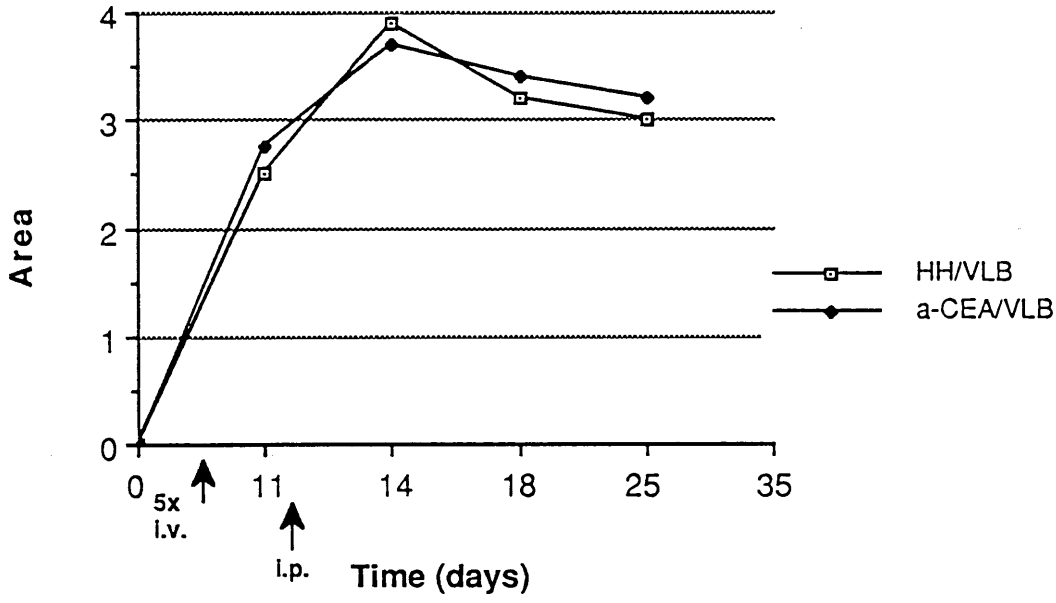


Figure 7.17: Mean anti-mouse Ig response, of rabbits i.v. immunized for 5 consecutive days (days 0-4) with either the hybrid-hybrid antibody mixed with vinblastine (HH/VLB) or the anti-CEA antibody mixed with vinblastine (a-CEA/VLB) (6 rabbits in each group). Both groups were i.p. boosted with the anti-CEA antibody on day 11.
The response was followed for 25 days, and the results are expressed as areas under the ELISA curves (see text for details).

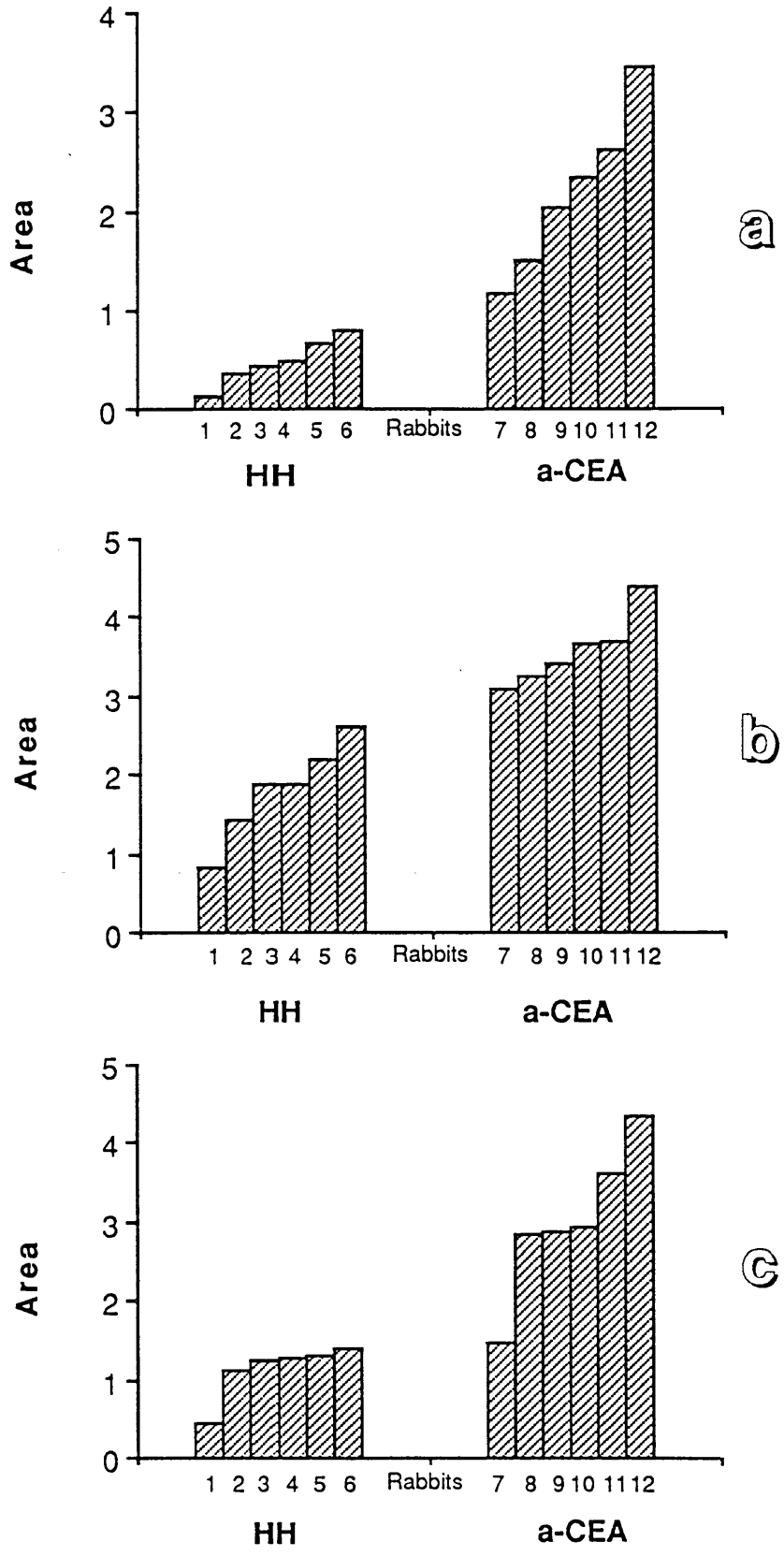


Figure 7.18: Primary anti-mouse Ig response, when the hybrid-hybrid (HH) and the anti-CEA (a-CEA) antibodies were administered i.v. to 2 groups of six rabbits, under constant infusion of vinblastine (using a subcutaneously implanted osmotic mini-pump). The response was measured on days 7 (a), 12 (b) and 60 (c) post administration of the antibodies, and results are expressed as area under the ELISA curves (see text for details).

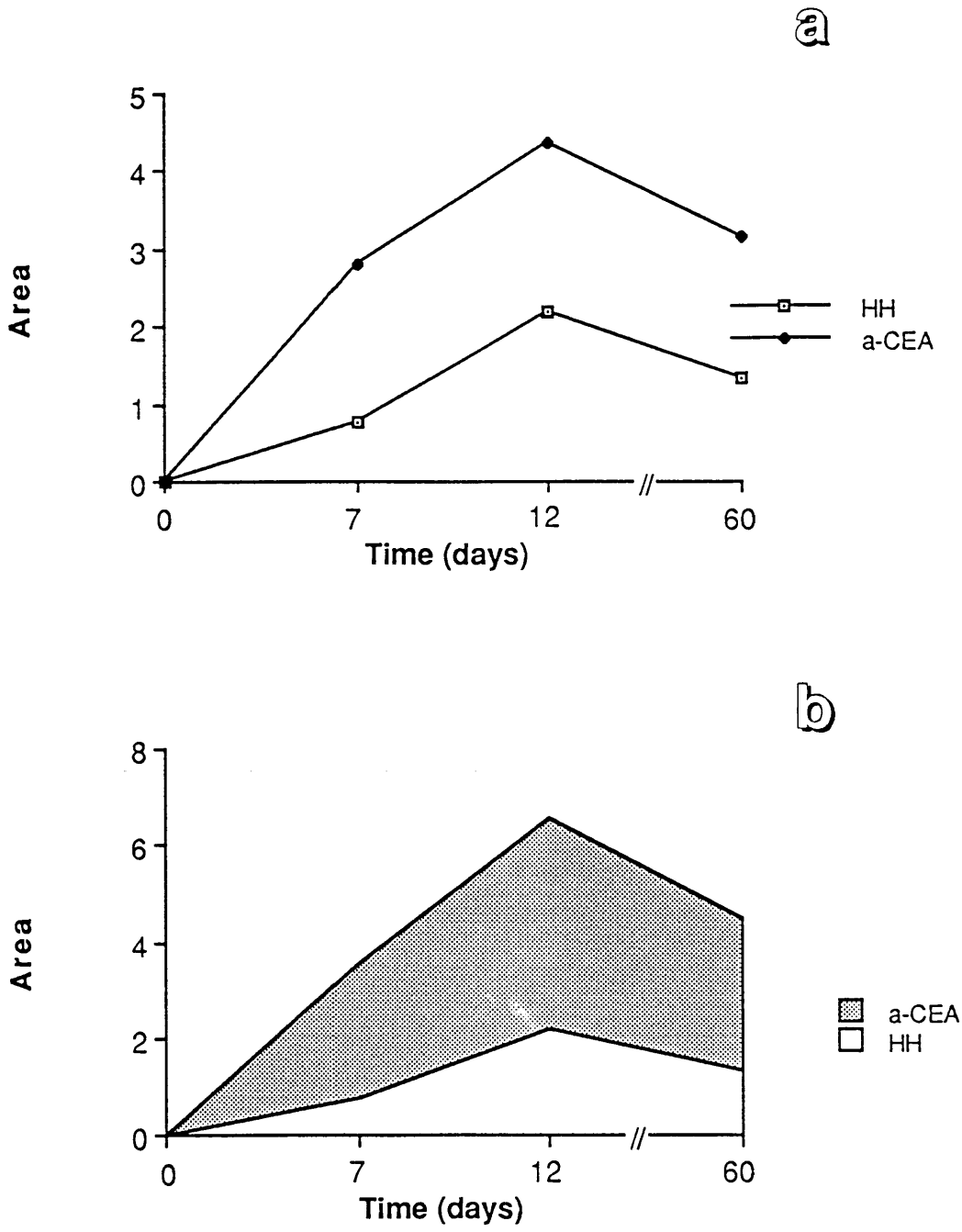


Figure 7.19: a) Mean primary anti-mouse Ig response, when the hybrid-hybrid (HH) and the anti-CEA (a-CEA)antibodies were administered i.v. to 2 groups of six rabbits, under constant infusion of vinblastine (using a subcutaneously implanted osmotic mini-pump). The response was measured on days 7, 12 and 60 post administration of the antibodies, and is expressed as area under the ELISA curves (see text for details).
b) is the a above, showing the two areas under the curves that were calculated.

above). We examined whether the actual immunoglobulin-specific B-cell population was also the same. PBLs and/or spleen cells of the above groups of rabbits (on day 64) were incubated with either FITC-HH or FITC-anti-CEA, and analyzed by flow cytometry. Unfortunately, the signal was too weak to permit any analysis.

7.3.3.6 Anti-ovalbumin and anti-vinblastine response: In the first experiment (Fig. 7.7a), rabbits were also immunized with OVA, as an irrelevant antigen. We followed the anti-OVA response throughout the experiments and found that, despite variability, there was no significant difference in the animals' ability to make antibodies against OVA, irrespective of the immunoglobulin preparation that they had simultaneously received. All animals (from all 3 experiments) were also tested for an anti-VLB response. We did not observe anti-VLB antibodies.

7.3.3.7 Measurement of circulating immunoglobulin and vinblastine: In the third antigen "suicide" experiment, where the drug was delivered by an osmotic mini-pump, we measured the levels of HH and anti-CEA still in circulation, on day 7 and 12 after administration. As shown in Table 7.2, 5 out of the 6 rabbits that received the HH antibody (and responded significantly less to it), still had detectable levels of the antibody in circulation. The 6th rabbit was the one that developed the highest anti-mouse Ig response of this group (Fig. 7.18a). In addition, in 5 out of 6 rabbits that were injected with the anti-CEA antibody (and responded very well to it), we did not detect any anti-CEA in the circulation. In only one rabbit could we detect circulating mouse Ig, and that was the one that developed the lowest anti-mouse Ig response of this group (Fig. 7.18a). By day 12 there was no mouse immunoglobulin in circulation. The VLB levels in the blood were also measured on day 12. As shown in Table 7.2, there was still VLB in circulation. There appears to be no relationship between the kind of immunogen administered and the amount of circulating VLB.

Rabbit	Immunogen	ANTIBODY Day 7 (µg/ml)	ANTIBODY Day 12 (µg/ml)	VINBLASTINE Day 7 (ng/ml)	VINBLASTINE Day 12 (ng/ml)
1	HH	108	-	N.T.	4.5
2		237	-	N.T.	<0.4
3		83	-	N.T.	4.3
4		26	-	N.T.	N.T.
5		77	-	N.T.	<0.4
6		-	-	N.T.	7.8
7	a-CEA	124	-	N.T.	<0.2
8		-	-	N.T.	N.T.
9		-	-	N.T.	1.3
10		-	-	N.T.	<0.13
11		-	-	N.T.	27
12		N.T.	N.T.	N.T.	1.3

Table 7.2: Concentration of the hybrid-hybrid (HH) and the anti-CEA (a-CEA) antibodies in rabbits' sera, 7 and 12 days after i.v. administration, together with the concentration of vinblastine in rabbits' sera at the same time points. The drug was constantly infused into the rabbits by an osmotic mini-pump, subcutaneously implanted 5 days before administration of the antibodies.

7.3.4 Discussion

It has previously been shown by many investigators that when an antigen is covalently linked to plant toxins or drugs, it can specifically inactivate lymphocytes that bind to it *in vitro* (Volkman et al., 1982; Vitetta et al., 1983; Morimoto et al., 1983; Killen and Lindstrom, 1984; Takeuchi et al., 1986; Brust et al., 1987; Shelton et al., 1988). Moreover, in certain cases antigen-toxin/drug conjugates were found to abrogate specifically the response of animals against the administered antigen (Shek et al., 1986; Diener et al., 1986; Brust et al., 1987; Shelton et al., 1988; Durrant et al., 1989). We have used a novel drug-conjugate consisting of a bispecific mouse monoclonal antibody, that recognises Vinca alkaloids with one binding site and CEA with the other. This hybrid-hybrid antibody when mixed with vinblastine, forms a complex that is stable *in vitro*, even in the presence of serum (Figs. 7.10a and b, Figs. 7.11a and b). We investigated the possible *in vivo* immunosuppressive effects of this monoclonal antibody/VLB complex, when it was administered to rabbits "loaded" with vinblastine.

We found that when this bispecific monoclonal antibody was administered i.v. to rabbits simultaneously with VLB, at a MR of 1:1, the primary anti-mouse Ig response was reduced by approximately 50% (Fig. 7.15). The immunosuppression observed was specific for the mouse Ig, since the response against an irrelevant antigen (OVA) was normal. Further proof that the amount of VLB injected was not responsible for a general, non specific immunosuppression, was that when the drug was co-injected with the control anti-CEA antibody, the anti-mouse Ig response was not reduced (Fig. 7.15). Interestingly, when the hybrid-hybrid antibody was covalently linked to VLB, it did not show any immunosuppressive effect (Fig. 15). It would therefore appear, that the hybrid-hybrid/VLB complex is captured by B-cells that have receptors specific for the mouse Ig administered. The VLB subsequently

dissociates and inactivates the mouse Ig-specific B-cells, which eventually die before they can proliferate, maturate and secrete their surface Igs. Dissociation of the drug from the antibody must be necessary for this action, since the covalently linked antibody-VLB complex is inactive (Fig. 7.15).

However, this effect is by no means tolerance. The suppressed animals responded equally well to a subsequent challenge with anti-CEA antibody, although their anti-mouse Ig antibody levels start to fall earlier than those in the control groups (Fig. 7.16). Moreover, a further challenge with the immunosuppressive HH/VLB mixture (MR 1:1), did not seem to affect the immune response of the previously primed animals. For over two months, the levels of anti-mouse Ig antibodies were the same in the animals that received the HH/VLB complex (1st and 3rd immunization, anti-CEA 2nd and 4th immunization), and the control groups. Unfortunately, we were unable to show that the actual mouse Ig-specific B-cell population also remained the same.

If the theory of the "lethal" antigen is correct, why did we observe only a 50% reduction of immunogenicity, instead of total abrogation and perhaps tolerance? One explanation is that since the toxic compound (VLB) is not covalently linked to the antibody, it is in constant association and dissociation with it. As soon as VLB dissociates, it is rapidly removed from the circulation (Fig. 7.12a), whereas bound onto the antibody, its half life slightly increases (Fig. 7.12b). We found that 5 hr after i.v. administration of the HH/VLB complex at a MR 1:1, almost all the antibody was present in the circulation, but associated with only 2% of the initial amount of VLB (Fig. 7.13a). B-cells that escape the first "attack" initially, may later capture the unconjugated mouse Ig, proliferate, and eventually secrete their anti-mouse Ig antibodies. Since VLB acts only on proliferating cells, we attempted to inactivate this population of B-cells that are clonally expanding, by five consecutive injections of the "immunosuppressive" HH/VLB mixture. It was expected that the effect of VLB

would be more profound to cells that are already activated and are undergoing proliferation. Unfortunately, we did not observe any immunosuppression at all (Fig. 7.17). This may result from high levels of circulating HH, after VLB had already dissociated and cleared from the blood stream.

It became clear, that insufficient drug was being delivered to the B-cells. We found that by increasing the amount of VLB administered, more VLB remained in circulation and associated with the monoclonal antibody (Fig. 7.13b). Since the non-covalently linked antibody and VLB would be constantly associating and dissociating, we tried to keep the antibody's binding sites occupied with the drug, by maintaining a constant level of VLB in the blood, over a given period of time. This was done using subcutaneously implanted osmotic mini-pumps that slowly released the drug into the circulation (Fig. 7.12c).

Under these conditions, the rabbit anti-mouse Ig primary response was further reduced, giving an overall reduction of 80% (Fig. 7.18a). The relative absence of anti-mouse Ig antibodies was also supported by the fact that 7 days after administration, there was still HH in circulation, at a time when all anti-CEA in the controls had been catabolized (Table 7.2). On days 12 and 60, the reduction of the immune response was 55-63% (Figs. 7.18b and c), an increase in the response, relative to that at day 7, of 20-25%. This was not expected, since by day 12 there was no Ig remaining in the circulation to stimulate the B-cells (Table 7.2), although there was presumably a reservoir of trapped antigen in/on APCs. In addition, we did not observe any anti-VLB response.

It would be interesting to investigate whether the anti-mouse Ig response of the partially immunosuppressed animals is against the constant or variable region. Would they respond normally to a subsequent challenge with the mouse Ig alone? It would also be important to find out if the HH/VLB complex retains its immunosuppressive effect, even after multiple injections.

The limited time of this thesis did not allow us to identify the organs into which free VLB or the HH/VLB complex accumulate in rabbits. From previous clinical data, we suspect that the main organ involved in catabolism is the liver (Nelson, 1982). In addition, it would be important to identify the type of cells that bind our immunogen *in vivo*. Is it only B-cells, or do other APCs (eg. macrophages and dendritic cells) and other mononuclear Fc-receptor-bearing cells also bind our xenogeneic immunoglobulin?

In conclusion, it appears that in our animal model the mouse monoclonal antibody-drug complex is less immunogenic, due to specific inactivation of anti-mouse Ig B-cells. Our data therefore support the antigen "suicide" theory, and we believe that a carefully designed molecule can be toxic to immunocompetent cells and hence become non-immunogenic.

Our drug-conjugate triggered a low immune response. Similar conjugates have been reported to be totally immunosuppressive (Durrant et al., 1989). This may be attributed to our particular antigen or animal used, and to the amount or route by which the Ig was administered. We designed our experiments to be as close to the clinical situation as possible. The bispecific antibody used as antigen is an anti-tumour monoclonal antibody, that will soon undergo clinical trials. It was administered to rabbits at a concentration equivalent to that desired in clinical therapy, and at this dose it was very immunogenic, immunogenicity that was enhanced by the fact that it binds to a cell surface antigen in rabbits. Nevertheless, we achieved a high degree of specific suppression, under conditions where the antibody fully retained its anti-tumour properties. We appreciate that our animal experiments could be improved further by modifying the amount of drug-complex injected, and by changing the route of administration or even the cytostatic drug used.

Ultimately, what remains to be seen is whether our monoclonal antibody/VLB complex is also less immunogenic in patients with cancer, something that would be of great value, even if total abrogation of the anti-Ig immune response cannot be achieved.

7.4 General discussion

Twenty five years ago, one of the major areas of investigation for immunologists was the identification of the mechanisms of immune responses, and the nature of the cells involved. Very soon it became apparent that certain lymphocytes could specifically bind antigen to a higher or a lower degree (Naor and Sulitzeanu, 1967; Humphrey and Keller, 1969). This very important observation was followed by a second one: since the only way of detecting antigen-specific cells was by radiolabelling the antigens, it was found, that radiation could irreversibly and specifically damage the cells bearing receptors for the particular antigen. Quite unexpectedly therefore, the concept of the antigen "suicide" was introduced, and in parallel with new evidence on the involvement of thymus and bone marrow derived cells (and their interactions) in immune responses, our knowledge of immune suppression and tolerance was increased. Thus, over the last two decades, it has been shown that, after *in vitro* incubation, various antigens attached to toxic compounds could specifically eliminate the subpopulation of immunocompetent cells, that were destined to capture them (Ada and Byrt, 1969; Humphrey et al., 1971; Roelants and Askonas, 1971; Golan and Borel, 1972; Volkman et al., 1982; Vitetta et al., 1983; Killen and Lindstrom, 1984; Takeuchi et al., 1986; Shelton et al., 1988).

In vitro experiments are fundamental, but very rarely have direct clinical applications. The construction of a "lethal" antigen that would cause specific

immune suppression *in vivo* is a difficult task, yet more exciting and of paramount importance, not only in serotherapy of cancer, but also in allergy, autoimmune diseases and transplantation. The main parameters that influence the development of such an immunosuppressive antigen are: a) the toxic compound conjugated onto the antigen, b) the *in vivo* stability of the antigen-conjugate, and c) the availability of the toxic compound at the site of the action.

Radioisotopes were the first to be employed in antigen "suicide" experiments, and these radiolabelled "lethal" antigens were shown to induce specific suppression, when administered *in vivo* to animals (Humphrey and Keller, 1969; Filipp et al., 1981; Csaba et al., 1985). The selected radioisotope should be a high energy, short range β-emitter, ideally with a physical half life of 12 hours to 4 days. Thus, a compromise can be achieved between effective B-cell inactivation (at the initial stages of encounter and clonal expansion) and unnecessary tissue irradiation. Despite its long half life (59.9 days), ^{125}Iodine has been exclusively used so far, because it combines many useful features: high β-ray emission, simple labelling method at very high specific activities, reasonably good *in vivo* stability (<10% dehalogenation/day), and immediate toxic effect on the cell.

As radiochemistry has progressed, a whole family of radioisotopes have become available, such as the β-emitters : ^{90}Yttrium, ^{186}Rhenium, ^{69}Copper, ^{32}Phosphorus, or the α-emitters ^{211}Astatine, ^{109}Palladium, ^{212}Lead and its daughter product ^{212}Bismouth. In addition, ^{111}Indium, a γ-emitter, may be useful, since it has been shown to be particularly toxic to lymphocytes (Kraal and Geldof, 1979; Ten Berge et al., 1983), mainly because when it is internalized, it crystallizes at the low pH of the endosomes; hence it remains in the cytoplasm (iodines are exocytosed), and the Auger electrons produced, damage the cell.

However, radioactivity is generally not user-friendly, its toxic effect is limited to lymphocytes undergoing proliferation, and there is always the danger of non-specific irradiation of neighbouring cells. Moreover, not all antigens can be labelled as efficiently as others, and the labelling method is not a controlled process. The radionuclide is distributed/bound onto the antigen in a normal Gaussian manner, resulting in a mixed population which includes highly radiolabelled molecules (that may undergo radiolysis) and molecules with little or no radioactivity (that will promote the immune response), without any way of separating them out. We believe that the latter were the main reason that we failed to induce suppression in our animal experiments. For immunoglobulins there is a limit to the amount of radionuclide that can be incorporated, and this limit may simply not be enough. In contrast to ^{125}Iodine, ^{111}Indium-labelled Ig slightly reduced the primary anti-Ig response in mice, an interesting phenomenon considering that the specific activity with this isotope can be improved. However, it is likely that studies on antigen "suicide" using radiolabelled antigens will be limited to *in vitro* experiments. Their clinical application seems remote, since the total amount of isotope required to cause immunosuppression is probably in excess of the ethically permitted dose.

Natural toxic compounds derived from bacteria or plants offer an alternative to radioactivity. Various toxins are extremely potent, but those most often used are diphtheria toxin, and the plant toxins ricin, abrin and gelonin. Their toxic effect is linked with the termination of the cell's protein synthesis: diphtheria toxin inactivates the elongation factor 2, whereas ricin, abrin and gelonin damage the 60S subunit of the ribosomes. This property is advantageous for the construction of "lethal" antigen, since the toxin-antigen conjugate paralyses resting (eg. macrophages) as well dividing immunocompetent cells. Theoretically, one toxin molecule is enough to damage the cell. Toxin-antigen linkage involves a rather more complicated method than that for radiolabelling, and the conjugates may not be so stable *in vivo*. Improvements

of biochemistry and genetic engineering can produce conjugates that are more resistant to enzymatic cleavage whilst in circulation (Thorpe et al., 1987; Knowles and Thorpe, 1987; Griffin et al., 1988; Thorpe et al., 1988; Ramakrishnan et al., 1989; Chaudhary et al., 1989).

There are, however, difficulties in the use of immunotoxins. Firstly, toxins have their own binding sites for virtually all cell types. Attempts to block this side effect are either incompatible with *in vivo* use (excessive amount of sugars) (Volkman et al., 1982, Killen and Lindstrom, 1984), or dramatically reduce the toxin's ability to be transported from the endosome into the cytoplasm and "hit" the ribosomes (use of toxin subunits) (Press et al., 1988a; Shen et al., 1988). Secondly, they are generally large molecules, that may easily exceed the size of the antigen itself. Thirdly, and most important, is that very potent toxins (such as ricin) are also found to be very immunogenic (Harkonen et al., 1987; Spittler et al., 1987; Hertler et al., 1988; Pimm et al., 1989). Chemical modifications of the molecule (Pietersz et al., 1988c; Blakey et al., 1988b) improve the kinetics and may reduce immunogenicity, but as the state of the art is now, toxins are not the best candidates for *in vivo* antigen "suicide".

A further option is to use chemotherapy drugs to form immunoconjugates. These drugs (or their active metabolites) are cytoxic, and their effects range from direct action on the cell membrane (adriamycin), to blockade of protein, DNA or RNA synthesis (methotrexate, 5-fluoro-uracil, cytosine arabinoside), DNA intercalation (epirubicin, adriamycin, daunorubicin,, cisplatin, chlorambucil, cyclophosphamide) and destruction (bleomycin, busulfan), or inhibition of mitosis (vinblastine, vindesine, vincristine). Over the past decade, progress in biochemistry has enabled the effective conjugation of many anti-neoplastic drugs to immunoglobulins and other molecules. However, the use of drug conjugates as "lethal" antigens was limited due to: a) the rather complex conjugation method, b) the low stability of the conjugate *in vivo*, and c) loss of the drug's effectiveness (Kanellos et al., 1985; Rowland et al., 1986).

The covalent linkage between the antigen and the drug must be especially designed not only to survive enzymatic cleavage in the circulation, but also to ensure prompt release in the target-cell. Drugs, being so small, may easily lose their reactivity during conjugation. Moreover, it is possible that the drug is not transferred to the cytoplasm in its native and active configuration (after endocytosis of the drug-conjugate), although the use of pH sensitive linkages may improve the drug's intracellular release (Diener et al., 1986; Dillman et al., 1988; Durrant et al., 1989).

We have used a novel drug-antibody complex as "lethal" antigen. This comprised of a bispecific mouse monoclonal antibody that recognises a tumour associated antigen (CEA) with one binding site, and Vinca alkaloids with the other. The antibody, mixed with Vinca alkaloids and administered *in vivo*, was able to retain the drug on its binding site. The advantage of using the antibody's binding site instead of a covalently bound linker, is: a) *in vivo* breakdown of the conjugate is avoided and b) the drug is delivered to the target-cell intact, unprocessed and in its native and most effective form.

Vinca alkaloids are known to disrupt the mitotic spindle of dividing cells, by non-covalent binding to tubulin. By inhibiting assembly/disassembly of microtubules, Vinca alkaloids disrupt cellular functions in a variety of ways, including inhibition of endocytosis and antigen modulation (De Rie et al., 1988). We envisaged therefore that our bispecific monoclonal antibody, bound to specific anti-Ig B-cells, would capture and deliver drug molecules to these cells.

Indeed, we observed up to 80% reduction of the primary anti-mouse Ig response in rabbits, when there was sufficient vinblastine to occupy the binding site of the antibody. The fact that there was no change in the immune response when the drug was covalently linked onto the antibody demonstrates the importance of the non-covalent linkage for the release of active drug. In

addition, we detected no anti-VLB antibodies, although the drug does become immunogenic when covalently linked to a carrier (Corvalan and Smith, 1987). The fact that we did not fully suppress the immune response may be because the drug is not effective against non proliferating antigen presenting cells, such as macrophages. Whereas T-cell independent responses can be totally abrogated using a drug-conjugated "lethal" antigen *in vivo* (Abu-Hadid et al., 1987; Abu-Hadid et al., 1988), our immunoglobulin, like any other thymus-dependent antigen, can be effectively processed by APCs, and exposed to T-cells, which in turn may trigger low affinity mouse Ig specific B-cells, that escaped the first "attack". This is also supported by our data showing that when the "immunosuppressive" VLB/Ig complex is present in the circulation, the anti-Ig response is limited to 20% of the normal. However, when the complex is fully catabolized, the anti-Ig response increases to 40-45% of the normal.

Our current data therefore suggest that non-covalent linkage of a small drug molecule to a therapeutic antibody may provide the most effective means by which both tumour target and the specific anti-Ig immune response can be deleted. The system clearly requires further study and optimization in areas such as the correct choice of drug, dose and route of administration, before the concept of *in vivo* antigen "suicide" can be realized.

Chapter 8

In vivo immunoregulation by anti-lymphocyte antibody

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8.1 Introduction

As discussed in the previous chapter (chapter 7), the binding of an antigen to the surface-immunoglobulin (Ig) of antigen-specific B-cells, activates and "drives" them to proliferation and maturation. We have attempted to induce specific unresponsiveness by blocking this clonal expansion, and the subsequent Ig secretion, using a "lethal" antigen. However, for the majority of antigens (T-dependent antigens), specific Ig secretion does not occur without stimuli from a subset of T-cells. This subpopulation of mature T-cells, referred to as T-helper or -inducer cells, are "key" cells for the immune system, since they promote both humoral (chapter 7) and cellular immunity (Dialynas et al., 1983a; Dialynas et al., 1983b; Wilde et al., 1983) (Fig. 8.1). Although it is proposed that there is more than one type of T-helper cell, depending on the lymphokines they secrete upon activation (Mosmann et al., 1986a; Cherwinski et al., 1987), they are essentially all characterized a) functionally, by their ability to respond to antigen in association with class II Major Histocompatibility molecules (MHC class II) (Dialynas et al., 1983b; Wilde et al., 1983; Swain, 1983; Swain et al., 1984), and b) phenotypically, by a cell surface marker, the CD4 molecule (T4 or Leu3a in primates and man, L3T4 in mice, W3/25 in rats) (reviewed by Dalgleish, 1986).

Before being recognised by T-cells, antigens must be processed by antigen-presenting cells (APCs) such as dendritic cells, macrophages and B-cells (Thomas et al., 1981; Unanue, 1984), and presented on the cell surface as peptide fragments in association with MHC class I or II (Erb and Feldmann, 1975; Watts et al., 1984; Babbitt et al., 1985; Yewdell and Bennink, 1989; Nuchtern et al., 1989). A combination of a lymphokine (interleukin 1, IL-1, secreted by APCs) and binding to the processed antigen/class II MHC complex, activates T-helper cells (Unanue and Allen, 1987) (Fig. 8.1). This results in clonal expansion, and release of a variety of growth and maturation factors that critically affect T- and B-cells (Palacios, 1982; Hagiwara et al., 1987).

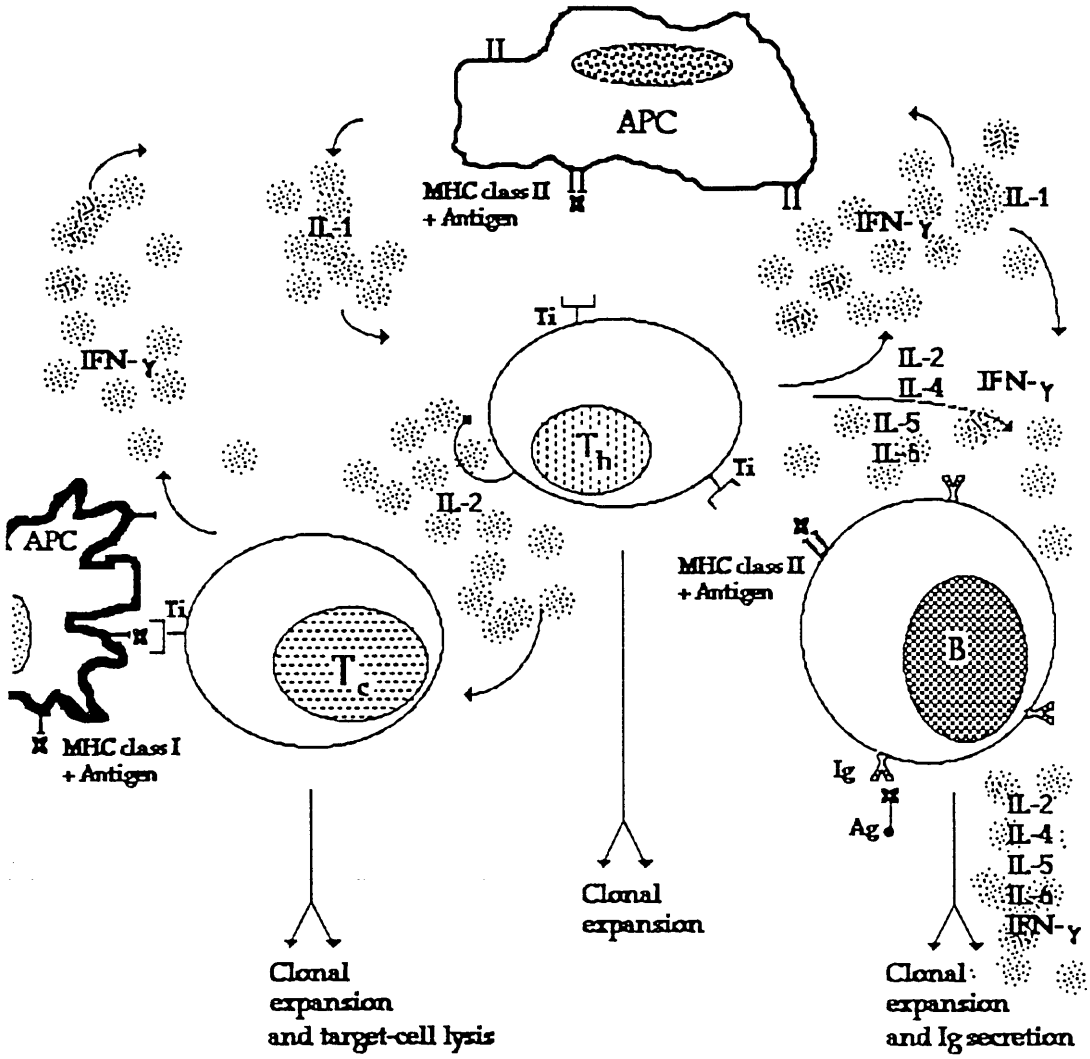


Figure 8.1: Interactions of immunocompetent cells in immune responses.
Antigen presenting cells (APC) secrete interleukin 1 (IL-1), and present the processed antigen, in association to Major Histocompatibility Complex (MHC) class II, to T-helper cells (T_h).
T-helper cells bind to the antigen via their antigen-receptor (T_i), secrete interferon γ (IFN-γ) and interleukin 2 (IL-2), and undergo proliferation.
T-cytotoxic cells (T_c) recognise the antigen in association to MHC class I. IL-2, secreted by T_h-cells, stimulates the T_c-cells to a) release IFN-γ, b) proliferate and c) lyse the "target" cell.
B-cells bind the antigen via their specific membrane-immunoglobulins (Ig), and are stimulated to proliferation and differentiation by a combination of various lymphokines (IL-1, IL-2, IL-4, IL-5, IL-6, IFN-γ) and T_h-cells.

Among all lymphokines secreted, interleukin 2 (IL-2) may be one of the most important, because it is responsible for the proliferation of T-helper (T_h), as well as T-cytotoxic (T_c) cells (Watson, 1979). As shown in Figure 8.1, IL-2 binds to IL-2 receptors (IL-2R) on T_h in an autocrine and on T_c-cells in a paracrine fashion (Cantrell and Smith, 1983). A variety of other lymphokines are also produced, with multiple actions on immunocompetent cells, their primary role being to amplify the immune response. Interleukin 3 (IL-3) facilitates the growth and differentiation of pluripotent haematopoietic stem cells and progenitors, particularly those that generate the myeloid lineages (Nicola and Vadas, 1984). Interleukin 4 (IL-4) is a pleiotropic lymphokine, that induces proliferation to both T- (Mosmann et al., 1986b; Fernandez-Botran et al., 1986; Hu-Li et al., 1987) and B-cells (Howard et al., 1982) (Fig. 8.1). In particular, it enhances MHC class II and CD23 (low affinity Fc_ε receptor) expression on the B-cell surface (Roehm et al., 1984), as well as facilitates IgG1 (Vitetta et al., 1985) and IgE secretion (Coffman et al., 1986). Interleukin 5 (IL-5), causes eosinophil differentiation, but also supports B-cell activation, growth and IgA secretion (Sanderson et al., 1988) (Fig. 8.1). Recently it has been shown that IL-4 and IL-5 may act synergistically to promote IL-2 receptor expression on murine B-cells (Loughnan and Nossal, 1989). Interleukin 6 (IL-6) is secreted by activated T-cells, and its role is primarily to facilitate the B-cell's differentiation (Gearing, 1989) (Fig. 8.1), whereas interleukin 7 (IL-7) supports pre B-cell growth, and generally act as B- and T-cell stimulant (Gearing, 1989). Finally, interferon γ (IFN-γ), is responsible for the induction of MHC class II and enhanced IL-1 production of macrophages (Schreiber et al., 1985; Adams and Hamilton, 1987), as well as for the proliferation and differentiation of B-cells (Gordon and Guy, 1987) (Fig. 8.1).

It is clear therefore, that *in vivo* blocking or depletion of T-cells would paralyze the immune system, and induce a state of almost total unresponsiveness. Early studies demonstrated that administration of anti-lymphocyte serum (ALS), primarily directed against T-lymphocytes, was closely

associated with a rapid depletion of T-cells (Levey and Medawar, 1966a; Denman and Frenkel, 1968; Gill, 1969; Martin, 1969). The immunosuppressive capacity of ALS was measured by a) inhibition of mainly primary antibody responses (James and Anderson, 1968; Mackaness and Hill, 1969; Argyris and Plotkin, 1970) to antigens such as flagellin (Shellam, 1969), sheep erythrocytes (Monaco et al., 1966; James and Anderson, 1968) and bovine serum albumin (James and Jubb, 1967), and b) prolongation of graft survival (Woodruff and Anderson, 1964; Levey and Medawar, 1966b; Kinne and Simmons, 1967; Howard et al., 1968a; James, 1969; Hagl et al., 1972). Very soon it became apparent that the optimal time of ALS administration was simultaneous with or a few days before the injection of the antigen. However, despite the general immunosuppressive effects, xenogeneic ALS was itself found to be immunogenic. Anti-ALS antibodies generated by the host reduced the ALS-effect and resulted in "serum sickness" phenomena (Clark et al., 1967; Kashiwagi et al., 1968; Butler et al., 1969; James, 1969; Butler et al., 1970). Various attempts to induce tolerance to ALS, mainly by deaggregation, failed (Golub and Weigle, 1969; Gewurz et al., 1970; Rossen et al., 1971).

The main difficulty with ALS was its polyclonality. The fact that it was raised in different animals and was of virtually unknown specificity, limited the evaluation of its effects, and led to conflict concerning its mode of action (Levey and Medawar, 1966a; Monaco, 1966; Martin and Miller, 1967; Mandel and Asofsky, 1968; Gill, 1969; Martin, 1969). The introduction of the monoclonal antibody technique (Köhler and Milstein, 1975) enabled the development of numerous antibodies against T- and B-cell surface molecules; this new development increased our knowledge of the antigen-recognition, activation, differentiation, and function of immunocompetent cells. It soon became possible to study the effects of antibodies directed against a specific receptor expressed on lymphocyte subsets.

As discussed earlier, T_h-cells are cells of major importance in the onset of the immune response. Many cell surface molecules are involved in the cell's "key" role in immunity. CD4 is one of these, recognizing the MHC class II on APCs (Meuer et al., 1982; Krensky et al., 1982; Biddison et al., 1983) (Fig. 8.2a). Initially it was thought that CD4 played an important role in antigen-recognition and T_h-cells adhesion to APCs (Dalgleish, 1986), but later studies showed that CD4 also delivers a signal for IL-2 production (Inaba and Steinman, 1987), and/or may be responsible for T-cell discernment of self from non-self (Benjamin et al., 1988).

Antibodies to CD4 inhibit *in vitro* T-cell activation and proliferation (but have no effect on T_h-APC adhesion; Inaba and Steinman, 1987) to both antigen and mitogens (Wassmer et al., 1985; Malek et al., 1985). When administered *in vivo*, these monoclonal antibodies are highly immunosuppressive, and have therefore been used in animal models for therapy of autoimmune diseases (Brostoff and Mason, 1984; Wofsy and Seaman, 1985; Waldor et al., 1985; Ranges et al., 1985; Hafler et al., 1988), prevention of graft rejection (Cobbold et al., 1984; Villemain et al., 1986; Cobbold et al., 1986) and tolerance induction against various antigens (Gutstein and Wofsy, 1986; Goronzy et al., 1986; Gutstein et al., 1986; Benjamin and Waldmann, 1986; Benjamin et al., 1986). The immunosuppressive effects observed were due to $CD4^+$ cell depletion (Cobbold et al., 1984; Waldor et al., 1985; Benjamin and Waldmann, 1986; Gutstein et al., 1986; Benjamin et al., 1986), inhibition of signal transmission in T_h-cells (Wassmer et al., 1985; Gutstein and Wofsy, 1986; Benjamin et al., 1988), or blockade of binding between T_h-cells and APCs (Wilde et al., 1983; Gutstein and Wofsy, 1986). The *in vivo* immunosuppressive effect of anti-CD4 antibodies can be enhanced, when a "cocktail" of antibodies, directed against different epitopes of the CD4 (Qin et al., 1987), or a combination of anti-CD4 and anti-CD8 (the "counterpart" molecule on the T_c-cells, Fig. 8.2b) are used (Cobbold et al., 1986).

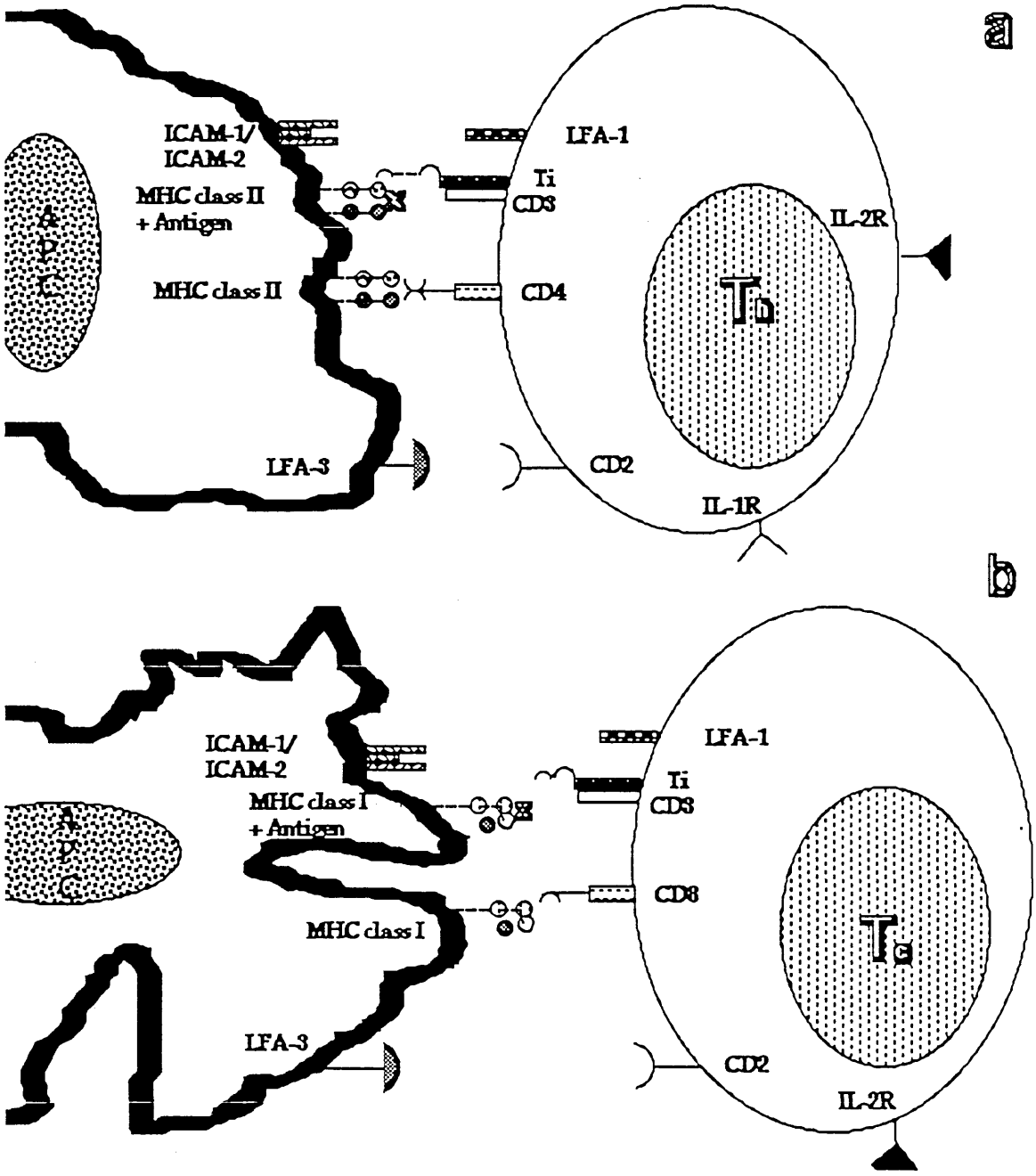


Figure 8.2: T_h- (a) and T_c-cell (b) interactions with antigen.
MHC : Major Histocompatibility Complex
ICAM : Intercellular Adhesion Molecule
LFA : Lymphocyte Function-associated Antigen
T_i : T-cell receptor
IL-1R : Interleukin 1 receptor
IL-2R : Interleukin 2 receptor
CD2, CD3, CD4, CD8 : Molecules that facilitate T-cell recognition of antigen and activation.

Induction of suppression, or tolerance, is not a unique property of CD4 and CD8. It has been shown that antibodies to other accessory molecules, such as CD2 and CD11a/CD18 (LFA-1) can elicit or facilitate similar effects. CD2 is the binding site for sheep red blood cells (Meuer et al., 1984), and the receptor for the lymphocyte function-associated antigen 3 (LFA-3, Fig. 8.2a and b) (Shaw et al., 1986; Selvaraj et al., 1987). It plays a role in T-cell adhesion (Shaw et al., 1986; Moingeon et al., 1989) and activation (Meuer et al., 1984; Bierer et al., 1988; Moingeon et al., 1989), therefore antibodies directed against CD2 may cause T-cell inactivation (Tadmori et al., 1986; Hafler et al., 1988). LFA-1 (Fig. 8.2a and b) is important for the interaction of lymphocytes with other cells (Springer et al., 1987). LFA-1 is the receptor for the intercellular adhesion molecule 1 (ICAM-1, CD54), and a closely related molecule designated as ICAM-2 (Staunton et al., 1989); its role is not limited to promotion and facilitation of cell adhesion (Inaba and Steinman, 1987; Dustin et al., 1988), since it may also be essential for lymphocyte maturation (Benjamin et al., 1988). LFA-1 is present on $CD4^+$ and $CD8^+$ T-cells (as well as B-cells, Mishra et al., 1986), therefore, antibodies directed against it can inhibit a wide range of T-cell responses (Inaba and Steinman, 1987; Benjamin et al., 1988).

CD3 is another important molecule present on both T_h and T_c subsets (Fig. 8.2a and b), playing a role in T-cell activation (Imboden and Stobo, 1985; Acuto and Reinherz,1985; Ledbetter et al., 1986). CD3 is closely associated with the T-cell receptor (T_i) (Van den Elsen et al., 1984), and is vital for the functioning of T-cells (Van Wauwe et al., 1980). Monoclonal antibodies against CD3 block *in vitro* cytotoxicity of T-cells (Chang et al., 1981; Goldstein, 1987); *in vivo* they remove $CD3^+$ cells from the circulation (Miller et al., 1981), "cover-up" the $CD3/T_i$ complex (Miller et al., 1981), or induce CD3 modulation (CD3 shedding and/or internalization, Chatenoud et al., 1982; Press et al., 1988b), thus rendering the lymphocytes ineffective. Administration of anti-CD3 antibodies was highly effective in reversing acute renal and hepatic allograft rejection

(Ortho Multicenter Transplant Study Group, 1985; Fung et al., 1987), treating kidney transplant rejection (Norman et al., 1987; Thistlethwait et al., 1987) and preventing rejection in cardiac allograft recipients (Bristow et al., 1988).

Additional experiments using different pan-T monoclonal antibodies showed that these reagents can inhibit activation and proliferation of lymphocytes *in vitro* (Yagita et al., 1986; Yagita and Hashimoto, 1986). Moreover, in preliminary *in vivo* studies, promising results were obtained in the treatment of autoimmune diseases (using anti-Thy1.2, Seaman et al., 1983; Wofsy et al., 1985) and acute renal transplant rejection (using anti-CD6, Kirkman et al., 1983; Hafler et al., 1986).

Since MHC class II is so important for antigen presentation and recognition by T_h-cells (Fig. 8.2a and b), it offers a potentially good target for "immunosuppressive" monoclonal antibodies. Animal experiments have shown that anti-MHC class II antibodies delayed the onset of autoimmune diseases (Steinman et al., 1981; Adelman et al., 1983) and suppressed humoral immune responses (Rosenbaum et al., 1981). This was due to prevention of the binding of class II restricted T-cells to APCs (Hodes et al., 1980; Longo and Shwartz, 1981; Nepom et al., 1981) and to MHC class II modulation (Aberer et al., 1986).

However, despite all these immunosuppressive properties, xenogeneic monoclonal antibodies directed against T-cell surface markers eventually become immunogenic, when administered *in vivo*. This paradox is not entirely a mystery, since B-cells and APCs could effectively capture the monoclonal antibodies, preventing them from reaching their T-cell "target". Antibody fragments could then be presented to T-cells that have escaped serotherapy, or have been newly "recruited" from the thymus. It has been found that some anti-T-cell antibodies are more immunogenic (i.e. less immunosuppressive) than others: generally, pan-T (Brostoff and Mason, 1984;

Wofsy et al., 1985; Lowder et al., 1987; Hafler et al., 1988), anti-CD3 (Ortho Multicenter Transplant Study Group, 1985; Goldstein et al., 1986; Shield et al., 1987a; 1987b; Shield and Norman, 1988), anti-CD2 (Lowder et al., 1987; Hafler et al., 1988) and anti CD8 (Cobbold et al., 1986; Benjamin et al., 1986), trigger the host's immune response more easily than do anti-CD4 antibodies (Cobbold et al., 1986; Benjamin et al., 1986; Lowder et al., 1987; Hafler et al., 1988). Nevertheless, even when total suppression was observed (with anti-CD4), the development of anti-idiotypic antibodies was not prevented, thus rendering the antibody less effective (Benjamin et al., 1986).

We have followed another strategy: we used a mouse monoclonal antibody, MR6, that recognises a structure related to the human IL-4 receptor (IL-4R). As discussed earlier, interleukin 4 promotes activation and proliferation of both B- and T-cells. It also induces MHC class II expression of B-cells and macrophages, thus enhancing antigen presentation. Therefore, the prevention of binding between IL-4 and its receptor, or a block in its signal transduction pathway, should inhibit crucial lymphocyte functions. *In vitro* studies, performed by colleagues in our Department (M. Larché, N. Imami-Shita, J. Lamb, R. O'Hehir), revealed that the MR6 antibody partially inhibits the proliferative response of human peripheral blood mononuclear cells and cloned T-cells to mitogens, specific antigen and IL-2 (Larché et al., 1988a). Moreover, MR6 totally abrogated the proliferative response of T-cell clones to IL-4, and the IL-4 dependent allergen-specific induction of IgE secretion by polyclonal B-cells (Larché et al., 1988b; O'Hehir et al., 1988).

The exact nature of the MR6 antigen is unclear. Recent experiments (Foxwell et al., 1989, Larché et al., unpublished data) indicate that the IL-4 binding site of the IL-4 receptor is composed of three polypeptide chains, 65, 70 and 110KD, and that the 200KD MR6 antigen may be a signal transduction molecule for the IL-4 system. Similar molecules have been reported for the receptors of IL-2 (Taniguchi et al., presented at the 7th International Congress of Immunology,

Berlin, 1989), IFN-γ (Schreiber et al., presented at the 7th International Congress of Immunology, Berlin, 1989), and IL-6 (Taga et al., 1989).

In order to test the clinical potential of MR6 as an immunosuppressive agent, it was necessary to establish an *in vivo* experimental system. Of several species tested, only the rabbit showed staining with MR6 antibody. Therefore, using the rabbit as an *in vivo* model, we tested the ability of MR6 to inhibit the immune response to itself, to a hapten (FITC) coupled to MR6, and to an independent antigen (human haemoglobin) administered simultaneously with MR6 (but uncoupled). We also had an additional interest in the MR6 antigen, since it has been shown to be upregulated on many carcinomas (Al Jabaari et al., 1988; Mat et al., 1989). Radiolabelled or drug/toxin conjugated MR6 antibody might therefore provide a unique target for *in vivo* serotherapy, that would not only destroy the tumour by targeting the radioisotope/drug/toxin to the tumour site, but would also suppress the immune response to itself, and avoid the limiting human anti-mouse Ig response.

8.2 Materials and Methods

8.2.1 Animals: Female, Balb/c mice (6-8 weeks old) (Olac, UK), were used for the production of ascitic fluid. Male , 1/2 "lop" rabbits (Froxfield Farm, UK), 3.0-4.0 Kg, were used for immunization with various immunoglobulin preparations, after which their immunocompetence was studied.

8.2.2 Immunogens: Rabbits were immunized with the following preparations: MR6, a mouse monoclonal IgG1 that recognises a structure related to the human IL-4 receptor (Larché et al., 1988b), raised by Dr. M. Ritter in our Department (De Maagd et al., 1985); HMFG1 (Taylor-Papadimitriou et al., 1981), H17E2 (Travers and Bodmer, 1984) and AUA1 (Spurr et al., 1986), all mouse monoclonal IgG1 that recognise human tumour-associated antigens (a

mucin on the Milk Fat Globule membrane, Placental Alkaline Phosphatase and a 40KD glycoprotein respectively), kindly provided by Unipath-Oxoid, UK; MR6 and HMFG1 antibodies conjugated to fluorescein isothiocyanate (method described below, 8.2.2); Human haemoglobin (Sigma).

8.2.3 Production and purification of the monoclonal antibodies: Unipath-Oxoid kindly provided the control antibodies (HMFG1, AUA1 and H17E2) in purified form; these were purified by protein A affinity and ion exchange chromatography, from large volumes of tissue culture supernatants.

The MR6 antibody was purified in our Department as follows: the hybridoma cell line producing the MR6 antibody was cultured in tissue culture flasks (Flow Laboratories, UK) in RPMI-1640 (Flow Laboratories) supplemented by 2mM L-glutamine, 100 IU/ml penicillin/streptomycin and 10% foetal calf serum (Gibco, UK), and the supernatant collected and stored at 4°C until required. In addition, mice were injected i.p. with 0.5 ml 2,6,10,14-tetra methyl pentadecane (pristane) (Aldrich, UK), and three weeks later they received another intraperitoneal injection of approximately 10^6 MR6-producing hybridoma cells. The accumulated MR6 ascitic fluid was removed, centrifuged and stored at -20°C, until use.

The monoclonal antibody was purified by Protein A and Protein G affinity chromatography (Pharmacia) as follows: a) A Protein A column was equilibrated with 0.1M Phosphate buffer, pH 8.0. Tissue culture supernatant/ascites preparations were filtered (0.45 μm filter, Millipore, France), adjusted to pH 8.0 (with 0.1M Phosphate buffer, pH 8.0), and loaded to the column at a constant flow rate, regulated by a peristaltic pump (P-1, Pharmacia) that did not exceed 10 ml/hr. The protein concentration was monitored by a UV monitor (UV-1, Pharmacia), and 1 ml fractions were automatically collected (Frac-100, Pharmacia). The signal (as absorbance at 280 nm) was recorded on graph paper by a two channel chart recorder (Rec-482,

Pharmacia). The column was washed with 0.1M phosphate buffer, pH 8.0, and the antibody eluted with 0.1M citrate buffer, pH 3.5. The protein obtained was immediately neutralized (0.1M Tris), dialyzed overnight in phosphate buffered saline (PBS), pH 7.4, and filter-sterilized (0.22 μm filter, Millipore). The concentration was spectrophotometrically estimated (280 nm, described in chapter 4) and the antibody stored at 4°C. However, we found that under these conditions only a very small percentage of MR6 binds onto Protein A. This is shown in Figure 8.3a, where the first peak represents the unbound material, whereas the second contains the bound and eluted antibody. In this particular experiment, where 250 μl ascitic fluid was loaded onto the column, only 59 μg of MR6 were eluted. Indirect immunoperoxidase on human thymus (method described below in 8.2.6) revealed that there was a lot of immunoreactive material remaining in the unbound fraction. We therefore slightly modified the conditions: 2.5M NaCl was added to the sample before loading, and the pH raised to 8.6 (with 0.1M carbonate/bicarbonate buffer, pH 9.0). The rest of the procedure remained the same, as described above. As shown in Figure 8.3b, the amount of bound and eluted antibody was increased to 193 μg (out of 250 μl ascitic fluid). Nevertheless, after indirect immunoperoxidase, we found that there was still a substantial amount of antibody in the unbound fraction. b) The Protein G column was equilibrated in PBS, pH 7.4, and the tissue culture supernatant/ascites preparations were added as above. The column was washed with PBS and eluted with 0.1M glycine/HCL, pH 2.7. The antibody was neutralized immediately with 0.1 M Tris, dialyzed and stored as described above. As shown in Figure 8.3c, we estimated that the eluted MR6 was 520 μg (out of 250 μl ascites). Indirect immunoperoxidase on the unbound fraction revealed that there was no remaining reactivity. In addition, the immunoreactivity of the purified material was also confirmed by indirect immunoperoxidase. Protein G affinity chromatography was therefore used for the purification of MR6 in all subsequent experiments.

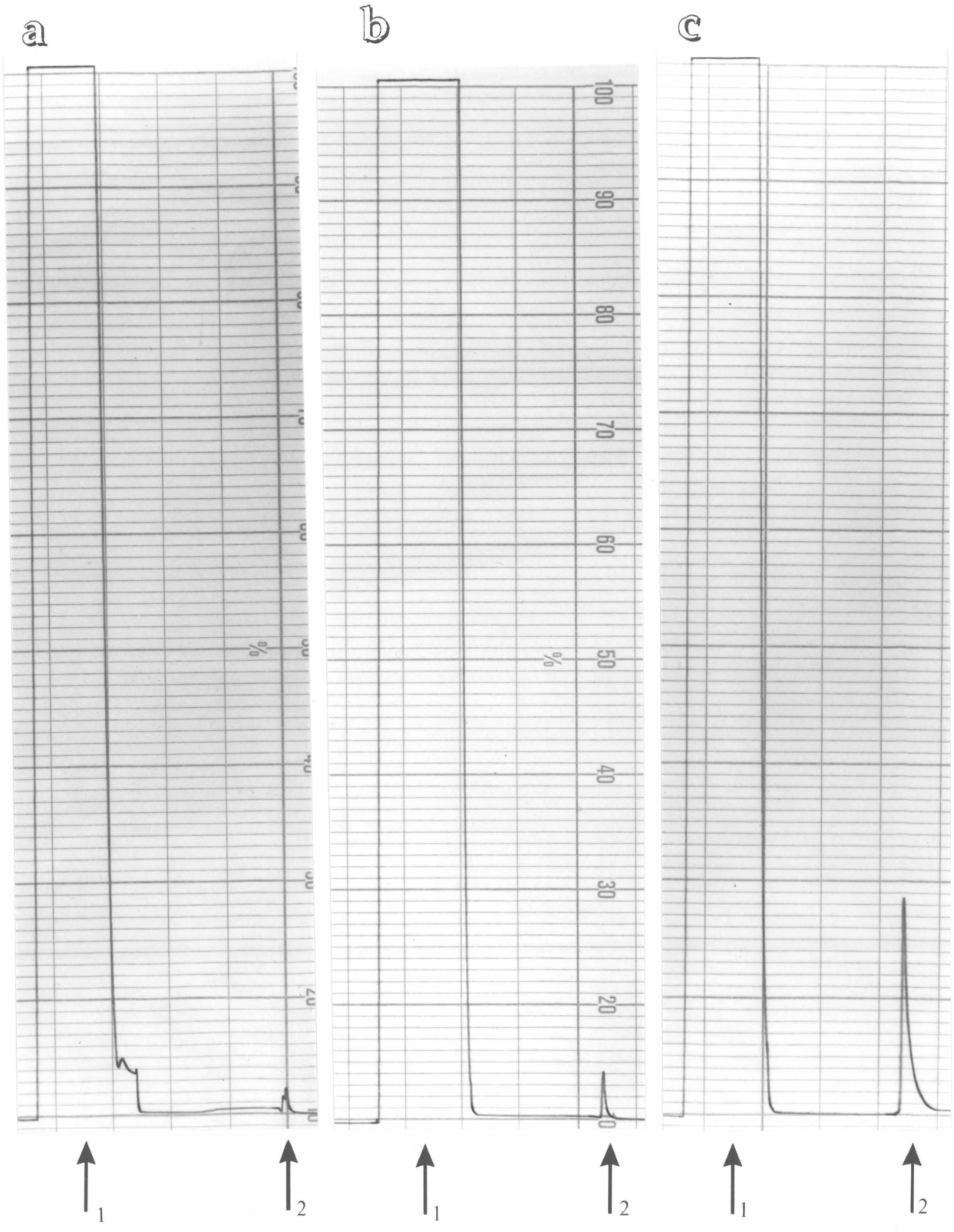


Figure 8.3: Purification of the MR6 mouse monoclonal antibody by: a) Protein A, b) Protein A under higher salt and pH conditions, and c) Protein G, affinity chromatography. The first peak (1) corresponds to unbound material, whereas the second peak (2) is bound and eluted MR6.

8.2.4 Conjugation to fluorescein isothiocyanate: The method for the conjugation of fluorescein isothiocyanate (FITC) onto antibodies is described in chapter 7 (Chapter 7, section 7.3.2.7). Briefly, the MR6 and HMFG1 antibodies and egg albumin (OVA), were concentrated to 10 mg/ml, dialyzed in 0.1M carbonate/bicarbonate buffer, pH 9.0, and mixed with FITC (Sigma). The mixture was stirred for 2 hr at RT, and free FITC was separated from protein-bound FITC, by size exclusion chromatography (Sephadex G25, Pharmacia). The protein concentration and the molar ratio (MR) FITC/antibody was estimated by dual spectroscopy at 280 nm and 495 nm., as described in chapter 7. The MR was found to be 5.3 and 4.9 for the MR6-FITC and the HMFG1-FITC conjugate respectively. The immunoreactivity of the MR6-FITC was measured by direct immunofluorescence, as described below (8.2.6). Similarly, the MR for OVA was found 7.3 FITC/OVA.

8.2.5 Quality control of the immunoglobulin preparations: Before each immunization, all immunoglobulin preparations were tested and found to be free of aggregates or other irrelevant proteins. This was performed by: a) size exclusion chromatography using a Superose 6 column connected to an FPLC (Pharmacia, Sweden), as described in chapter 3, b) SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Mighty Small II, Hoefer Scientific Instruments, USA), as described in chapter 3, and c) isoelectric focusing (IEF), using the Phast System from Pharmacia. The Phast System is a relatively new product in the market. Pre-cast gels are used (SDS-PAGE or IEF), that are automatically loaded with the samples, stained and destained by the apparatus. At the end, the dried gel appears as a slide, ready to be projected on a slide-projector. The whole procedure takes less than 2 hours (without loss of separating efficiency). It is highly reproducible, since all parameters (gel, current, voltage, temperature, solutions) are standardized and computer-controlled. Figure 8.4a shows a Coomassie blue stained IEF gel with a pH gradient from 3 (top) to 9 (bottom). Lane 1 contains the MR6, lane 2 the H17E2 and lane 3 the HMFG1. Figure 8.4b

shows a silver stained IEF gel (pH gradient 3 to 9), where lane 1 contains the HMFG1, lane 2 the H17E2 and lane 3 the AUA1 mAbs. Differences in glycosylation are reflected in the closely packed bands observed, but we found no evidence of contaminating non-immunoglobulin proteins. Moreover, the PIs of MR6, HMFG1, AUA1 and H17E2 were found to be approximately 7.3, 7.4, 7.0 and 7.2 respectively.

All antibodies and antibody-FITC conjugates were stable in sterile PBS at 4°C, or frozen at -20°C. Aliquots were tested regularly (SDS-PAGE, size exclusion chromatography), and found to remain free of aggregates and fully immunoreactive (see section 8.2.7) for a long time (>8 months). Aggregate formation was only observed after 8 months' storage at -20°C, and repeated thawing and freezing. Figure 8.4c shows a Coomassie blue stained, 6% SDS-PAGE of these samples. In lane 1 are prestained molecular weight markers (Sigma), and lanes 2-7 contain the MR6, MR6-FITC, HMFG1, HMFG1-FITC, AUA1 and H17E2 respectively. Macromolecules of molecular weight higher than 150KD were present at very low concentration, and only just visible. This was also confirmed by size exclusion chromatography (Superose 6, FPLC): as shown in Figure 8.5, a very small, and similar, percentage (first peak) of the MR6 (a), HMFG1 (b), AUA1 (c) and H17E2 (d) antibodies tested here, had aggregated. The sensitivity of the system had to be increased 10 fold (maximum absorbance 0.2), in order to detect these macromolecules.

8.2.6 Immunohistochemistry and flow cytometry: The immunoreactivity of the MR6-FITC was tested by direct immunofluorescence as follows: 5 µm sections of fresh frozen human thymus were fixed for 10 min in acetone, and incubated for 1 hr at RT with 1 µg in 100 µl MR6-FITC (or HMFG1-FITC as negative control). The slides were washed with PBS, mounted in a water soluble mountant (Cityfluor, UK), and viewed using a UV microscope equipped with epilumination optics (Dialux 20, Leitz, E. Germany).

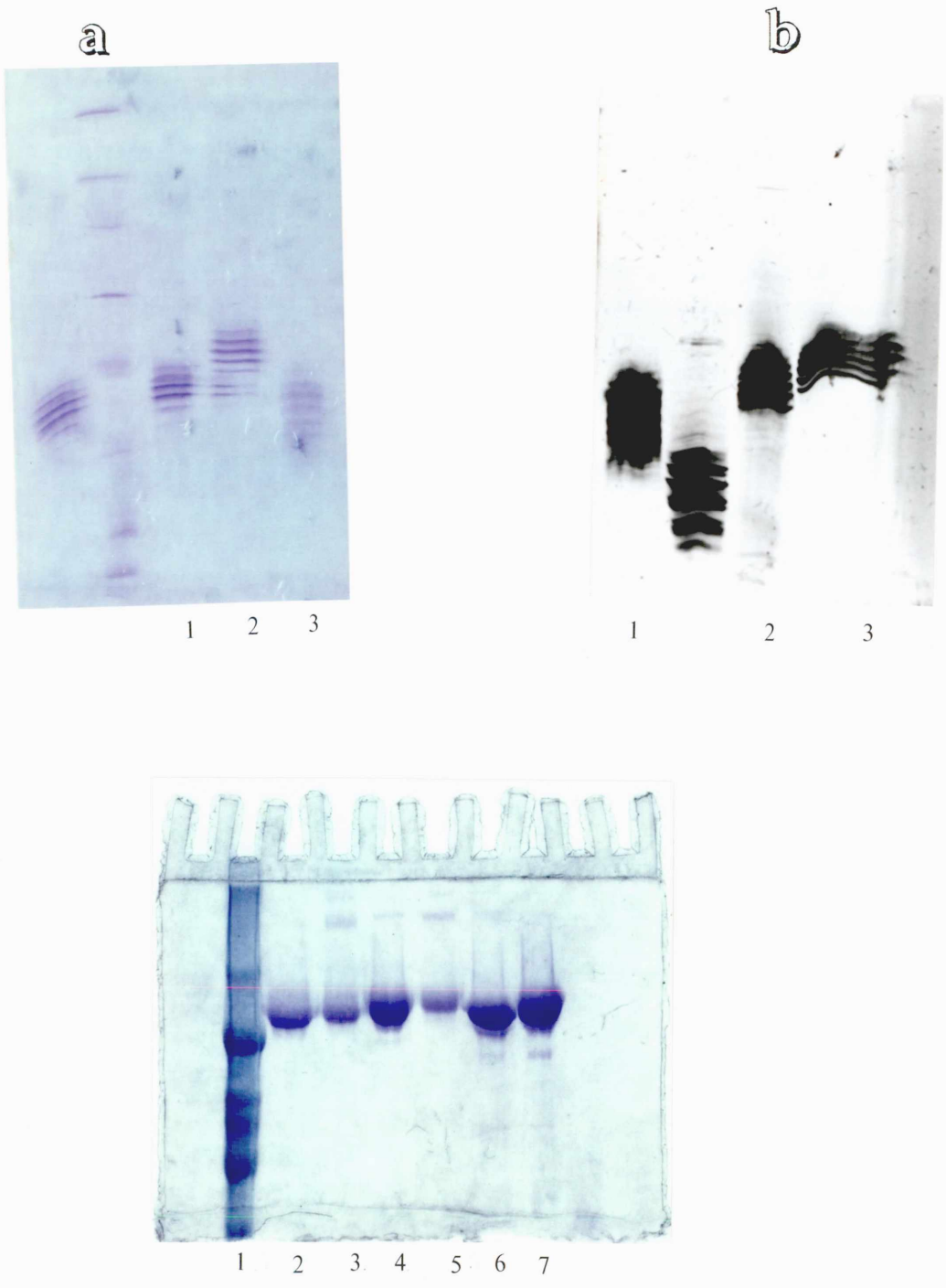


Figure 8.4: a) Coomassie blue stained isoelectric focusing (IEF) gel of the mouse monoclonal antibodies used as immunogens. Lane 1: MR6; lane 2: H17E2; lane 3: HMFG1. b) Silver stained IEF gel of HMFG1 (lane 1), H17E2 (lane 2) and AUA1 (lane 3). c) Coomassie blue stained polyacrylamide gel (SDS-PAGE) of the mouse monoclonal antibody preparations used as immunogens, after 8 months' storage at -20°C, and repeated thawing and freezing. Lane 1: molecular weight (MW) markers; lane 2: MR6; lane 3: MR6-FITC; lane 4: HMFG1; lane 5: HMFG1-FITC; lane 6: AUA1; lane 7: H17E2.

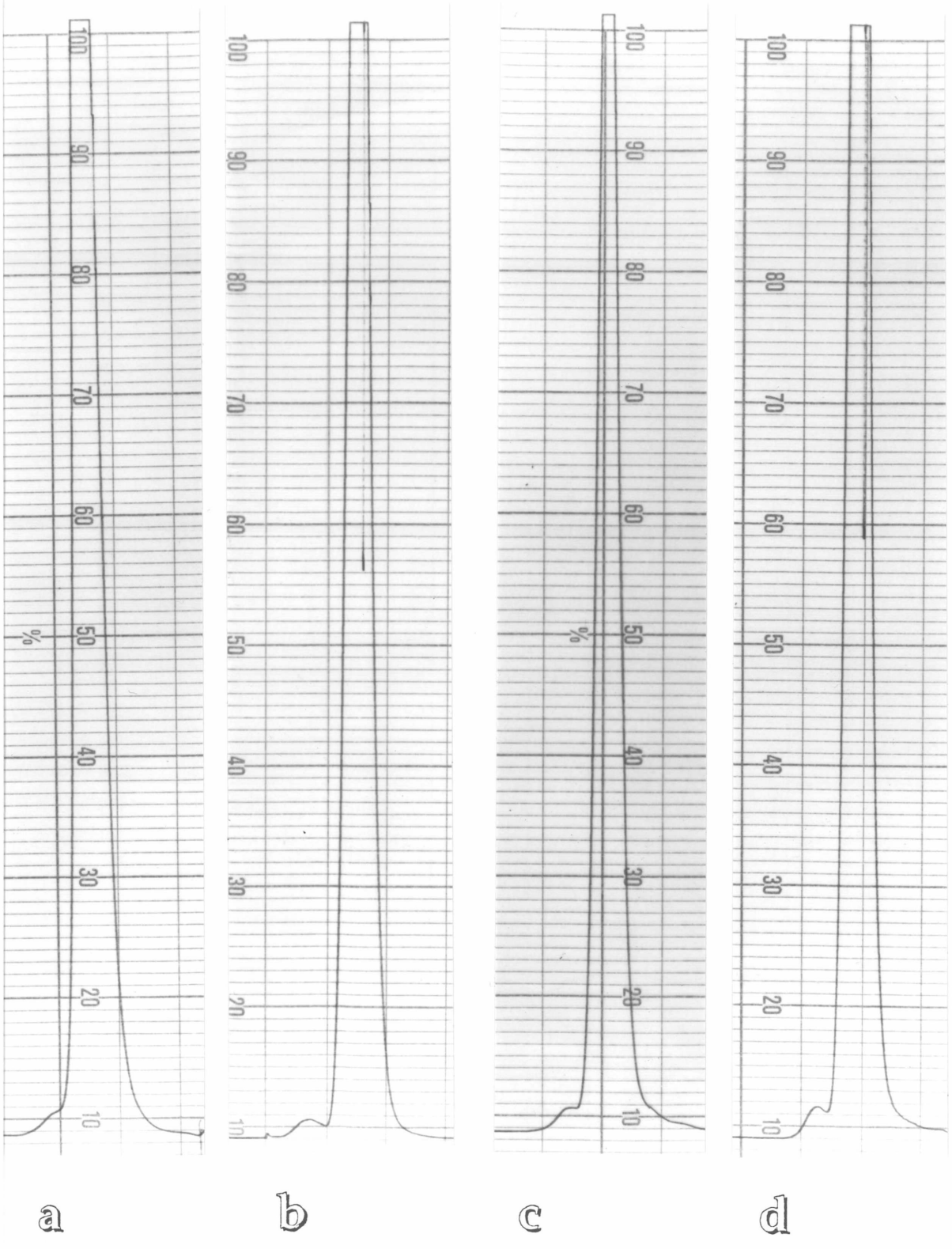


Figure 8.5: Fast Protein Liquid Chromatography (FPLC) profile of the MR6 (a), HMFG1 (b), AUA1 (c) and H17E2 (d), after 8 months' storage at -20°C, and repeated thawing and freezing.

The MR6 antibody was also tested for binding onto rabbit splenocytes by indirect immunofluorescence in cell suspension. The spleen was removed from five rabbits, and the spleen cells were teased out and washed in RPMI-1640. Approximately 5 x 10^5 - 10^6 cells were incubated with 2 μg MR6 in 200 μl (and HMFG1 as a negative control), for 1 hr on ice. The cells were washed, and a rabbit anti mouse Ig-FITC (DAKO, Denmark) added. After 45 min incubation on ice, the cells were again washed, and analyzed by flow cytofluorimetry (EPICS C, Coulter, USA), after red blood cells were gated out.

All immunoglobulins used as antigens were tested for binding to rabbit thymus, colon, stomach and liver, by indirect immunoperoxidase. The method is described in chapter 7 (Chapter 7, section 7.3.2.3), but briefly, tissue was removed from very young or adult rabbits, snap frozen, and 5 μm sections incubated with approximately 1 μg of the antibodies. Positive reaction was detected by a rabbit anti-mouse Ig-horseradish peroxidase (DAKO) secondary reagent, followed by diaminobenzidine (DAB) (Sigma) as substrate.

8.2.7 Immunoreactivity of monoclonal antibodies: Prior to each administration, the monoclonal antibodies were tested for immunoreactivity as follows: a) The MR6 and MR6-FITC by indirect immunoperoxidase or direct immunofluorescence, on human paediatric thymus, as described above (8.2.6); b) the HMFG1 and HMFG1-FITC, by indirect and direct immunofluorescense respectively, using the T47D cell line (kindly provided by Dr. J. Taylor-Papadimitriou, ICRF, UK). Briefly, the cells were cultured in RPMI-1640, supplemented by 10% FCS. Cytospins were prepared, and the cells fixed for 10 min in acetone. Approximately 1 μg (100 μl) of the antibodies was added for 60 min at RT, and for the indirect immunofluorescence, a rabbit anti-mouse Ig-FITC (DAKO), followed the HMFG1 incubation; c) the AUA1 and H17E2 by indirect immunofluorescence, as described above, using the LoVo (kindly

provided by Dr. J. Taylor-Papadimitriou, ICRF) and the Hep-2 cell lines (kindly provided by Dr. D. Tucker, ICRF) respectively. All antibodies were found to retain their immunoreactivity even after long storage (>1 year) at 4°C.

8.2.8 Antigen determination: We tried to identify the cell surface antigen, that the MR6 recognises in rabbits, by radiolabelling the surface of thymus-cells, lysing the cells, and immunoprecipitating the antigen. An adult rabbit thymus was teased apart; the cells (stroma cells including lymphocytes) were incubated for 15-20 min with lactoperoxidase (Sigma), H_2O_2 (Sigma) and approximately 1.5 mCi ^{125}Iodine (Amersham, UK), and then washed in cold PBS. The cells were very gently resuspended in lysis buffer (10mM Tris, pH 7.4, 1mM $MgCl_2$, 10 µg/ml aprotonin, 1mM PMSF and 0.5% nonident P-40 - all from Sigma), and left on ice for 10-20 min. The lysate was centrifuged and dialyzed (0.15M NaCl, 10mM Tris, pH 7.4) at 4°C.

The lysate was again centrifuged, and "pre-cleared" with non-specific rabbit serum as follows: normal rabbit serum was incubated with Protein A Sepharose beads (Pharmacia) for 1 hr at room temperature (RT). The beads were washed three times with PBS, 0.5% nonident P-40, and incubated with the lysate at 4°C. After 3 hr, the beads were centrifuged, and washed twice with washing buffer (0.5% nonident P-40, 0.1% SDS, 0.5M NaCl, 10mM EDTA and 50mM Tris/HCl, pH 8.0). Subsequently, the beads were incubated with MR6, washed with PBS, 0.5% nonident P-40, and added to the lysate. After 3 hr at 4°C, the beads were washed twice with washing buffer, centrifuged, and the supernatant boiled for 1 min, and loaded onto a 7.5% SDS-polyacrylamide gel. The gel was stained, dried, and autoradiography was performed as described in chapter 3.

Alternatively, we attempted to identify the thymic molecule that MR6 recognises, by Western blotting. A cell lysate was prepared as described above,

and loaded onto a gradient SDS-polyacrylamide gel (3-15%). The material was then transferred onto nitrocellulose paper, using a blotting apparatus (Biorad). The paper was rinsed in blotto solution (as described earlier, chapter 4, section 4.2.4), and incubated with 4 ml (20 μg/ml) of MR6 for 1 hr at RT. The paper was washed and incubated for a further 1 hr with anti-mouse Ig-HRP (DAKO). The enzyme colour was developed using DAB (DAKO) as substrate.

8.2.9 Immunization protocol: Six groups of 5 rabbits were immunized i.v. (ear vein), with 1 mg of the following antigens: MR6, MR6-FITC, HMFG1, HMFG1-FITC, H17E2 and AUA1. The rabbits that received the MR6, MR6-FITC, HMFG1 and HMFG1-FITC preparations also received 4 mg human haemoglobin (Hb), that served as a control antigen. Seven days later, the animals were bled (ear vein), and boosted with 1 mg of the corresponding immunoglobulins (i.e. as on day 0). The 4 groups that were primed with human haemoglobin, were also boosted for a second time with 4 mg Hb. All animals were bled again on day 14.

The rabbits that were primed and boosted with the HMFG1 antibody (days 0 and 7 respectively), were subsequently challenged three times with 1 mg of HMFG1 (i.v.), on days 18, 25 and 33. On day 40 they received 1 mg of HMFG1-FITC. The anti-HMFG1 and anti-FITC response was monitored by bleeding the animals at regular time points, before and after the administration of the FITC conjugate.

8.2.10 Anti-immunoglobulin response: The humoral response to the administered mouse Ig, was measured by ELISA, as previously described (Chapter 2). Briefly, the antigen (MR6 for the MR6 and MR6-FITC immunized animals, HMFG1 for the HMFG1 and HMFG1-FITC immunized animals, AUA1 and H17E2 for the AUA1 and H17E2 immunized animals respectively) was coated onto a 96-well plates (0.5 μg/well), and the sera were added. The positive (or negative) reaction was detected by a species-specific anti-rabbit Ig-horseradish peroxidase conjugate, followed by ABTS as substrate. The absorbance was measured with a Titertek multiscan spectrophotometer at 405

nm. The results are expressed as areas under the sigmoidal titration curve of the test sera (see chapter 2). Statistical analysis was performed using the Student's t test for the logs of the areas. Prior to any immunization, only those animals that did not have any pre-existing anti-mouse Ig antibodies were selected .

8.2.11 Anti-fluorescein isothiocyanate response: The rabbit anti-FITC response was measured by ELISA. 96-well plates (Sterilin) were incubated overnight with 20 μg/well OVA-FITC, in carbonate/bicarbonate buffer, pH 9.6. The optimal concentration of antigen was determined by a chequer board ELISA (as described in chapter 2), using rabbit anti-OVA (DAKO), and rabbit anti-FITC (Biogenesis, USA). The plates were subsequently washed, and the test sera, second layer and substrate was added as described above (8.2.10). On the same plate, test sera were also checked for reactivity against OVA alone (20 μg/well), in order to avoid any false positive results. Statistical analysis was performed using the Student's t test for the logs of the areas. The results are expressed as areas under the titration curve. Prior to each administration, only those animals that did not have any pre-existing anti-FITC and anti-OVA antibodies were selected .

8.2.12 Anti-haemoglobin response: The rabbit anti-Hb response was measured by ELISA. 96-well plates were coated with 0.5 μg/well human Hb, in carbonate/bicarbonate buffer, pH 9.6, overnight. The optimal concentration of antigen was determined by a chequer board ELISA (see chapter 2), using a rabbit anti-human Hb antiserum (DAKO). The plates were subsequently washed, followed by titration of the test sera, and application of the anti-rabbit Ig reagent and substrate as described above (8.2.10 and 8.2.11). The results are given as areas under the sera titration curves. Statistical analysis was performed using the Student's t test for the logs of the areas. Prior to each administration, only those animals that did not have any pre-existing anti-human Hb antibodies were selected .

8.2.13 Reduction of antigenicity after fluorescein isothiocyanate conjugation: Since the anti-MR6 response was found to be greater when rabbits were immunized with MR6 than with MR6-FITC (section 3.3), we examined whether FITC masks the immunoglobulin molecule, rendering it less accessible to anti-Ig antibodies. ELISA plates were coated with MR6, MR6-FITC, HMFG1 and HMFG1-FITC (all on the same plate). Immune sera from the rabbits that were injected twice with MR6 or HMFG1 was incubated for 1 hr at 37°C, and the anti-rabbit Ig-horseradish peroxidase reagent was added, followed by the substrate ABTS. The absorbance was measured at 405 nm, and the results were expressed as areas under the sera titration curves.

For these experiments, we initially checked that all ELISA plates were coated with the same amount of the respective antigen. After the immunoglobulin preparations (MR6, MR6-FITC, HMFG1, HMFG1-FITC, AUA1, H17E2) were incubated overnight in carbonate/bicarbonate buffer, pH 9.6, the wells were then carefully emptied and the protein concentration in the supernatant estimated by the Bradford assay, as follows. 50 µl of the test solution was mixed with 50 µl of the Bradford protein assay reagent (40% in distilled water) (Biorad, UK), in 96-well plates, and after 10-15 min incubation at RT, the absorbance at 620 nm was measured in a multiscan. A standard curve using known amounts of the mouse Igs was plotted (1-0.05 µg/ml), in order that the absorbance of the test samples could be quantitated. We found no detectable protein in the supernatant within the detection limits of the method (0.05 µg/ml).

8.3 Results

8.3.1 Immunohistochemistry and flow cytometry: After the MR6 was conjugated to FITC, its immunoreactivity was tested on human thymus by direct immunofluorescence. As shown in Figure 8.6a, after conjugation, the

antibody retained its ability to bind cortical thymic epithelium and macrophages. We suspect that lymphocyte labelling is too weak to see in tissue sections by immunofluorescence. Figure 8.6b is the negative control (HMFG1-FITC). Indirect immunoperoxidase showed that MR6 also recognised adult rabbit thymus, with a staining pattern very similar, if not identical, with the one we observed in human thymus. All control antibodies (HMFG1, H17E2 and AUA1) were negative. However, MR6 did not react with young rabbit thymus. In addition, HMFG1 was found to be positive on rabbit colon, recognizing primarily muscle (Fig. 8.6c), whereas MR6, AUA1 and H17E2, were negative (MR6 in Fig. 8.6d). Finally, none of the antibodies was found to bind to rabbit stomach and liver.

FACS analysis showed that MR6 also bound to rabbit splenocytes. As shown on the left column in Figure 8.7, the percentage of cells that were positive with MR6 varied among animals, from 4.5 to 21.2% (individual percentage counts to the 5 animals, from the top: 6.8, 10.4, 21.2, 4.5 and 13.7%). On the right, is the control antibody (HMFG1) for each spleen cell population. The background levels never exceeded 2.7% (individual percentage counts, from the top: 2.7, 1.7, 2.3, 1.5 and 2.7%).

8.3.2 Antigen determination: Unfortunately, until this moment we were unable to identify the molecule that MR6 recognises in the rabbit thymus. Both immunoprecipitation and Western blotting techniques produced negative results. We feel that the lysis buffer used, was either too harsh and therefore destroyed the antigen, or too weak, thus unable to solubilize the appropriate molecule. We are currently employing alternative buffers, as well as more sensitive variations of the above techniques (eg. radiolabelled second-layer antibodies).

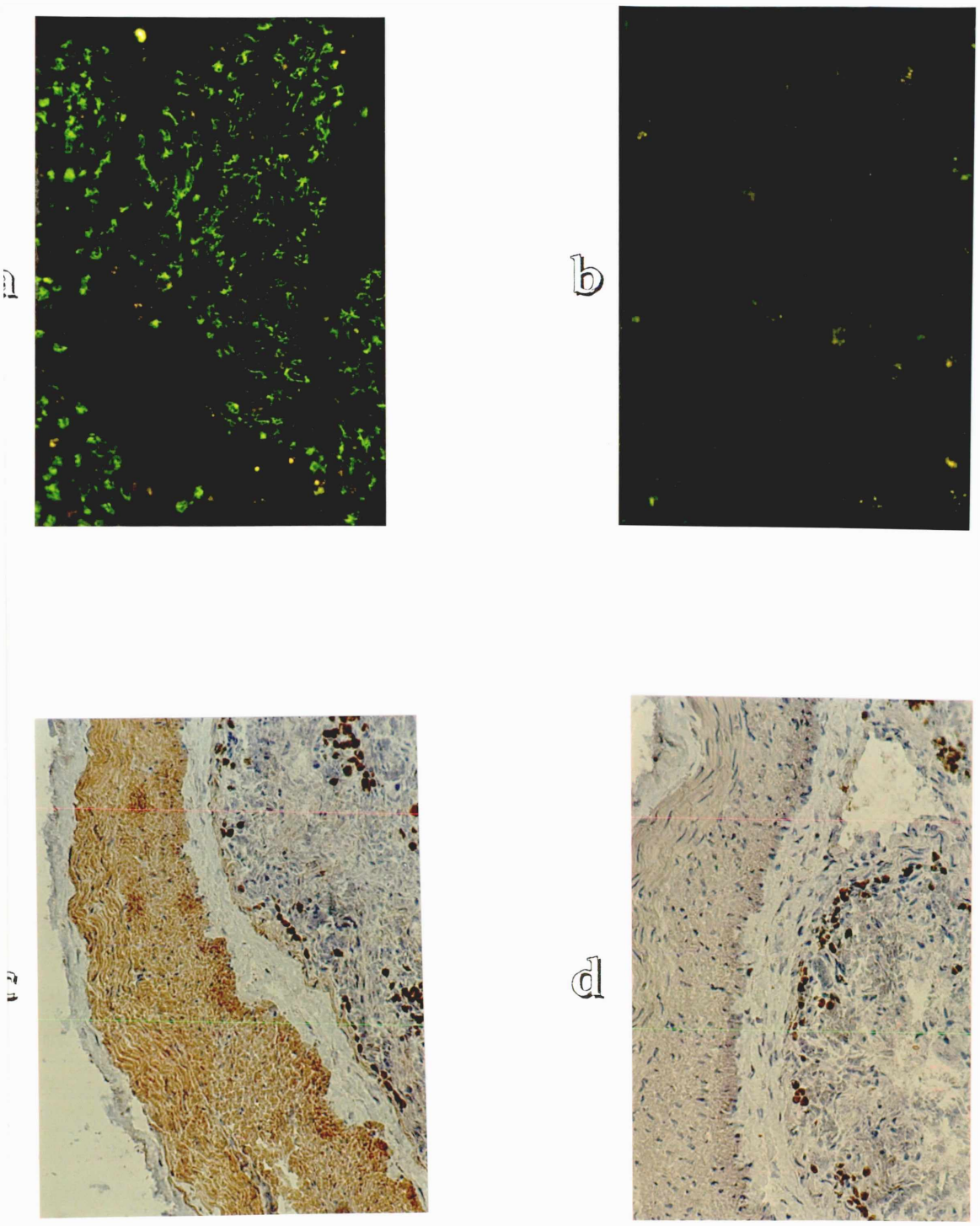
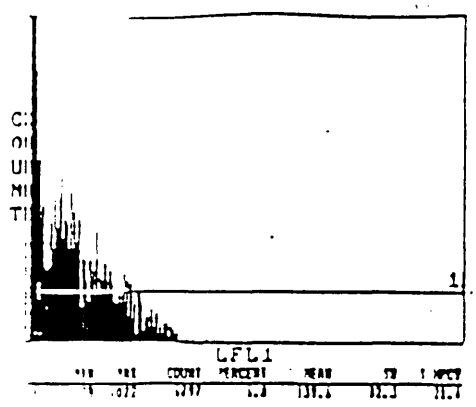
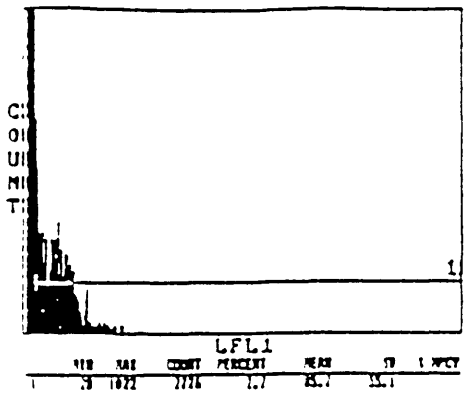
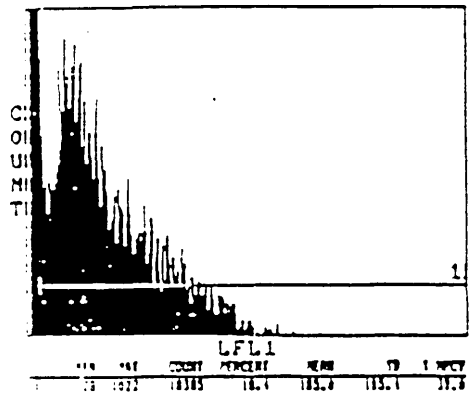


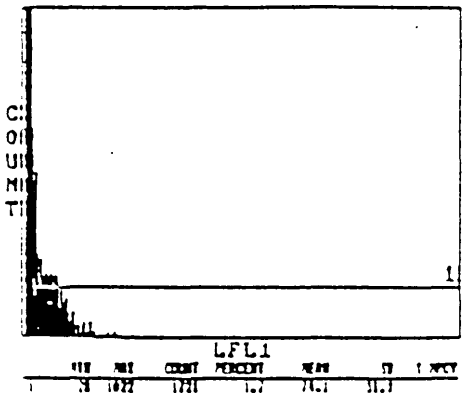
Figure 8.6: Direct immunofluorescence staining of MR6-FITC (a) and HMFG1-FITC (b) on adult rabbit thymus, and indirect immunoperoxidase staining of HMFG1 (c) and MR6 (d) on normal rabbit colon.

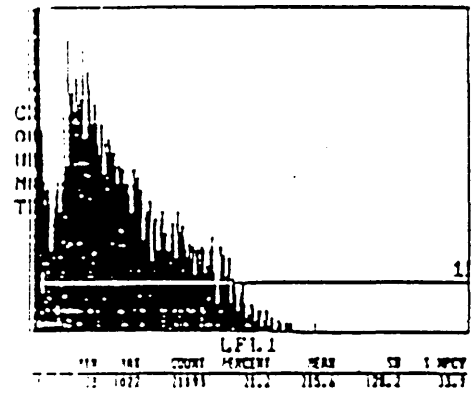
Figure 8.7: Flowcytometric analysis of MR6 (left-hand panels) and HMFG1 (negative control, right-hand panels) immunolabelling of splenocytes from 5 different rabbits. The horizontal axis represents the intensity of staining vs. the number of positive cells (vertical axis).

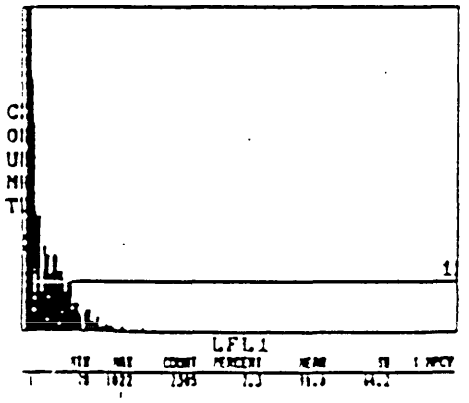

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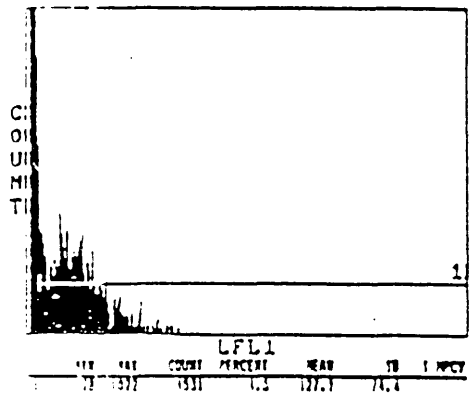

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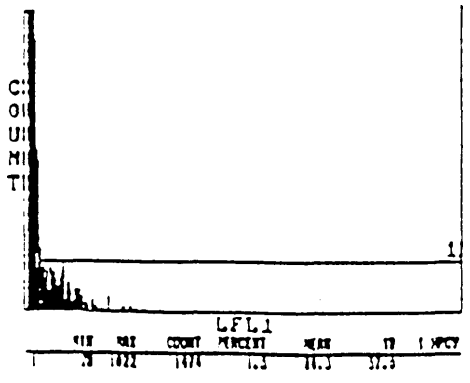

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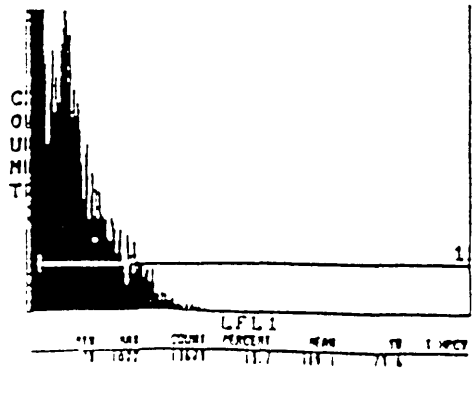

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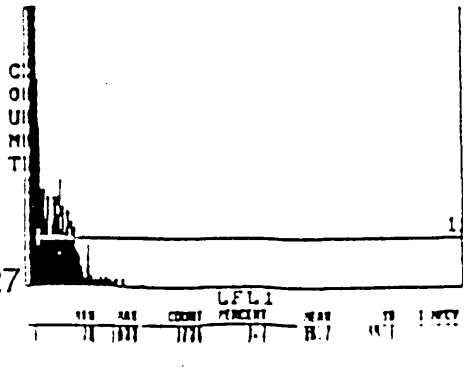

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8.3.3 Anti-immunoglobulin response: Figure 8.8a, shows the rabbit anti-mouse Ig primary response, as measured 7 days after administration of the mouse antibodies. The anti-MR6 response (from rabbits immunized with MR6, group 1, $mean_{MR6} = 1.354 \pm 0.639$) was 76% higher than the anti-AUA1 (group 5, $mean_{AUA1} = 0.328 \pm 0.252$) ($p=0.011$) and 91.2% higher than the anti-H17E2 (group 6, $mean_{H17E2} = 0.094 \pm 0.093$) ($p=0.005$), but not statistically significantly higher than the anti-MR6 response from rabbits immunized with MR6-FITC (group 2, $mean_{MR6\text{-}FITC} = 0.606 \pm 0.362$) ($p=0.066$). The anti-MR6 response of this latter group (group 2), was 81.2% higher than the anti-H17E2 ($p=0.030$); however there was no significant difference with the anti-AUA1 response ($p=0.086$). Finally, although the mean anti-AUA1 response was found to be 65.2% higher than the anti-H17E2, this was of marginal statistical significance ($p=0.052$). Interestingly, the rabbits did not respond at all against HMFG1, irrespective of whether the antigen was HMFG1 or HMFG1-FITC.

The rabbit anti-mouse Ig secondary response, as measured 7 days after the second boosting (day 14 after initial immunization), is shown in Figure 8.8b. The anti-MR6 response (rabbits immunized with MR6, group 1, $mean_{MR6} = 3.836 \pm 0.384$), was 29.6% higher than the anti-MR6 from rabbits immunized with MR6-FITC (group 2, $mean_{MR6\text{-}FITC} = 2.721 \pm 0.699$) ($p=0.024$), 67.9% higher than the anti-HMFG1 (group 3, $mean_{HMFG1} = 1.249 \pm 0.954$) ($p=0.002$), 86.4% higher than the anti-HMFG1 from rabbits immunized with HMFG1-FITC (group 4, $mean_{HMFG1\text{-}FITC} = 0.532 \pm 0.669$) ($p<0.001$), 76.9% higher than the anti-AUA1 (group 5, $mean_{AUA1} = 0.859 \pm 0.551$) ($p<0.001$) and 73.8% higher than the anti-H17E2 response (group 6, $mean_{H17E2} = 1.025 \pm 0.624$) ($p<0.001$). In addition, the anti-MR6 response from rabbits immunized with MR6-FITC (group 2), was 54.4% higher than the anti-HMFG1 ($p=0.001$), 80.6% higher than the anti-HMFG1 (from rabbits immunized with HMFG1-FITC, group 4) ($p<0.001$), 67.2% higher than the anti-AUA1 ($p<0.001$) and 62.7% higher than the anti-H17E2 ($p=0.002$). No other significant differences in the responses were observed.

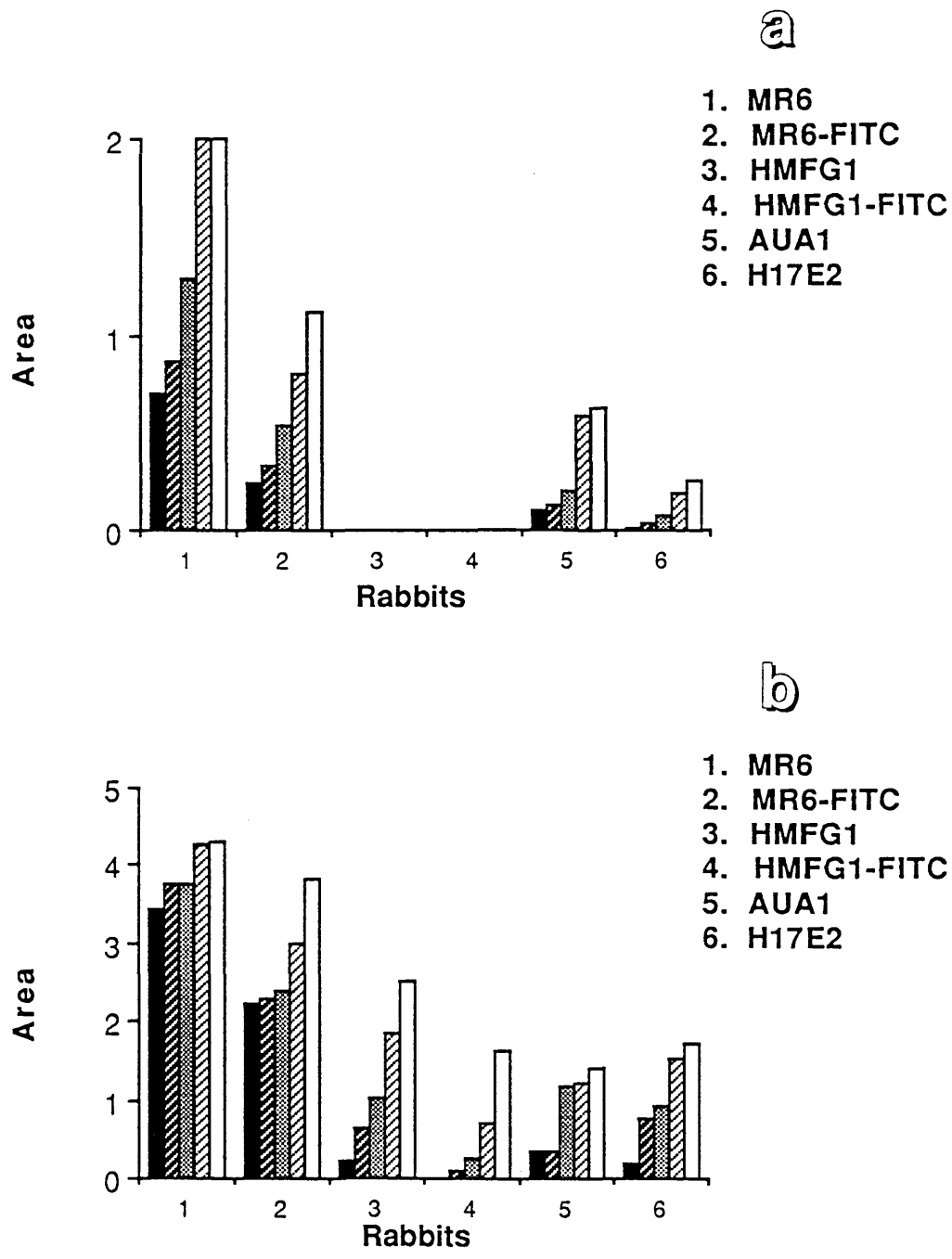


Figure 8.8: Humoral primary (a) and secondary (b) rabbit anti-mouse immunoglobulin responses, when the immunogens were : MR6 (group 1), MR6-FITC (group 2), HMFG1 (group 3), HMFG1-FITC (group 4), AUA1 (group 5) and H17E2 (group 6). Each group contained 5 rabbits; each represented by a separate bar on the histogram. The results are expressed as areas under the sigmoidal ELISA curve (vertical axis).

8.3.4 Anti-fluorescein isothiocyanate response: The rabbit primary response against FITC was measured, when FITC was conjugated onto MR6 and HMFG1. As shown in Figure 8.9a, 4 out of 5 rabbits that received the FITC conjugated onto MR6 (group 1), developed a response against it. In contrast even when the animals were immunized for a second time, only 2 out of 5 rabbits that were injected twice with HMFG1-FITC developed a low level of anti-FITC antibodies, whereas those given MR6-FITC, showed a very high response (Fig. 8.9b).

The rabbits that were immunized twice with HMFG1 (group 3, Fig. 8.8b), were further challenged 3 times, with 1 mg HMFG1, i.v.. The immune response against the mouse Ig, over a period of 40 days, is shown in Figure 8.10a. When all animals had established a reasonably high response, they were injected with 1 mg of HMFG1-FITC on day 40. Seven days later, we tested the sera for the anti-mouse Ig and anti-FITC response. The anti-Ig levels on day 47, are shown in Fig. 8.10a. The primary anti-FITC response is shown in Figure 8.10b. Only one out of five rabbits developed low levels of anti-FITC antibodies, compared with the primary response when FITC was covalently linked to MR6 (Fig. 8.9a).

8.3.5 Reduction of antigenicity after fluorescein isothiocyanate conjugation: Ten sera from rabbits that were immunized twice with either MR6 (5 rabbits) and HMFG1 (5 rabbits), and had developed good anti-MR6 and anti-HMFG1 responses (groups 1 and 3, Fig. 8.8b), were tested for their ability to recognise MR6-FITC and HMFG1-FITC respectively, in an ELISA assay. As shown in Figure 8.11, none of the anti-MR6 (a) or anti-HMFG1 (b) immune sera were able to recognise the FITC-conjugated Ig as well as the "naked" molecule.

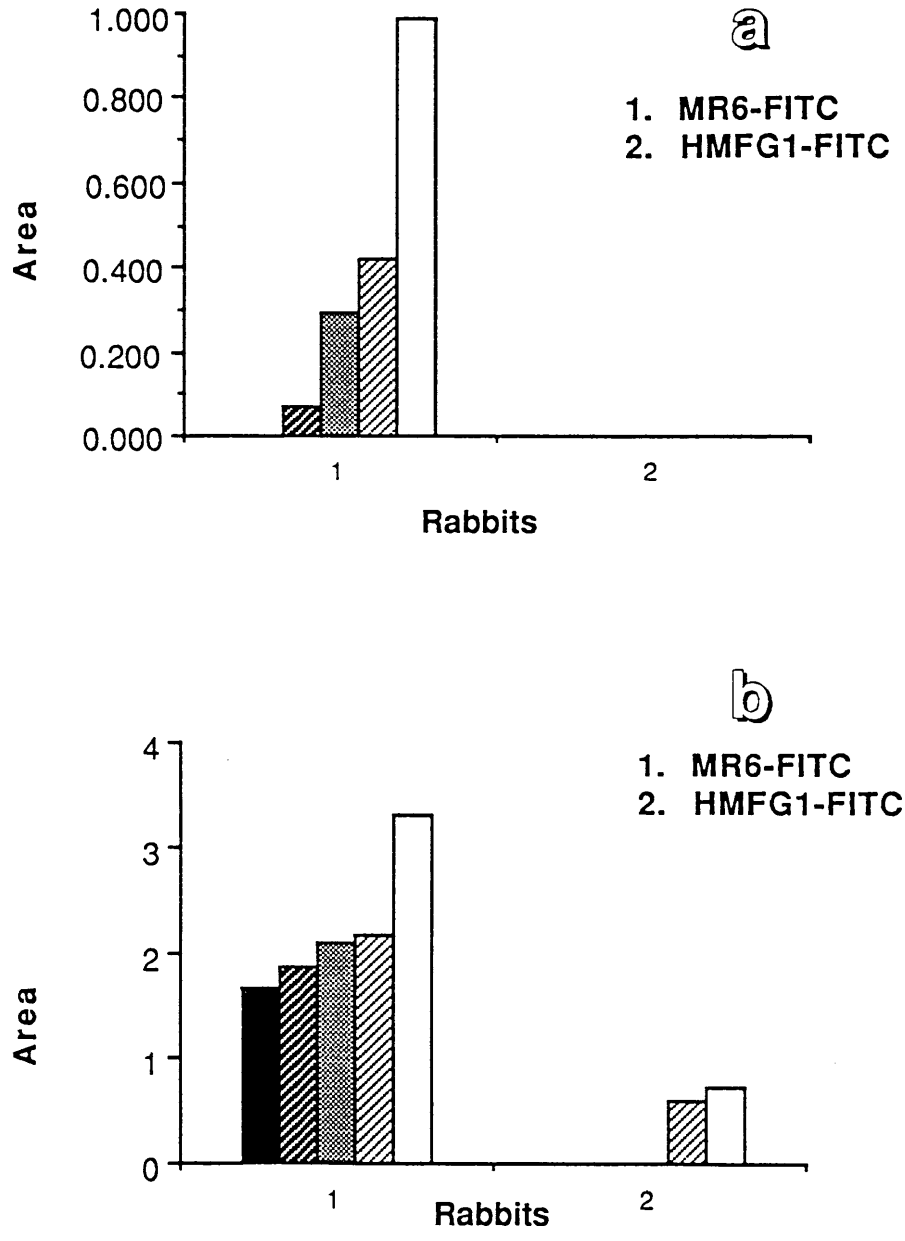


Figure 8.9: Humoral primary (a) and secondary (b) rabbit anti-FITC responses when FITC was covalently linked to MR6 (group 1) and HMFG1 (group 2). Each group contained 5 rabbits, each represented by a separate bar on the histogram. The results are expressed as areas under the sigmoidal ELISA curve (vertical axis).

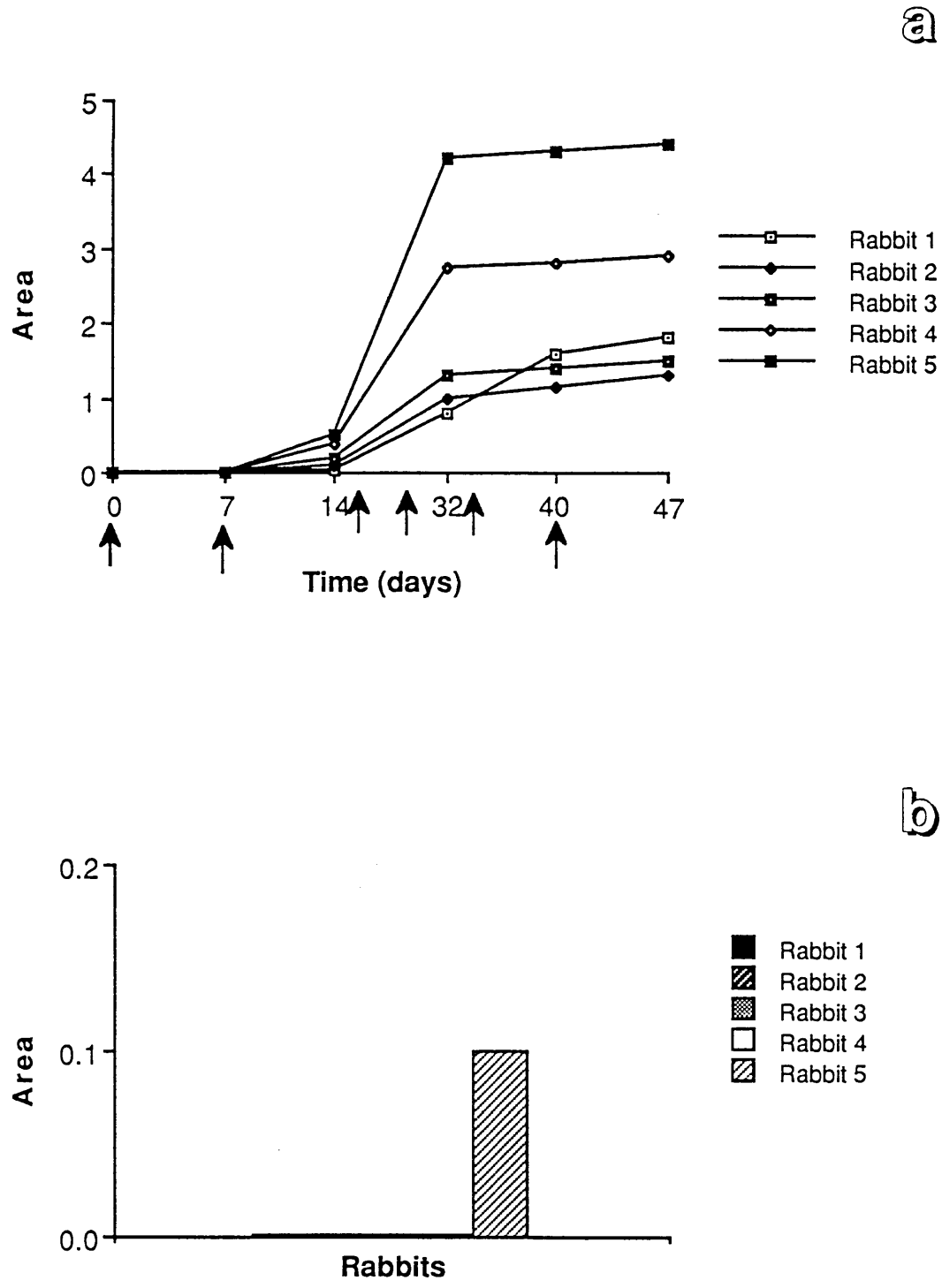


Figure 8.10: a) Humoral anti-mouse immunoglobulin responses of 5 rabbits, followed up for a period of 47 days (horizontal axis), after they had been challenged i.v. with HMFG1 (days 0, 7, 18, 25, 33) and HMFG1-FITC (day 40). b) The primary anti-FITC response of the 5 rabbits, as measured on day 47.
The results for both a) and b) are expressed as areas under the sigmoidal ELISA curve (vertical axis).

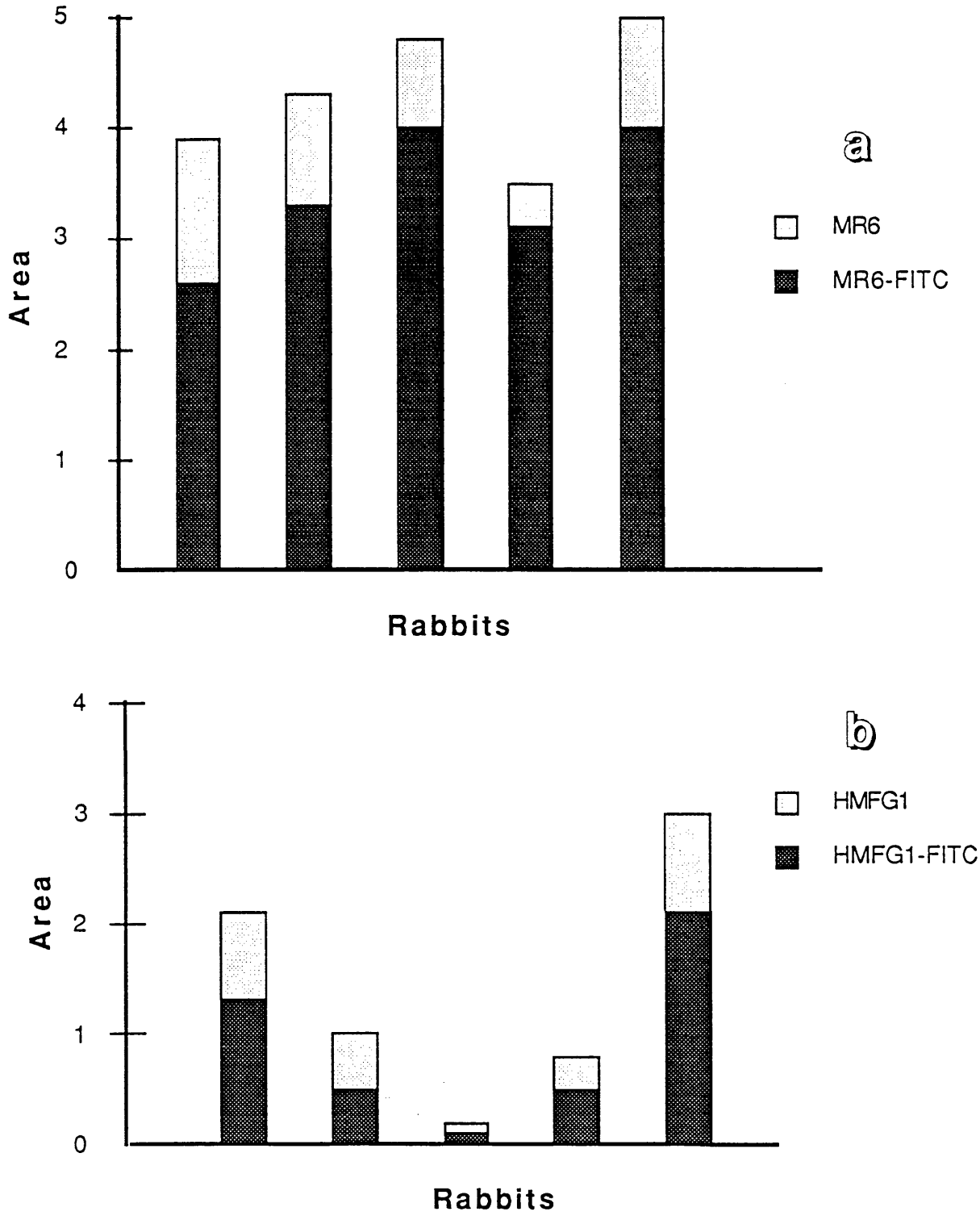


Figure 8.11: The ability of serum from 5 rabbits sensitized with MR6 (a) and HMFG1 (b), to recognise MR6 and MR6-FITC (a), or HMFG1 and HMFG1-FITC (b), in an Enzyme Linked Immunosorbent Assay. The results are expressed as areas under the sigmoidal ELISA curve (vertical axis).

8.3.6 Anti-haemoglobin response: Rabbits that received MR6, MR6-FITC, HMFG1 and HMFG1-FITC, were also injected with human Hb as an irrelevant control antigen. We found that none of the four groups of rabbits primed with 4 mg of human Hb, i.v., developed antibodies against it. However, after the second immunization, all members of the group of animals co-injected with MR6 responded against Hb, as did 4 out of 5 from the group co-injected with MR6-FITC (Fig. 8.12). In contrast, from the 10 animals co-immunized with HMFG1 and HMFG1-FITC, only 5 showed an anti-Hb response (Fig. 8.12).

8.4 Discussion

In these experiments, we have used a mouse monoclonal antibody (MR6) that recognises a human lymphocyte-associated antigen, functionally related to IL-4 receptor. The demonstrated *in vitro* T- and B-cell inhibitory effects of the MR6 antibody (Larché et al., 1988a; Larché et al., 1988b; O'Hehir et al., 1988), and the wide expression of its antigen on human B-, T-cells and APCs, make it a prime candidate for use in *in vivo* immunosuppressive experiments. We chose the rabbit as our animal model because we found that MR6 also recognises rabbit thymic epithelium and macrophages, as well as spleen-cells. The distribution pattern and the degree of expression, was remarkably similar to the human one (Larché and Ritter, 1989).

To our surprise, when this antibody was administered i.v. it was not immunosuppressive, but instead elicited a significantly higher response against itself than did 3 other isotypically matched mouse monoclonal antibodies. The increase in immunogenicity ranged from 76-100% for both the primary and secondary responses (Fig. 8.8a and b). Examination of the Ig preparations showed that there were no aggregates at the time of injection. In addition, although MR6 had affinity for a cell-surface antigen (and hence would

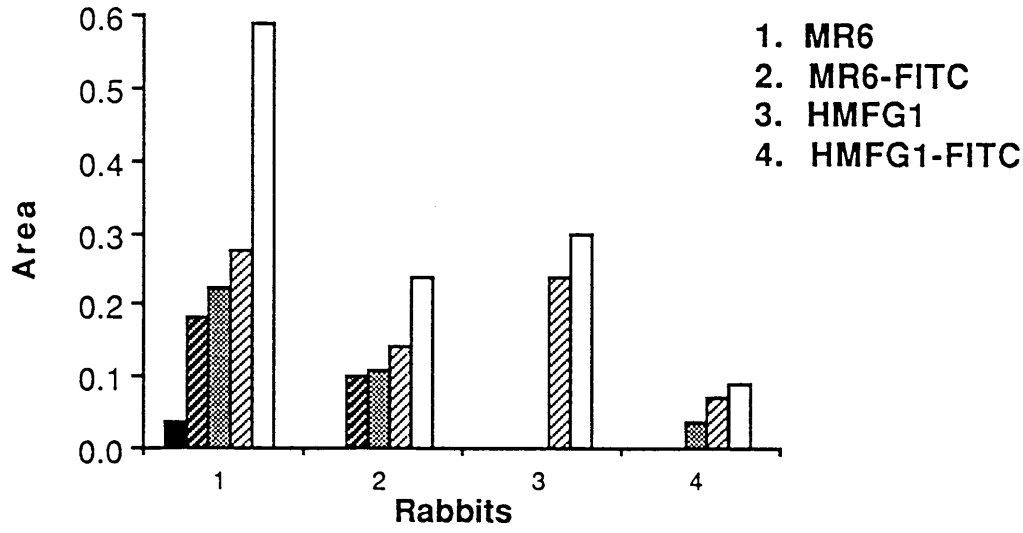


Figure 8.12: Rabbit antibody responses to human haemoglobin, when it was simultaneously administered with MR6 (group 1), MR6-FITC (group 2), HMFG1 (group 3) and HMFG1-FITC (group 4). Each bar represents an individual rabbit. The results are expressed as areas under the sigmoidal ELISA curve (vertical axis).

aggregate *in vivo* and become more immunogenic), so also did HMFG1, one of the control antibodies (Fig. 8.6c, d). There was nothing unusual about the animals used, since they were randomly chosen from 30 rabbits that did not have any pre-existing antibodies against either mouse Ig, FITC or human Hb. Besides, all animals injected with the control antibodies showed a lower (than MR6) but very similar anti-mouse Ig response (Fig. 8.8b). It is therefore unlikely that the phenomenon observed was due to an artifact in the ELISA, since this is a very reliable method (chapter 2), and we could detect no difference in the amount of the different antigens coated onto the 96-well microtitre plates.

The most probable explanation for the augmentation of the anti-Ig response by MR6 is that the specific binding of the antibody onto its antigen on the surface of APCs (macrophages, dendritic cells and B-cells), facilitates more effective antigen-processing, and subsequent T-cell recognition/activation. This was first described by Howard et al., who found that Igs become more immunogenic when directed against lymphocytes (Howard et al., 1968b), and our observation is also in agreement with more recent data, where antigens conjugated onto monoclonal antibodies directed against MHC class II were more efficiently presented by APCs in mice, because the antigen was actively "targeted" onto these cells (Carayanniotis and Barber, 1987; Carayanniotis et al., 1988). In similar experiments, antigen covalently linked to Tuftsin (a tetrapeptide derived from the heavy chain of Ig) augmented humoral immune responses in mice, since it enhanced phagocytic activity of macrophages, and promoted IL-1 synthesis and secretion (Dagan et al., 1988).

The fact that a hapten (FITC) becomes immunogenic only when covalently conjugated to MR6, supports the above theory. We found that rabbits did not respond to an initial injection of FITC when conjugated onto the control IgG1, HMFG1. Even after a second challenge, only 2 out of 5 animals responded, and these produced only low levels of anti-FITC antibodies (Fig. 8.9a and b). In

contrast, when FITC was conjugated onto MR6, 4/5 rabbits responded to the primary stimulation and all 5 responded to secondary challenge. All animals produced high levels of anti-FITC antibodies, especially in the secondary response (Fig. 8.9a and b). Theoretically it is possible that the anti-FITC response is enhanced when one carrier (MR6) is for various reasons more antigenic than another (HMFG1). We tested the possibility by challenging rabbits with HMFG1 until we had established a reasonably high anti-mouse Ig response (Fig. 8.10a). However, when these animals were then injected with HMFG1-FITC, we found that only 1 out of 5 developed anti-FITC antibodies (Fig. 8.10b), and at extremely low levels compared with the primary anti-FITC response that was generated when MR6-FITC was administered (Fig. 8.9a). Thus antigenicity *per se* could not explain our data.

Our results therefore suggest that the MR6 (and MR6-FITC), specifically "targets" onto immunocompetent cells, enhancing the anti-MR6 and anti-FITC response. Moreover, the response to an antigen simultaneously administered with MR6 (human haemoglobin) was higher overall than when it was co-injected with the control HMFG1 (Fig. 8.12). Nine out of 10 rabbits developed anti-Hb antibodies when MR6 was co-administered, whereas only 5 out of 10 showed an anti-Hb response when Hb was co-injected with HMFG1. These results suggest that in addition to its direct "targeting" role, MR6 may also have a "bystander" effect, activating APCs, and subsequently T-cells, that "enrich" their microenvironment with lymphokines, thus potentiating a general immune responsiveness in the area.

Surprisingly, MR6 conjugated to FITC triggered a lower (30%) anti-Ig response, than MR6 alone (Fig. 8.8b). If our theory of enhanced antigen-processing is correct, we might have expected to see similar responses. However, the explanation probably lies in the fact that FITC "masks" certain epitopes on the Ig molecule (Fig. 8.11a and b), making them inaccessible to B-cell surface receptors and, in our assay, soluble antibody molecules. A similar reduction in

immunogenicity was also seen when HMFG1 was coupled to FITC (Fig. 8.8b). This phenomenon of reduced immunogenicity when antigen is coupled to a macromolecule, has been observed in other systems. Asparaginase was rendered less immunogenic to rabbits and guinea-pigs, when linked to Dextran (Wileman et al., 1986), and mice were tolerized to human IgG when it was covalently conjugated to polyethylene glycol (Wilkinson et al., 1987). However, exclusion of water from the antigen's microenvironment (hence inhibition of cell-antigen interaction) might account for the latter observation. It is probable, therefore, that only a proportion of the potentially anti-mouse Ig reactive B-cells are activated by our FITC conjugated reagents since many immunoglobulin epitopes are covered by the fluorochrome. This is supported by our data showing that fewer anti-MR6 and anti-HMFG1 antibodies bind in an ELISA when the coating antigen has been fluoresceinated (Fig. 8.11).

Ideally, we would like to investigate the exact nature of the antigen to which MR6 binds, in order to understand its *in vitro* and *in vivo* function. Lack of appropriate rabbit cell lines, and the fact that we have so far found no other MR6$^+$ animal model, has limited further *in vitro* experiments. In the future, the MR6 antibodies should be compared with a larger number of mouse monoclonal IgG1s, and in combination with various haptens and peptides. Alternative routes of administration could also be tried, as well as a wide range of doses. However, until the rabbit MR6- antigen has been identified and fully characterized, the *in vivo* effects of MR6 in man (immunogenic or immunosuppressive) can only be speculated upon.

Ironically, our study, which was initially aimed at developing an immunosuppressive reagent, revealed an immunostimulatory molecule. In contrast to its *in vitro* inhibitory effects in the human system, MR6 augmented the immune response *in vivo* in rabbits, probably by specifically targeting to immunocompetent cells. Monoclonal antibody-mediated enhancement of the immune response might prove extremely useful in

vaccination and immunization using poor immunogens, in the absence of other adjuvants, in many clinical situations. In addition, if cancer epithelial cells internalize MR6 as rapidly and efficiently as APCs seem to do, this antibody could facilitate the intracellular delivery of cytotoxic compounds conjugated to it. The use of MR6 in serotherapy will clearly be limited if it also shows enhanced immunogenicity in man. However, the system could be manipulated by either "humanizing" the antibody (Riechmann et al., 1988), or by conjugating toxin molecules (ricin A-chain, Vinca alkaloids etc.) to it, in order to inactivate rather than activate the antigen processing cells.

Chapter 9

Final discussion

Passive administration of antiserum with anti-malignant cell specificities for *in vivo* tumour localization and growth inhibition is not a new idea. The introduction of monoclonal antibodies (mAbs) improved the specificity of immunodiagnosis and immunotherapy and abolished the restriction of reagent availability. Thus, over the last decade cancer manipulation has taken on a new dimension following the extensive study and documentation of the anti-tumour properties of mAbs in several *in vitro* and *in vivo* experiments. This initiated phase I, II and III clinical trials, that were performed with increasing frequency but moderate results. It soon became apparent that antibody administration was associated with host sensitization against the mAb, resulting in increased risk immune-complex mediated type III hypersensitivity and contributing to the low tumour uptake. This was not totally unexpected since most mAbs used were of rodent origin. In the work described in this thesis we attempted to reduce the immunogenicity of the foreign antibody, and, if possible, to induce specific anti-immunoglobulin (Ig) suppression in the host.

In our studies we have shown that mouse tumour-associated IgG mAbs administered systemically to patients with cancer, remained in circulation for a relatively long time (3-4 days). Even if they were given i.p. their catabolism was not rapid; in this latter case we also found that antibodies were not restricted by the peritoneum, but that a large proportion (~30%) migrated into the blood stream within the first two days. Thus, foreign antibodies easily become a target for immunocompetent cells *in vivo*, and elicit immune responses. In addition, the presence of pre-existing antibodies against the mAb, found in almost all patients to a greater or lesser extent (Davies et al., 1986; Courtenay-Luck et al., 1987), may further contribute to enhancement of the human anti-Ig response.

Immunoglobulins are unique molecules; the level of their xenogeneity is not typical and cannot be a paradigm of other foreign antigens. No other molecule

possesses the structural variety and the complexity of *in vivo* immunoregulatory properties that antibodies do. Firstly, their accumulation onto the cell-surface via their antigen-binding site may facilitate degradation and expression in a form that triggers cellular (and subsequently humoral) responses. Secondly, the affinity of the Fc region for receptors on monocytes/macrophages and B-cells may further enhance immunity (Guyre et al., 1983; Akiyama et al., 1984; Lubeck et al., 1985), but may alternatively lead to unresponsiveness, by interfering with the differentiation pathway of B-cells (Kölsch et al., 1980). Interestingly, it was also found that in experimental animals the anti-xenogeneic Ig antibodies are of lower relative affinity than antibodies against other antigens (Steward et al., 1973c). Does this imply that anti-Ig responses are restricted under some genetic rules? Is it possible that anti-antibodies may induce autoimmune disorders due to recognition of "self" determinants close to foreign epitopes? Such questions are still awaiting an answer. In view of the above we wish to emphasize therefore, that specific properties render each Ig a unique antigen; *in vivo* studies on immunogenicity and induction of anti-Ig suppression or tolerance can only be achieved after the various Ig interactions with immunocompetent cells are well understood and appreciated.

The first data suggesting that deaggregation (by ultracentrifugation) might render Ig tolerogenic were published 27 years ago (Dresser, 1962). However, the degree of unresponsiveness depended upon the mouse strain and deaggregation method used. Apparently there were tolerance "susceptible" and "resistant" mice defined according to their total anti-Ig antibody production (Golub and Weigle, 1969; Lukic´et al., 1975a; Hosono and Fugiwara, 1979a), and as shown in later studies, low responders also made antibodies of relatively low affinity (Alpers et al., 1972; Steward et al., 1973a). We therefore examined whether monomeric (not aggregated) murine mAb would be less immunogenic in man. We first performed animal experiments where deaggregated human IgG (hIgG) was administered to 3 different mouse strains.

Monomeric hIgG was produced by size exclusion chromatography column connected to a FPLC, since more stringent deaggregation methods induced unresponsiveness even to tolerance "resistant" mice (Golub and Weigle, 1969; Lukic´et al., 1975a). We believe that gel filtration provided a fast and most efficient way of aggregate separation, and thus was more advantageous over other methods.

We found that hIgG became less immunogenic after deaggregation, in a fashion that was genetically controlled: CBA and C57BL/6 mice (tolerance "susceptible") responded significantly less when primed with deaggregated than partially aggregated or heavily (heat) aggregated Ig, whereas there was either marginal or no difference in Balb/c mice (tolerance "resistant"). The primary response of the high responder Balb/c mice was reduced only when cyclosporin A (CSA) was concomitantly administered with hIgG at 7-15 mg/kg/day. After a second challenge however, monomeric IgG not only elicited a reduced anti-Ig response in CBA and C57BL/6 but also in Balb/c mice. Interestingly, CSA showed no further immunosuppressive effect on the secondary response of Balb/c mice, except when the animals were immunized twice with either aggregated or deaggregated material.

The overall magnitude of the anti-Ig response observed in the above three inbred mouse strains, showed that for a given antigen some animals are sensitized more than others. Thus, our data confirm and extend previous reports that immunocompetence to Ig is under genetic control (Biozzi et al., 1968; Oldstone and Dixon, 1969; Alpers et al., 1972; Lukic´et al., 1975b; Benacerraf and Katz, 1975; Roitt et al., 1985). However, the most important finding was that in all cases monomeric Ig was less immunogenic than aggregated Ig. The mechanism for this phenomenon is not yet clear. We favour the concept that large Ig-aggregates are more readily processed by antigen presenting cells. This is also supported by previous *in vitro* experiments showing that Ig polymers are internalized more efficiently by

macrophages than are monomers or dimers (Finbloom, 1986), triggering interleukin 1 production (Staruch and Wood, 1983; Weigle et al., 1987), which as known facilitates immunity (Unanue and Allen, 1987). Cyclosporin A reduced the primary anti-Ig response via its well known inhibitory effect on lymphokine production (Homan et al., 1980; Lindsey et al., 1980). It also reduced the secondary response when the immunogen was aggregated IgG, possibly by interfering with interleukin 1 as well as interleukin 2 synthesis and secretion. However, the mechanism of the additive effect between CSA and deaggregation observed in secondary responses remains unclear.

When we examined whether deaggregated Ig was also less immunogenic in man, we found no difference in the anti-Ig response of patients with cancer when the therapeutic radiolabelled mouse monoclonal antibody administered was either monomeric (^{131}Iodine-labelled) or partially aggregated (^{90}Yttrium-labelled). This failure to reproduce our animal data may be due to the fact that the mAb had affinity for a membrane antigen. *In vivo* aggregation on the cell surface may have influenced the development of immunity. Moreover, antibody-guided irradiation of neoplasms is associated with a degree of general immunosuppression, due to bone marrow irradiation. We have data to indicate that ^{90}Yttrium (the radiolabel for aggregated mAb) is associated with higher myelosuppression than ^{131}Iodine (radiolabel for monomeric mAb). This may therefore have obscured differences in the immune responses to the two mAb preparations. In future experiments, the immunogenicity of di/polymeric and monomeric antibodies should be examined when they are either unlabelled, or both labelled with the same isotope (^{131}Iodine). Finally, although genetic differences in antigen processing can be demonstrated in inbred mice, the outbred nature of the human population may obscure such effects.

In summary, deaggregated Ig was less immunogenic in mice, but in no case induced specific tolerance, as previously suggested. Even the low responder

CBA and C57BL/6 strains developed anti-Ig antibodies irrespective of whether they had been primed or boosted with deaggregated or aggregated material. One explanation for this low but demonstratable response may be that our monomeric polyclonal human IgG preparation reaggregated *in vivo* (Gronski et al., 1988a; Gronski et al., 1988b). However, we believe that the discrepancy of our results with earlier studies is more likely to be due to the anti-Ig detection method. The assay previously used was biological clearance of radiolabelled antigen (Ig). We now know that antigen clearance does not exactly correspond with the actual presence of anti-Ig antibodies (Rossen et al., 1971; Steward, 1979b). Low affinity antibodies could bind antigen without causing rapid blood clearance. In fact, there are cases reported, where the half life of certain antigens was prolonged due to formation of low affinity antibody/antigen complexes (Schifferli and Taylor, 1989). Such antibodies however, can be detected by our ELISA. Thus, what we called a "reduced response" based on ELISA findings and actual antibody production, was called "tolerance" by others based on the rate of antigen clearance.

Thus, our animal experiments suggest that the induction of unresponsiveness to deaggregated Ig may be due to the long term production of low affinity antibodies. Would a similar situation be desirable in man? At first it seems an attractive idea, because a monoclonal antibody would have a half life similar to a human Ig, thus it would be allowed sufficient time to localize and damage the target-cell. In practice however, this may prove very dangerous, since it might be associated with serious immune complex disease (Steward, 1979; Steward et al., 1981). Although epitope density plays an important role, in addition low antibody affinity (Lew and Steward, 1984), induction of a state where the host is unable to produce high affinity antibodies to subsequent challenges of the immunogen (as in patients with cancer receiving multiple antibody therapy), could be disastrous, since such immune complex formation may lead to severe glomerulonephritis and impaired renal function (Steward, 1979a; Steward et al., 1981; Devey et al., 1984).

In the past it was shown that passive administration of antiserum against a given antigen resulted in antigen-specific unresponsiveness due to "masking" of certain epitopes or faster immunogen catabolism (Freda et al., 1967; Murgita and Vas, 1972; Brüggeman and Rajewsky, 1982; Heyman and Wigzell, 1984). Thus, in patients with ovarian cancer receiving i.p. radiolabelled mouse mAb therapy, we administered affinity purified human anti-mouse Ig antibodies (HAMA) i.v., in order to "chase out" the excess of unwanted circulating murine antibody. We found that the antigen (mouse Ig) was rapidly cleared from the circulation after injection of the HAMA. In addition, a rapid but short duration reduction in complement levels was observed. The *in vivo* formed antibody/antibody complexes transiently accumulated in the liver, but generally no serious side effects were observed. On the contrary, administration of HAMA resulted in a substantial reduction of the mAb concentration and subsequently the total radioactivity in the blood, thus reducing the non-specific bone marrow irradiation 4-fold. However, the 20% reduction in circulating immunogen (mAb) observed within the first two days of injection, did not seem to result in lower anti-mouse Ig antibody production in the small number (five) of patients investigated.

Our failure to reduce the anti-Ig response may have been due to genetic variations among patients, different levels of pre-existing anti-mouse Ig antibodies and the fact that most of the mAb antigen (~80%) remained in the peritoneal cavity, inducing an immune response which may have been enhanced by aggregation of the mAb on the tumour-cell surface. Nevertheless, we found that by administering homologous high affinity anti-mAb antiserum, one could substantially reduce the amount of xenogeneic Ig in circulation. Therefore, we believe that it is possible to reduce, although perhaps not abolish, the level of host sensitization against the mAb. For future experiments, it will be critical to increase the amount of infused HAMA, together with careful monitoring of complement level, size and solubility of

immune complexes. This approach to antigen clearance may prove more successful in reducing immunogenicity after both mAb and anti-mAb are administered i.v., providing that the exogenous HAMA does not lower the efficiency of the anti-tumour antibody. For this approach to be of clinical usefulness, a reliable large scale supply of HAMA is required. Experiments are currently in progress to immortalize HAMA-secreting B-cells from previously "immunized" patients.

If circulating anti-cancer mAbs are suitably "equipped" to kill malignant cells *in vivo,* there is no reason why they should not also inactivate the immunocompetent cells with which they interact. This concept of antigen "suicide" was first introduced about 20 years ago, when it was found that a highly radiolabelled antigen could induce specific unresponsiveness to itself by binding to and killing antigen-specific B-cells (Humphrey and Keller, 1969). Since then it has been successfully used in *in vitro* and *in vivo* inhibition and suppression experiments with various antigens (Humphrey et al., 1971; Basten et al., 1971; Golan and Borel, 1972; Filipp et al., 1981; Csaba et al., 1985).

Initially we investigated the possibility that a radioactive antibody, labelled at very high specific activity with a suitable radioisotope, could cause specific anti-Ig unresponsiveness when administered to animals. A mouse mAb was therefore labelled with ^{125}Iodine at 180 mCi/mg, but when administered to rabbits, we found that it elicited a normal humoral response. We believe that the reactivity of this mouse antibody for a rabbit cell-surface antigen, along with inadequate radiolabelling compared with similar previous studies (albeit the maximum possible for this Ig), were the reasons for our failing to induce specific immunosuppression. In contrast, ^{111}Indium-labelled human IgG (15 mCi/mg) administered to mice, showed a small, but statistically significant reduction of the primary response anti-Ig, although it was ineffective after a second challenge. The toxicity of ^{111}Indium for lymphocytes is well documented (Chisholm et al., 1979; Ten Berge et al., 1983) but this is the first

time that it has been in antigen "suicide" experiments. Since the specific activity can be substantially improved using better techniques developed recently, it is possible that our initial promising data on the specific immunosuppressive effect of ^{111}Indium-labelled antibodies can be further improved in the future.

In general we feel that the main limitation to constructing a radioactive "lethal" molecule is the isotope-conjugation method and the nature of the antigen itself. Existing radiolabelling techniques do not allow controlled incorporation of isotopes on each Ig moiety. This results in the production of a heterogeneous mixture of conjugates ranging from highly radioactive molecules to unlabelled ones. Moreover, Igs like any other antigen, cannot incorporate an unlimited amount of radioisotope. There will always be a maximum specific activity above which radiolysis will occur, and this maximum may simply not be sufficient for the induction of specific immunosuppression. We feel that this, in addition to the hazardous nature of radioisotopes, will hinder further extensive *in vivo* experimentation with these reagents.

We therefore continued our experiments on antigen "suicide" using an antibody-drug complex. This reagent was a mouse mAb that recognised Vinca alkaloids with one binding site and a tumour associated antigen (CEA) with the other. In previous *in vitro* and *in vivo* experiments it was shown that when this antibody was mixed with vinblastine (VLB) or vindesine it could specifically kill CEA-expressing cells (Corvalan et al., 1987a; Corvalan et al., 1987b). Thus, we examined whether it was equally toxic to immunocompetent cells when administered to rabbits. We found that this bispecific mouse antibody when associated with VLB specifically reduced the rabbit primary anti-Ig response by up to 80%. One possible reason that we did not achieve total abrogation of the response may be because immunocompetent cells captured antibody molecules that had released their VLB, since the drug was

not covalently linked to the antibody but in a constant association/dissociation with it. However, covalent linkage of VLB to the antibody binding site abolished the immunosuppressive activity of this reagent. This is probably due to the fact that the drug is only toxic when delivered in its native form to the target cell. An alternative explanation might be that once bound to the target-cell surface, the bispecific mAb acts as a "pump", such that one mAb molecule can introduce many drug molecules into the cell. This "amplification" of toxicity would not be possible for the covalently linked mAb/VLB reagent.

We have shown therefore, that it is possible to construct a mAb suitable for cancer therapy, that has both immunosuppressive and anti-tumour activity. Provided that such immunoconjugates are stable *in vivo* and that their toxic portion is readily available at the site of action, one could use alternative drugs, such as protein-synthesis inhibitors, so that macrophages and other slowly-dividing antigen presenting cells would also be affected. Thus, we believe that in the future it may be possible to totally abolish the anti-Ig response, constructing bispecific mAb/drug complexes.

It has been shown that antibody-mediated *in vivo* T-cell blocking or depletion results in the induction of specific unresponsiveness against antigens simultaneously given with the anti-T-cell antibody (Martin, 1969; Gill, 1969; Gutstein and Wofsy, 1986; Benjamin et al., 1986). This phenomenon was most profound when anti-T_h-cell (anti-CD4) antibodies were used (Benjamin and Waldmann, 1986; Gutstein et al., 1986) due to the "key" role of T_h-cells in immune responses (Dialynas et al., 1983a; Dialynas et al., 1983b; Wilde et al., 1983). In this study we used a mouse mAb (MR6) that recognises a molecule associated with the human interleukin 4 receptor, normally expressed on human epithelial, B-, T- and antigen presenting cells (Larché and Ritter, 1989). We selected this reagent since it has been shown *in vitro* to have an inhibitory effect on T- and B-cell activation (Larché et al., 1988a; 1988b), we studied its possible *in vivo* immunosuppressive effects in rabbits, since this

was the only animal found to express a similar epitope.

To our surprise we found that MR6 antibody enhanced the response not only against itself but also to a hapten covalently linked to it. The exact mechanism of this phenomenon is not clear and needs further *in vivo* and *in vitro* experimentation. The most likely explanation however is that the mAb specifically bound to the surface of antigen presenting cells (B-cells, macrophages and dendritic cells) on sites "sensitive" to internalization, facilitating antigen processing and presentation, and thus enhancing T- and B-cell responses. Although the aim of this thesis was the induction of specific unresponsiveness, we believe that the specific immunopotentiation observed is of great importance. Adjuvant-free, antibody-mediated immunoregulation can be used in vaccination and other similar situations where the immunogen is a small and poorly immunogenic moiety such as synthetic peptides (Brown et al., 1984; Thanavala et al., 1986; Vandenbark et al., 1989). Naturally, from the point of view of direct clinical application, the enhanced anti-mouse Ig response is undesirable. However, a suitably "tailored" chimaeric antibody composed of the mouse hypervariable region in an otherwise human molecule (Jones et al., 1986; Riechmann et al., 1988), may produce a future novel "vehicle" for vaccination in man. Furthermore, since the "vehicle" alone would not be immunogenic (apart from its idiotype), and yet would still be rapidly internalized, this reagent might be a highly effective *in vivo* targeting molecule for those tumours that are MR6$^+$ (eg. 25% of breast carcinoma; Mat et al., 1989)

In the future, the development of human anti-tumour monoclonal antibodies may provide an alternative way to relieve immunotherapy of the problem of anti-mouse Ig responses. However, although the technique of producing rodent mAbs has been improved considerably since its introduction in 1975

(Köhler and Milstein, 1975), great obstacles are yet to be overcome for the production of human mAbs. The two main limiting factors are the availability of tumour-specific human B-cells and the techniques of immortalizing them. The source of human B-cells is usually peripheral blood lymphocytes obtained from patients with cancer who have developed anti-tumour antibodies. Their number, however, in no case can be compared with the spleen population derived from a hyperimmunized mouse or rat, and they are probably not at an appropriate stage of activation. In addition, immortalizing human B-lymphocytes in order to indefinitely produce their antibody, has proved to be more difficult than initially thought. Human lymphocytes can be fused with human myeloma cell lines (Sikora, 1984; Locniskar et al., 1988; Zumla et al., 1988) or infected with Epstein-Barr virus (EBV) (Sikora, 1984). However, this generally results in short lived, poor-producing (mainly IgM) cell lines. Various attempts at stabilizing these cells by a subsequent fusion with more stable murine myeloma lines, "diluted" the human genome and have not been very successful. EBV transformation appears to be the most successful method so far.

However, significant advantages have come through genetic engineering. Recently developed techniques enabled the production of human chimaeric monospecific antibodies, in which the variable or hypervariable regions of the human heavy and light chain genes have been replaced by those of a mouse mAb with predefined specificity (Morrison et al., 1984; Boulianne et al., 1984; Jones et al., 1986; Neuberger et al., 1987; Nishimura et al., 1987; Riechmann et al., 1988). Through gene manipulation it will soon be possible to design and produce chimaeric human Igs of the desired specificity and Fc function, in unlimited amounts. In addition, the toxicity of such genetically engineered molecules may be enhanced by recombinant attachment of toxic compounds (i.e. drugs, toxins), thus rendering such reagents particularly attractive in cancer immunotherapy. It may also be possible to design novel immunoconjugates with toxic compounds whose efficiency will not be

dependent upon internalization: for example, antibody-complement or antibody-allo-MHC chimaeras could be used to trigger tumour cell lysis or rejection upon simple binding to the cell surface.

Paradoxically, a promising way for cancer immunotherapy may be through immunopotentation against the administered Ig, and not through suppression. It has been shown that part of the humoral response that a mAb elicits *in vivo* is directed against its antigen binding site (Herlyn et al., 1986; Jaffers et al., 1986; Bosslet et al., 1988; Courtenay-Luck, 1989). Some of the anti-idiotypic antibodies that are generated have binding sites that are complementary to the paratope of the initial antibody, thus mimicking the primary structure of the antigen (anti-idiotypic antibody 1, Fig. 9.1). There are numerous studies where such anti-idiotypic antibodies elicited a further anti-paratopic response when administered to animals (anti-idiotypic antibody 2, Fig. 9.1) (Raychaudhuri et al., 1986; Viale et al., 1987; Raychaudhuri et al., 1987). These second generation anti-idiotypic antibodies mimic the structure of the original antigen epitope. Thus, through the idiotypic network recipients could produce their own antibodies against a variety of antigens. The development of such anti-idiotypic antibodies can prevent the onset of diseases in animals (Binz et al., 1982; Hahn and Ebling, 1984; Gaulton et al., 1986; Nelson et al., 1987; Dunn et al., 1987; Percy and Harn, 1988; Walker et al., 1988) and may also prove to be effective in inhibiting cancer growth in man (Herlyn et al., 1987; Steinitz et al., 1988; Courtenay-Luck et al., 1988).

Administration of the whole anti-idiotypic molecule may not be necessary, since just the hypervariable region could be sufficient to induce an anti-idiotypic response. Thus anti-idiotypic antibodies could be replaced by synthetic peptides chosen for their complementarity for certain antigen epitopes, as previously reported for the hepatitis B antigen (Brown et al., 1984; Thanavala et al., 1986) and recently for a peptide representing a region from the T-cell receptor of anti-myelin specific T-cells (Vandenbark et al., 1989).

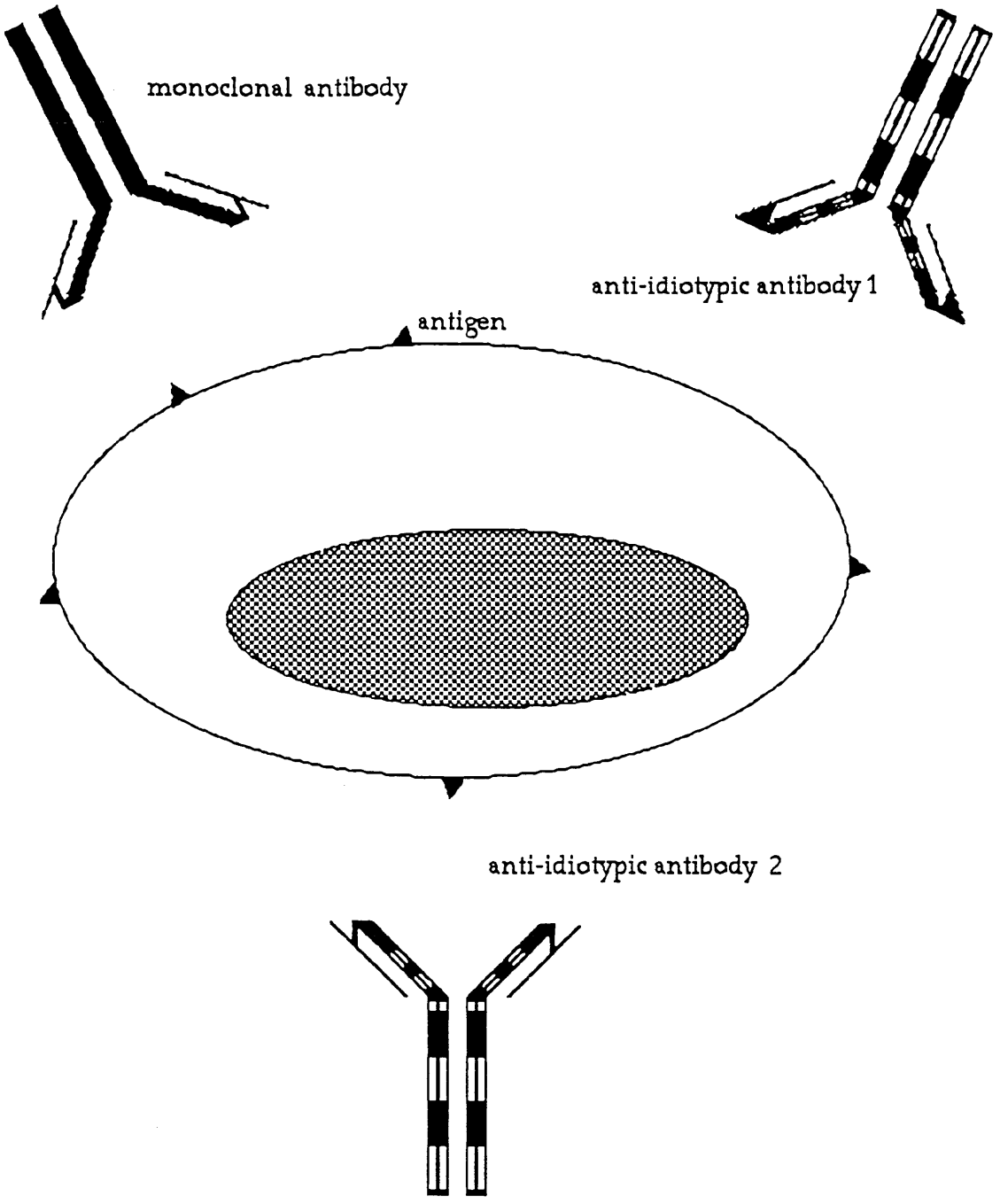


Figure 9.1: Administration of a mAb gives rise to a generation of antibodies whose binding sites mimic the primary structure of the antigen (anti-idiotypic antibody 1). Such anti-idiotypic antibodies may further trigger the production of a second generation of anti-idiotypic antibodies that directly recognize the same antigen as the original monoclonal antibody (anti-idiotypic antibody 2).

Synthetic peptides are therefore likely to form the new generation of vaccines, and since "mapping" of the structure of various antigens is becoming a routine procedure, sequences of aminoacids can be chosen that would bind to and sensitize immunocompetent cells in the same way as native antigens. The role of synthetic peptides however, is not limited to vaccination, and may be extended to direct immunotherapy. As recently reported by Ward et al., the genomic DNA of the variable heavy chain domain of an anti-lysozyme antibody was isolated, inserted and expressed by *Escherichia coli*. This resulted in the continuous secretion of this heavy chain that also retained its original binding specificity (Ward et al., 1989). In the future, even smaller peptides may be synthesized that would "recognise" complementary structures on antigens, thus radically changing our perception of immunotherapy.

In summary, successful immunotherapy of neoplasia greatly depends on tumour accessibility and antibody specific toxicity for malignant cells, associated with no adverse reactions from the host. With the current state of the art, antibody-mediated cancer therapy is faced with restrained optimism, partly because of the mAb's immunogenicity. Before human mAbs or other alternatives become readily available, the immunogenicity of the xenogeneic Ig can be reduced by removing large aggregates or by clearing the vast majority of mAb still in circulation immediately after maximum tumour localization occurs. However, we believe that the best approach would be to "arm" the therapeutic mAb with a toxic compound in order to make it "lethal" not only to the tumour target-cells, but also to immunocompetent cells, thus inducing specific unresponsiveness to the injected Ig, while providing maximum anti-tumour activity.

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