THE ROLE OF ACCESSORY CELLS IN CELL-MEDIATED IMMUNE RESPONSES IN VITRO, AS STUDIED USING MACROPHAGE-ACTIVE AGENTS

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ABSTRACT

Two culture systems were used to study cell-mediated immune responses *in vitro*, namely the generation of murine alloreactive cytotoxic T lymphocytes, and the expression of non-specific spontaneous cytotoxic activity by cultured human peripheral blood mononuclear cells. Optimal culture conditions were determined for each system, and the role played by macrophage-like accessory cells was examined using cell depletion and separation techniques based on the adherent properties of these cells. In the mouse system, the adherent population was found to have a dual role, acting as allogeneic stimulator cells and providing an accessory function; in the latter capacity, there appeared to be no requirement for histocompatibility between the responder cells and the accessory population. In contrast, removal of the adherent fraction from cultures of human mononuclear cells resulted in increased cytotoxic activity, depending on the interval between adherent cell depletion and assay, and it is suggested that the effector cells in this system are undergoing a continuous process of maturation, which is regulated by the adherent population.

Both systems were further investigated by the addition of some known macrophage-active agents. The results obtained with silica were consistent with the hypothesis that this material destroys phagocytic cells. Carrageenan and zymosan suppressed cell-mediated cytotoxicity in both culture systems, apparently acting via the accessory population without causing the death of such cells. Addition of latex beads to the cultures did not affect cytotoxicity.

The role of cyclo-oxygenase metabolites of polyunsaturated fatty acids in mediating carrageenan- and zymosan-induced suppression was investigated using exogenous prostaglandin E1 and prostacyclin, together with antisera against these compounds. In the mouse system, the

drugs indomethacin and flurbiprofen were also studied, and evidence is presented that cyclo-oxygenase products, particularly prostaglandins of the E series, are involved in regulating the generation of cytotoxic effector cells in vitro.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ADCC	antibody-dependent cell-mediated cytotoxicity
Ag	antigen
ALK	activated lymphocyte killing
APC	antigen-presenting cell
ATP	adenosine triphosphate
B cell	lymphocyte derived from bone marrow in mammals, Bursa of Fabricius in birds
BCG	Bacille Calmette Guerin
BSA	bovine serum albumin
C	serum complement
cAMP	cyclic adenosine monophosphate
^{51}Cr	radioactive sodium chromate
CTL	cytotoxic T lymphocyte
DMSO	dimethyl sulphoxide
EB	ethidium bromide
E:T	effector to target ratio
Fc	crystalline fragment of immunoglobulin
FCS	foetal calf serum
FDA	fluorescein diacetate
FDA-EB	viability stain
G	gentamicin
HBSS	Hanks' balanced salt solution

HEPES	N-2-hydroxyethyl-piperazine-N-2-ethane sulphonic acid
HETE	hydroxyeicosatetraenoic acid
HMP	hexose monophosphate
$HO_2\cdot$	perhydroxyl radical
H_2O_2	hydrogen peroxide
HPETE	hydroperoxyeicosatetraenoic acid
IFN	interferon
Ig	immunoglobulin
IL-1	interleukin 1
IL-2	interleukin 2
IL-2R	interleukin 2 receptor
i.p.	intraperitoneal
Ir	immune response (genes)
i.v.	intravenous
K	killer (cell)
LAK	lymphokine-activated killer (cell)
LFA	lymphocyte function-associated
LGL	large granular lymphocyte
LPS	lipopolysaccharide
LT	leukotriene
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
MLC	mixed leucocyte culture
MLR	mixed leucocyte reaction
MPS	mononuclear phagocytic system
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NK	natural killer (cell)
O_2	molecular oxygen
O_2^-	superoxide anion

$^1O_2$	singlet oxygen
OH·	hydroxyl radical
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PG	prostaglandin
PGI_2	prostacyclin
PLA_2	phospholipase A_2
PUFA	polyunsaturated fatty acid
PVPNO	poly-2-vinyl-pyridine-N-oxide
R cell	responder cell in MLC
rbc	erythrocyte (red blood cell)
RES	reticuloendothelial system
ROI	reactive oxygen intermediate(s)
R:S	responder to stimulator ratio
S cell	stimulator cell in MLC
SRS-A	slow reacting substance of anaphylaxis
T cell	lymphocyte derived from bone marrow and subsequently "processed" or "educated" in the thymus
T_C	cytotoxic T lymphocyte
T_H	helper T lymphocyte
TGC	thioglycollate
TX	thromboxane

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CHAPTER ONE

General introduction

1.1. ACCESSORY CELLS IN IMMUNE RESPONSES

Progress in understanding mammalian cellular immunology has occurred largely through identification of sub-populations of cells, and analysis of their functions and interactions. Specific effector mechanisms in the immune system develop via maturation of precursor cells into operational effector lymphocytes, and this process frequently involves additional accessory cells, which may serve a regulatory role. For the purposes of this study, an accessory cell may be defined as any mononuclear cell other than a T or B lymphocyte which is capable of enhancing or suppressing an immune reaction. Clearly, the classical tissue macrophage falls into this category; however, this definition also embraces other cell types, and the accessory population as a whole therefore displays considerable heterogeneity.

1.1.1. TYPES OF ACCESSORY CELL

Experimentally, accessory cells are usually included in the glass- or plastic-adherent fraction obtained from the lymphoid tissues or from peripheral blood. Many of these cells are actively phagocytic, and are therefore classified as "mononuclear phagocytes;" such nomenclature has replaced the earlier "reticuloendothelial system" proposed by Aschoff to describe phagocytic cells sequestering vital dyes within their cytoplasm, as further study revealed that this term embraced cells whose phagocytic activity was not directly related to host defence, such as the endothelial cells lining blood

sinuses and the reticular cells of connective tissue (van Furth et al, 1975; Klein, 1982).

Mononuclear phagocytes are released from the bone marrow as monocytes, which travel round the body in the circulatory system and ultimately migrate into the tissues. Here they mature into tissue macrophages, and can survive for several months as a slowly cycling population; although able to divide, mature macrophages rarely undergo mitosis, and new cells are recruited primarily from a monocytic precursor pool (Carr, 1975; van Furth et al, 1979, 1980; North, 1969, 1970).

Mononuclear phagocytes are found throughout the mammalian body, in bone marrow, peripheral blood and lymphoid organs, connective tissue, liver, lungs and components of the nervous system. Many of these cells retain the ability to mobilise in response to appropriate stimuli (Gallin et al, 1980; Hahn, 1975; Wilkinson and Allan, 1980), and they can accumulate at sites of inflammation and granuloma formation, being attracted and activated by factors released by other cells and by chemotactic complement components, for which they possess specific surface receptors. Receptors for the Fc fragment of immunoglobulin G (IgG) are also present on the surface of macrophages and related cells, and binding of antibody to these sites can confer antigenic specificity on the effector functions of the cell.

Cells included in the mononuclear phagocytic system (MPS) are heterogeneous both in function and morphology. They display variable phagocytic activity, and can also be highly active secretory cells, manufacturing and releasing digestive enzymes and other biologically active products. The secretory activity of these cells is difficult to demonstrate and quantify in vivo; however, in vitro studies have

revealed a variety of biochemical and functional responses. The classical tissue macrophage has been most extensively studied, and has been identified as a crucial element in iron and lipid metabolism. Some of the products synthesised and secreted by macrophages are described in more detail in section 1.3..

Studies on the role of accessory cells in immune responses are frequently hampered by the confusing nomenclature used to describe these cells. As previously mentioned, the term "mononuclear phagocyte" has been adopted to include tissue macrophages and their monocytic precursors, and to distinguish these from the polymorphonuclear cells which originate from the same progenitor in the bone marrow. However, immune accessory functions can also be served by cells which are apparently unrelated to the monocytic series.

Dendritic cells were first isolated from the adherent fraction of mouse spleen, and most studies of these cells have employed this species. Dendritic cells have since been identified in other tissues, including lymph nodes, rarely exceeding one per cent of the total leucocyte count (Steinman and Cohn, 1973). Few mature dendritic cells are found in bone marrow; however, a proliferating pool of precursor cells is present in both bone marrow and in the non-adherent spleen population (Steinman et al, 1974). Maturation of these cells is independent of thymic influence (Steinman and Nussenzweig, 1980). Dendritic cells display little endocytic activity, and do not possess surface receptors for immunoglobulins; however, in support of their inclusion in the MPS, they have been shown to stain positively with an antibody raised against macrophages in the mouse (S. Gordon, personal communication). In

common with most monocytes and macrophages, dendritic cells also express products of class II histocompatibility genes at their surface. These molecules have been shown to play a vital role in T cell activation, and will be described in more detail in section 1.1.2..

The properties and characteristics of dendritic cells both *in vivo* and *in vitro* have been extensively reviewed (Steinman and Cohn, 1973, 1974; Steinman *et al*, 1974; Steinman and Nussenzweig, 1980), particularly with respect to their role in T cell activation (see section 1.1.2.). Considerable variation in these properties is apparent between cells obtained from different species, and indeed between cells obtained from different lymphoid tissues within an animal. Thus it has been shown that dendritic cells isolated from rat lymph nodes are frequently non-adherent, though highly mobile (Klinkert *et al*, 1980); in contrast, murine splenic dendritic cells are strongly adherent following explant, but gradually detach and become non-adherent, while murine thymic dendritic cells do not adhere at any stage (Steinman and Nussenzweig, 1980). Such variation may reflect further heterogeneity of this population; indeed, it has been suggested that the epidermal Langerhans cell may represent a sub-population of dendritic cells (Steinman and Nussenzweig, 1980). Langerhans cells constitute up to eight per cent of mammalian epithelial cells (Katz *et al*, 1979). They may play a role in resistance to some infections, and have been implicated as the passenger leucocytes responsible for inducing rejection of skin allografts (Stingl *et al*, 1980). Like dendritic cells, Langerhans cells are replenished from a mobile precursor pool derived from the bone marrow; the relationship of these precursors to early cells in the monocytic lineage is not clear, although human Langerhans cells

express surface antigens found on tissue macrophages (N. Hogg, personal communication). Langerhans cells are not avid phagocytes, nor are they firmly adherent; however, they do possess Fc receptors, and express surface class II histocompatibility gene products (Frelinger et al, 1979). Work with skin-sensitising agents has demonstrated that, following interaction with these antigens in the epidermis, Langerhans cells migrate via the afferent lymph to the lymph nodes in the form of veiled cells (Balfour et al, 1981; Drexhage et al, 1980; Knight et al, 1982, 1983; S. Knight, personal communication). They therefore represent a means for transporting antigen from the site of contact to the immunocompetent T lymphocytes in the paracortical areas of the lymph node. On reaching this site, veiled cells lose their characteristic cytoplasmic "veils" and become relatively sessile interdigitating cells, whose branching processes form an ideal substratum for T cell interaction (Knight et al, 1982, 1983). Morphologically similar interdigitating cells have been demonstrated in the thymic medulla (van Furth, 1980), and it is conceivable that all tissues possess a small percentage of class II positive accessory cells of dendritic type (Stingl et al, 1980). However, caution must be exercised in ascribing all such cells to the same lineage. For example, the follicular dendritic cell originally isolated from mouse spleen, and subsequently identified in rat spleen and lymph nodes, has been shown to arise independently of the haemopoietic bone marrow ontogeny (Humphrey and Grennan, 1982; Humphrey et al, 1984; Humphrey, 1985), and may be derived from the reticular cells which form the intercellular skeleton in peripheral lymphoid organs (Hoefsmit, 1975; Nossal et al, 1968). Sometimes referred to as dendritic reticular cells, follicular dendritic cells comprise less

than one per cent of mouse spleen leucocytes (Humphrey and Grennan, 1982). They have branching cytoplasmic processes which interdigitate between lymphocytes, particularly B cells (Nossal et al, 1968), and are difficult to isolate intact; study of their close association with B lymphocytes suggests that these cells may play an important role in the generation of B memory cells (Klaus et al, 1980; Klaus and Kunkl, 1981). Although displaying a similar enzyme profile to dendritic cells, follicular dendritic cells also possess surface receptors for immunoglobulin and for the third component of complement (C3); however, they do not usually express class II histocompatibility antigens, and are not phagocytic (Humphrey and Grennan, 1982; Humphrey et al, 1984; Klaus et al, 1980).

From this brief survey, it is apparent that several types of cell can contribute to immune responses in an "accessory" manner. In a system where the accessory cell has not been phenotypically identified, it is perhaps the safest course to refer to it with respect to its functional characteristics; accordingly, throughout this study the accessory cells are not described as macrophages, but rather as adherent cells, as this was the method by which they were isolated.

1.1.2. ANTIGEN PRESENTATION

In addition to their phagocytic role as scavengers of pathogens and effete or abnormal cells, mononuclear phagocytes are responsible for processing and presenting antigen to responding lymphocytes. As discussed in section 1.1.1., several other cell types may also serve in antigen presentation; the term "antigen-presenting" or "accessory" cell is therefore used to include all such cells. As the current study concentrates on cell-mediated immune responses,

presentation of antigen to T lymphocytes only will be considered here.

Thymus-educated or T lymphocytes form the antigen-specific effector arm of the cell-mediated immune response. Morphologically indistinguishable from B lymphocytes, they can be identified on the basis of their surface proteins, and have been divided into four sub-populations using functional and phenotypic criteria, namely helper, suppressor, cytotoxic and delayed-type hypersensitivity T cells. An additional lymphoid population has been isolated which possesses some T cell markers, but also shares some characteristics with monocytic cells; the exact lineage of these cells is not clear, but they are believed to include non-specific effector cells such as those responsible for spontaneous cytotoxicity (see section 3.1.) and antibody-dependent cytotoxicity.

T lymphocytes are committed to a single antigenic specificity. Following binding of the appropriate antigen in the presence of accessory cells, naive T cells undergo clonal expansion to produce mature effector cells, whose activity varies according to the T cell subset. Unlike B lymphocytes, T cells do not usually respond to antigen which is free in solution, but are only activated by antigen present on cell surfaces. The T cell receptor responsible for such interactions has until recently proved elusive; however, it was assumed that antigen was recognised by a specific surface receptor on the T lymphocyte in a manner analogous to that demonstrated for B cell-antigen interactions. Subsequent structural clarification of the T cell receptor (reviewed by Collins and Owen, 1985) has led to improved understanding of the interactions of these cells with other elements of the immune network, and it has been shown that activation of T lymphocytes depends on simultaneous recognition of

antigen and a second surface molecule, encoded by genes within a region of the genome termed the major histocompatibility complex (MHC). This region comprises a group of closely linked loci, which were originally identified as crucial elements in determining allograft survival.The MHC has subsequently been found to play a central role in immune responses, acting as the identifying signal for self-nonself discrimination and regulating the response to antigen. To date, analogous histocompatibility systems have been found in all mammalian species; in man, the MHC is termed the HLA system and is located on chromosome 6, while the murine MHC is called H-2 and is found on chromosome 17. Genes within the MHC code for a number of proteins and display varying degrees of polymorphism. They are currently divided into three classes, of which class I and class II play a role in T cell activation. Class III MHC genes code for some of the proteins of the serum complement system, and will not be considered here.

The requirement for T cells to see antigen in association with an MHC molecule is termed MHC restriction. Helper T lymphocytes are usually restricted by class II MHC molecules (Kappler and Marrack, 1976), while the majority of cytotoxic T cells are restricted by class I MHC products (Zinkernagel and Doherty, 1975); however, structural similarities between the two classes of MHC molecule, and between the receptors on the two T cell populations, suggest that a common mechanism may apply. Suppressor T cells, while phenotypically resembling cytotoxic T lymphocytes, have been reported to be restricted by either class I (Parham, 1984) or class II MHC molecules (Alberts et al, 1983; Qvigstad et al, 1985), and it has also been suggested that this T cell population may be able to

respond to free antigen in solution (Alberts et al, 1983; Collins and Owen, 1985).

For the majority of immune responses, a critical step in initiation is the generation of helper T cells. The precise nature of the ligand recognised by the receptor on this T lymphocyte remains to be finalised, although it is now accepted that determinants from both the antigen and a class II MHC molecule must be recognised (Qvigstad et al, 1985). However, while most nucleated cells within the mammalian body express surface class I MHC molecules, class II gene products show a much narrower tissue distribution, being largely confined to some lymphoid subsets (B lymphocytes and some activated T cells) and to cells ascribed to the MPS. Thus only a few cell types are able to effectively "present" antigen on their surface to helper T cells and consequently induce a specific response. Such antigen presentation has been demonstrated for most of the cell types described in the previous section (section 1.1.1.). In the case of actively phagocytic cells such as monocytes and macrophages, endocytosed antigen may be partially digested, the remnants being re-expressed at the cell surface in association with class II molecules (Unanue and Benacerraf, 1984a). However, cells belonging to the MPS show varying degrees of endocytic activity, and some may merely trap antigen on their plasma membranes, which then interacts with class II gene products to form an immunogenic configuration; the relatively high concentration of class II molecules on the surface of dendritic cells (Knight et al, 1983) and Langerhans cells (Katz et al, 1979) would favour such associations. In addition to the mononuclear phagocytes, presentation of antigen has also been demonstrated using B lymphocytes (Lanzavecchia, 1985) and transfected mouse fibroblasts (Malissen et al, 1984); in all cases

such activity is dependent on expression of class II MHC products by the accessory cell, and a similar mechanism of antigen-class II interaction at the cell surface may apply.

Quantities of class II protein vary greatly between different cell types (Cowing et al, 1978); up to one million class II molecules have been estimated on the surface of Langerhans cells (R. Lechler, personal communication), while murine peritoneal exudate macrophages have been shown to synthesise and express class II molecules only transiently (Beller and Unanue, 1981). The T cell-derived lymphokine gamma interferon (gamma-IFN) can increase expression of class II molecules on mononuclear phagocytes, and can even induce synthesis of these gene products by cells which are usually class II negative (Steeg et al, 1982). Such findings suggest that many cells have the potential to express class II molecules; however, the importance of weakly class II positive cells in vivo is not clear. Such cells may be unable to serve in immune induction under normal physiological conditions, as there is evidence that a threshold density of class II molecules may be required for effective T cell stimulation (Lechler et al, 1985). However, inappropriate expression of class II molecules, resulting in aberrant immunogenic presentation of surface components, has been implicated in some autoimmune disorders. Thus pancreatic islet cells expressing class II molecules have been reported in insulin-dependent diabetes mellitus (Bottazzo et al, 1985), while studies of autoimmune thyroid disorders have revealed class II positive epithelial cells in both Hashimoto's thyroiditis and Graves' disease (Todd et al, 1986). Whether expression of class II molecules is a causative factor in such disorders, or merely contributes to a disease process initiated by some other agent is still under investigation. However, once stimulated by an antigen-

class II complex, autoreactive T cells will release lymphokines, including gamma-IFN, and may therefore induce further expression of class II molecules; the autoimmune response can therefore amplify itself.

In contrast, presentation of self antigens to T lymphocytes in the thymus may be a critical stage in the development of MHC restriction and self tolerance. The thymus is a primary lymphoid organ where T lymphocytes mature in the absence of exogenous antigen, thus there would seem to be no requirement for antigen-presenting cells. However, a population of class II positive interdigitating follicular cells has been identified in the thymic medulla (Kyewski et al, 1986). These cells are rich in MHC gene products, and may in fact be presenting self components in the context of these molecules to the developing T lymphocytes in a manner favouring recognition of self MHC (Beller and Unanue, 1980; Unanue and Benacerraf, 1984b), with subsequent deletion or suppression of strongly autoreactive clones, or the induction of antigen-specific suppressor cells (Kyewski et al, 1986).

Overall, it is clear that cell-mediated responses to antigen are under the strict control of class II MHC genes; indeed, these loci were originally defined as immune response or Ir genes before the proteins which they encode were identified on cell surfaces (Unanue and Benacerraf, 1984c). Many studies have described the absolute requirement for class II positive antigen-presenting cells in immune responses of both T and consequently B cells, and these have been adequately reviewed elsewhere (Pierce, 1980; Unanue, 1972, 1981). However, there remain a few antigens to which antibody responses can be initiated in the apparent absence of T cells. These "thymus-independent" antigens are usually bacterial products or

polysaccharides, with repeating antigenic determinants, and may induce polyclonal B cell stimulation. Such responses are usually somewhat limited, and are less subject to genetic regulation; it is possible that they represent a less specialised form of immunity which evolved before the complex interactive system now in operation.

1.2. MACROPHAGE-ACTIVE AGENTS

Many of the cells included in the MPS are actively phagocytic, and will avidly ingest both soluble and particulate substances. Most of these will be digested by lysosomal enzymes; however, some may not be susceptible to enzymic action, and may persist as inclusions within the phagolysosomes of the cell. The nature of the ingested material and its fate within the cell may therefore influence the behaviour of the phagocyte. A number of materials have been reported to interact with phagocytic cells in a manner which may alter their activity, both *in vivo* and *in vitro*, and some of these are described below.

1.2.1. SILICA

A mineral with low chemical reactivity, silica is known to be fibrogenic in man and experimental animals. This material has been shown to react with lipoprotein in aqueous solution, and can damage cell membranes as a result of hydrogen bonding between silicic acid and lipid and protein components (Allison *et al*, 1966; Nash *et al*, 1966; Stalder and Stober, 1965). A number of substances have been

found to reduce the toxic effects of silica *in vivo* and *in vitro*. For example, the polar polymer poly-2-vinyl pyridine N-oxide (PVPNO) is a strong proton acceptor (Allison *et al*, 1966); by forming hydrogen bonds with silicic acid it prevents interaction of this material with cellular components, so maintaining the integrity of the cell membranes and protecting the cell from autolysis (Allison *et al*, 1966). PVPNO is particularly effective in suppressing silicosis in animals, where it considerably reduces collagen production (Nash *et al*, 1966). Silica particles coated with lipid or protein interact less avidly with phospholipid membranes than do uncoated particles (Stalder and Stober, 1965), and protein-coated silica has been shown to be selectively toxic for phagocytes; following ingestion, proteolytic enzymes remove the protective protein to expose silicic acid, which then damages the phagolysosomal membrane, resulting in release of the lysosomal contents into the cytoplasm and subsequent cell death (O'Rourke *et al*, 1978). *In vitro*, sub-lethal doses of silica have been shown to impair the bactericidal activity of mouse peritoneal macrophages (Allison and Hart, 1968; Sheagren *et al*, 1975), suggesting that low doses of this material may impair intracellular systems in the absence of any detectable leakage of cellular components into the surrounding medium.

Several workers have used silica to remove phagocytes from experimental systems and to determine the role of such cells without recourse to separation techniques which may result in loss of other cell types. Most experiments using this material have been performed *in vivo*, as cell cultures can suffer indirect toxicity following death of the phagocytic fraction. Zisman *et al* (1971) demonstrated impaired liver macrophage function in mice given silica i.v. or

i.p., resulting in decreased resistance to i.v. inoculation of yellow fever virus; inefficient removal of circulating virus by the liver permitted early invasion of the brain, before an effective antibody response had developed. Prolongation of skin graft survival has been achieved in mice by i.p. injection of silica two or three days before or after grafting (Rios and Simmons, 1972), suggesting that macrophages may function as non-specific effectors in allograft rejection, while a similar role for phagocytes is indicated by the abrogation of resistance to bone marrow grafts in irradiated mice following i.v. administration of silica (Lotzova and Cudkowicz, 1974). In both these studies, the effects of silica could be prevented by prior administration of PVPNO. It is believed that silica particles given *in vivo* become coated with plasma protein, rendering them specifically toxic for phagocytes. Particles released from damaged cells can then be ingested by other phagocytes, resulting in cumulative destruction of this population and providing a strong fibrogenic stimulus. Silica has also been used as a selective macrophage-toxic agent *in vitro* (O'Rourke *et al*, 1978; Parkhouse and Dutton, 1966; Sanderson and Taylor, 1976).

1.2.2. CARRAGEENAN

The carrageenans form a heterogeneous mixture of sulphated polygalactans, which vary in total sulphur content and distribution. They are extracted from cell walls of marine algae of the family *Rhodophyta*, the main source being *Chondrus crispus*. Although soluble in aqueous solution, carrageenan preparations become viscous when cold or in the presence of potassium; kappa and lambda carrageenans may be separated on the basis of their different gelling properties while a third type, designated iota carrageenan, has been isolated

from algae of the genus Eucheuma. Carrageenan molecules have a double helical conformation, with covalent inter-chain bonds forming a structure which is resistant to biochemical degradation.

Carrageenan displays a variety of biological properties (reviewed by DiRosa, 1972; Thomson et al, 1979). It has been widely used as an inflammatory agent, inducing both acute and chronic inflammation (DiRosa et al, 1971; Monis et al, 1968; Willis, 1969); lambda carrageenan appears to be the most potent in this respect. Carrageenan has been shown to potentiate endotoxicity, a property shared by other agents which impair macrophage function (Becker and Rudbach, 1978). Several workers have described a macrophage-toxic effect of carrageenan. Allison et al (1966) reported rapid ingestion of carrageenan by macrophages and subsequent cell death, but stated that the toxic effect of ingested carrageenan was slower than that of silica. Similarly, Catanzaro et al (1971) and Catalona et al (1978) described a toxic effect of ingested carrageenan in vitro which was specific for adherent mononuclear cells; it was suggested that the lysosome was the site of initial damage, with destruction of the cell proceeding as for silica. This hypothesis was supported by the work of Rios and Simmons (1972), who demonstrated carrageenan-mediated lysis of macrophages both in vivo and in vitro, which was prevented by addition of PVPNO. In view of such reports, carrageenan was adopted by some as a specific anti-macrophage agent and was employed in much the same way as silica. However, further studies with this material have challenged the assumption that carrageenan is toxic for macrophages. Aschheim and Raffel (1972) observed increased phagocytic activity of macrophages from carrageenan-treated mice, while Sawicki and Catanzaro (1975) suggested that although carrageenan is ingested by all phagocytic

cells, it may only be toxic for a sub-population. The type and concentration of carrageenan used must also be considered; at high doses, toxic effects may become non-specific (Kiessling et al, 1977; Yung and Cudkowicz, 1978b). However, Simon and Jones (1979) failed to demonstrate significant reduction of either viability or phagocytic activity of mouse peritoneal cells following incubation with different types of carrageenan, although they could demonstrate histochemically that this material was present within the cells. Consequently, while it is clear that carrageenan and silica exert their effects on the same population of cells, they probably act via different mechanisms (Cudkowicz and Yung, 1977). It has been suggested that partial or prolonged phagolysosomal digestion of carrageenan may affect the cell in a non-toxic manner (Abraham et al, 1972), stimulating synthesis and release of lysosomal enzymes and other biologically active factors (Davies and Allison, 1976a; Rumjanek et al, 1977); some of the substances which may be released under these circumstances are described in section 1.3.

The effects of carrageenan on immune responses in vivo and in vitro have been widely studied (reviewed by Thomson et al, 1979). Administration of carrageenan either i.v. or i.p. has been shown to reversibly depress MPS activity in vivo, as measured using blood clearance techniques or organ uptake of particulate materials (Chaouat and Howard, 1976; Fowler and Thomson, 1978; Rumjanek et al, 1977). Suppression of antibody responses to thymus-dependent antigens has been described in carrageenan-treated mice (Aschheim and Raffel, 1972; Ishizaka et al, 1977; Rumjanek et al, 1977; Wong and Herscowitz, 1979); in contrast, in vivo humoral responses to T-independent antigens are not affected by carrageenan (Ishizaka et al, 1977; Wong and Herscowitz, 1979). Adjuvant activity has also

been ascribed to carrageenan (Turner and Higginbotham, 1977).

Carrageenan has been shown to suppress cell-mediated responses, acting during initiation of the response by interfering with antigen processing and presentation to T lymphocytes (Schwartz and Catanzaro, 1973; Yung and Cudkowicz, 1978b). Prolongation of skin allograft survival in mice treated with carrageenan has been reported by Rios and Simmons (1972) and Rumjanek and Brent (1978); both studies also demonstrated reversal of this effect by PVPNO. However, Cudkowicz and Yung (1977) and Yung and Cudkowicz (1977) were unable to protect against carrageenan-induced abrogation of resistance to foreign bone marrow grafts using PVPNO.

Similar results have been obtained using carrageenan in vitro. Suppression of both mixed leucocyte reactions (Pawelec and Brons, 1978; Rumjanek and Brent, 1978) and cytotoxic T cell responses have been reported (Yung and Cudkowicz, 1978b). Carrageenan has also been shown to interfere with spontaneous cytotoxic activity in vitro (Kiessling et al, 1977), and evidence has been presented that some carrageenans may induce suppressor cells (Bash and Cochran, 1980; Yung and Cudkowicz, 1978b).

1.2.3. ZYMOSAN

Zymosan is an insoluble protein-polysaccharide complex, and is isolated from fungal cell walls, particularly of the yeast Saccharomyces cerevisiae (Burger et al, 1980). The molecule shares some properties with bacterial endotoxins such as lipopolysaccharide; however, it does not appear to have any toxic effects in vivo (Cutler, 1960).

Zymosan has been widely used to activate the MPS in vivo (Cutler, 1960; Warr and Sljivic, 1974) and phagocytic cells in vitro

(Schnyder and Baggiolini, 1978, 1980), inducing enhanced phagocytic activity and stimulating rapid release of lysosomal enzymes (Baggiolini et al, 1982; Dean et al, 1979; Schnyder and Baggiolini, 1980). However, in contrast to the enzyme release induced by silica, which appears to be non-specific and reflects leakage of intracellular components from damaged cells (Schnyder and Baggiolini, 1978), zymosan has no toxic effect on phagocytes in vitro and has been shown to stimulate selective enzyme secretion. Thus there is no change in the rate of lysozyme secretion following ingestion of zymosan (Schnyder and Baggiolini, 1980), and while lysosomal hydrolases and some non-lysosomal neutral proteinases are released (Dean et al, 1979), enzymes such as lactate dehydrogenase accumulate intracellularly (Schnyder and Baggiolini, 1980). As zymosan is not digested within the phagocyte, its effects are persistent and do not depend on simultaneous phagocytosis (Dean et al, 1979); cells storing zymosan particles will not only release their current enzyme content, but may also synthesise and secrete other proteins and biologically active compounds, including oxygen metabolites and fatty acid oxidation products (Baggiolini et al, 1982; Gemsa, 1981; Seaman et al, 1982). Some of these materials and their relevance to immunological systems are described in section 1.3..

The stimulatory effect of zymosan on phagocytes is not due to the phagocytic process per se (Schnyder and Baggiolini, 1978), as ingestion of inert substances of comparable size is not stimulatory (Baggiolini et al, 1982; Dean et al, 1979; Riggi and DiLuzio, 1961). Ingestion of zymosan triggers a respiratory burst in phagocytic cells, accompanied by elevated glucose metabolism via the hexose monophosphate (HMP) shunt; using redox compounds which drive this

metabolic pathway in the absence of phagocytosis, Baggiolini et al (1982) and Schnyder and Baggiolini (1978, 1980) have shown that it is the increased shunt activity rather than the phagocytic event which activates the cell. Consequently, cells which already display elevated HMP shunt activity and are therefore in an activated state cannot be further stimulated by ingestion of zymosan (Baggiolini et al, 1982).

The effects of zymosan on immune responsiveness in vivo largely reflect its stimulatory activity on the MPS. Administration of zymosan to rats and rabbits has been shown to enhance humoral responses to sheep erythrocytes (Cutler, 1960, 1961); zymosan was still effective if given up to two days after the antigen, but could not affect the response after four days, by which time antibody was detectable. Increased spleen weight was observed in these animals, but whether this reflected cell recruitment or proliferation in situ was not determined. Lotzova and Gutterman (1979) demonstrated reduced spontaneous cytotoxic activity in mice treated with glucan, the active component of zymosan (Riggi and DiLuzio, 1961), and Seaman et al (1982) confirmed this suppressive effect in vitro, demonstrating that it was dependent on a phagocytic-adherent population.

1.2.4. LATEX

Latex particles are widely used as a biologically inert material in phagocytic assays and tests for mononuclear phagocyte function. Their ingestion has not been shown to influence phagocyte behaviour or metabolism, and consequently they are frequently used as negative control particles in studies on the effects of macrophage-active agents (Baggiolini et al, 1982; Dean et al, 1979; Schnyder and

Baggiolini, 1980).

1.3. SECRETORY PRODUCTS OF MONONUCLEAR PHAGOCYTES

From the preceeding section on macrophage-active agents, it is clear that the immunomodulating effects of carrageenan and zymosan cannot be solely attributed to direct toxicity towards the phagocytic population. Mononuclear phagocytes synthesise large amounts of protein, producing an array of enzymes and factors, and many of these are released from the cell, together with other metabolites. Some of these products are listed in Table 1.3.1.. Conditions for secretion vary; some enzymes are destined for intracellular functions, and may only be released from intact cells by non-specific leakage, while other products are released continuously, regardless of the functional state of the cell. In addition, some substances are only manufactured in response to specific stimuli such as phagocytosis, and are secreted shortly after synthesis. These secretory products, particularly those associated with phagocytosis, have been implicated as mediators of carrageenan- and zymosan-induced effects on immune responses, and some of them will now be considered in more detail.

1.3.1. BIOLOGICALLY ACTIVE PROTEINS

Phagocytosis and subsequent intracellular killing form one of the most primitive means of defence. In multicellular animals, this process has become a property of specialised cells; one of the major functions of a phagocytic cell in a higher organism is to engulf and degrade effete cells and invading micro-organisms. Phagocytes are

Table 1.3.1. Secretory products of mononuclear phagocytes

Biologically active proteins

arginase
lipoprotein lipase
lysozyme
lysosomal acid hydrolases: beta glucoronidase
beta galactosidase
acid phosphatase
acid ribonuclease
acid deoxyribonuclease
cytoplasmic neutral proteinases: plasminogen activator
collagenases
elastase
coagulation factors
complement components: C1, C2, C3, C4, C5
factor B
factor D
properdin
enzyme inhibitors
interferons (mainly alpha-IFN)
interleukin 1 (endogenous pyrogen)
tumour necrosis factor (cachectin)

Reactive metabolites of oxygen

superoxide anion O_2^-
hydrogen peroxide H_2O_2
free hydroxy radical $OH\cdot$
singlet oxygen $^1O_2$
hypohalous acids and hypohalide ions

Metabolites of polyunsaturated fatty acids

prostaglandins
thromboxanes
leukotrienes
HPETES (reactive intermediates with short half-life)

Low molecular weight products

cyclic AMP
nucleotide metabolites
platelet activating factor (lipid)

(Gordon, 1980; Nathan, 1987; Takemura and Werb, 1984; Unanue and Benacerraf, 1984d).

therefore equipped with a battery of digestive enzymes, specific for proteins, lipids, polysaccharides and nucleic acids, and these are contained within membrane-bound lysosomal vesicles to ensure efficient intracellular digestion without damaging the cell (Unanue and Benacerraf, 1984d). The process of phagocytosis is essentially similar to that observed in the Protozoa, the engulfed particle being enclosed in a portion of the plasma membrane to form a cytoplasmic inclusion known as a phagocytic vacuole or phagosome. On encountering a lysosome, the membranes of the two organelles fuse to yield a phagolysosome, in which the ingested particle is exposed to enzyme attack. Lysosomal contents may be released from the cell during phagocytosis if the two vesicles fuse before ingestion is complete; similarly, a process of regurgitation or reverse endocytosis may occur when phagocytes encounter non-ingestible material and extrude their lysosomes onto its surface (Goldstein et al, 1973; Unanue and Benacerraf, 1984d). Neither of these mechanisms is dependent on cell death. As noted in section 1.2., some ingested materials are resistant to degradation, and may persist within the phagolysosome for long periods. Cultured mouse macrophages storing zymosan particles or short fibre asbestos (but not latex beads) have been shown to release lysosomal enzymes in the absence of further extra-cellular particles (Dean et al, 1979), demonstrating that enzyme discharge may also be independent of the phagocytic event.

In addition to the leakage of lysosomal contents described above, it has been observed that mononuclear phagocytes may selectively release different biologically active proteins in response to particular stimuli. A number of factors may affect the biochemical characteristics of these cells; the animal species employed, the location of the cells in vivo, and the functional state of the

cells, which may be immature, resident, elicited or immunologically activated (Allison and Davies, 1975). In view of their availability and the ease with which they may be manipulated in vivo, many studies have employed cells isolated from the murine peritoneal cavity; however, rat and guinea pig peritoneal cells have also been used, together with alveolar cells from these species (Allison and Davies, 1975). Comparison of these populations and human peripheral blood monocytes has revealed considerable variation with respect to their secretory profiles, reflecting the heterogeneity of these cells mentioned in section 1.1..

Regulation of the synthetic and secretory behaviour of mononuclear phagocytes is poorly understood, although it is apparent that secretion of different products is controlled independently (Gordon, 1980). A major secretory product of mononuclear phagocytes is lysozyme, which may form up to twenty-five per cent of total extracellular protein, and is released at a constant rate independent of the functional state of the cell. This enzyme does not appear to be stored intracellularly, and its manufacture only stops after inhibition of all protein synthesis. Constitutive lysozyme secretion serves as a marker for mononuclear phagocytes and polymorphonuclear leucocytes as it is absent from lymphoid cells (Gordon, 1980). In contrast, secretion of neutral proteinases such as plasminogen activator, collagenase and elastase is elevated following stimulation of the cell, and may serve as a marker for activation of the phagocytic population (Gordon, 1980). Neutral proteinases released from the cell in vivo can attack extracellular substrates, and if generated constitutively like lysozyme, or in excess of plasma inhibitors which limit their range of action, would cause extensive tissue damage, degrading connective tissue and

matrix proteins (e.g. collagen and myelin). Their production must therefore be regulated, secretion being induced in response to specific stimuli. This restriction does not apply to lysozyme, which attacks microbial cell walls and has no substrate among self components.

In the absence of any stimulation, lysosomal acid hydrolases are predominantly intracellular. However, prolonged selective release of lysosomal enzymes from viable phagocytes *in vitro* has been shown to be induced by agents associated with chronic inflammatory responses, such as asbestos, carrageenan, zymosan and immune complexes (reviewed by Allison and Davies, 1975; Davies and Allison, 1976b). This may reflect an important mechanism in the pathogenesis of such inflammation *in vivo*, generating chemotactic and inflammatory factors such as kinins and cleavage products of complement, and contributing to the degradation of connective tissue and cartilage (Davies and Allison, 1976a). Zymosan in particular may induce enzyme release by generating C3b, which can then bind to and activate cells bearing the appropriate receptor (Goldstein *et al*,1973). It has been noted that enzyme release following exposure to carrageenan is delayed; this may reflect the ingestion of solubilised carrageenan by pinocytosis, which is less rapid than phagocytic engulfment (Allison and Davies, 1975; Davies and Allison, 1976b). In contrast, ingestion of silica particles *in vitro* results in massive non-specific release of enzymes from all intracellular compartments, including the cytoplasmic enzyme lactate dehydrogenase, reflecting death of the phagocyte and release of cellular contents (Davies *et al*, 1974).

Phagocytes can also be induced to release lysosomal enzymes in the absence of a phagocytic stimulus, following exposure to products

from activated lymphocytes. Such secretion may reflect the tissue damage produced during delayed hypersensitivity reactions, in which factors released by T lymphocytes are responsible for recruitment and activation of mononuclear phagocytes at the site of inflammation (Pantalone and Page, 1975).

Conversely, cytokines released by mononuclear phagocytes influence the behaviour of surrounding leucocytes. Several such factors promoting growth and differentiation of other cell types have been identified, including interferon (largely alpha-IFN although some production of gamma-IFN has been reported) (Balkwill, 1985), and factors acting on fibroblasts, endothelial cells, and smooth muscle cells. Thymocyte differentiation factor is produced by mononuclear phagocytes, and proliferation and differentiation of various haemopoietic lineages are induced by glycoprotein regulators released from these cells, collectively referred to as colony stimulating factors (Moore et al, 1979; Watson and Prestidge, 1983). However, there is some controversy over the relationship of these molecules, and it has been suggested that some monokines may display more than one biological activity (Unanue and Benacerraf, 1984d). A good illustration of this phenomenon is provided by interleukin 1 (IL-1), previously known as lymphocyte activating factor (LAF). Originally identified by its ability to activate thymocytes whilst being unable to support the growth of IL-2-dependent cell lines, IL-1 is now known to have stimulatory effects on the growth and differentiation of numerous cell types involved in inflammation and development (Oppenheim and Gery, 1982), and is believed to be identical to endogenous pyrogen, the monocyte-derived mediator of fever (Dinarello, 1984). Interleukin 1-alpha and IL-1-beta are now recognised, encoded by two distinct genes, and a third IL-1 species

has been reported; whether these share the same biological activities is not yet clear, although there is evidence that the alpha and beta forms share the same receptor (reviewed by Oppenheim et al, 1986). Production of IL-1 is not restricted to cells of the MPS, but has been demonstrated for many other cell types, including normal and transformed B lymphocytes, keratinocytes and epithelial cells. Most of these cells only synthesise and release IL-1 following stimulation (Durum et al, 1985), including direct cell contact and exposure to other cytokines, and secretion can be inhibited by some immunosuppressive agents such as corticosteroids and prostaglandins (Oppenheim et al, 1986). Specific modulators of IL-1 have also been described, including an inhibitor produced by macrophages, suggesting that feedback mechanisms may exist to regulate IL-1 activity. In view of its diverse effects throughout the body, IL-1 should perhaps be regarded not only as a local cytokine, but also as a systemic intercellular signal, and may therefore be classified as a hormone even though it is not produced by discrete endocrine glands (Dinarello, 1984; Oppenheim et al, 1986).

1.3.2. REACTIVE METABOLITES OF OXYGEN

The process of phagocytosis is associated with dramatic changes in oxidative metabolism and an increase in glucose catabolism via the HMP shunt (Rossi et al, 1980). This process is referred to as the respiratory burst, and reflects perturbation of the plasma membrane; internalisation of the triggering stimulus is not an absolute requirement, as a similar response is provoked by membrane-active agents in the absence of phagocytosis (Rossi et al, 1980) or by attachment of particles to the plasma membrane (Roos and Stijl-

Neijenhuis, 1980). Similarly, some parasites such as Toxoplasma and Leishmania can be ingested without triggering a respiratory burst (Berton and Gordon, 1983).

The enzyme system primarily responsible for the respiratory burst is NADPH oxidase (Adams and Hamilton, 1984), although NADH oxidation may also occur to a lesser extent (Rossi et al, 1982; Takemura and Werb, 1984). The enzyme is bound to the plasma membrane of the phagocyte, and it has been suggested that enzyme activation will only occur in those parts of the membrane receiving appropriate stimulation (Rossi et al, 1982). De novo synthesis of NADPH oxidase in response to stimulation is unlikely in view of the rapid appearance of oxygen metabolites, which can be detected within seconds and may be produced for up to three hours (Adams and Hamilton, 1984; Berton and Gordon, 1983). However, mouse peritoneal macrophages have been shown to enter a refractory state for several hours following stimulation, and this may reflect modulation or exhaustion of the membrane enzyme (Berton and Gordon, 1983).

Following activation, NADPH oxidase reduces molecular oxygen (O_2) to water by donating electrons from an electron transport chain; this is reflected in the increased oxygen consumption of phagocytosing cells (Weiss and LoBuglio, 1982). However, partial reduction of oxygen gives rise to a battery of highly reactive intermediates including hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl and perhydroxyl radicals ($OH\cdot$ and $HO_2\cdot$) and singlet oxygen ($^1O_2$) (Klebanoff, 1980); these may be released from the cell and have been implicated as both intracellular and short-range extracellular mediators of anti-microbial (Nathan, 1982; Rossi et al, 1980) and anti-neoplastic activity (Takemura and Werb, 1984; Weiss and LoBuglio, 1982).

Mononuclear phagocytes display a heterogeneous profile with respect to their release of reactive oxygen intermediates (ROI), and enhanced generation of these metabolites may be a marker of activation for these cells (Berton and Gordon, 1983; Nathan, 1982). Thus murine peritoneal macrophages require activation *in vivo*, for example by microbial vaccines, before they will release ROI in response to a phagocytic stimulus *in vitro*, while phagocytosing monocytes freshly isolated from human peripheral blood release H_2O_2 at rates comparable to those obtained using granulocytes (Nathan, 1980, 1982). Following activation of the phagocyte, for example by infection with BCG, levels of ROI generated may exceed those obtained from resting cells in response to the same phagocytic challenge (Johnston *et al*, 1980), in parallel with increased cytotoxicity towards both intra- and extracellular targets (Nathan, 1982); however, such enhanced activity is not induced by non-microbial inflammatory agents such as thioglycollate (Nathan, 1982; Weiss and LoBuglio, 1982). The capacity to produce ROI in response to stimulation is also increased following exposure to gamma-IFN (Adams and Hamilton, 1984), and may therefore be manifest during immune responses involving T lymphocytes. However, there is evidence that the enhanced respiratory burst induced by lymphocyte factors may reflect differentiation of the existing phagocyte population, rather than a shift in this population as observed in inflammatory states (Nathan, 1982). Lymphokine-mediated enhancement of the respiratory burst is a transient event, being sustained for a maximum of five days *in vitro* (Nakegawara *et al*, 1982); the self-limiting nature of this response may reflect a regulatory feedback mechanism. The oxidative metabolism of mononuclear phagocytes may also be down-regulated; long-term exposure to physiological doses of

hydrocortisone has been shown to decrease production of H_2O_2 by human monocytes, and this may be one factor in the reduced host resistance to microbial pathogens observed with pharmacological doses of this anti-inflammatory drug (Nakegawara et al, 1982).

The importance of ROI in protection against microbial disease is demonstrated by the susceptibility to infection of individuals with a defect in this pathway, for example in chronic granulomatous disease (Takemura and Werb, 1984). The mechanisms of ROI-mediated cytotoxicity have been most widely studied using *in vitro* stimulation of polymorphonuclear leucocytes, and care should be exercised both in extrapolating these findings to the *in vivo* situation and in applying them to other cell types. In particular, it should be noted that the presence of a metabolite need not imply that it has an active role, but may reflect concomitant production of more active intermediates. Much attention has been focussed on production of the superoxide anion by mononuclear phagocytes, although the activity of this species in aqueous media is restricted by its rapid dismutation; up to eighty per cent of the H_2O_2 generated during the respiratory burst is now believed to derive from spontaneous or catalytic dismutation of O_2^- (Weiss and LoBuglio, 1982). A strong oxidant, H_2O_2 shows slow reactivity with organic substrates, but reacts rapidly with transition metal complexes to yield free radicals. Stimulation of monocytes has been shown to induce discharge of the lysosomal enzyme myeloperoxidase, which in the presence of H_2O_2 can catalyse oxidation of halide ions to generate toxic oxyhalides and hypohalous acids (Takemura and Werb, 1984; Weiss and LoBuglio, 1982). These powerful oxidising compounds react rapidly with organic molecules including sulphydryl groups, amino-acids and unsaturated carbon groups, and can

halogenate susceptible target molecules in bacterial cell walls (Weiss and LoBuglio, 1982). Adjacent host cells and tissue components will be non-specifically exposed to ROI from activated phagocytes, and there is clearly potential for inadvertent damage; for example, collagen and hyaluronic acid are particularly susceptible to attack by O_2^- (Freeman and Crapo, 1982). Anti-oxidant defences have therefore evolved to protect cells from damage, including enzyme systems to degrade the reactive species, such as catalase, peroxidase, superoxide dismutase and the glutathione redox enzyme system (Freeman and Crapo, 1982; Weiss and LoBuglio, 1982). However, extracellular fluids contain little catalase or dismutase activity to destroy radicals, and protection must therefore be provided by free radical scavengers such as vitamin E, ascorbate and beta-carotene (Freeman and Crapo, 1982). Reactivity of the different oxygen metabolites determines their diffusion distances outside the cell of origin; the highly reactive hydroxyl radical will not diffuse far, while H_2O_2 is more likely to escape and exert toxic effects distal to the phagocyte. There is some evidence for systemic protection by scavengers circulating in the plasma, such as uric acid, which can protect haemoglobin against oxidation by H_2O_2 (Freeman and Crapo, 1982). It is interesting to note that some neoplastic cells also possess active anti-oxidant enzyme systems; inhibition of these enzymes renders the tumour cell more susceptible to lysis by small numbers of phagocytes or small amounts of H_2O_2 (Nathan, 1982; Weiss and LoBuglio, 1982). Similarly, some antibiotics and anti-neoplastic drugs can generate ROI, and their chemotherapeutic effects and cytotoxic side-effects may reflect this activity (Freeman and Crapo, 1982). Manipulation of this phenomenon may be of value in the therapy of neoplastic and infectious disease.

To date, little study has been made of the effects of ROI on lymphocyte responses. As mentioned previously, these intermediates can alter or destroy cellular function, and H_2O_2 has been shown to suppress some lymphocyte responses *in vitro*; proliferation of murine splenocytes induced by the mitogen Concanavalin A is suppressed by peritoneal macrophages secreting H_2O_2, and can be restored by addition of catalase to the system (Metzger *et al*, 1980). Whether this reflects toxicity towards the responding lymphocytes or forms part of a more complex regulatory mechanism is unclear at present. Oxygen metabolites have also been implicated in spontaneous phagocyte-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). Expression of membrane receptors for the Fc fragment of immunoglobulin enables mononuclear phagocytes to bind to antibody-coated (opsonised) target cells in a specific manner; interaction of the Fc receptor and its ligand triggers a respiratory burst, the products of which may be concentrated towards the target cell by virtue of its apposition to the activated enzyme site (Weiss and LoBuglio, 1982). A similar juxtaposition exists during interactions between lymphocytes and antigen-presenting cells, again involving receptor-ligand binding and plasma membrane activity, and it is reasonable to propose that ROI may be generated at this stage of the immune response. However, there is currently no direct evidence for this, and the possible role of these secretory products during immune induction remains to be determined.

1.3.3. METABOLITES OF POLYUNSATURATED FATTY ACIDS

Compared with other mammalian cells, the phospholipids comprising the membranes of mononuclear phagocytes are unusually rich in

esterified arachidonic acid (AA) (Takemura and Werb, 1984); up to twenty per cent of the fatty acid contained in rabbit alveolar macrophages and human peripheral blood monocytes is AA (Bonney et al, 1978; Davies et al, 1980), while twenty-five per cent of the total fatty acid in murine peritoneal macrophages is of this type (Scott et al, 1980). In view of the role of AA as a major substrate for the generation of prostaglandins and related compounds, much attention has therefore focussed on the ability of mononuclear phagocytes to produce such metabolites.

Arachidonic acid can be obtained directly from the diet, or can be synthesised from linoleic acid by desaturation and chain elongation (Moncada, 1982). Little free AA exists within normal cells (Davies et al, 1980; Stenson and Parker, 1980); however, during phagocytosis large areas of plasma membrane are internalised and brought into contact with intracellular structures such as lysosomes. The lysosomal enzyme phospholipase A_2 (PLA_2) (and to a lesser extent phospholipase C, which is probably more important in platelets and mast cells [Stenson and Parker, 1980]) can attack phospholipids released by such membrane perturbation, liberating free AA (Bonney et al, 1980; Metcalfe and Kaliner, 1981; Moncada, 1982). Phospholipase enzymes are activated by changes in their chemical environment (Moncada, 1982) and are dependent on calcium ions (Ninneman, 1984). Liberation of fatty acid from the phospholipid pool is inhibited by anti-inflammatory steroid hormones, and it has been suggested that these drugs induce peptides which inhibit phospholipases (Hong and Levine, 1976; Samuelsson, 1983).

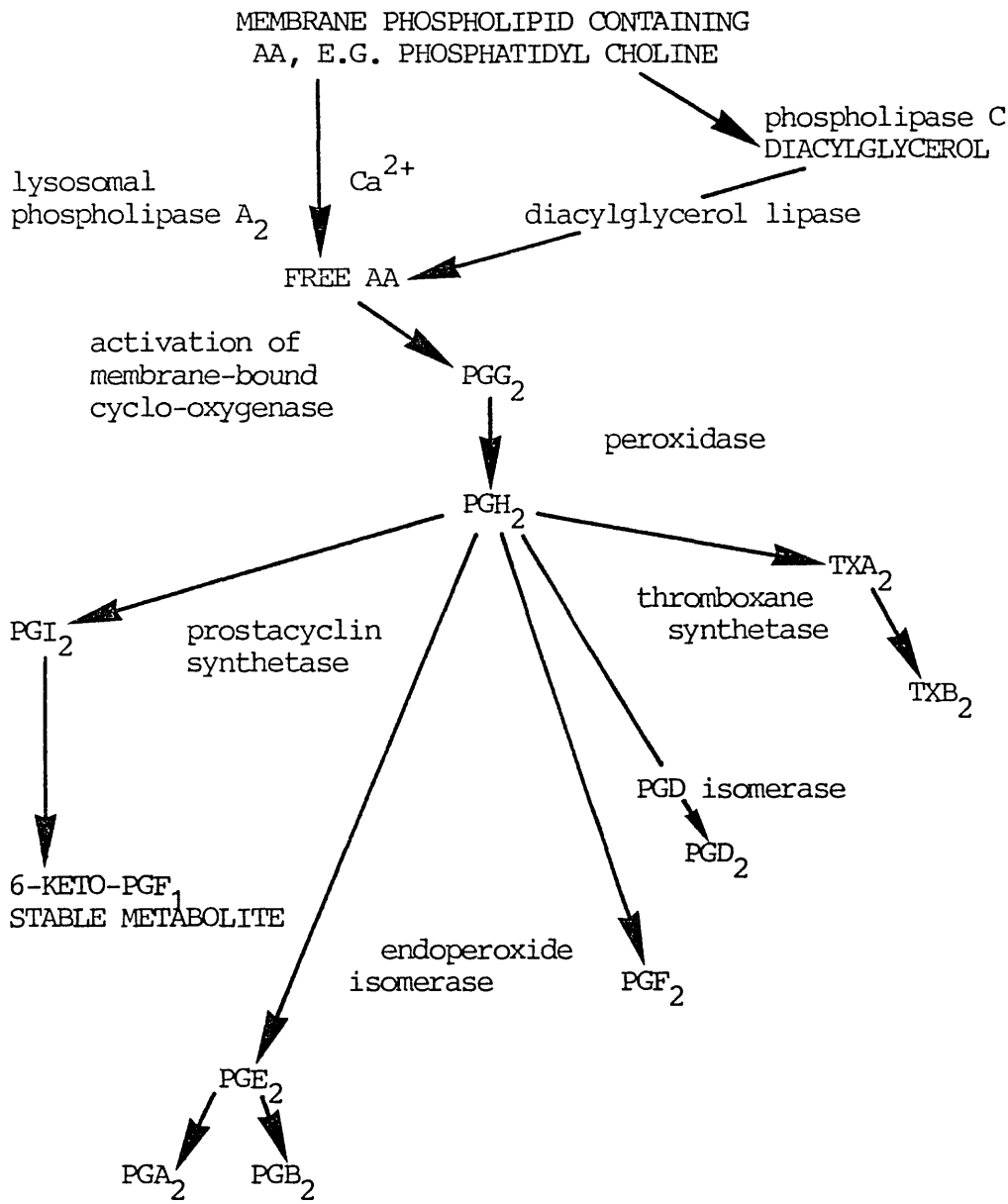
Free AA can be re-esterified, or may be metabolised via two separate oxidative enzyme pathways. The presence of AA induces substrate activation of membrane-bound cyclo-oxygenase, believed to

be located on the endoplasmic reticulum (Brune et al, 1978; Metcalfe and Kaliner, 1981). This enzyme catalyses oxygenation and cyclisation of fatty acids to the unstable endoperoxide intermediates PGG_2 and PGH_2, which then give rise to the prostaglandins (PG) and thromboxanes (TX) (Higgs and Salmon, 1979; Moncada and Vane, 1979); this metabolic pathway is represented in Figure 1.1.. Conversion of PGH_2 to prostaglandins can proceed spontaneously; however, production of prostacyclin (PGI_2) and the thromboxanes is dependent on the relevant catalytic pathway (Moncada and Vane, 1979), and some cells may therefore be unable to generate these metabolites. Cyclo-oxygenase is present in all mammalian cells and tissues except erythrocytes, although its activity may vary (Moncada, 1982). It is interesting to note that this enzyme is inactivated by peroxides (Metcalfe and Kaliner, 1981), and it may therefore promote formation of its own inhibitors. Cyclo-oxygenase is also inhibited by aspirin-like drugs (non-steroidal anti-inflammatory drugs), and this mechanism is believed to account for the therapeutic effects of these agents (Samuelsson, 1983; Vane, 1971).

Alternatively, oxidation of AA may be catalysed by a lipoxygenase enzyme. Several lipoxygenases exist, which catalyse peroxidation of different carbon atoms in the fatty acid molecule. These enzymes show a more restricted tissue distribution than cyclo-oxygenase, lipoxygenases specific for different positions being found in lung, platelets, blood vessel walls, epicardium and leucocytes (Moncada, 1982; Moncada and Vane, 1979). Mononuclear phagocytes contain 5-lipoxygenase (Takemura and Werb, 1984); the non-cyclised hydroxy acid generated in these cells is therefore called 5-hydroxy-eicosatetraenoic acid (5-HETE). The unstable hydroperoxy

Figure 1.1.

Arachidonic acid metabolism via the cyclo-oxygenase pathway



Adapted from
Moncada, 1982;
Stenson and Parker, 1980.

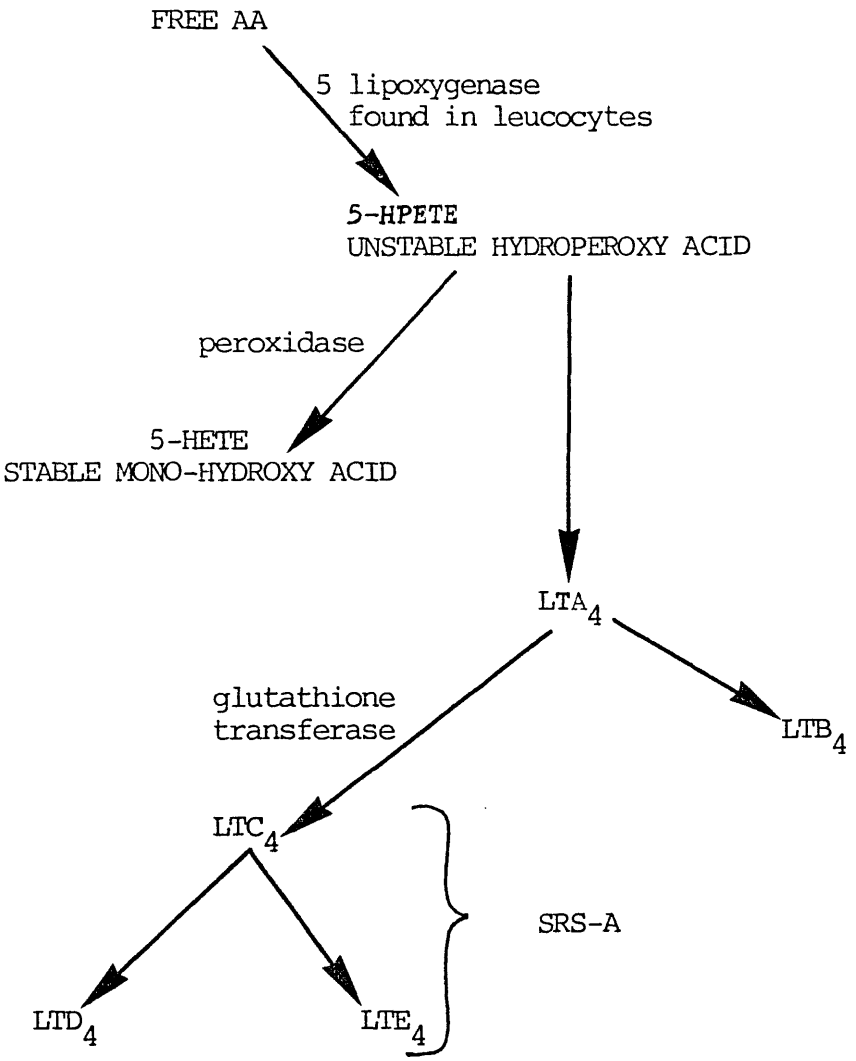
intermediate in this reaction can also be metabolised to the non-cyclised triene substances known as leukotrienes (LT), some of which are now known to account for the activity of slow-reacting substance of anaphylaxis or SRS-A (Moncada, 1982). This metabolic pathway is represented in Figure 1.2.; for the sake of clarity, the activity of 5-lipoxygenase only is considered. The term "eicosanoids" has been proposed to include both cyclo-oxygenase and lipoxygenase metabolites of 20-carbon atom fatty acids (Moncada, 1982).

The AA molecule (eicosatetraenoic acid) contains four double bonds, and is the most common substrate for the cyclo-oxygenase and lipoxygenase enzyme systems, giving rise to the bisenoic prostaglandins and thromboxanes such as PGE_2 and TXA_2, and leukotrienes of the 4-series such as LTC_4 (Metcalfe and Kaliner, 1981; Moncada, 1982). However, alternative natural substrates exist; thus dihomo-gamma-linoleic acid (eicosatrienoic acid) gives rise to prostaglandins and thromboxanes with only one double bond (e.g. PGE_1), while eicosapentaenoic acid generates trienoic cyclo-oxygenase products (e.g. TXA_3) (Metcalfe and Kaliner, 1981; Moncada and Vane, 1979). In general, these products show similar activities to the bisenoic metabolites of AA, although they may be less active (Moncada and Vane, 1979). As most studies have utilised bisenoic eicosanoids, the remainder of this section will concentrate on the oxidation products of AA.

From the brief outline above, it is clear that mononuclear phagocytes possess the catalytic machinery to generate bioactive lipid metabolites. The frequent membrane perturbations to which these cells are exposed through their phagocytic and antigen presenting activities provide ideal conditions for liberation of

Figure 1.2.

Arachidonic acid metabolism via the 5-lipoxygenase pathway



Adapted from
Moncada, 1982;
Stenson and Parker, 1980.

substrate and activation of the oxidative enzymes, and it is therefore not surprising to find that mononuclear phagocytes synthesise large amounts of PG, TX and LT. It has been estimated that up to fifty per cent of the total AA present in a mononuclear phagocyte may be oxygenated and released with no effect on the viability of the cell (Rouzer et al, 1980; Scott et al, 1980). Oxidative metabolites are not stored within the cell, but are released immediately after synthesis, and so form a major group of inducible phagocyte secretory products.

Most studies have concentrated on fatty acid metabolism by human peripheral blood mononuclear cells or macrophages isolated from rodent alveolar and peritoneal fluids. Human monocytes produce mainly PGE_2 and TXA_2 in response to a range of stimuli (Lewis, 1983), while mouse peritoneal macrophages phagocytosing asbestos, antigen-antibody complexes or zymosan particles in vitro have been shown to secrete PGE_2 and 6-keto-PGF_1, the stable break-down product of PGI_2 (Bonney et al, 1978, 1980; Davies et al, 1980; Humes et al, 1977). Ingestion of latex beads failed to induce PG secretion. Similarly, rabbit alveolar macrophages released PGE_2 together with smaller amounts of PGD_2, PGF_2 and 6-k-PGF_1 in response to in vitro challenge with zymosan or heat-killed bacteria; the time-course of PG secretion paralleled that of lysosomal enzyme release (Hsueh et al, 1980). In all cases, PG release was dependent on internal events subsequent to ingestion rather than on phagocytosis per se, and did not reflect cell damage. This observation is supported by studies using the fungal metabolite cytochalasin B, which has been shown to induce synthesis of PG and TX by mouse peritoneal macrophages (Brune et al, 1978). Within the cell, membrane structures are isolated from each other by a network of microfilaments; destruction of this

cytoskeleton using cytochalasin B inhibits phagocytosis but promotes intracellular membrane interactions. Thus arachidonate-rich phospholipids may be exposed to lysosomal phospholipases and cyclo-oxygenase on the endoplasmic reticulum, resulting in oxidative metabolism and generation of eicosanoids. In the experiments referred to above, secretion of eicosanoids was accompanied by the formation of non-endocytic intracellular vacuoles consistent with cytoskeletal disruption (Brune et al, 1978).

There appears to be a parallel between release of fatty acid metabolites and discharge of lysosomal contents (as described in section 1.3.1.), both events being specifically stimulated by inflammatory agents (Baggiolini et al, 1982; Bonney et al, 1980; Davies et al, 1980). This is not unexpected, as fatty acid oxidation is dependent on the rate-limiting step of substrate release, catalysed by phospholipase enzymes released from lysosomes. In contrast, mononuclear phagocytes exposed to silica particles have been found to produce massive amounts of fatty acid oxidation products accompanied by generalised membrane catabolism, and leakage of intracellular contents preceeding cell death (Gemsa et al, 1978). Interestingly, resident murine peritoneal macrophages generate more fatty acid metabolites in response to challenge with zymosan than do cells elicited with thioglycollate, releasing up to five times as much AA from the phospholipid pool (Bonney et al, 1978, 1980; Scott et al, 1982). It is possible that elicited cells have already undergone a burst of fatty acid metabolism in vivo in response to the irritant, and are subsequently refractory to further stimulation, suggesting that the capacity for fatty acid metabolism may be a function of cell activation. This may reflect activation and subsequent inactivation or exhaustion of phospholipases (Bonney

et al, 1978, 1980) and the oxidative enzymes (Davies et al, 1980); inhibition of cyclo-oxygenase by its own products may be of relevance here (Metcalfe and Kaliner, 1981).

Relative proportions of the different eicosanoids released following stimulation of a mononuclear phagocyte vary according to the physiological site of the cell. Thus while mouse peritoneal macrophages ingesting zymosan generate primarily cyclo-oxygenase products, stimulated pulmonary macrophages from the same species are a rich source of lipoxygenase metabolites (Rouzer et al, 1980, 1982). Release of PGE_2, 6-k-PGF_1 and LTC_4 from resident murine peritoneal macrophages stimulated with zymosan in vitro has been reported in the approximate ratio 50:15:5 (Rouzer et al, 1980), though this may vary according to the assay conditions and means of stimulation.

Eicosanoids are released in vivo following tissue irritation or injury, and are present in many inflammatory responses in mammalian species (Higgs, 1982). Stimulation of fatty acid oxygenation in mononuclear phagocytes following exposure to inflammatory agents in vitro is consistent with the presence of large numbers of such cells at sites of inflammation. In addition to their role as secretors of AA metabolites, mononuclear phagocytes are themselves sensitive to these products, providing a feed-back mechanism to control the inflammatory response. Inflammatory exudates have been shown to contain a range of eicosanoid activities, and it has emerged that while some of these are pro-inflammatory and exacerbate the response (e.g. PGE_2) (Moncada and Vane, 1979; Takemura and Werb,1984), others (e.g. PGF_2, TXA_2) have the opposite effect (Lewis, 1983). The course of an inflammatory response will therefore be dictated by the balance of these activities, providing considerable scope for

regulation.

In addition to phagocytosis, the oxidative enzymes are activated by some mitogens (endotoxin or LPS) and by lymphokines released from activated T lymphocytes (Higgs, 1982; Stenson and Parker, 1980). Mononuclear phagocytes are active producers of eicosanoids, particularly E-series PG (Lewis, 1983), and although there are reports of T lymphocyte-derived PGE, these probably reflect insufficiently rigorous cell depletion, with consequent contamination of the lymphoid fraction with monocytes or macrophages. Specific receptors for PG and LT have been demonstrated on mononuclear cells (Bourne et al, 1974; Gemsa, 1981; Goodwin et al, 1979; Lewis, 1983); cells of the immune system are therefore both sources of and sensitive to fatty acid metabolites, indicating that such products may be physiologically important modulators of immune responses (Goodwin et al, 1977; Leung and Mihich, 1980). To date, most studies in this area have focussed on cyclo-oxygenase products, particularly PG of the E and F series. Several workers have described the immunosuppressive properties of various PG (reviewed by Goodwin and Webb, 1980); however, it should be noted that different types or doses of PG may have different or even opposing effects, as suggested by Belldegrun et al (1981). For example, Mertin and Stackpoole (1981) have demonstrated that at low concentrations E-series PG are positive mediators of the inductive phase of cell-mediated immunity in vivo, while higher concentrations inhibit this process. Whether such effects are due to different target populations, for example activity towards helper or suppressor T lymphocytes, or whether they reflect opposing effects upon the same cell, perhaps via different receptors, remains to be determined.

1.4. OBJECTIVES OF THIS STUDY

In summary, it is clear that cells of the mononuclear phagocytic system play a complex and integral role in immune responses. To assess this role further, the involvement of such accessory cells in two *in vitro* systems was studied:

a) the generation of cytotoxic effector cells during a one-way murine mixed leucocyte culture (MLC)

b) the expression of non-specific spontaneous cytotoxic activity by human peripheral blood mononuclear cells

The accessory cells were functionally defined by their adherent properties, and the effects of macrophage-active agents and involvement of some cyclo-oxygenase products were examined.

CHAPTER TWO

The role of accessory cells in the generation of cytotoxic T lymphocytes in the murine mixed leucocyte culture

2.1. INTRODUCTION

As mentioned in section 1.1.2., T lymphocytes can be divided into four populations based on functional and phenotypic criteria. Of these, helper (T_H) and cytotoxic T lymphocytes (CTL) are most relevant to the current study.

The role of the major histocompatibility complex (MHC) in T cell activation has already been described (section 1.1.2.), restriction by class I or class II gene products correlating with T cell surface expression of T8 (murine Lyt 2, human CD8) and T4 (murine L3T4, human CD4) antigens respectively (Littman, 1987). In general, T lymphocytes expressing T4 display helper activity, proliferating in response to antigenic determinants on the surface of class II positive antigen-presenting cells (APC), while T8 positive T cells usually mediate effector functions (Wagner et al, 1980). However, T4 positive cytotoxic T lymphocytes restricted by class II MHC molecules have been reported, and it has recently been suggested that such cells may have an important regulatory function during later stages of an immune response, terminating T_H activation by destroying class II positive APC (Braakman et al, 1987). In addition, it should be noted that although both classes of T lymphocyte are specifically activated, they may subsequently co-operate in a non-specific manner; T_H activated by one antigen may therefore provide "bystander help" for cytotoxic cells responding to a different antigen.

In the whole animal, T lymphocytes are educated by MHC molecules expressed on the APC; consequently the T cell repertoire develops in the presence of syngeneic MHC gene products. For this reason, clones

of T cells which respond to antigen in the context of foreign (allogeneic) MHC molecules are not readily detected following conventional immunisation protocols (Unanue and Benacerraf, 1984a). However, T cells reactive with allogeneic MHC products per se, in the absence of associated antigen, are easily demonstrable. In contrast to the MHC restriction observed for other polypeptide antigens, recognition of these foreign MHC products does not require co-recognition of self MHC determinants, suggesting that at the level of the T cell antigen receptor, allogeneic MHC determinants may be seen as modified self molecules (Nabholz and MacDonald, 1983). Such alloreactivity is responsible for the transplantation reactions observed following grafting of incompatible tissue.

The strong reactivity of T lymphocytes for allogeneic MHC antigens is somewhat surprising; it has been estimated that approximately one T cell in every thousand will respond to antigen in association with syngeneic MHC molecules, compared with up to one hundred cells per thousand responding to foreign MHC molecules per se (Alberts et al, 1983; Ford et al, 1975). The reason for this high proportion of alloreactive T lymphocytes is unknown, but there is evidence that some self-restricted antigen-specific T cell clones show cross-reactivity for allogeneic targets (Levy et al, 1980; Unanue and Benacerraf, 1984a), supporting the hypothesis that incompatible MHC is recognised as altered self.

Although little specific cytotoxic activity is apparent in non-immunised animals (Nabholz and MacDonald, 1983; Nelson, 1981), antigen-specific CTL are rapidly generated following immunisation with allogeneic cells, with syngeneic cells modified by hapten or viral infection, or with syngeneic tumour cells or MHC-identical cells which differ at minor histocompatibility loci. Cytolytic

activity is transient, with concomitant development of a specific anamnestic response (Nabholz and MacDonald, 1983); triggering this immunological memory may be essential for detection of weak cell-mediated cytotoxic responses such as those directed against minor histocompatibility antigens (MacDonald, 1982). It has been suggested that acquisition of lytic activity is a state of terminal differentiation for CTL; however, single-cell experiments have demonstrated that a lymphocyte which has successfully lysed its target cell may go on to divide and form a clone, or may revert to a smaller non-lytic form which is responsible for antigen-specific memory (MacDonald, 1982; Nabholz and MacDonald, 1983).

2.1.1. THE MIXED LEUCOCYTE CULTURE

Cell-mediated cytotoxic responses have been implicated in the pathology of allograft and tumour rejection and some autoimmune diseases, in elimination of infection, and in immune surveillance against neoplastic transformation and senescence. As it is difficult to study such a complex phenomenon *in vivo*, several methods have been developed to assess this activity *in vitro* by measuring effector cell proliferation and cytotoxic activity in response to antigenic challenge. In view of the strong immunogenicity of incompatible MHC molecules, these are frequently used as test antigens to study the cellular and genetic bases of cell-mediated immunity.

Culture of naive T lymphocytes with cells differing at the MHC results in a proliferative response referred to as a mixed leucocyte reaction (MLR). Proliferation occurs largely in the T4+ population in response to incompatible class II molecules, and can be quantitated by measuring the uptake of radio-labelled precursors of

DNA, RNA or protein. In practice, proliferation of one population is inhibited by irradiation or treatment with mitomycin C; these cells then serve as the antigen (= stimulator cells) in a one-way mixed leucocyte culture (MLC) (Bradley, 1980; Nelson, 1981). Proliferation of the responder population in a primary MLC is maximal after 72-96 hours; subsequent exposure to the same stimulator cell antigens yields a more rapid maximal response in approximately 48 hours. This peak of T_H proliferation precedes maximal development of MHC class I-directed cytotoxic activity in the T8+ population by about 24 hours, after which the response diminishes in the absence of further stimulation (Nabholz and MacDonald, 1983). Specific effector cell competence in the form of cytotoxic activity is assessed by mixing the putative effector lymphocytes with a population of target cells and measuring target cell damage, either by vital staining or by radio-labelling; a reliable method which was adopted in the current study is to pre-label the target population with a suitable isotope such as sodium ^{51} chromate, which is released in a non-usable form after target cell death. The MLC and subsequent assay for cell-mediated cytotoxicity are believed to represent an <u>in vitro</u> analogue of events occurring during transplantation reactions, and form a convenient approach to determine the requirements for generation and activation of CTL.

2.1.2. MECHANISM OF T CELL-MEDIATED CYTOTOXICITY

Although most work has been done using alloreactive CTL, the results obtained regarding their lytic mechanism are believed to apply to self-restricted effector cells (Henney, 1980).

Cytolysis mediated by T lymphocytes can be divided into three stages (reviewed by Henkart, 1985). In the initial recognition or

binding stage, receptors on the CTL bind specifically to target cell antigens, and a strong adhesion rapidly develops between the two cells. Accessory molecules on the surface of both effector and target cells are believed to enhance adhesion by non-specific interaction (Henkart, 1985); these include T8, which may bind monomorphic determinants on MHC class I molecules (Littman, 1987), and the lymphocyte function-associated (LFA) antigens (Springer et al, 1987). Binding to the target cell is independent of antibody and serum complement, but requires magnesium ions. Target cell adhesion is a metabolically active process, and experiments with cytochalasin B and colchicine indicate microtubular involvement (Henney, 1980). Binding is accompanied by rearrangement of cytoplasmic organelles within the effector cell (Henkart, 1985), polarisation of CTL actin, and rapid movements of the CTL plasma membrane in the contact area (Nabholz and MacDonald, 1983).

During the second stage of the lytic process, the CTL inflicts irreversible damage on the target cell; this has been referred to as the lethal hit. This process is dependent on calcium ions, but does not require protein synthesis (Unanue and Benacerraf, 1984b). As lysis is specific and unidirectional, with non-involved bystander cells and the CTL itself remaining undamaged, target cell killing is unlikely to result from release of a soluble toxin from the effector cell (Nabholz and MacDonald, 1983); however, it has recently been demonstrated that binding to the target cell induces local exocytosis of cytoplasmic granules by the CTL (Henkart, 1985). These granules are rich in a pore-forming protein known as perforin or cytolysin, which shows some homology with terminal complement components and can bind to membrane lipids (Henney, 1980). Following detachment of the CTL from its target, polymerisation of this

protein within the plasma membrane of the target cell results in formation of ring-shaped lesions or pores (Dennert and Podack, 1983; Podack and Konigsberg, 1984; Young et al, 1986), which allow non-specific electrolyte exchange and ion equilibration across the plasma membrane, leading to osmotic swelling of the cell. In the final stage of the cytotoxic mechanism, referred to as killer cell-independent lysis, such permeability changes ultimately cause rupture of the plasma membrane and death of the target cell (Henkart, 1985; Henney, 1980; Unanue and Benacerraf, 1984b). By this stage, the effector lymphocyte is available to enter a new lytic cycle, and such cells have been shown to interact sequentially with several targets (Nabholz and MacDonald, 1983).

2.2 MATERIALS AND METHODS

2.2.1. MATERIALS

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2.2.1. MATERIALS

2.2.1.1. Animals

Inbred CBA female mice were used to provide responder cells for the mixed leucocyte culture (MLC), and to provide peritoneal and splenic macrophage populations.

Inbred Balb/c female mice were used to provide stimulator cells for the MLC, peritoneal and splenic macrophage populations, and thioglycollate-elicited peritoneal cells for use as target cells in the cytotoxicity assay.

Inbred C57BL female mice were used to provide stimulator cells for the MLC, peritoneal and splenic macrophage populations, and thioglycollate-elicited peritoneal cells for use as target cells. These mice were also used to passage EL4 lymphoma target cells.

Inbred DBA/2 male and female mice were used to passage P815Y mastocytoma target cells.

All mice were bred and maintained in the Animal Department at St. Mary's Hospital Medical School, and were used between 12 and 20 weeks of age.

2.2.1.2. Hibitane

0.5% chlorhexidine in 70% industrial methylated spirit ("Hibitane") was obtained from the Pharmacy at St. Mary's Hospital.

2.2.1.3. Culture media

2.2.1.3.1. Hanks' balanced salt solution

Hanks' balanced salt solution (HBSS) powder, without sodium bicarbonate, was obtained from Flow Laboratories, Irvine, Scotland. This was dissolved in deionised distilled water at a final concentration of 9.91 grams per litre. The pH was adjusted to 7.2 with either 0.1 molar hydrochloric acid or 0.1 molar sodium hydroxide, and the medium

was sterilised by filtration through a Sartorius filter, pore size 0.2 μm. The medium was then aliquoted into 500 ml bottles and stored at 4°C until required, when foetal calf serum (FCS) was added to give a final concentration of 5% v/v.

2.2.1.3.2. RPMI 1640

RPMI 1640 powder with glutamine, without sodium bicarbonate, was obtained from Flow Laboratories, and was made up as follows:

RPMI 1640 powder	10.43 g
Sodium bicarbonate	2.00 g
HEPES	3.50 g
Deionised distilled water	1000.00 ml

After all the solids were dissolved, the pH was adjusted to 7.2 and the medium was sterilised by filtration, as described in section 2.2.1.3.1. It was then aliquoted into 500 ml bottles and stored at 4°C until required, when FCS was added to give a final concentration of 5%, 10%, or 20% v/v. For sterile work, the antibiotic gentamicin was added to the medium.

2.2.1.3.3. Gentamicin

Gentamicin sulphate was obtained as Cidomycin from Roussel Laboratories Limited, London, at 80 x 10^3 I.U. in 80 mg in 2.0 ml for injection. This solution was added to culture medium at 0.125 ml per 500 ml medium, to give a final concentration of 10 I.U. per ml.

2.2.1.4. Foetal calf serum

Sterile foetal calf serum (FCS) was supplied by Sera-Lab, Crawley Down, Sussex. Several batches were screened before one was selected, on the basis of its lack of toxicity and its ability to support murine cells in culture without influencing their immune responses.

Frozen batches of the chosen serum were thawed and heat-inactivated at 56°C for 30 min, before being aliquoted into 12.5 ml fractions for storage at -20°C until required.

2.2.1.5. White cell counting fluid

A 1% solution of glacial acetic acid in distilled water was prepared, to which was added a pinch of gentian violet, sufficient to lightly colour the solution. This was stored at room temperature. White cell counting fluid prepared in this manner lyses red blood cells, while fixing and lightly staining nucleated cells.

2.2.1.6. Viability stain

Viability was assessed as described by Takasugi (1971). Fluorescein diacetate-ethidium bromide stain was prepared by dissolving a small quantity of fluorescein diacetate (FDA) in a few drops of acetone, adding this dropwise to saline until the solution just turned cloudy, and then adding an equivalent amount of ethidium bromide (EB). This mixture was prepared shortly before use, and was then stored at 4°C in the dark.

2.2.1.7. 2-mercaptoethanol

2-mercaptoethanol was obtained from BDH Chemicals Limited, Poole, Dorset. A solution of 5×10^{-4} molar 2-ME in HBSS or RPMI was prepared for use in cell cultures, and this was further diluted on addition to give a final concentration of 5×10^{-5} molar 2-ME in the culture fluid. A fresh preparation of 2-ME was made up for each experiment.

2.2.1.8. Saline

2.2.1.8.1. Physiological saline

Sodium chloride was dissolved in distilled water to give a 0.85% w/v solution. This was sterilised by autoclaving at 120°C and

15 p.s.i. for 15 min.

2.2.1.8.2. Phosphate buffered saline

Phosphate buffered saline was prepared as follows:

Solution A	NaCl	8.00 g
	K Cl	0.20 g
	Na_2HPO_4	1.15 g
	KH_2PO_4	0.20 g
	Distilled water	800.00 ml
Solution B	$Mg\ Cl_2\ 6H_2O$	0.10 g
	Distilled water	100.00 ml
Solution C	$Ca\ Cl_2$	0.10 g
	Distilled water	100.00 ml

The three solutions were autoclaved separately at 120°C, 15 p.s.i. for 15 min, and were then mixed at the ratio of 8:1:1 v/v, to give one litre of sterile PBS.

2.2.1.9. Macrophage-active agents

2.2.1.9.1. Silica

Fumed silica, particle size 0.014 μm, batch number 95C-0319, was obtained from Sigma Chemical Company, St. Louis, U.S.A., and was stored at room temperature. For *in vitro* use, silica was hot-air sterilised at 160°C for 60 min, and was suspended at 1.0 mg/ml or 2.0 mg/ml in RPMI 1640 supplemented with 10% FCS. It was then incubated at 4°C for 24 hours to coat the particles with protein. As this protein coating is lost within a few days, particles were only treated 24 hr before required (O'Rourke *et al*, 1978).

2.2.1.9.2. Carrageenan

Types I to V carrageenan were obtained from Sigma Chemical Company as follows:

Type I	natural mixture of approximately 80% κ, 20% λ Batch number 116C-0394	C1013
II	mainly ι form Batch no. 84C-02651	C1138
III	purified κ form Batch no. 87C-0375	C1263
IV	purified λ form Batch no. 48C-0094	C3889
V	purified ι form Batch no. 68C-0074	C4014

Weighed quantities of the relevant type were suspended in physiological saline at 1.0 mg/ml or 2.0 mg/ml, and were dissolved and sterilised by heating in a boiling water bath for 60 min. Carrageenan preparations were stored at room temperature; however, more concentrated solutions tended to gel under these conditions, and had to be warmed before use. Further dilutions were made in sterile saline or culture medium.

2.2.1.9.3. Zymosan

Zymosan A was obtained from Sigma Chemical Company. The powder was stored dessicated at 4°C. For addition to cultures zymosan was suspended in physiological saline and sterilised by autoclaving at 120°C, 15 p.s.i. for 15 min. Some batches of zymosan were found to be unsatisfactory, and in one case (batch no. 11F-0190) the problem was identified as contamination of the zymosan preparation with live yeast.

Sterilised zymosan suspensions were stored at room temperature, and further dilutions were made in sterile saline or culture medium.

2.2.1.9.4. Latex

Monodispersed latex particles, diameter 0.789 μm, lot no. 4-1618, were obtained as a 2.5% suspension in water from Polysciences Limited, Moulton Park, Northampton. The stock suspension, containing approximately 1×10^{11} particles/ml, was stored at room temperature, and was diluted in culture medium before use. No sterilisation procedures were employed, and any contaminants present in the latex suspension were apparently sensitive to the antibiotic contained in the culture medium.

2.2.1.10. Anti-inflammatory drugs

2.2.1.10.1. Indomethacin

Indomethacin powder (batch no. 67C-0279) was obtained from Sigma Chemical Company. As this material was not very soluble in saline, it was first dissolved in ethanol at a concentration of 4.0 mg/ml, and was stored at -20°C in this state. Shortly before use the solution was further diluted in culture medium.

2.2.1.10.2. Flurbiprofen

Flurbiprofen (batch no. DB/2537) was obtained in powder form from Boots Pure Drug Company Limited, Nottingham, and was stored at room temperature, away from light. Solutions were prepared shortly before required. Due to its low solubility in saline, flurbiprofen was initially dissolved in ethanol at a concentration of 4.0 mg/ml, and was then diluted in culture medium.

2.2.1.11. Exogenous prostanoids

2.2.1.11.1. Prostaglandin E_1

Prostaglandin E_1 (Sigma Chemical Company), dissolved at 1.0 mg/ml in ethanol, was a gift from Dr. V. Rumjanek, Department of

Clinical Pharmacology, Cardiothoracic Institute, London. This stock solution was stored at -20°C, and was diluted to 1 x 10^{-3} molar in ethanol shortly before use. Further dilutions were made in culture medium.

2.2.1.11.2. Prostacyclin and carbacyclin

Prostacyclin (sodium salt) and the stable synthetic analogue carbacyclin were donated by Dr. G. Higgs, Department of Prostaglandin Research, Wellcome Research Laboratories, Beckenham. The solid materials were stored dessicated at 4°C until required, when each was dissolved in 1.0 molar Tris buffer, pH 8.4, to give a concentration of 1.0 mg substance/ml. These stock solutions were stored at -20°C, and were further diluted in culture medium before use.

2.2.1.12. Anti-prostanoid antisera

2.2.1.12.1. Anti-prostaglandin E antiserum

Rabbit anti-PGE_1 antiserum was donated by Dr. J. Mertin, Transplantation Biology Section, Clinical Research Centre, Harrow. This antiserum was prepared by repeatedly injecting rabbits with PGE_1 coupled to BSA, in complete Freund's adjuvant. It was found to be specific for prostaglandins of the E series, and bound to both PGE_1 and PGE_2 (J. Mertin, personal communication). The antiserum was stored at -20°C until required, when it was diluted 1:1 in culture medium and sterilised by filtration through a Millipore filter, pore size 0.22 µm. Control rabbit serum from the same source was treated similarly.

2.2.1.12.2. Anti-prostacyclin antiserum

Rabbit anti-PGI_2 antiserum was a gift from Dr. G. Higgs, Wellcome Research Laboratories. This antiserum was prepared by injecting rabbits once weekly for 4 weeks with 5,6-dihydroprostacyclin

(PGI_1) coupled to BSA, in complete Freund's adjuvant, then once monthly with the PGI_1-BSA conjugate in incomplete Freund's adjuvant. It was found to neutralise both PGI_1 and PGI_2, and at higher doses it also inhibited PGE_1 and PGD_2. However, 10-30 times more antiserum was needed to neutralise PGE_1, PGI_1 and PGD_2 than was required to inhibit the activity of PGI_2 (Bunting et al, 1978; S. Bunting, personal communication). The antiserum was stored at -20°C until required, when it was diluted 1:4 in culture medium and sterilised by filtration through a Millipore filter, pore size 0.22 μm. Control rabbit serum was treated similarly.

2.2.1.13. Complement

Normal mouse serum was used as a source of complement to opsonise yeast for phagocytosis; this was stored at -80°C.

2.2.1.14. Complement fixation diluent

Complement fixation diluent tablets were obtained from Oxoid Limited, Basingstoke, Hampshire. Diluent was prepared by dissolving one tablet in 100 ml warm distilled water.

2.2.1.15. Yeast

2.2.1.15.1. Sterilised yeast

Bakers' yeast was suspended in PBS and autoclaved at 120°C, 15 p.s.i. for 30 min. It was then washed thoroughly in PBS, and resuspended in PBS containing 0.1 molar 2-ME. Following a 2-hr incubation at 37°C, the yeast was washed, and resuspended in 0.85% saline containing 0.02 molar iodoacetamide for 2 hr at room temperature. It was then washed in PBS, re-autoclaved, and finally resuspended in complement fixation diluent containing 0.1% sodium azide (Lachmann et al, 1973). This stock yeast suspension was stored at 4°C.

2.2.1.15.2. Stained yeast

Yeast suspensions prepared as above were stained as described by Shaala et al (1979). Procion Rubine powder, obtained from ICI Limited, Macclesfield, was dissolved in PBS at 0.02 g/ml, and 5.0 ml of this solution was mixed with 2 x 10^{10} washed yeasts in 1.0 ml saline, the mixture being rolled for 2 hours at room temperature. The yeast was then washed thoroughly in saline, resuspended in PBS + 0.1% azide, and stored at 4°C.

2.2.1.16. Carbonyl iron powder

Carbonyl iron powder was obtained from G.A.F. (U.K.) Limited, Chemical Division, Wythenshawe, Manchester. Particle size was of the order of 3-4 μm.

2.2.1.17. Nylon wool

Leuko-Pak Leukocyte Filters were obtained from Fenwal Laboratories, Illinois, U.S.A. The nylon wool was removed from these filters, and prepared as described by Julius et al (1973). The wool was washed thoroughly by soaking in saline for 2 hr at 37°C, then rinsing in several changes of deionised distilled water over a period of one week. Sterile nylon wool columns were prepared by packing 30 ml and 60 ml disposable syringe barrels with 2.0 g and 4.0 g respectively of washed and dried nylon wool. The columns were then sterilised by autoclaving at 120°C, 15 p.s.i. for 15 min.

2.2.1.18. Tissue culture plates

Cell cultures were set up in sterile Linbro 24-well plates, supplied by Flow Laboratories, catalogue no. 76-033-05.

2.2.1.19. CO_2 incubator

A Salamander incubator (Vindon Scientific Limited, Oldham, Lancashire) was used for all cell cultures. This was gassed with CO_2

to generate an atmosphere of 5% CO_2 in air, and was maintained at 37°C. Humidity was maintained by a reservoir of distilled water in the base of the incubator, and an additional reservoir of copper II sulphate solution was included to discourage growth of contaminating microorganisms.

2.2.1.20. Thioglycollate

Thioglycollate powder was obtained from Difco Laboratories, East Mollesey, Surrey. A 10% w/v solution of thioglycollate in distilled water was prepared, and was sterilised by autoclaving at 120°C, 15 p.s.i. for 15 min. The solution was kept in the dark, and was allowed to "age" for at least 2 weeks before use. This step is essential to permit oxidation of the solution, whereupon the colour changes from green to brown.

2.2.1.21. Radioactive sodium chromate

^{51}Cr-sodium chromate, code number CJS 4, was obtained as a saline solution from Amersham International Limited, Buckinghamshire. Specific activity, radioactive concentration and chromium concentration varied slightly between batches, and each batch was diluted to 7.0 µg Cr/ml with saline before use, to give a solution at 2.5 - 4.5 mCi/ml. This solution was stored at 4°C.

2.2.1.22. Target cells

P815Y mastocytoma cells were maintained *in vitro*. If these cells were in poor condition, either through infection or because they had overgrown, they were passaged *in vivo* in DBA/2 mice. Stocks of frozen P815Y cells were also kept, and these were stored in liquid nitrogen.

EL4 lymphoma cells were maintained *in vitro* and were passaged in C57BL mice when necessary. EL4 cells were also stored frozen in

liquid nitrogen.

In some experiments, peritoneal exudate cells elicited by injection of thioglycollate were used as target cells.

2.2.1.23. Reagents for protein determination

Reagents used to assess the protein content of cell digests were prepared according to a modification of the Lowry method, as described by Nerurkar et al (1981).

Solution A	Na_2CO_3	2.00 g
	NaK tartrate	0.02 g
	Na lauryl sulphate	0.50 g
	Distilled water	100.00 ml
Solution B	$CuSO_4.5H_2O$	1.00 g
	Distilled water	100.00 ml

Solution A was stored in a dark bottle. Immediately before use, 50 volumes of solution A were mixed with one volume of solution B.

Folin and Chiocalteu's phenol reagent was obtained from BDH Chemicals Limited, and was diluted 1:2 (v/v) in distilled water immediately before use.

2.2.2. METHODS

2.2.2.1. Preparation of cell suspensions

2.2.2.1.1. Peritoneal cell suspensions

Mice were killed by cervical dislocation, and were immersed in Hibitane to dampen and cleanse the fur. They were then pinned ventral surface uppermost on a cork board. Using sterilised instruments, the abdominal skin was reflected, exposing the abdominal wall. If there was any evidence of bleeding into the peritoneal cavity, the mouse was not used as a source of peritoneal cells.

The abdominal wall was then swabbed with alcohol, and 2.0 - 5.0 ml HBSS was injected into the peritoneal cavity along the mid-line. The abdomen was gently massaged for 2-3 min, then the fluid was withdrawn from the side. It was possible to recover up to 80% of the injected fluid in this way. The cell suspension was then topped up with HBSS, and the cells were washed and resuspended in RPMI + 5% FCS + G. They were then counted and adjusted to the required concentration, and were kept on ice to prevent loss of the adherent population.

2.2.2.1.2. Spleen cell suspensions

Mice were killed as before and immersed in Hibitane. The body cavity was opened laterally, and the spleen was removed aseptically and placed in a Petri dish containing 5.0 - 10.0 ml sterile HBSS + 5% FCS. After being dissected free of connective tissue and fat, the spleen was gently teased apart using curved forceps, liberating the cells into the medium. Larger tissue fragments and clumps of cells were allowed to settle out for about 2 min, then the supernatant fluid was aspirated into a sterile Universal container and topped up with HBSS + 5% FCS. This cell suspension was then spun at 400 x g for 5 min, and the cells were resuspended in 10.0 ml RPMI + 5% FCS + G. They were then counted

and adjusted to the required concentration in this culture medium, and were kept at room temperature until required.

2.2.2.2. Determination of cell numbers

2.2.2.2.1. White cell counts

To determine the number of nucleated cells, 0.1 ml of the cell suspension was added to 0.9 ml white cell counting fluid. A sample of this mixture was placed on a haemocytometer, and the cells were counted using a Zeiss microscope. Cell counts were expressed as $\chi \times 10^6$ cells per millilitre of suspension.

2.2.2.2.2. Viable cell counts

Cell viability was assessed using fluorescein diacetate-ethidium bromide stain. Equal volumes of the cell suspension and the staining fluid were mixed, a sample of this was placed on a haemocytometer, and the cells were counted using a Leitz SM-LUX microscope with incident ultra-violet illumination.

Using this method, live cells fluoresce green, due to hydrolysis of FDA, while non-viable cells take up EB and are stained red.

2.2.2.3. Cell depletion using carbonyl iron powder

Phagocytic and adherent cells can be removed from a cell suspension by treatment with carbonyl iron powder (Sjöberg et al, 1972). In this study, the method of Lundgren et al (1968) was followed in essence. The cell suspension to be depleted was adjusted to 20×10^6 cells/ml. Carbonyl iron powder was weighed into suitable aliquots, allowing 20 mg for each millilitre of suspension. The powder was then hot-air sterilised at 160°C for 60 min, and was washed 5x with sterile saline to remove any soluble toxic material. An appropriate volume of

the cell suspension was then added to the damp carbonyl iron, and the mixture was rolled at 37°C for 60 min. After this incubation period, the bottle was placed over a magnet, which retained the carbonyl iron and its associated cells, while the supernatant fluid containing the non-adherent fraction was decanted. The sediment was then resuspended in culture medium and mixed, then placed over the magnet again, and this supernatant was added to the previous non-adherent fraction. The recovered cells were then washed and resuspended at the required concentration in RPMI + 5% FCS + G.

Carbonyl iron treatment resulted in good cell yields, with approximately 60-75% of the original cell number being recovered in the non-adherent fraction.

2.2.2.4. Cell separation using nylon wool

2.2.2.4.1. Loading nylon wool columns

Sterile nylon wool columns were prepared as described in section 2.2.1.17. Cell suspensions were depleted of the adherent fraction according to the method of Henry et al (1980).

Columns were clamped vertically, and thoroughly rinsed with culture medium pre-warmed to 37°C. The release tap was then closed, and the saturated column was left to equilibrate for at least 60 min before use in a CO_2 incubator. After this period the excess medium was drained from the column, and the tap was closed. The cell suspension to be depleted was adjusted to 50 x 10^6 cells/ml, and between 10.0 ml and 20.0 ml of this suspension was slowly loaded onto the column. This was washed into the nylon wool with a further 5.0 - 10.0 ml warm medium, and the column was sealed and returned to the incubator for 45-60 min.

2.2.2.4.2. Recovery of the non-adherent population

After the incubation period, the column tap was opened and the

nylon wool was washed slowly with warm medium, so as not to generate a fluid head. A flow rate of approximately 1.0 ml/min proved satisfactory. Between 50.0 ml and 100.0 ml medium was usually sufficient to flush out the non-adherent cells, which were then washed and resuspended at the required concentration in RPMI + 5% FCS + G.

Cell yields were frequently low, 15-25% of the original cell number being recovered in the non-adherent fraction.

The non-adherent population obtained by nylon wool treatment is depleted of B cells, plasma cells, and accessory cells, while being enriched for most T cell populations (Henry et al, 1980). Tada et al (1978) have reported that a T helper subpopulation may be retained on the nylon wool column.

2.2.2.4.3. Recovery of the adherent population

The cell populations mentioned in section 2.2.2.4.2., which were retained on the column, could be recovered by rinsing with ice-cold medium and compressing the nylon wool with a sterile plunger. These cells were then washed, resuspended, and kept on ice until required.

Nylon wool from used columns was recycled after soaking overnight in 0.1 molar hydrochloric acid, and washing as before. This procedure did not adversely affect the performance of the nylon wool (Henry et al, 1980).

2.2.2.5. Cell separation by adherence to plastic

Strongly adherent cells can be selectively removed by allowing them to adhere to plastic culture vessels for at least 60 min (Sjöberg et al, 1972).

Cell suspensions were adjusted to 2 x 10^6 cells/ml, and were dispensed into tissue culture plates. They were then incubated at 37°C for either 3 hr or 24 hr, after which time the non-adherent population

was resuspended by aspirating the total 1.0 ml volume 7 x using a 1.0 ml Finnpipette. All the fluid was then removed.

This procedure yielded sterile non-adherent cells in suspension, and sterile adherent cells already attached to a culture plate. These populations could then be cultured further, either separately or in combination. Where necessary, adherent cells attached to the culture wells could be resuspended and recovered. One millilitre of ice-cold HBSS was added to the drained wells, and the culture plates were then chilled on ice for 10 min. The total volume of fluid was then aspirated vigorously 10 x using a 1.0 ml Finnpipette, and was harvested into a chilled Universal container. Further manipulation of this adherent cell suspension was performed on ice. Cell recovery using this procedure varied between 50-100%, viability usually being in excess of 70%.

2.2.2.6. One-way mixed leucocyte cultures

Responder and stimulator spleen cell suspensions were prepared as described in section 2.2.2.1.2. The most usual strain combination was CBA responder cells stimulated by Balb/c cells, though in some experiments C57BL mice were used as the source of stimulator cells.

Proliferation of the stimulator population was prevented by X-irradiation using a Siemens Stabilipan unit, operated at 240 kV and 10 mA with a 0.5 mm copper filter. The stimulator cell suspension was exposed to 2000 rads at a dose rate of 301 rads/min. The cells were then aliquoted into multiwell culture plates, 1.0 ml of the suspension containing 2×10^6 cells being placed in each well. An equal volume of responder cells at the same concentration was added, to give a responder to stimulator ratio of 1:1. In some preliminary experiments, different R:S ratios and cell numbers were used.

Two hundred microlitres of 5×10^{-4} molar 2-ME were then added

to the cultures to give a final concentration of 5×10^{-5} molar. Any macrophage-active agents, drugs, et cetera were also added to the cultures at this stage, and the plates were then kept in a CO_2 incubator for the duration of the culture period. In most instances this was 5 days.

2.2.2.7. Addition of macrophage-active agents and drugs to cell cultures

Stock solutions and suspensions of these materials were prepared as described in sections 2.2.1.9. and 2.2.1.10. These were diluted in saline or culture medium to 10 x the desired final concentration, and were then added to cell cultures at one volume in 10 volumes culture fluid.

In the case of flurbiprofen, a stock solution at 400 x the desired final concentration was prepared in ethanol. This was then diluted 100 x in culture medium to give a solution at 4 x the desired final concentration, which was added to cultures at one volume in 4 volumes culture fluid. To facilitate this dilution, responder and stimulator cell suspensions were adjusted to twice the usual concentration, and only 0.5 ml of each was added.

2.2.2.8. Addition of exogenous prostanoids and anti-prostanoid antisera to cell cultures

2.2.2.8.1. Prostaglandin E_1

The stock PGE_1 solution was diluted in culture medium to give a range of concentrations from 1×10^{-5} molar to 1×10^{-8} molar. These preparations were then added to cultures at one volume in 10 volumes culture fluid, to give a range of final concentrations from 1×10^{-6} molar to 1×10^{-9} molar.

2.2.2.8.2. Prostacyclin and carbacyclin

Stock solutions of prostacyclin and carbacyclin were diluted in culture medium to give a range of concentrations from 1 x 10^{-3} molar (300 µg/ml) to 1 x 10^{-7} molar (0.03 µg/ml). These preparations were sterilised by filtration through a Millipore filter, pore size 0.22 µm, and were added to cultures at one volume in 10 volumes culture fluid, to give a range of final concentrations from 1 x 10^{-4} molar to 1 x 10^{-8} molar.

2.2.2.8.3. Anti-prostanoid antisera

Control, anti-PGE_1, and anti-PGI_2 antisera were added to cultures at one volume in 10 volumes culture fluid, to give a range of final dilutions from 1 in 20 to 1 in 200.

To maintain a constant final volume when more than one material was added to a culture, the concentrations of responder and stimulator cell suspensions were adjusted and smaller volumes were used, as mentioned in section 2.2.2.7. The final culture volume was then made up if necessary by adding culture medium.

2.2.2.9. Phagocytic assay

By placing 13 mm diameter round glass coverslips in the wells of culture plates, the adherent cells from mixed leucocyte cultures could be recovered. These cells were then studied microscopically, and their ability to phagocytose opsonised yeast was assessed.

2.2.2.9.1. Opsonisation of yeast

Yeast was prepared as described in section 2.2.1.15, and was suspended at 2 x 10^8 particles/ml. It was then opsonised with complement by incubating 0.5 ml of the suspension with 0.25 ml mouse serum at 37°C for 30 min. The yeasts were then washed 3 x in saline, and were

resuspended in culture medium at 1 x 10^7 particles/ml.

2.2.2.9.2. Phagocytosis

The non-adherent population was harvested from each well of the culture plate, leaving the adherent cells attached to the coverslip in the bottom of the well. Two hundred microlitres of the yeast suspension, containing 2 x 10^6 yeasts, was added to each well, followed by 0.5 ml culture medium. The plate was placed on a level surface and incubated at 37°C for 30-60 min, then the coverslips were removed from the wells. They were rinsed with saline, followed by foetal calf serum, and were then air dried and fixed in methanol.

The coverslips were subsequently stained with May Grünwald stain in the Haematology Department at St. Mary's Hospital Medical School, and were examined microscopically.

2.2.2.10. Maintaining cell lines

In vitro maintained tumour cells were generally used as targets for the cytotoxicity assay. These cells were cultured in 200 ml tissue culture flasks in RPMI + 5% FCS + G, and were kept in a CO_2 incubator. The rate of growth of the cells varied, but they generally needed to be sub-cultured at least twice a week. Excess dead cells and cell debris from a culture in poor condition could be removed by spinning the cells through neat FCS, as described by Shortman et al (1972). Frozen cells were stored in liquid nitrogen; when these were thawed, they were passaged in vivo in the syngeneic mouse strain before being cultured in vitro or used as targets.

2.2.2.11. Cryopreservation of cells

2.2.2.11.1. Freezing

Cells were suspended in RPMI + 20% FCS at 10^6 - 10^7 viable

cells/ml, and were cooled to 4°C. An equal volume of RPMI containing 20% DMSO was then added dropwise with gentle shaking, and the suspension was aliquoted into 2.0 ml freezing vials. These were placed in the vapour phase of a liquid nitrogen container, and were allowed to freeze to -70°C at a rate of 1°C/min. The vials were then immersed in liquid nitrogen for storage.

2.2.2.11.2. Thawing

Vials removed from storage were placed in a water-bath at 37°C and mixed until almost melted. They were then transferred to ice for 2 min, then decanted into a larger vessel and brought to room temperature. The freezing solution was then gradually diluted with RPMI + 20% FCS by doubling dilutions every 2 min, up to approximately 50 ml. The suspension was then left for 5 min, then the cells were washed and resuspended at the appropriate concentration.

2.2.2.12. Thioglycollate-induction of peritoneal cells

Thioglycollate broth was used to increase the yield of peritoneal cells, when these were required as targets for the cytotoxicity assay. The broth was prepared as described in section 2.2.1.20.

Male or female mice of the relevant strain (either Balb/c for the CBA v Balb/c combination, or C57BL for the CBA v C57BL combination), aged 16-20 weeks, were injected intraperitoneally with 0.5-1.0 ml thioglycollate 3 days before the cells were required. The peritoneal exudate was subsequently harvested as described in section 2.2.2.1.1.

2.2.2.13. Cell-mediated cytotoxicity assay

At the end of the 5-day mixed leucocyte culture, the cells were resuspended and harvested using a Finnpipette. They were then

washed, resuspended in RPMI + 5% FCS, and counted for viability as described in section 2.2.2.2.2. The concentration was adjusted to 8×10^6 viable cells/ml where possible, and to 4×10^6 or 2×10^6 viable cells/ml when cell recovery was low. The cells were then plated out in 100 µl aliquots into V-form microtitre plates, using doubling dilutions to give a range of cell numbers per well, from 80×10^4 (or 40 or 20×10^4) down to 1.25×10^4. Each cell culture was plated out in triplicate in this manner.

In vitro grown tumour cells or thioglycollate-induced peritoneal cells were used as targets in the cytotoxicity assay. These cells were washed and resuspended at $100\text{-}200 \times 10^6$ cells/ml; 100 µl of this suspension was added to 100 µl ^{51}Cr-sodium chromate solution (radioactive concentration 2.5-4.5 mCi/ml), and the mixture was incubated at 37°C for 60 min. The cells were layered on neat FCS for 5 min, then spun through fresh FCS (200 x g, 10 min) and washed once in RPMI + 5% FCS. They were then counted and adjusted to 1×10^5 cells/ml.

One hundred microlitres of the radiolabelled target cell suspension was added to each well of the microtitre plate, to give a range of effector to target ratios from 80:1 (or 40:1 or 20:1) down to 1.25:1. Target cells were also added to wells containing medium only, to give a value for the spontaneous release of radioactivity. The plates were then spun gently (100 x g, 3 min) to sediment the cells and bring the effector cells and their targets into contact, and were then placed in a CO_2 incubator for 3-4 hr. This slight variation in the length of the assay did not appear to affect the results.

After the incubation period, the cells were spun down (400 x g, 10 min), and 100 µl of the supernatant was withdrawn from each well. This was placed in a Beckman 0.4 ml reaction vial (Sterilin

Limited, Teddington, Middlesex), and was subsequently counted for radioactivity in a Packard Auto-Gamma Scintillation Spectrometer.

One hundred microlitre aliquots of the original target cell suspension were also counted to give a value for the total incorporation of radioactivity. However, as some of the incorporated activity may not be available for release upon death of the cell, a value for the total releasable activity was also required. This figure was determined by diluting an aliquot of the target cell suspension 1:1 in distilled water, then freezing and thawing this mixture 3 x in an acetone-dry ice bath. The mixture was then centrifuged (400 x g, 10 min), and 100 µl samples of the supernatant were counted.

2.2.2.14. Calculation of specific cytotoxicity

Percent specific lysis was calculated for each effector to target ratio from the following formula:

$$\% \text{ specific lysis} = \left(\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \right) \times 100$$

Using a programmable calculator, this value was determined as the mean of replicate samples ± standard error. Values for percent specific lysis, or percent cytotoxicity, were then plotted against the logarithm to base 10 of the effector to target ratio. Standard errors rarely exceeded 10%, and are not included in the Figures.

2.2.2.15. Determination of protein content of cell digests

The amount of cell protein present in culture wells was determined using a modification of the Lowry method described by Nerurkar et al (1981).

Adherent cells attached to culture wells were digested by adding 0.2 ml of 1.0 molar NaOH solution to the wells, and incubating overnight at 37°C in a humidified incubator. The cell digest was then harvested; 0.1 ml of this was added to 1.0 ml of a freshly prepared mixture of solutions A and B (section 2.2.1.23.), and these were mixed in test-tubes by Vortex, then left to stand at room temperature for 30-45 min. One hundred microlitres of diluted Folin and Ciocalteu's phenol reagent was then added to each tube; these were immediately mixed by Vortex, and left to stand at room temperature for at least 30 min.

Absorbance at 750 nm was measured in a Unicam Spectrophotometer using microcuvettes with a light path of 1.0 cm. Where appropriate, readings were corrected against the absorbance of samples prepared from culture wells containing medium only.

A series of 11 dilutions of BSA (Sigma, Cohn fraction V) in 1.0 molar NaOH, ranging from 5 µg/ml to 200 µg/ml, were used to construct a standard curve for absorbance. Corrected values for experimental absorbance were compared to this standard curve, and results were calculated from the regression line and expressed as µg protein (BSA equivalent) per well.

2.3. PRELIMINARY WORK ON THE MURINE MIXED LEUCOCYTE CULTURE

2.3.1. CULTURE CONDITIONS

2.3.1.1. HBSS for handling cells

2.3.1.2. Preparation of spleen cell suspensions by teasing and sieving

2.3.1.3. Lysis of red blood cells

2.3.1.4. Comparison of tissue culture flasks and multiwell culture plates as vessels for mixed leucocyte cultures

2.3.1.5. Cell concentrations and responder to stimulator cell ratios

2.3.1.6. Requirement for 2-mercaptoethanol

2.3.1.7. Specificity of the cytotoxic response

2.3.2 ROLE OF ACCESSORY CELLS

2.3.2.1. Cell depletion using carbonyl iron powder

2.3.2.2. Cell separation using nylon wool

2.3.2.3. Cell separation by adherence to plastic

2.3.2.4. Time-course of the adherent cell requirement

2.3.1. CULTURE CONDITIONS

2.3.1.1. HBSS for handling cells

In a few preliminary experiments, cell suspensions were prepared in RPMI culture medium, supplemented with 5% FCS and gentamicin. This medium contains both bicarbonate and HEPES buffering systems; bicarbonate ions are essential for the metabolism of the cultured cells, and serve to maintain physiological pH in an atmosphere of 5-10% CO_2, while HEPES controls the pH of the medium with air as the gas phase (Paul, 1975). However, when both of these systems are used in one preparation, the efficiency of HEPES is reduced, and the pH can only be controlled for a limited period of time. Prolonged exposure to air may therefore result in an alkaline solution, particularly when using small volumes. This change in pH may be observed as a change in the colour of the medium, which becomes pink under alkaline conditions.

As cells were frequently kept in medium exposed to air for several hours before culture, it was decided that a bicarbonate-free phosphate-buffered balanced salt solution should be substituted for RPMI during this period. Hanks' BSS was selected as the most suitable preparation, and the cells were subsequently handled in this medium prior to incubation. They were then resuspended in RPMI culture medium before plating out.

The viability of cell suspensions prepared in either RPMI or HBSS was compared, as shown in Table 2.3.1. Two methods of spleen cell release were used; these are discussed further in section 2.3.1.2. In each case, one suspension was handled solely in RPMI, the other solely in HBSS, and viability was assessed at several stages of the procedure. The suspension prepared in HBSS showed enhanced viability throughout, and this was most marked after incubation of the suspensions at room temperature for 3 hr, in medium exposed to air; this incubation

Table 2.3.1. Preparation of cell suspensions and effect on cell viability

Spleen cell suspensions prepared either by sieving or teasing were handled throughout in either RPMI or HBSS. Two spleens were used for each suspension, taken from 12-week female CBA mice. The cells were treated with rbc lysis fluid for 15 min, then layered on neat FCS and washed twice with the relevant medium. Aliquots of each suspension were then incubated for 3 hr, either at room temperature in medium exposed to air, or at 37°C in a CO_2 incubator. Cells suspended in HBSS were not incubated in a CO_2 atmosphere, as this medium rapidly becomes acidic under such conditions. Cell viability was assessed at each stage in the procedure, and is presented as the mean percentage viability of five samples taken from each cell suspension. Standard errors did not exceed 10%, and are not included in the Table.

Table 2.3.1. Preparation of cell suspensions and effect on cell viability

Percent viable cells using FDA-EB stain	Sieved spleen		Teased spleen	
	RPMI	HBSS	RPMI	HBSS
Immediately after preparation of suspension	54	73	67	89
Immediately after lysis of rbc	30	37	35	48
After layering on FCS and washing 2 x in medium	59	74	79	93
After incubation at room temperature for 3 hr, in medium exposed to air	43	69	49	88
After incubation at 37°C for 3 hr, in medium exposed to 5% CO_2	58	-	77	-

reproduced the conditions frequently imposed on the cells during an experiment.

These results confirm that prolonged exposure to RPMI culture medium which is not buffered by a CO_2 atmosphere is detrimental to the viability of the cells. In the remainder of the experiments discussed in this study, HBSS supplemented with 5% FCS was substituted for RPMI in all cell manipulations performed outside a CO_2 atmosphere.

2.3.1.2. Preparation of spleen cell suspensions by teasing and sieving

In some early experiments, spleen cell suspensions were prepared by gently grinding the tissue through a fine stainless steel sieve, released cells being washed through the sieve by repeatedly pipetting medium over the working area. However, as freshly prepared suspensions were sometimes found to have low viability, this procedure was abandoned, and in all subsequent experiments spleen cell suspensions were prepared by gently teasing the organ apart in a dish of medium. As shown in Table 2.3.1., this treatment resulted in a more viable cell preparation, and this increased viability was maintained during handling of the cells prior to incubation. Teasing also improved the cell yield from individual spleens, as fewer cells were lost in tissue clumps and debris, and consequently this was adopted as standard procedure for cell preparation, as described in section 2.2.2.1.2.

2.3.1.3. Lysis of red blood cells

In preliminary experiments to this study, spleen cell suspensions were exposed to ammonium chloride lysis fluid to remove the red blood cells from the preparation, as described by Boyle (1968). However, as shown in Table 2.3.1., this treatment resulted in a marked reduction in viability of the remaining nucleated cells, and this step was therefore omitted from the standard procedure in future experiments.

2.3.1.4. Comparison of tissue culture flasks and multiwell culture plates as vessels for mixed leucocyte cultures

In a few preliminary experiments, mixed leucocyte cultures (MLCs) were set up in 50.0 ml tissue culture flasks. However, cultures established in multiwell plates proved more satisfactory, and consequently these were used throughout this study.

Parallel cultures were set up, using the same original cell suspension, to compare the efficiency of flasks and plates. As shown in Table 2.3.2., cell recovery and viability from cultures in plates were considerably better than recovery and viability from corresponding cultures set up in flasks. This may be due in part to more efficient gassing of plates in a CO_2 atmosphere, so reducing exposure of the cells to alkaline conditions. Cytotoxicity assays were subsequently set up with cells from each culture, cell numbers being adjusted to give different ratios of viable effector cells to radiolabelled target cells. As shown in Figure 2.3.1., specific cytotoxicity generated in plates was equivalent to that obtained from cultures in flasks.

A further important advantage conferred by the use of plates is a reduction in the number of cells required. Using the optimal cell numbers for this system, a culture of 4 wells can be set up with 8×10^6 responder cells, compared with 25×10^6 responder cells for each flask used. In addition, the risk of contamination is reduced by using plates; a few wells may become contaminated without the rest of the culture being affected, whereas with flasks, replicates must be set up to avoid loss of the culture through infection.

2.3.1.5. Cell concentrations and responder to stimulator cell ratios

Various cell concentrations and responder to stimulator cell (R:S) ratios were compared in multiwell plates before the optimal combination was found. From previous experience with culture flasks,

Table 2.3.2. Comparison of cell recovery and viability from cultures set up in tissue culture flasks and multiwell culture plates

Cultures in flasks were set up using 25×10^6 responder cells and 12.5×10^6 stimulator cells in 10.0 ml per flask, 3 flasks per culture.

Cultures set up in plates consisted of 2×10^6 responder cells and 2×10^6 stimulator cells in 2.0 ml per well, 8 wells per culture.

In each experiment, replicate cultures were established. At the end of the 5-day culture period, the cells from each culture were pooled, and cell recovery and viability were assessed.

Table 2.3.2. Comparison of cell recovery and viability from cultures set up in tissue culture flasks and multiwell culture plates

	Tissue culture flasks		Multiwell culture plates	
	% recovery	% viability	% recovery	% viability
Experiment 1	66	62	144	78
	59	60	119	79
Experiment 2	64	63	137	77
	72	64	108	83
	52	59	97	75

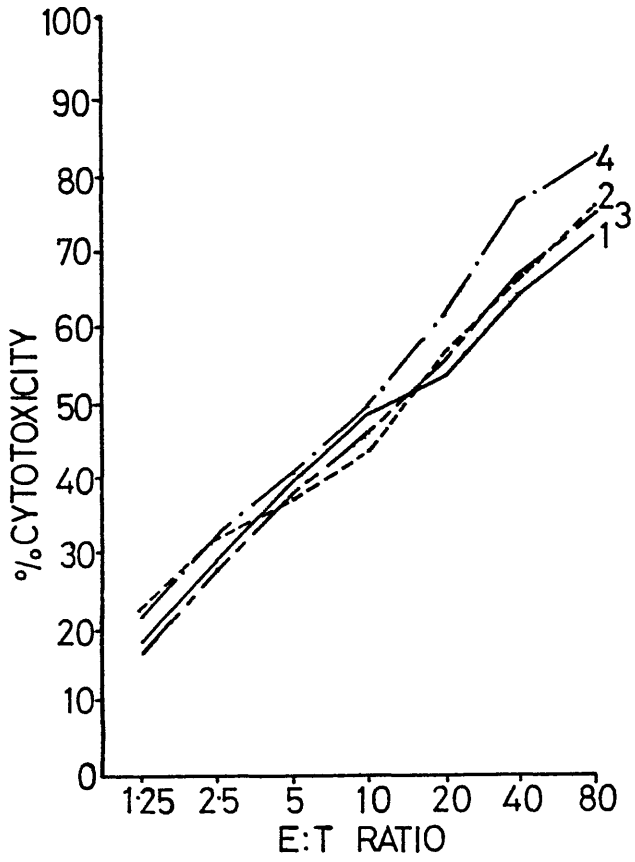


Figure 2.3.1.

Generation of CTL in the MLC: comparison of flasks and plates

Cytotoxicity data were obtained in experiment 1, Table 2.3.2. Each point represents the mean of triplicate samples; standard errors were generally less than 10% of the mean, and are not included.

Lines 1 and 2: 25×10^6 responder cells cultured in flasks, at an R:S ratio of 2:1

3 and 4: 2×10^6 responder cells cultured in plates, at an R:S ratio of 1:1

CULTURE	1	2	3	4
% recovery	66	59	144	119
% viability	62	60	78	79

TARGET CELLS P815Y in vitro cell line

Spontaneous release 10%

optimal results were obtained with 25 x 10^6 responder cells cultured in a total volume of 10.0 ml. As the culture volume in multiwell plates is approximately 2.0 ml per well, concentrations of 2 x 10^6 and 4 x 10^6 responder cells per well were studied, at R:S ratios of 1:2, 1:1, and 2:1. As shown in Figure 2.3.2., a good cytotoxic response was obtained with 2 x 10^6 responder cells per well at R:S ratios of 1:2 and 1:1. At the higher cell concentration, an equally good response was obtained at R:S ratios of 1:1 and 2:1. However, cell recovery varied, and was maximal with 2 x 10^6 responder cells per well at an R:S ratio of 1:1. For this reason, the combination of 2 x 10^6 responder cells and 2 x 10^6 irradiated stimulator cells per well was used throughout this study, as described in section 2.2.2.6.

2.3.1.6. Requirement for 2-mercaptoethanol

The reducing agent 2-mercaptoethanol (2-ME) was routinely added to MLCs to give a final concentration of 5 x 10^{-5} molar. The absolute requirement for this agent in such cultures is shown in Figure 2.3.3.; in the absence of 2-ME, no cytotoxic activity developed in the culture, and there was a marked fall in cell recovery and viability.

2.3.1.7. Specificity of the cytotoxic response

In order to test the specificity of the cell-mediated cytotoxic response, MLCs were set up in which CBA responder cells were stimulated by either Balb/c or C57BL cells. Effector cells harvested from each culture were then assayed against both P815Y (Balb/c H-2^d haplotype) and EL4 (C57BL H-2^b haplotype) target cells. As shown in Figure 2.3.4., both cultures gave a good response to the stimulating target, while little or no cytotoxic activity was directed against third party target cells.

In a separate experiment cultures were set up in which CBA

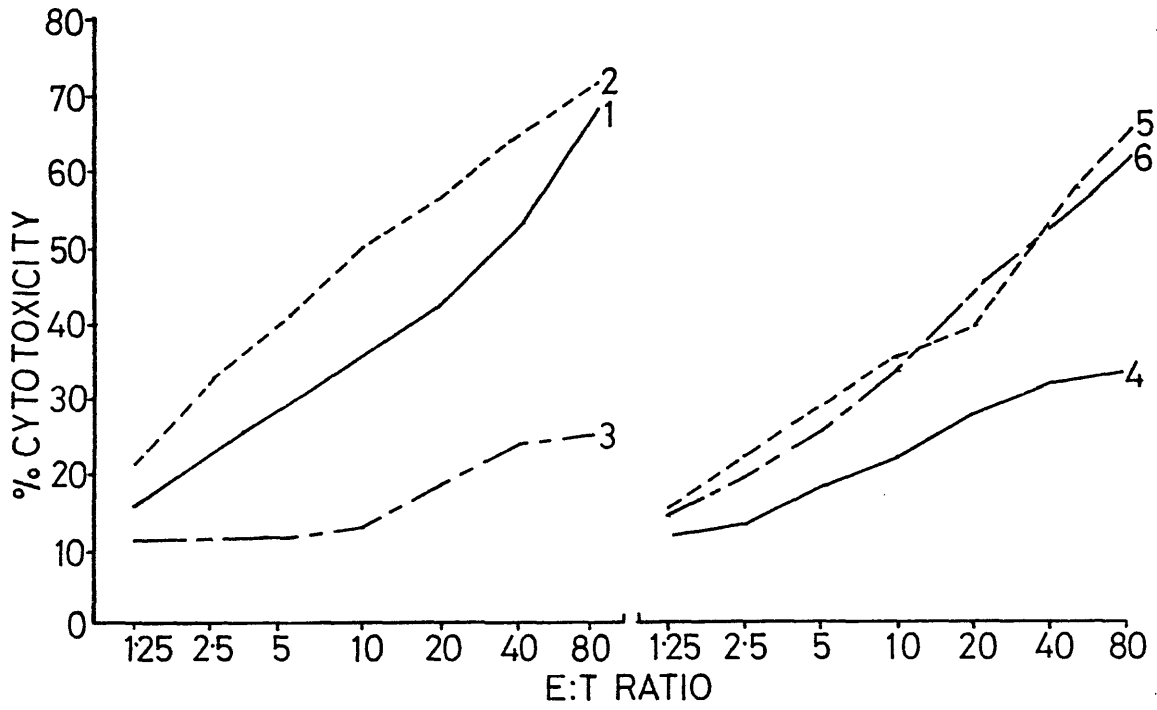


Figure 2.3.2.

Generation of CTL in multiwell plates: cell concentrations and R:S ratios

Parallel cultures were set up using the same original cell suspension.

Line 1: R:S = 1:2)
2: R:S = 1:1) 2 x 10^6 responder cells per well
3: R:S = 2:1)

4: R:S = 1:2)
5: R:S = 1:1) 4 x 10^6 responder cells per well
6: R:S = 2:1)

CULTURE	1	2	3	4	5	6
% recovery	123	136	65	62	94	85
% viability	83	87	71	69	78	73

TARGET CELLS P815Y in vitro cell line

Spontaneous release 8%

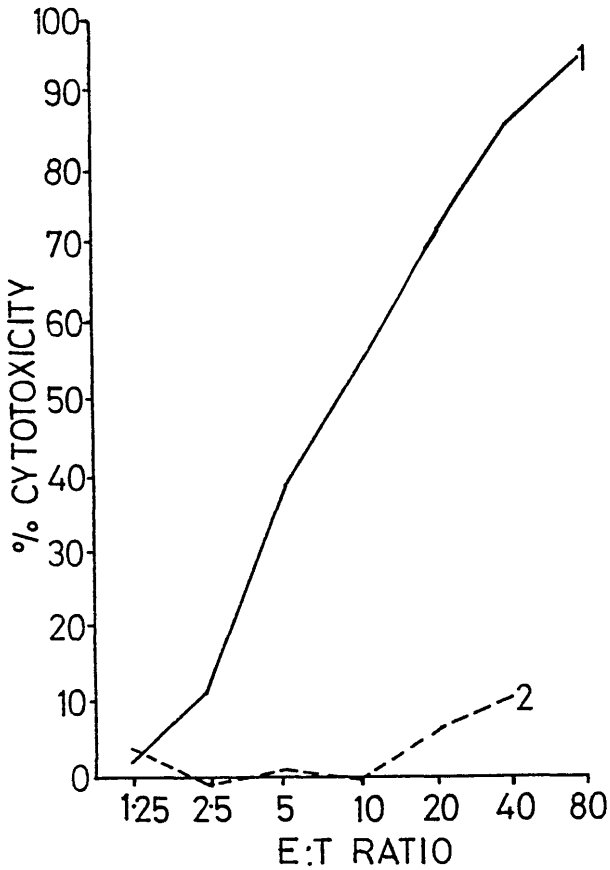


Figure 2.3.3.

2-mercaptoethanol requirement for generation of CTL in the MLC

Cultures were set up with or without 2-ME at a final concentration of 5×10^{-5} molar.

Line 1: cells cultured in the presence of 2-ME

2: cells cultured without 2-ME

CULTURE	1	2
% recovery	138	63
% viability	69	44

TARGET CELLS P815Y in vitro cell line

Spontaneous release 24%

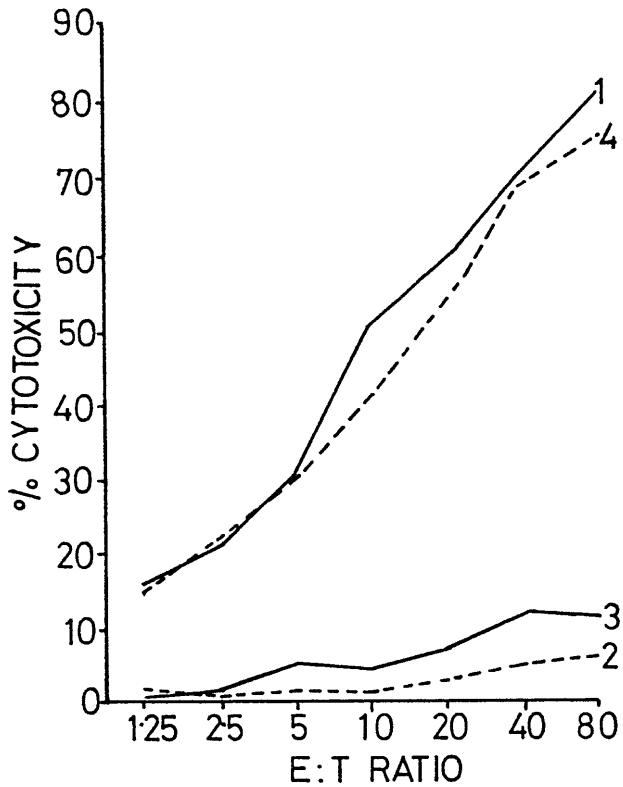


Figure 2.3.4.

Specificity of the cytotoxic response

Lines 1 and 4: responder cells assayed against target cells of the original stimulator strain

2 and 3: responder cells assayed against third party target cells

CULTURE	1, 2	3, 4
% recovery	127	131
% viability	81	79
TARGET CELLS	P815Y and EL4 *in vitro* cell lines	
Spontaneous release	10%	11%

responder cells were cultured alone, or with irradiated syngeneic cells. Control cultures consisted of CBA responder cells and Balb/c stimulator cells, and all cultures were assayed against P815Y target cells. As shown in Figure 2.3.5., no cytotoxic activity developed in the autologous or unstimulated cultures, while a high level of killing was observed in cells taken from control cultures.

These results indicate that the effector cells generated during the MLC are specific for the sensitising alloantigen, previously encountered on the stimulator cells.

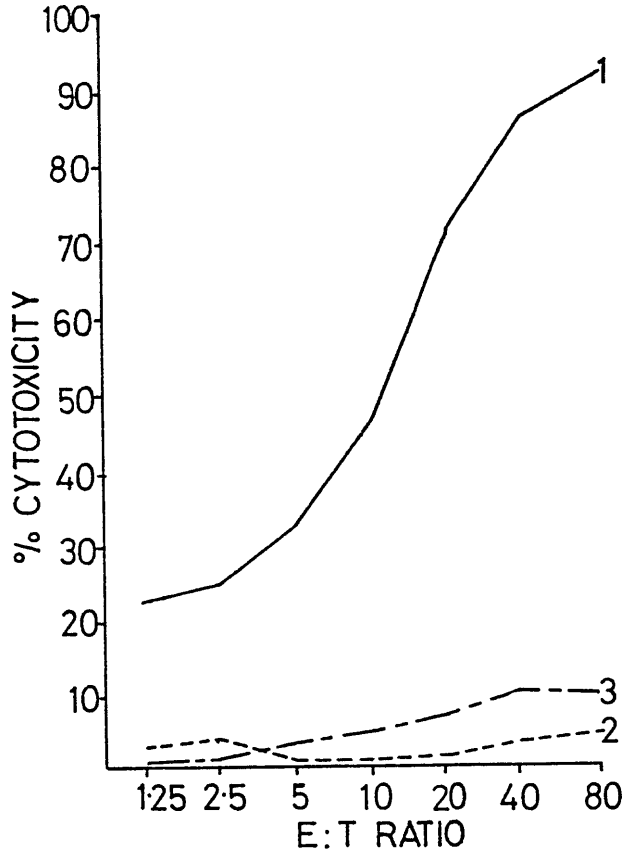


Figure 2.3.5.

Generation of CTL in unstimulated or self-stimulated cultures

Cells cultured alone, or in the presence of irradiated syngeneic or allogeneic cells, were assayed against allogeneic target cells.

Line 1: control; CBA responder cells cultured with Balb/c stimulator cells

2: CBA cells cultured alone

3: CBA cells cultured with irradiated syngeneic cells

CULTURE	1	2	3
% recovery	112	97	89
% viability	89	91	81

TARGET CELLS P815Y in vitro cell line

Spontaneous release 10%

2.3.2. ROLE OF ACCESSORY CELLS

2.3.2.1. Cell depletion using carbonyl iron powder

Carbonyl iron powder and a magnetic field can be used to deplete cell suspensions of macrophage-like accessory cells. This process was initially thought to depend on phagocytosis of the iron powder; however, it is now believed that adherence of the accessory cells to the iron particles is probably the basis for depletion (Sjöberg et al, 1972).

In the experiments described here, carbonyl iron powder was used to deplete responder and/or stimulator cells of the adherent/phagocytic fraction, as described in section 2.2.2.3. Cell suspensions were adjusted to 20 x 10^6 cells/ml and incubated with carbonyl iron powder, then the supernatant fluid containing the non-adherent cells was decanted. After washing, these non-adherent cells were resuspended in 10 x the volume of the supernatant fluid, such that their concentration was equivalent to that of an undepleted cell suspension at 2 x 10^6 cells/ml. Cell counts at this stage revealed that between 25-40% of the original cell number had been removed by carbonyl iron treatment. In some experiments, these cells were replaced by irradiated "filler" spleen cells of the responder strain. Control or depleted responder and stimulator cells were then cultured together in an MLC for 5 days, after which the effector cells were harvested and assayed against ^{51}Cr-labelled target cells.

As shown in Figure 2.3.6., carbonyl iron depletion of the responder population alone had little effect on the cytotoxic activity generated in the culture, and neither cell recovery or viability were markedly altered. However, if the stimulator population alone was depleted, the level of killing was suppressed. Little effect was observed on cell recovery and viability, and as the cytotoxicity assay is set up using the ratio of viable effector cells to targets, suppression

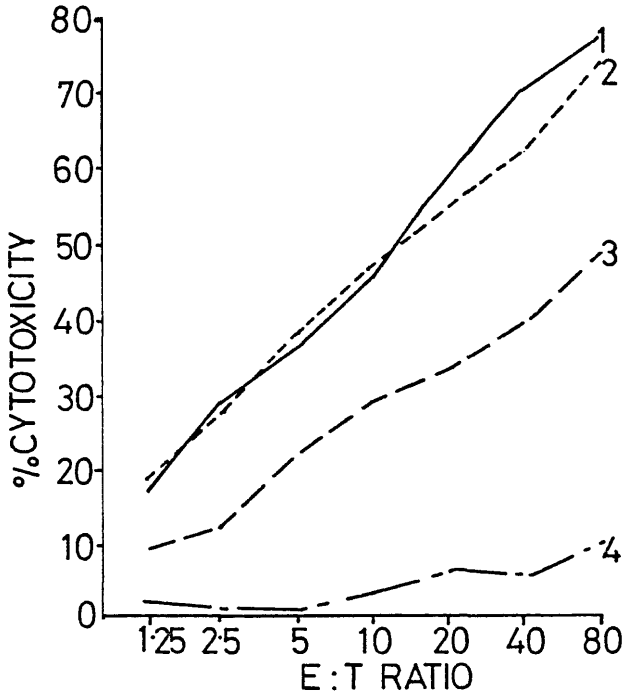


Figure 2.3.6.

Generation of CTL after depletion of the responder and stimulator cells using carbonyl iron powder

Line 1: control; undepleted responder and stimulator cells

2: responder cells depleted, stimulator cells undepleted

3: responder cells undepleted, stimulator cells depleted

4: both responder and stimulator cells depleted

CULTURE	1	2	3	4
% recovery	117	97	86	92
% viability	78	81	74	83

TARGET CELLS TGC-induced peritoneal cells

Spontaneous release 10%

could not be due to dilution of the cytotoxic effectors by non-viable cells. It is therefore likely that the observed suppression results from interference with the generation of cytotoxic activity in this culture. Carbonyl iron treatment of both the responder and stimulator populations led to total abrogation of the cytotoxic response, again without affecting the number of viable cells recovered from the culture.

As the cell numbers were not restored to 2×10^6 cells/ml after carbonyl iron treatment, it was suggested that the subsequent failure of the MLC to generate cytotoxic cells may be a density-dependent effect, perhaps due to a sub-optimal number of allogeneic stimulating cells. An identical culture was therefore set up with carbonyl iron-depleted responder and stimulator cells, in which the deficit in cell numbers was made up using either irradiated adherent cell-depleted CBA or irradiated adherent cell-depleted Balb/c filler cells. As shown in Figure 2.3.7., neither responder or stimulator filler cells were able to restore the cytotoxic activity of the culture, indicating that suppression of target cell lysis is not merely a reflection of the change in cell numbers. Hence it appears that carbonyl iron depletion of the responder and stimulator cells before the MLC is removing a population of cells which is essential for the generation of cytotoxic activity. However, this population is apparently not essential for the survival of the cells in culture, as depleted cultures did not show a concomitant fall in cell recovery and viability. Depletion of the responder cells alone indicates that the adherent accessory population required in this system need not be syngeneic to the responder cells.

To further investigate this requirement for a macrophage-like accessory cell, cultures were set up in which adherent peritoneal or

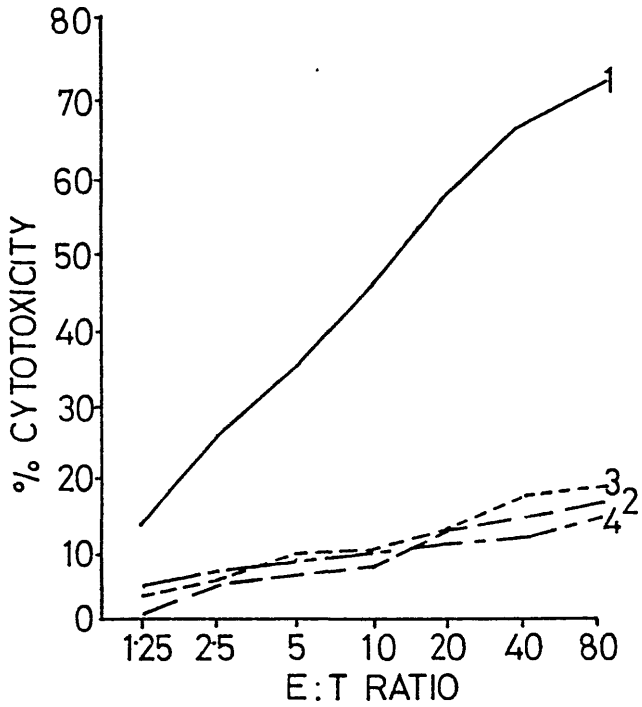


Figure 2.3.7.

Reconstitution of carbonyl iron-treated cells with irradiated depleted filler cells

Line 1: control; undepleted R and S cells

2: depleted R and S cells

3: depleted culture with added filler cells of responder strain

4: depleted culture with added filler cells of stimulator strain

CULTURE	1	2	3	4
% recovery	104	118	97	101
% viability	82	80	84	79

TARGET CELLS P815Y in vitro cell line

Spontaneous release 11%

spleen cells of the responder strain were added back to depleted cultures. In each case, the adherent fraction of the cell suspension was obtained by allowing the cells to adhere to culture wells for 3 hr, then rinsing off the non-adherent cells, as described in section 2.2.2.5. Figure 2.3.8. shows the results of a typical experiment in which the cultures were reconstituted with varying numbers of adherent peritoneal cells. The peritoneal cell suspension was found to consist of approximately 50% adherent cells under these conditions. Reconstitution of the depleted cultures using these cells led to restoration of the response in a dose-dependent manner, supporting the hypothesis that carbonyl iron depletion is selectively removing a macrophage-like adherent cell, which is required for the development of cytotoxic cells. That this increased lytic activity was not due to a direct cytotoxic effect of the adherent cells themselves is shown in Figure 2.3.8.; no cytotoxic activity developed in a culture consisting of irradiated depleted stimulator cells and adherent peritoneal cells of the responder strain, with no responder lymphocytes included. However, very few cells were recovered from this culture, presumably as few stimulator cells would still be present, and most of the adherent cells would remain attached to the culture plate at harvest.

Similar results were obtained from depleted cultures reconstituted with adherent spleen cells of the responder strain. As shown in Figure 2.3.9., depletion of the responder cells alone again had no suppressive effect on the level of the response, while depletion of the stimulator cells alone resulted in partial suppression to approximately 50% of the control response at the higher E:T ratios. Fully depleted cultures showed no cytotoxic activity; however, partial restoration was obtained in the presence of adherent spleen cells of the responder strain. The number of adherent cells added to the cultures was not assessed directly. However, as these cells were

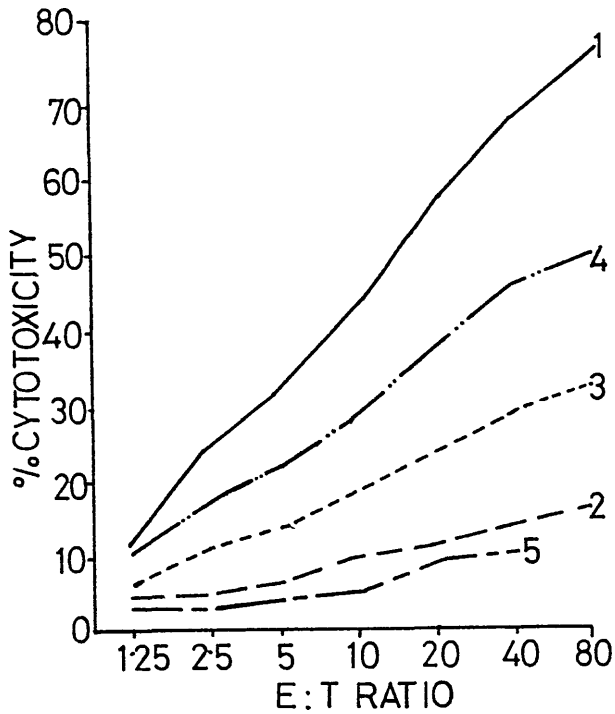


Figure 2.3.8.

Restoration of the cytotoxic response of carbonyl iron-treated cells by the addition of adherent peritoneal cells of the responder strain

Line 1: control; undepleted culture

2: fully depleted culture

3: depleted culture with added adherent cells (responder : peritoneal cells = 10:1)

4: depleted culture with added adherent cells (responder:peritoneal cells = 2:1)

5: negative control; adherent cells added to irradiated stimulator cells, in the absence of responder lymphocytes (stimulator:peritoneal cells = 2:1)

CULTURE	1	2	3	4	5
% recovery	101	79	79	81	-
% viability	78	77	82	80	60
TARGET CELLS	TGC-induced peritoneal cells				
Spontaneous release	10%				

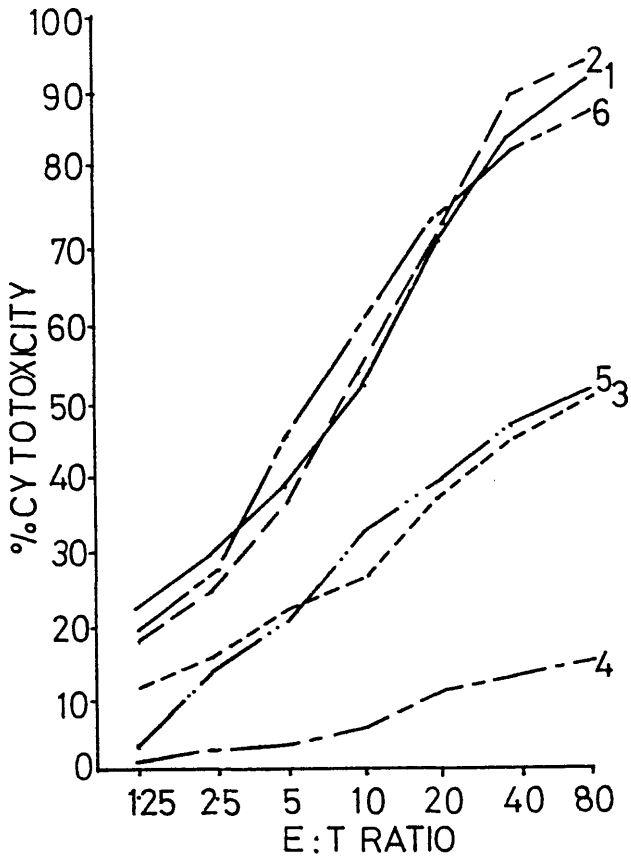


Figure 2.3.9.

Restoration of the cytotoxic response of carbonyl iron treated cells by the addition of adherent spleen cells of the responder strain

Line 1: control; undepleted culture

2: responder cells depleted, stimulator cells undepleted

3: responder cells undepleted, stimulator cells depleted

4: fully depleted culture

5: fully depleted culture with added spleen adherent cells

6: undepleted culture with added spleen adherent cells

CULTURE	1	2	3	4	5	6
% recovery	121	119	98	104	101	97
% viability	81	84	80	86	83	80

TARGET CELLS P815Y in vitro cell line

Spontaneous release 12%

obtained by allowing 2 x 10^6 untreated responder spleen cells to adhere to culture wells, it is likely that the number of adherent cells acquired was roughly equivalent to that found in an undepleted suspension, and to the number of such cells removed by carbonyl iron treatment.

It is interesting to note that even when large numbers of responder strain adherent peritoneal cells were added, the response of fully depleted cultures could not be completely restored to control levels. The maximum restoration obtained was to the level of cultures where only the stimulator cells were treated with carbonyl iron, the responder cells being undepleted. This suggests that part of the allogeneic stimulus may actually be removed from the stimulator population by carbonyl iron treatment, such that reconstitution with adherent cells of the responder strain alone cannot fully restore the cytotoxic response. To test this possibility, a similar experiment was set up in which adherent spleen cells of the stimulator strain were used to reconstitute carbonyl iron-treated cultures. As shown in Figure 2.3.10., addition of responder strain adherent cells again led to only partial restoration of cytotoxicity, while reconstitution with adherent cells of the stimulator strain restored the response almost to control levels. When the adherent cells used were not of the responder strain, irradiation was necessary to maintain a unidirectional MLC. Cell suspensions were irradiated before plating out for 3-hour adherence; this procedure did not seem to affect the adherent properties of the cells. Comparison of the restorative capacity of irradiated and non-irradiated responder adherent cells indicated that such treatment did not influence the accessory function of these cells in the MLC.

Irradiated adherent cells of a third party strain were added to some depleted cultures to investigate the requirement for histocompatibility between responder cells and the accessory population;

Figure 2.3.10.

Restoration of the cytotoxic response of carbonyl iron-treated cells using responder, stimulator, and third party splenic adherent cells

Line 1: control; undepleted culture

2: depleted culture

3: depleted culture with added non-irradiated adherent cells of the responder strain

4: depleted culture with added irradiated adherent cells of the responder strain

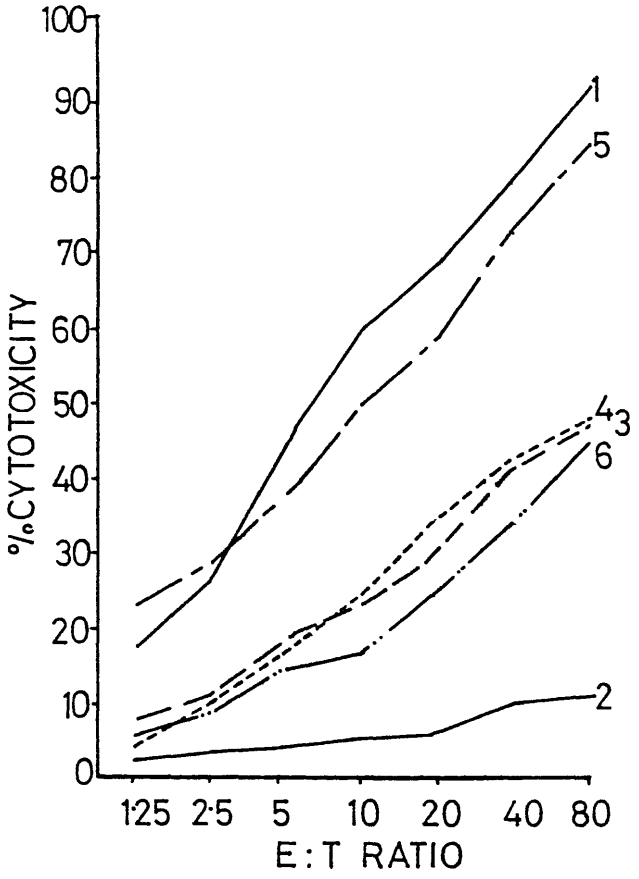
5: depleted culture with added irradiated adherent cells of the stimulator strain

6: depleted culture with added irradiated adherent cells of an unrelated third party strain

CULTURE	1	2	3	4	5	6
% recovery	132	119	124	106	111	109
% viability	83	86	79	82	83	85

TARGET CELLS P815Y *in vitro* cell line

Spontaneous release 17%


100
90
80
70
60
50
40
30
20
10
0
%CYTOTOXICITY
1
5
4
3
6
2
1·25
2·5
5
10
20
40
80
E:T RATIO

these cells were able to restore the cytotoxic response as efficiently as adherent cells of the responder strain, again indicating that there is no genetic restriction in this system.

2.3.2.2. Cell separation using nylon wool

The results of cell separation experiments involving nylon wool are presented in this section. Mixed leucocyte cultures were set up as before, using nylon wool non-adherent responder and/or stimulator cells. The results of such experiments are essentially similar to those obtained using carbonyl iron powder, as demonstrated in Figure 2.3.11. Fully adherent cell-depleted cultures, where both responder and stimulator cell suspensions were passed through a nylon wool column, generated no cytotoxic activity, while depletion of the stimulator cell suspension alone was less suppressive. However, using adherent cell-depleted responder cells and undepleted stimulator cells led to some suppression of the level of killing, in contrast to the corresponding experiment using carbonyl iron-treated responder cells, where no effect on cytotoxicity was observed (Figure 2.3.6.). This may be due to greater loss after nylon wool treatment, which yielded only 15-25% of the original cell number in the non-adherent fraction, compared with 60-75% recovery after carbonyl iron treatment. A similar experiment was performed in which the concentration of the nylon wool non-adherent cells was re-adjusted to 2×10^6 cells/ml, rather than being adjusted to a volume equivalent to that of an unseparated suspension, as described in section 2.3.2.1. As shown in Figure 2.3.12., this adjustment of cell numbers after separation did not restore the cytotoxic potential of the depleted cells, indicating that the reduction in cytotoxic activity was not merely due to the change in cell numbers. Consequently the cell concentration was not adjusted in future experiments, suspensions being made up to the equivalent volume as for carbonyl iron-treated cells (section 2.3.2.1.).

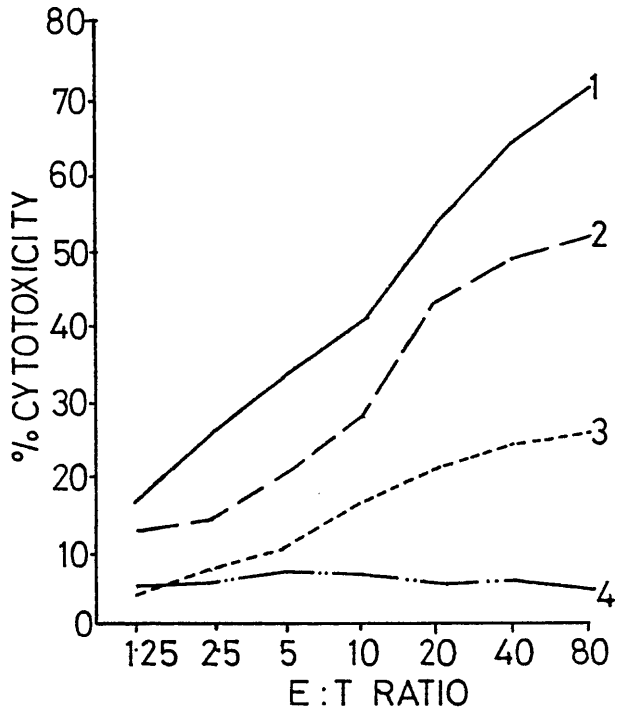


Figure 2.3.11.

Generation of CTL after nylon wool treatment of the responder and stimulator cells

Line 1: control; unseparated R and S cells

2: nylon wool non-adherent R cells, unseparated S cells

3: unseparated R cells, nylon wool non-adherent S cells

4: nylon wool non-adherent R and S cells

CULTURE	1	2	3	4
% recovery	110	89	81	74
% viability	87	79	74	79

TARGET CELLS P815Y in vitro cell line

Spontaneous release 11%

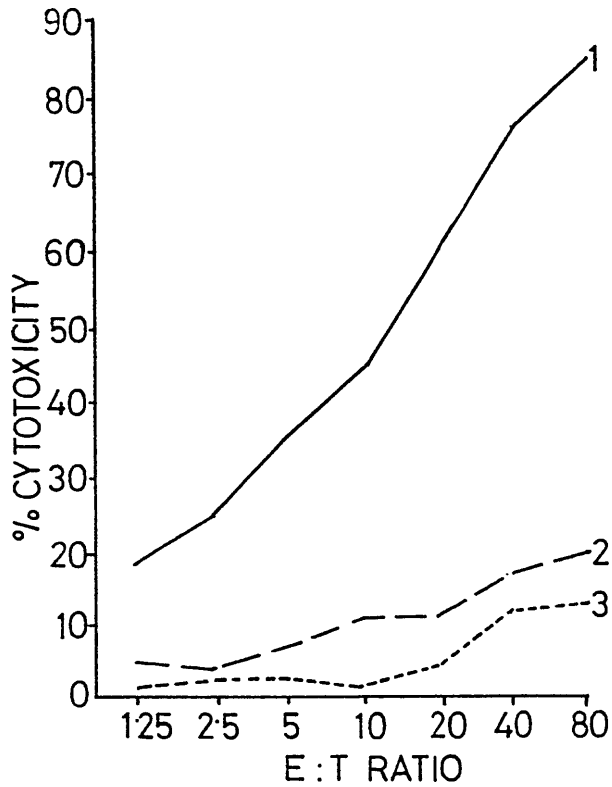


Figure 2.3.12.

Generation of CTL after nylon wool treatment of the responder and stimulator cells

Line 1: control; unseparated R and S cells

2: nylon wool non-adherent R and S cells; cell concentrations adjusted to 2 x 10^6 cells/ml

3: nylon wool non-adherent R and S cells; volume of cell suspensions adjusted to equal that of control suspensions

CULTURE	1	2	3
% recovery	112	79	68
% viability	87	82	79

TARGET CELLS TGC-induced peritoneal cells

Spontaneous release 11%

It is possible that some other cell types are removed by nylon wool in addition to the strongly adherent accessory cells. For example, the alloreactive cells themselves may be depleted, and this may explain the observed fall in activity after depletion of the responder population alone. There also appeared to be a slight reduction in cell recovery and viability of cultures set up using nylon wool-treated cells; the reason for this is not clear. However, the overall results confirm previous observations using carbonyl iron-treated cells, by demonstrating the requirement for an adherent cell in this system, which need not be histocompatible with the responding lymphocytes. There is also support for the earlier hypothesis that the adherent fraction of the stimulator population is providing a significant portion of the allogeneic stimulus in the MLC.

Further examination of the accessory cell requirement in this system was performed by reconstituting adherent cell-depleted cultures with adherent cells. The cells retained on the nylon wool column could be recovered, as described in section 2.2.2.4.3.; such adherent populations were then added back to fully depleted cultures for the duration of the MLC. As shown in Figure 2.3.13., reconstitution of fully depleted cultures with nylon wool-adherent cells of the stimulator strain alone led to considerable restoration of the response, the level of killing attained being equivalent to that found in cultures where only the responder population was depleted (Figure 2.3.11., line 2). Reconstitution with nylon wool-adherent cells of the responder strain alone was less effective at restoring the cytotoxic response; however, the results obtained are comparable to those found in cultures where the stimulator cells alone were depleted (Figure 2.3.11., line 3). By adding back the nylon wool-adherent cells of both responder and stimulator strains, the cytotoxic activity of the culture could be restored almost to control levels.

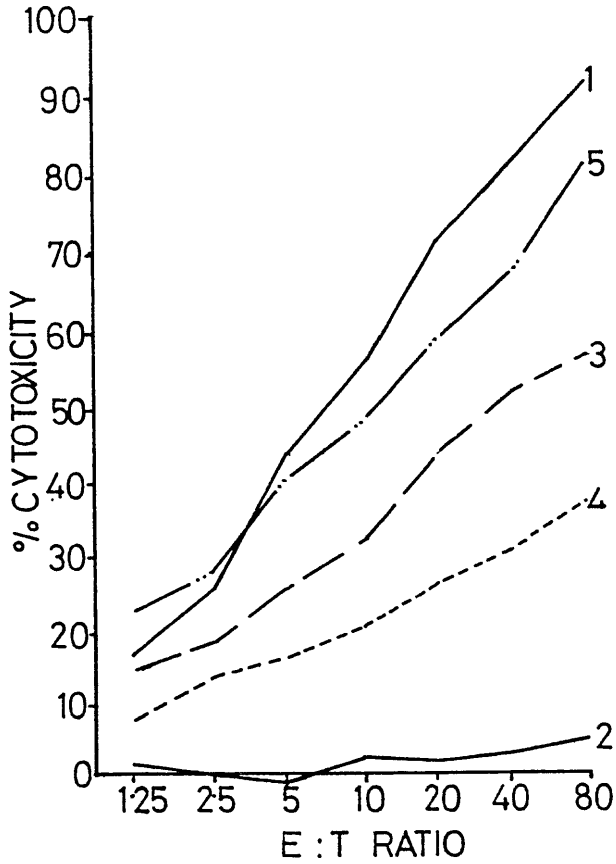


Figure 2.3.13.

Restoration of the cytotoxic response of nylon wool-treated cells by the addition of nylon wool-adherent cells

Line 1: control; unseparated R and S cells

2: nylon wool non-adherent R and S cells, i.e. adherent cell-depleted culture

3: depleted culture with added nylon wool-adherent cells of the stimulator strain

4: depleted culture with added nylon wool-adherent cells of the responder strain

5: depleted culture with added nylon wool-adherent cells of both R and S strains

CULTURE	1	2	3	4	5
% recovery	117	68	76	71	93
% viability	91	74	79	77	89
TARGET CELLS	TGC-induced peritoneal cells				
Spontaneous release	14%				

These results indicate that the adherent population removed by passing cells through a nylon wool column is essential for the development of cytotoxicity in the MLC, and show that this population can be recovered in an active form from the column, and can then be added back to cultures to restore the response of depleted cells. A similar experiment was set up in which depleted cultures were reconstituted with adherent peritoneal cells of the responder strain. Even using relatively high numbers of peritoneal cells (1×10^6 adherent peritoneal cells per 2×10^6 responder cells), only partial restoration of the response was obtained, as shown in Figure 2.3.14. This is in agreement with previous observations indicating that adherent cells of the stimulator strain are required for maximal stimulation of the cytotoxic response. A repeat experiment was therefore set up, using adherent peritoneal cells of both responder and stimulator strains. As shown in Figure 2.3.15., addition of stimulator strain adherent cells led to improved restoration of cytotoxic activity, giving a response equivalent to that obtained using depleted responder cells and undepleted stimulator cells in the MLC (Figure 2.3.11., line 2). That this response was not fully restored to control levels is in agreement with the previous suggestion that nylon wool treatment of the responder population removes an additional sub-population of cells, which is not present in the 3-hour plastic-adherent population studied here. Consequently it is not surprising that the addition of equal numbers of adherent peritoneal cells of both responder and stimulator strains did not lead to any further restoration of the response.

2.3.2.3. Cell separation by adherence to plastic

The strongly adherent fraction can be separated from a cell suspension by allowing the cells to attach to a culture plate, as

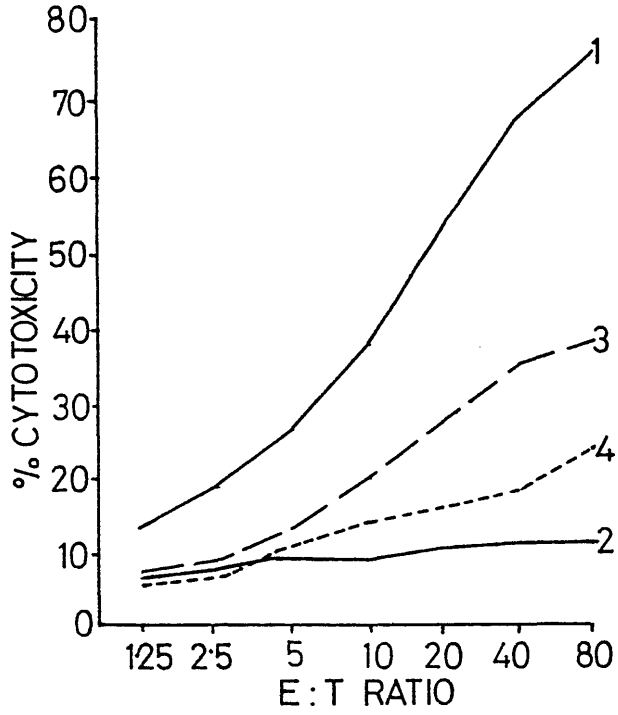


Figure 2.3.14.

Restoration of the cytotoxic response of nylon wool-treated cells by the addition of adherent peritoneal cells of the responder strain

Line 1: control; unseparated R and S cells

2: adherent cell-depleted culture

3: depleted culture with added adherent cells (responder: peritoneal cells = 2:1)

4: depleted culture with added adherent cells (responder:peritoneal cells = 20:1)

CULTURE	1	2	3	4
% recovery	98	63	75	71
% viability	79	72	86	79

TARGET CELLS TGC-induced peritoneal cells

Spontaneous release 13%

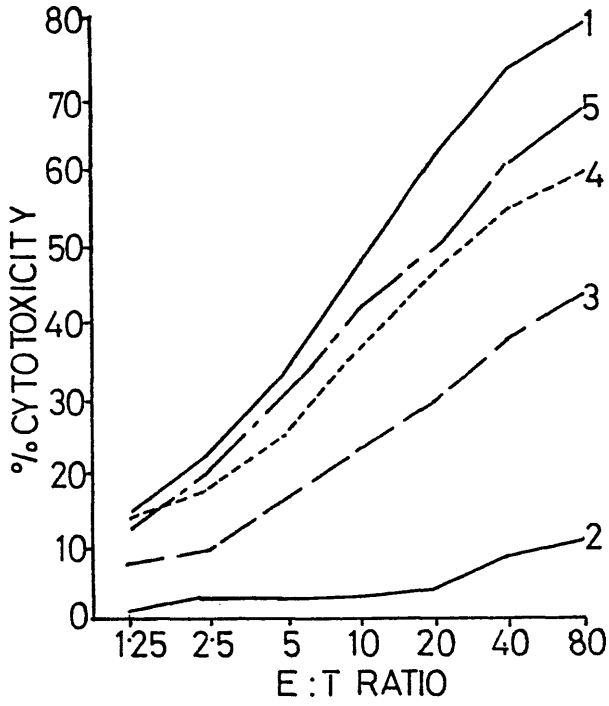


Figure 2.3.15.

Restoration of the cytotoxic response of nylon wool-treated cells by the addition of adherent peritoneal cells of both responder and stimulator strains

Line 1: control; unseparated R and S cells

2: adherent cell-depleted culture

3: depleted culture with 1 x 10^6 R strain peritoneal cells per well

4: depleted culture with 1 x 10^6 S strain peritoneal cells per well

5: depleted culture with 1 x 10^6 R strain and 1 x 10^6 S strain peritoneal cells per well

CULTURE	1	2	3	4	5
% recovery	98	79	83	84	88
% viability	89	78	81	79	83

TARGET CELLS P815Y in vivo tumour cells

Spontaneous release 11%

described in section 2.2.2.5. The results of a typical experiment using this method are presented in Figure 2.3.16.; after 3 hr in a CO_2 incubator, a sufficient number of adherent accessory cells had been removed to interfere with the generation of cytotoxic activity in a subsequent MLC. If both responder and stimulator populations were depleted in this way, the cytotoxicity developed in culture was markedly reduced, while cell recovery and viability were apparently unaffected. Depletion of the responder population alone had little suppressive effect on the response, while treatment of the stimulator cells alone led to pronounced suppression, approaching that obtained in a fully depleted culture. From these results it appears that plastic adherence is a less efficient means of depleting a cell suspension of the adherent accessory fraction, as sufficient accessory cells are still present to support a limited cytotoxic response in cultures consisting of depleted responder and stimulator cells. However, as depletion of the stimulator population alone still led to a marked fall in cytotoxic activity, it is possible that this method is equally effective at removing the alloantigen-presenting cells.

Overall, these results indicate a general agreement with previous findings using carbonyl iron powder and nylon wool; depletion of both responder and stimulator cell suspensions led to a reduction in the cytotoxic response, while removal of the adherent cells from only one population indicates that the accessory cell requirement may be fulfilled by cells of either strain. The stimulatory function of the allogeneic adherent cells is again demonstrated.

2.3.2.4. Time-course of the adherent cell requirement

Attachment of the adherent cells to culture wells during the MLC can be exploited to assess the time-course of the adherent cell requirement in this system.

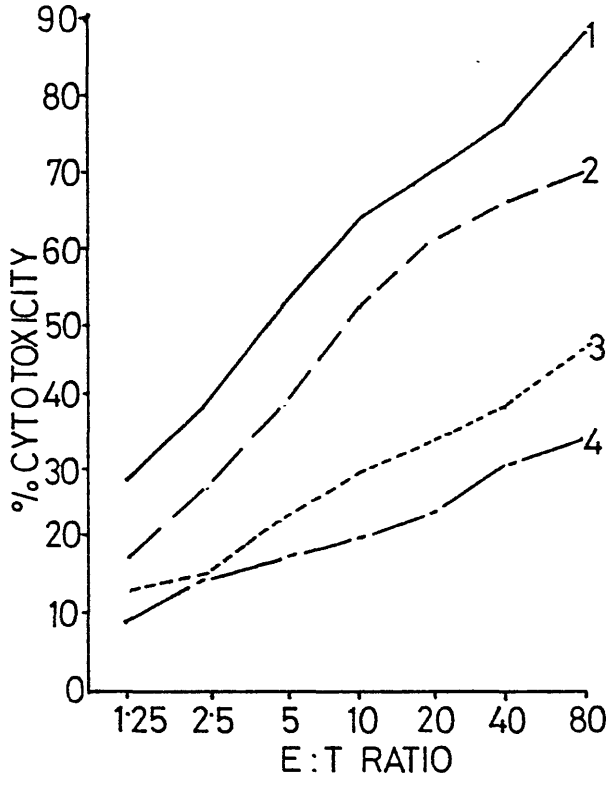


Figure 2.3.16.

Generation of CTL after adherent cell-depletion of the responder and stimulator cells by 3-hour plastic adherence

Line 1: control; undepleted R and S cells

2: depleted R cells, undepleted S cells

3: undepleted R cells, depleted S cells

4: both R and S cells depleted

CULTURE	1	2	3	4
% recovery	89	79	84	82
% viability	84	81	91	86

TARGET CELLS P815Y in vitro cell line

Spontaneous release 11%

Mixed leucocyte cultures were set up in the usual way, with undepleted responder and stimulator cell suspensions. Figure 2.3.17. shows the results obtained when the non-adherent cells of such cultures were subsequently resuspended and transferred to new culture wells. Cells transferred to fresh wells after 3 hr, without the addition of any further adherent cells, failed to generate a cytotoxic response. However, if these cells were transferred to wells containing adherent spleen cells of the responder strain, a limited cytotoxic response was obtained, while transfer to wells containing adherent stimulator spleen cells allowed development of a cytotoxic response comparable to that obtained from an undisturbed control culture. These results indicate that the adherent accessory population is still required in this system after the two cell suspensions have been in contact for 3 hr. The results also show that the mixed responder and stimulator populations can be manipulated after the initiation of culture without any adverse effects on their cytotoxic potential, and that the subsequent failure of cells transferred to fresh wells to generate cytotoxic effector lymphocytes is entirely due to the selective loss of their adherent accessory populations. The dual role of adherent cells in the stimulator cell suspension is again apparent; these cells serve to provide part of the allogeneic stimulus, and are also capable of co-operating with the responding lymphocytes in the generation and maturation of cytotoxic effector cells.

Having found that cells in the MLC can be disturbed and transferred to different culture wells without injury, further experiments were performed to ascertain the interval during which adherent cells were required for the generation of cytotoxicity in this system. In a typical experiment, cultures were established and after varying time intervals the non-adherent cells were resuspended and transferred to fresh culture wells, as described in section 2.2.2.5. The results

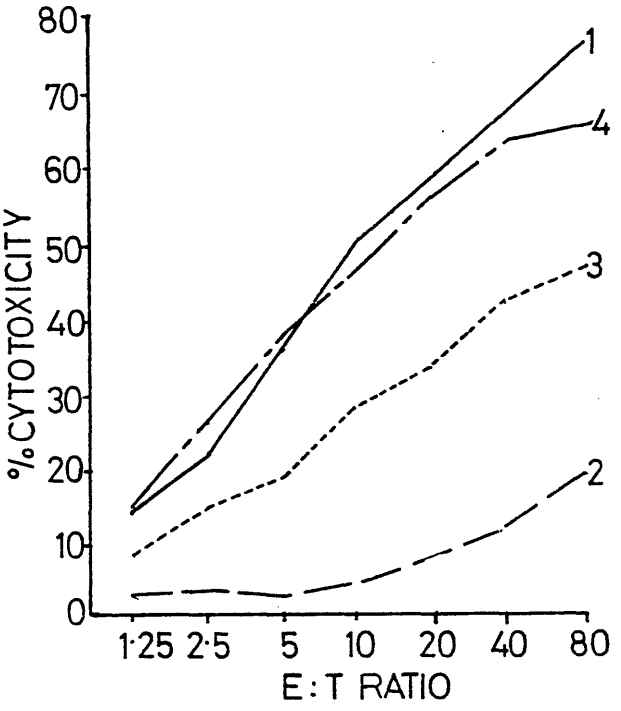


Figure 2.3.17.

Adherent cell requirement for the generation of CTL in the MLC

Line 1: control; undisturbed cultures

2: mixed leucocytes transferred to fresh wells after 3 hours

3: cells transferred after 3 hours to wells containing adherent R strain spleen cells

4: cells transferred after 3 hours to wells containing adherent S strain spleen cells

CULTURE	1	2	3	4
% recovery	103	93	94	97
% viability	88	79	82	85

TARGET CELLS TGC-induced peritoneal cells

Spontaneous release 16%

presented in Figure 2.3.18. show that there is no requirement for adherent cells after the first 24 hr of the MLC; cells transferred to fresh wells after this time, and so depleted of their adherent fraction, did not show a concomitant reduction in the level of the cytotoxic response. However, cells transferred only 4 hr after the initiation of the MLC had not yet discharged their requirement for an adherent accessory population, and when depleted of such cells by transfer they were unable to mount a cytotoxic response against the allogeneic stimulating cells. This failure to respond is unlikely to be entirely due to loss of the allogeneic adherent stimulating cells, as the transferred lymphocytes were capable of a partial response in the presence of syngeneic adherent cells, as shown in Figure 2.3.17., line 3.

It was suggested that the adherent properties of the accessory population may change during the initial 24 hr of culture, these cells detaching from the culture wells and so becoming available for transfer with the non-adherent fraction. Such behaviour would allow them to continue in their accessory role. To test this possibility, cultures were set up in which the non-adherent population was transferred after 4 hr into fresh wells with no further adherent cells added, or onto adherent cells obtained from an MLC after 4 hr or 24 hr. The results of such an experiment are given in Figure 2.3.19., and show that the early adherent cell requirement of the mixed non-adherent cells in this culture system can be fulfilled by both 4-hour and 24-hour adherent populations. The requirement for adherent cells of the stimulator strain in order to obtain an optimal cytotoxic response is again demonstrated. These results suggest that the cells adhering to culture wells after 4 hr and after 24 hr *in vitro* belong to the same functional population. In view of the results obtained in the previous time-course experiments, we may therefore conclude that the adherent accessory cells required for the generation of cytotoxic effector cells in the MLC

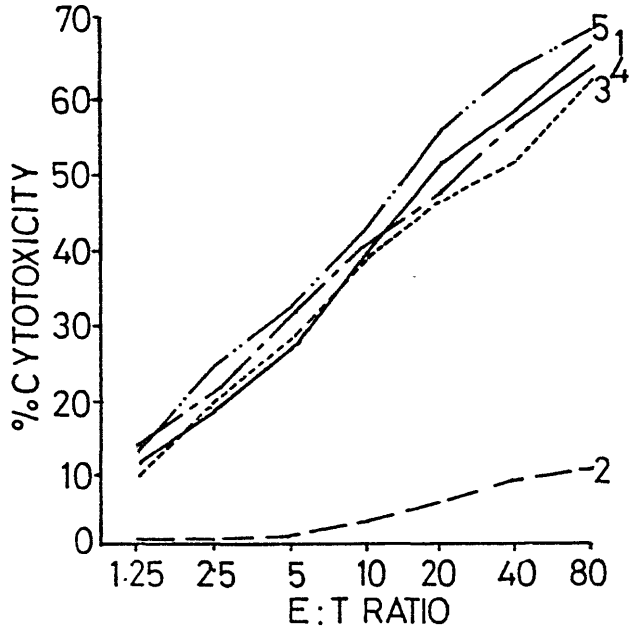


Figure 2.3.18.

Time-course of the adherent cell requirement for the generation of CTL in the MLC

Line 1: control; undisturbed cultures

2: cells transferred to fresh wells after 4 hours

3: cells transferred to fresh wells after 24 hours

4: cells transferred to fresh wells after 48 hours

5: cells transferred to fresh wells after 72 hours

CULTURE	1	2	3	4	5
% recovery	122	98	101	112	103
% viability	87	85	88	87	90

TARGET CELLS P815Y in vitro cell line

Spontaneous release 13%

Figure 2.3.19.

Adherent cell requirement for the generation of CTL in the MLC

Line 1: control; cells cultured undisturbed for 5 days

2: non-adherent cells transferred to fresh wells after 24 hours, and cultured for a further 4 days in the absence of adherent cells

3: non-adherent cells transferred to fresh wells after 4 hours, and maintained for the remainder of the culture period in the absence of further adherent cells

4: non-adherent cells transferred after 4 hours onto 4-hour-adherent cells from an identical culture

5: non-adherent cells transferred after 4 hours onto 24-hour-adherent cells from an identical culture

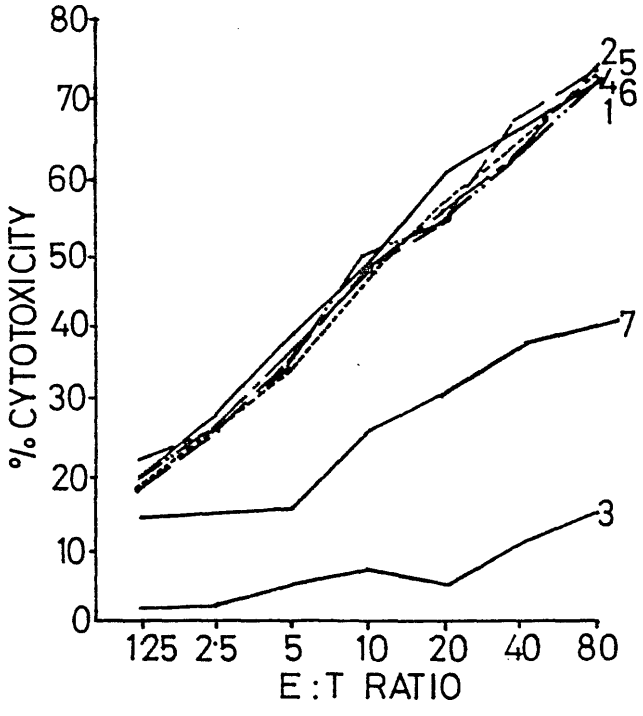
6: non-adherent cells transferred after 4 hours onto 24-hour-adherent cells of the stimulator strain

7: non-adherent cells transferred after 4 hours onto 24-hour-adherent cells of the responder strain

CULTURE	1	2	3	4	5	6	7
% recovery	148	134	119	149	113	136	117
% viability	66	65	70	67	68	67	69

TARGET CELLS P815Y *in vivo* tumour cells

Spontaneous release 16%


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
125 2·5 5 10 20 40 80
E : T RATIO
2
5
4
6
1
7
3

fulfill their function during the first 24 hr of the culture period, and that these cells presumably play some vital role during the initial sensitisation phase of the mixed leucocyte reaction.

2.4. EFFECTS OF MACROPHAGE-ACTIVE AGENTS ON THE GENERATION OF CYTOTOXIC T LYMPHOCYTES IN THE MURINE MIXED LEUCOCYTE CULTURE

2.4.1. SILICA

2.4.1.1. Effects of silica on the development of cell mediated cytotoxicity *in vitro*

2.4.1.2. Time-course of silica-induced suppression of the cytotoxic response

2.4.2. CARRAGEENAN

2.4.2.1. Different types of carrageenan

2.4.2.2. Time-course of carrageenan-induced suppression of the cytotoxic response

2.4.3. ZYMOSAN

2.4.3.1. Effects of zymosan on the development of cell-mediated cytotoxicity *in vitro*

2.4.3.2. Time-course of zymosan-induced suppression of the cytotoxic response

2.4.4. LATEX

2.4.4.1. Effects of latex on the development of cell-mediated cytotoxicity *in vitro*

2.4.5. TOXICITY OF SILICA, CARRAGEENAN AND ZYMOSAN TOWARDS ADHERENT CELLS *IN VITRO*

2.4.5.1. Viability of adherent cells cultured with macrophage-active agents

2.4.5.2. Effect of macrophage-active agents on the number of adherent cells remaining after culture

2.4.1. SILICA

2.4.1.1. Effects of silica on the development of cell-mediated cytotoxicity in vitro

Suspensions of protein-coated silica particles were prepared as described in section 2.2.1.9.1. Shortly before addition to MLCs, the silica suspensions were thoroughly mixed using a Vortex, or were exposed to ultrasonic vibration for 2 minutes to disperse the particles.

By adding a range of concentrations of silica to cell cultures, dose-response relationships were constructed for suspensions prepared by Vortex or by sonication. The results of such an experiment are shown in Figure 2.4.1., and clearly demonstrate the dose-dependent suppressive effect of silica in this system. Silica suspensions prepared by sonication were more potent in this respect than those mixed by Vortex; it is suggested that the former method may yield a more finely dispersed suspension, resulting in enhanced phagocytosis of silica particles, and consequently a lower effective dose. Further experiments were therefore performed using sonicated silica suspensions.

There appeared to be no concomitant fall in recovery of viable cells from cultures treated with either silica suspension, even at the highest dose tested. However, as complete suppression of the response was obtained at the slightly lower dose of 100 µg silica/ml, this concentration was used in future experiments.

2.4.1.2. Time-course of silica-induced suppression of the cytotoxic response

To investigate the time-course of silica-induced suppression, sonicated silica was added to MLCs at initiation of culture or at 24-hour intervals subsequent to this. The same dose (100 µg silica/ml culture fluid) was added to some cultures on day 5 for the duration of the cytotoxicity assay. As shown in Figure 2.4.2., silica administered

Figure 2.4.1.

Dose response relationships for Vortexed and sonicated silica suspensions

Suspensions of silica particles dispersed by Vortex or by sonication were added to MLCs at initiation, to give the following range of final concentrations.

Line 1: control; no silica added to culture

2: 10 µg silica/ml

3: 20 µg silica/ml

4: 50 µg silica/ml

5: 100 µg silica/ml

6: 150 µg silica/ml

TREATMENT	-	Vortex					Sonication				
CULTURE	1	2	3	4	5	6	2	3	4	5	6
% recovery	117	120	112	124	115	120	112	109	121	118	116
% viability	87	89	93	87	91	88	93	92	86	89	90

TARGET CELLS	P815Y *in vitro* cell line
Spontaneous release	12%

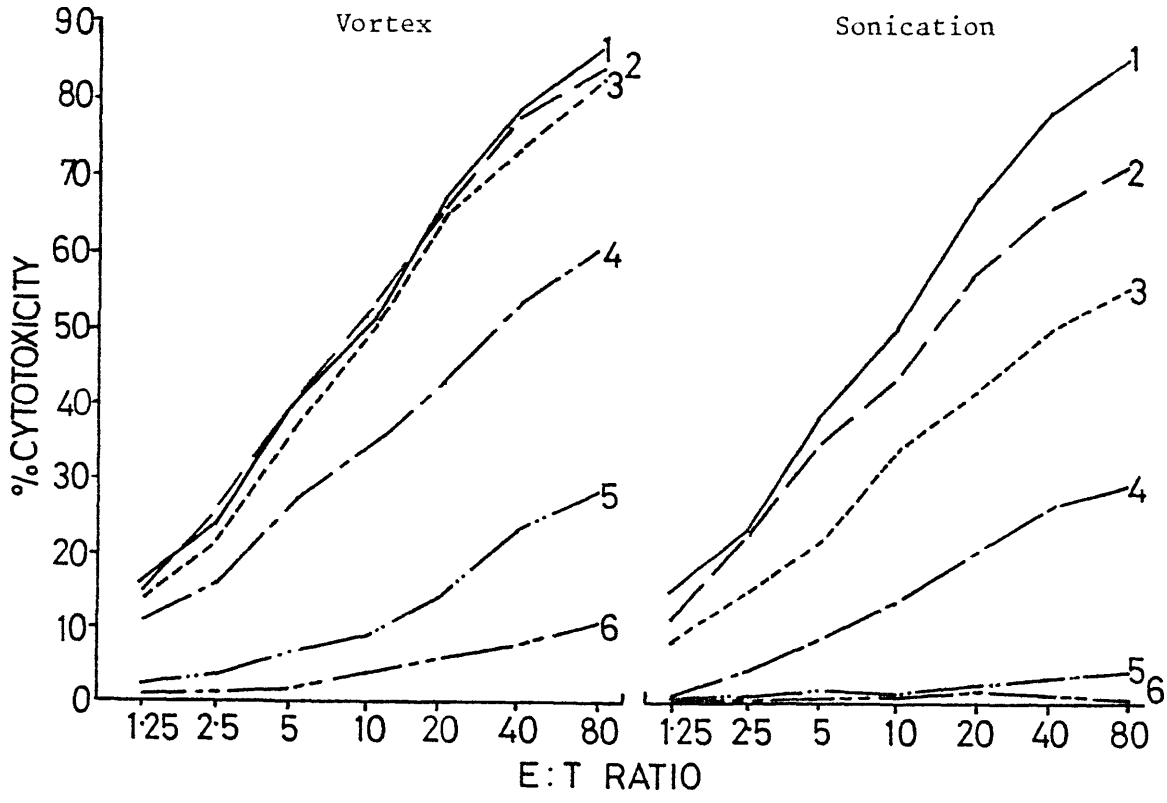

Vortex
Sonication
%CYTOTOXICITY
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
1·25
2·5
5
10
20
40
80
E:T RATIO

Figure 2.4.2.

Time-course of silica-induced suppression of the cytotoxic response in vitro

Sonicated silica was added to MLCs at initiation or at 24-hour intervals after this, to give a final concentration of 100 μg/ml. All cultures were harvested and assayed together at the end of the 5-day culture period, and silica was added to some previously untreated cells for the duration of the cytotoxicity assay.

Line 1: control; no silica added to culture

2: silica added on day 0

3: silica added on day 1

4: silica added on day 2

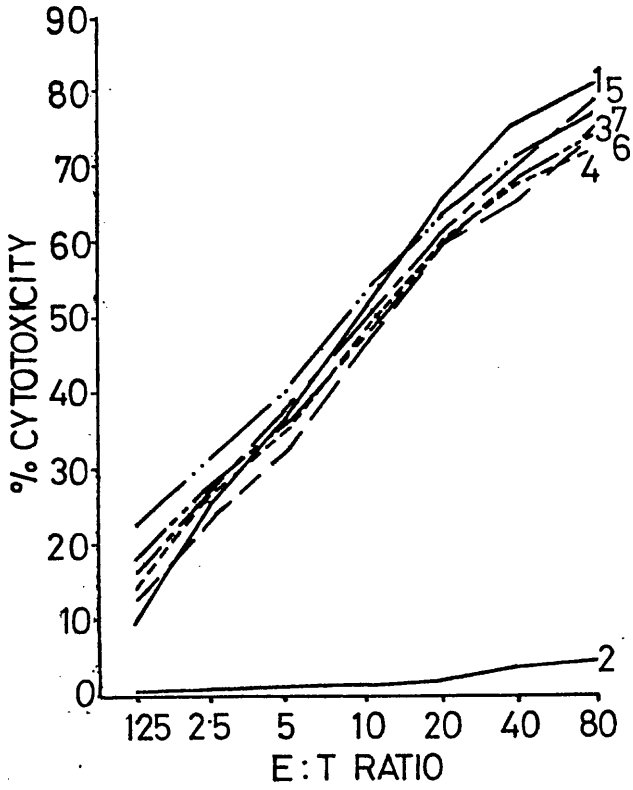
5: silica added on day 3

6: silica added on day 4

7: silica added to cytotoxicity assay on day 5

CULTURE	1	2	3	4	5	6
% recovery	128	121	123	117	113	120
% viability	89	92	90	94	93	88

TARGET CELLS	P815Y in vitro cell line
Spontaneous release	12%


90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
125
2·5
5
10
20
40
80
E:T RATIO
1
5
3
7
6
4
2

at the start of the culture period fully abrogated the cytotoxic response. However, if silica was added to cultures 24 hours later, it had no apparent effect on the level of killing, and subsequent additions were equally ineffective. These results indicate that the silica-sensitive phase of the MLC occurs sometime during the first 24 hours. The failure of subsequent silica treatment to induce suppression, accompanied by the apparent lack of toxicity towards the non-adherent cells harvested after the culture period, demonstrates that this material is not exerting its effects directly on the effector T lymphocytes. It is interesting to note that the time-course of silica-sensitivity exactly parallels the requirement for adherent accessory cells in this system, suggesting that the suppressive effects of this agent may be dependent on such cells. Silica particles have been reported to cause lysis of phagocytic cells without affecting lymphocyte viability (Lotzova and Cudkowicz, 1974), and such an effect would be compatible with the results observed here; macrophage-like accessory cells have been shown to be required in the initial stages of development of cytotoxicity in this system, and destruction of a large proportion of these cells after ingestion of silica would prevent generation of cytolytic effector cells.

After harvest of the non-adherent fraction, most of the cells remaining attached to the culture plate could be recovered, as described in section 2.2.2.5. Microscopic examination of such cells obtained after the MLC provided some support for the above hypothesis; few adherent cells were recovered from cultures treated with silica at any stage, and most of the cells which were present were non-viable according to FDA-EB stain (section 2.4.5.1.), and were unable to ingest opsonised yeast particles. In contrast, adherent cells recovered from control cultures appeared healthy, and were phagocytically competent. Indirect quantitation of the number of adherent cells surviving exposure to silica confirmed the toxicity of this material (section 2.4.5.2.).

The data presented in this section suggest that silica exerts its suppressive effects on cell-mediated cytotoxicity in the MLC by destroying phagocytic accessory cells. At initiation of the MLC, such destruction impedes development of the cytotoxic response; however, lysis of the accessory cells after the first 24 hours of culture does not affect the response, as the absolute requirement for such cells is over, and the sequence of events leading to generation of cytotoxic cells has commenced.

2.4.2. CARRAGEENAN

2.4.2.1. Different types of carrageenan

Several types of carrageenan of varying degress of purity are commercially available. In the following experiments, five types were studied, including three purified forms.

Type I and type II carrageenan (Sigma Chemical Company) are relatively unpurified, and consist mainly of κ- and ℓ-carrageenan respectively. By adding solutions of these agents to MLCs at the beginning of 5-day cultures, the effects of different doses of each type were determined.

As shown in Figure 2.4.3., type II carrageenan strongly inhibited the cytotoxic response, causing complete abrogation of cytotoxicity at a dose of 50 µg/ml culture fluid. In contrast, type I carrageenan had little effect on the response, with no evidence of suppression even at the highest dose tested (100 µg carrageenan/ml). Cultures exposed to this high concentration of either carrageenan yielded considerably fewer viable cells; this may indicate a lymphocyte-toxic effect at high doses. Full suppression of the response was obtained using apparently non-toxic doses of type II carrageenan, and as equal numbers of viable cells were assayed from each culture, this effect cannot be attributed to an increased number

Figure 2.4.3.

Dose-response relationships for type I and type II carrageenan

Type I and type II carrageenan were added to MLCs at initiation, to give the following range of final concentrations.

Line 1: control; no carrageenan/ml

2: 10 µg carrageenan/ml

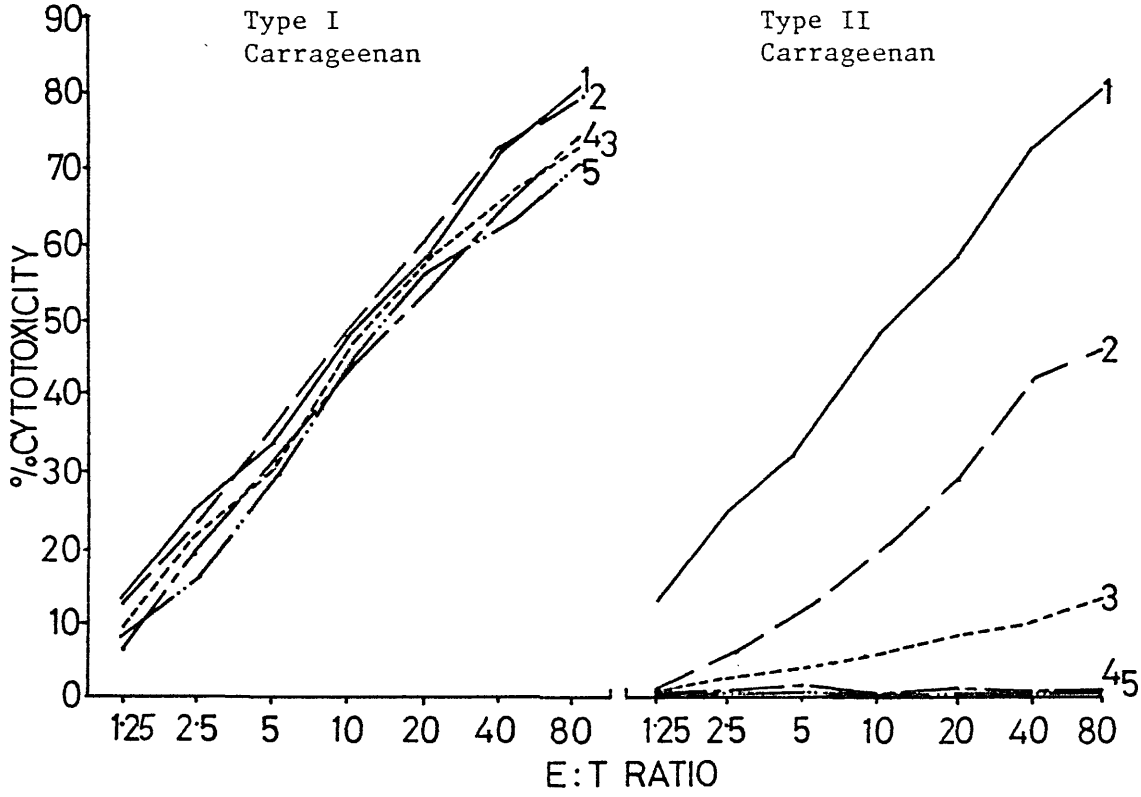
3: 20 µg carrageenan/ml

4: 50 µg carrageenan/ml

5: 100 µg carrageenan/ml

TREATMENT	-	Type I carrageenan				Type II carrageenan			
CULTURE	1	2	3	4	5	2	3	4	5
% recovery	141	150	137	146	90	147	153	149	84
% viability	89	90	92	88	79	91	94	90	74

TARGET CELLS	TGC-induced peritoneal cells
Spontaneous release	13%


Type I
Carrageenan
Type II
Carrageenan
%CYTOTOXICITY
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
1·25
2·5
5
10
20
40
80
E:T RATIO

of non-viable cells in the effector population.

From these results, 2 doses were selected for use with the more purified types of carrageenan, namely 20 μg/ml and 50 μg/ml culture fluid. The results of a typical experiment involving types III, IV, and V carrageenan are presented in Figure 2.4.4. Type III carrageenan is a purified κ form; the failure of this preparation to suppress cytotoxicity at either dose is compatible with the results obtained using type I. However, addition of type IV or type V carrageenan to cultures resulted in marked suppression of cytotoxic activity, with no concomitant fall in cell recovery and viability. Type V carrageenan is a purified ι form, and would therefore be expected to behave in a similar manner to type II, while type IV carrageenan consists of the purified λ form.

From these results it was possible to grade the different types of carrageenan in order of their suppressive potency in this system, as shown in Table 2.4.1. This order of activity is in agreement with that found by other workers, using a similar cell-mediated cytotoxicity system (Yung and Cudkowicz, 1978a), and using an inflammatory model (Davies and Allison, 1976a). It is interesting to note that the order of activity of the purified carrageenan preparations parallels their sulphate content, and it is possible that these groups have a biochemical function associated with the observed effects of different carrageenans on the immune system.

Both λ- and ι-carrageenan were active over the same dose range, as shown in Figure 2.4.5., and a dose was selected for further work which appeared to be fully suppressive without causing indiscriminate cell injury. Subsequent experiments were therefore performed using λ- or ι-carrageenan at a final concentration of 20 μg/ml culture fluid.

2.4.2.2. Time-course of carrageenan-induced suppression of the cytotoxic response

To investigate the time-course of carrageenan-induced suppression,

Figure 2.4.4.

Effect of different types of purified carrageenan on the generation of CTL in the MLC

Purified κ-, λ-, and ι-carrageenans were added to MLCs at initiation. Each type was tested at two concentrations previously shown to be suppressive using type II carrageenan.

Line 1: control; no carrageenan added to culture

2: 20 μg κ-carrageenan/ml

3: 50 μg κ-carrageenan/ml

4: 20 μg λ-carrageenan/ml

5: 50 μg λ-carrageenan/ml

6: 20 μg ι-carrageenan/ml

7: 50 μg ι-carrageenan/ml

CULTURE	1	2	3	4	5	6	7
% recovery	126	114	121	120	106	131	119
% viability	90	91	94	91	93	94	90

TARGET CELLS	P815Y in vitro cell line
Spontaneous release	11%

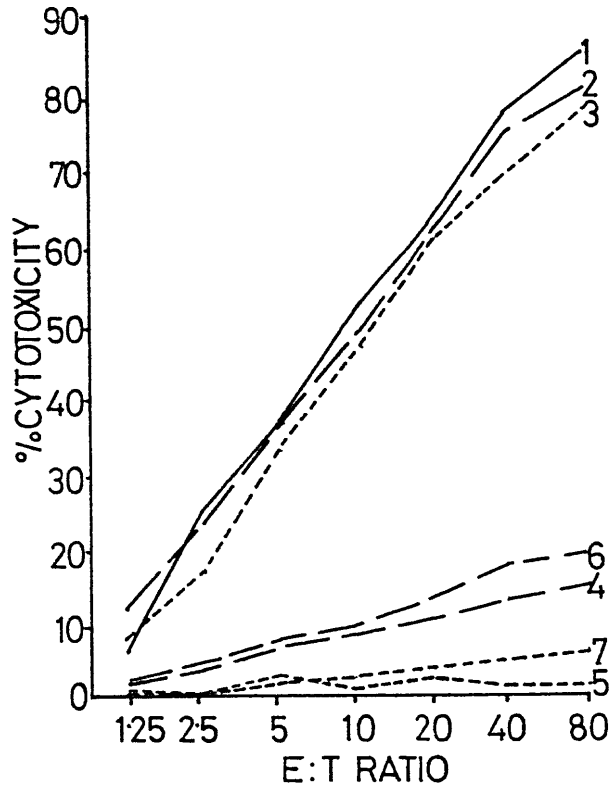

90
80
70
60
50
40
30
20
10
0
%CYTOTOXICITY
1
2
3
6
4
7
5
1·25
2·5
5
10
20
40
80
E:T RATIO

Table 2.4.1. Order of suppressive activity of purified carrageenans in the MLC

Carrageenan type	Composition	Sulphate content	Suppressive potency
I	Natural mixture, mainly κ form	-	Least suppressive
III	Purified κ form	1	
II	Mainly ν form	-	
V	Purified ν form	2	
IV	Purified λ form	3	Most suppressive

Sulphate content is presented as the number of sulphate groups per monomeric unit of the carrageenan molecule.

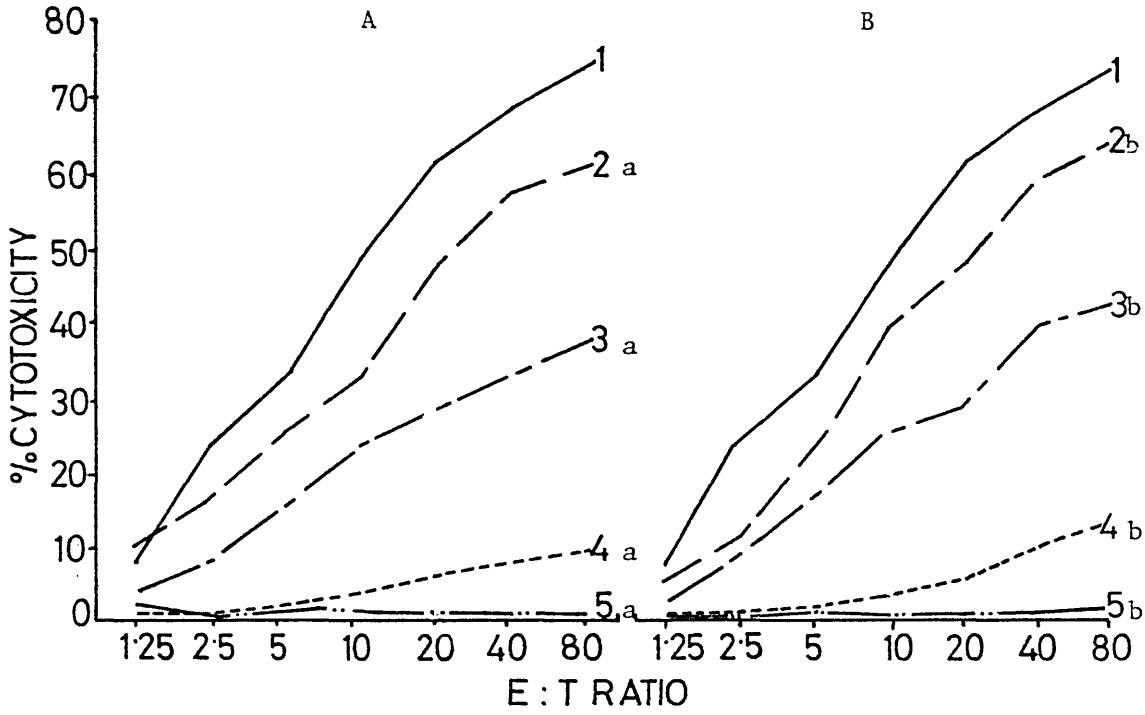


Figure 2.4.5.

Dose-response relationships for purified λ- and ι-carrageenan

Line 1: control; no carrageenan added to culture

2: 5 µg carrageenan/ml

3: 10 µg carrageenan/ml

4: 20 µg carrageenan/ml

5: 50 µg carrageenan/ml

TREATMENT	-	λ-carrageenan				ι-carrageenan			
CULTURE	1	2a	3a	4a	5a	2b	3b	4b	5b
% recovery	119	110	117	120	101	114	131	125	105
% viability	88	89	93	91	84	89	95	92	74

TARGET CELLS P815Y in vitro cell line

Spontaneous release 17%

ι-carrageenan (20 μg/ml) was added to MLCs at the start of culture or at 24-hour intervals subsequent to this. The same dose of carrageenan was added to some cells for the duration of the cytotoxicity assay on day 5.

As shown in Figure 2.4.6., carrageenan added at the initiation of culture fully abrogated the cytotoxic response. If carrageenan treatment was delayed for 24 hours, cytotoxic activity was still fully suppressed; however, addition of carrageenan subsequent to this was progressively less effective, and when added on day 3 or 4, or when incorporated into the cytotoxicity assay, this agent had no apparent effect on the cytotoxic response. Similar results were obtained using the same dose of λ-carrageenan, as shown in Figure 2.4.7.

These data indicate that ι- and λ-carrageenan are affecting an early stage in the MLC. Their decreasing suppressive potency as the culture progresses also provides evidence that these agents are not directly influencing the effector T lymphocytes in this system, which are present from approximately day 4 of culture (Simpson et al, 1975).

If carrageenan is only affecting the initial sensitisation phase of the MLC, then suppression may still be obtained if this agent is removed later in the culture period. To test this hypothesis, cells were exposed to carrageenan for a period of only 24 hours, at varying stages of culture. At the end of the 24-hour period, 2.0 ml of culture fluid (total volume 2.4 ml) was carefully removed from each well without disturbing the cells, and was replaced by fresh medium. After allowing at least 60 minutes for the cells to settle again, this process was repeated to ensure that any carrageenan remaining in the culture was greatly diluted. Cells were exposed to carrageenan in this way for the first, second or third day of the MLC; later stages were not tested, as carrageenan added from day 3 onwards had already been found to have no

Figure 2.4.6.

Time-course ι-carrageenan-induced suppression of the cytotoxic response *in vitro*

ι-carrageenan was added to MLCs at initiation or at 24-hour intervals after this, to give a final concentration of 20 μg/ml. Carrageenan was also added to some previously untreated cells for the duration of the cytotoxicity assay.

Line 1: control; no carrageenan added to culture

2: carrageenan added on day 0

3: carrageenan added on day 1

4: carrageenan added on day 2

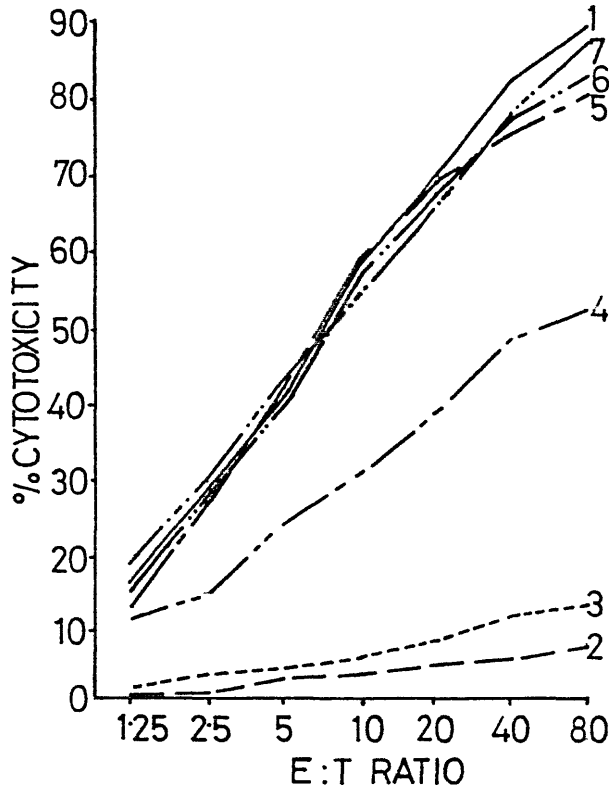
5: carrageenan added on day 3

6: carrageenan added on day 4

7: carrageenan added to cytotoxicity assay on day 5

CULTURE	1	2	3	4	5	6
% recovery	126	119	113	107	115	121
% viability	91	93	90	88	91	89

TARGET CELLS	P815Y *in vivo* tumour cells
Spontaneous release	8%


90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25
2·5
5
10
20
40
80
E:T RATIO
1
7
6
5
4
3
2

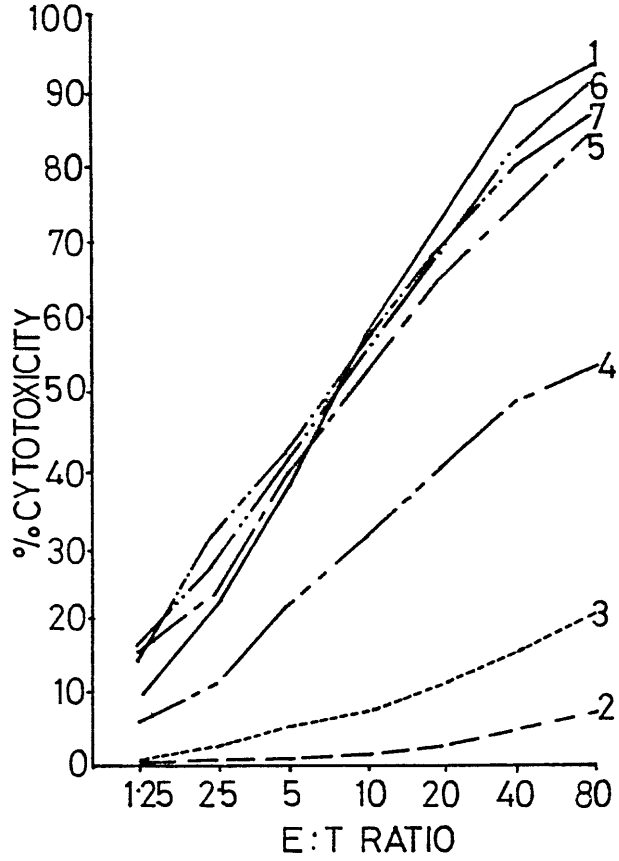


Figure 2.4.7.

Time-course of λ-carrageenan-induced suppression of the cytotoxic response in vitro

Line 1: control; no carrageenan added to culture

2: carrageenan added on day 0

3: carrageenan added on day 1

4: carrageenan added on day 2

5: carrageenan added on day 3

6: carrageenan added on day 4

7: carrageenan added to cytotoxicity assay on day 5

CULTURE	1	2	3	4	5	6
% recovery	134	141	137	131	143	128
% viability	86	89	92	86	91	90

TARGET CELLS P815Y in vitro cell line

Spontaneous release 19%

suppressive effect on cytotoxicity. Undisturbed control cultures and washed control cultures were also set up.

The results of such an experiment are presented in Figure 2.4.8. Exposure of cells to ι- or λ-carrageenan for the first 24 hours of culture resulted in full suppression of the response, comparable to that obtained when carrageenan was present throughout the culture period (Figures 2.4.6. and 2.4.7., line 2). If carrageenan was added on day 1, and so was present from 24-48 hours of culture, the subsequent cytotoxic response was still suppressed, though not to the same level as that found when carrageenan was added earlier. Carrageenan present from 48-72 hours of culture caused only partial suppression. Results obtained from washed control cultures confirmed that manipulation of cells in this manner did not contribute to the observed effects.

These results support conclusions drawn from the earlier time-course experiments, demonstrating that the stage of the MLC which is most sensitive to carrageenan occurs within the first 48 hours. However, exposure of cells to this agent for the first 24 hours of culture was sufficient to cause full suppression of cytotoxicity, indicating that continuous presence of carrageenan in the culture medium is not essential for this effect.

To further investigate this early effect of carrageenan on cells involved in the MLC, experiments were set up in which responder and/or stimulator cell suspensions were pre-exposed to carrageenan before mixing. Responder and stimulator cells were cultured separately for 24 hours, with or without ι-carrageenan. They were then resuspended, harvested and washed, and after irradiation of the stimulator cells the two suspensions were mixed and cultured together for a further 5 days. The adherent fraction of each suspension would become attached to the culture plate during the initial 24 hours *in vitro*. These cells are

Figure 2.4.8.

Effect of short-term exposure to carrageenan at different stages of the MLC

λ- or ι-carrageenan was added to MLCs

a) at initiation

b) after 24 hours

or c) after 48 hours, to give a final concentration of 20 μg/ml

The carrageenan was washed off again 24 hours after addition, without disturbing the cells.

Line 1: control; no carrageenan added to culture

2: washed control, no carrageenan added

3: carrageenan present 0-24 hours of culture

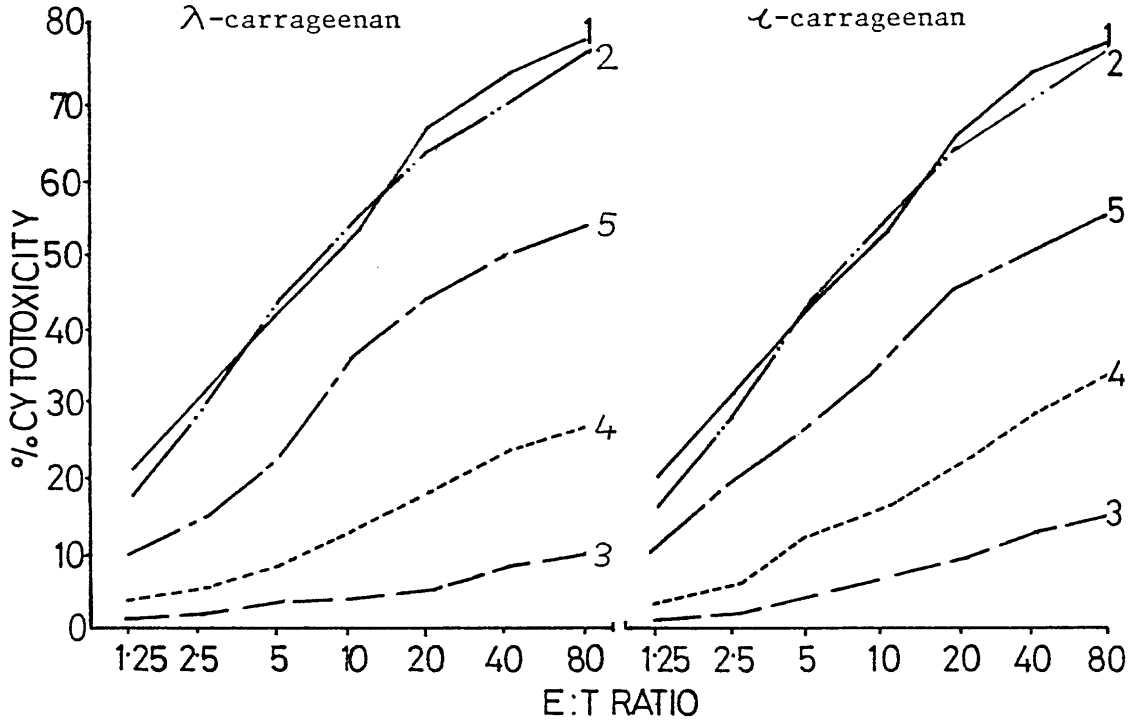
4: carrageenan present 24-48 hours

5: carrageenan present 48-72 hours

TREATMENT	—		λ-carrageenan			ι-carrageenan		
CULTURE	1	2	3	4	5	3	4	5
% recovery	117	119	120	122	111	119	113	109
% viability	87	89	85	91	83	90	88	94

TARGET CELLS P815Y in vitro cell line

Spontaneous release 12%


λ-carrageenan
ι-carrageenan
% CYTOTOXICITY
80
70
60
50
40
30
20
10
0
1
2
3
4
5
1·25
2·5
5
10
20
40
80
E:T RATIO

known to be vital for the generation of cytotoxic activity in this system; however, it had already been found that, in the absence of adherent cells of the responder strain, this function could be served by adherent cells of stimulator origin (section 2.3.2.). Mixed non-adherent cells were therefore returned to those culture wells in which the stimulator cells had originally been maintained, and to which the adherent fraction of this stimulator population had attached. No further carrageenan was added to any of the cultures.

The results of such an experiment are presented in Figure 2.4.9. Untreated cells cultured undisturbed for a total of 6 days gave a response of control levels, as did cells cultured separately for 24 hours before mixing. Negligible cytotoxic activity was generated by cells cultured undisturbed for 6 days in the presence of carrageenan. In cultures where one or both cell suspensions had been pre-exposed to carrageenan, the subsequent cytotoxic response was dependent on which suspension had been treated; pre-exposure of the responder cells alone had no effect on cytotoxicity, while treatment of the stimulator population resulted in partial suppression of the response. A similar degree of suppression was obtained if both responder and stimulator cells were pre-exposed to carrageenan, suggesting that this agent was exerting its effects through the stimulator cells alone.

Under the culture conditions imposed in this experiment, cells in the stimulator suspension were acting both as a source of alloantigen and as an accessory population. Previous experiments on the time-course of carrageenan-induced suppression have revealed that this agent is still effective after the first 24 hours of culture, suggesting that it does not interfere directly with recognition of the stimulatory antigenic signal. It was therefore suggested that the suppressive effects of carrageenan may be mediated by accessory cells in the stimulator population. To test this hypothesis, a similar

Figure 2.4.9.

Effect of pre-exposing responder and/or stimulator cells to ι-carrageenan before the MLC

Cells from responder and/or stimulator suspensions were exposed to 20 μg ι-carrageenan/ml for 24 hours. Non-adherent cells were then resuspended and washed, and were set up in MLC in the presence of adherent cells of the stimulator strain. No further carrageenan was added to the cultures.

Line 1: control; untreated cells cultured undisturbed for 6 days

2: cells cultured undisturbed for 6 days in the presence of 20 μg ι-carrageenan/ml

3: control; untreated responder and stimulator cells cultured separately for 24 hours, then mixed and cultured for a further 5 days

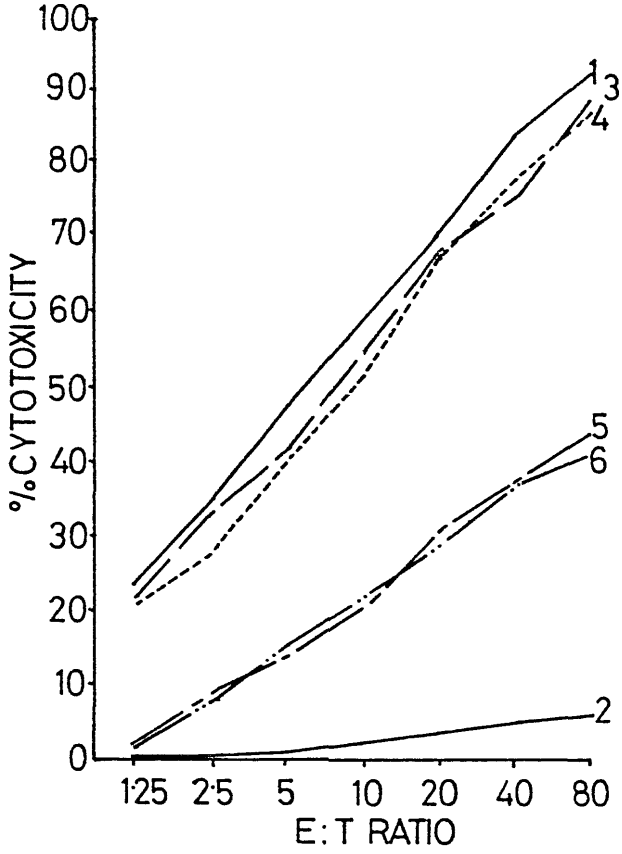
4: as for line 3, with responder cells exposed to carrageenan

5: as for line 3, with stimulator cells exposed to carrageenan

6: as for line 3, with both responder and stimulator cells exposed to carrageenan

CULTURE	1	2	3	4	5	6
% recovery	133	128	115	120	109	112
% viability	84	87	89	90	86	82

TARGET CELLS	P815Y *in vitro* cell line
Spontaneous release	12%


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
3
4
5
6
2
1·25
2·5
5
10
20
40
80
E:T RATIO

experiment was set up in which mixed non-adherent cells were returned to culture wells containing adherent cells of responder origin. Cytotoxic activity generated in control cultures was now reduced, due to the absence of adherent cells of the stimulator strain (as reported previously in section 2.3.2.). However, further suppression of the response was observed after pre-treatment of the responder cells with carrageenan, as shown in Figure 2.4.10. In this experiment, pre-exposure of the stimulator cells to carrageenan did not suppress the response relative to that of control cultures.

Taken together with the observed time-course of carrageenan-induced suppression, these results strongly suggest that this agent is acting through adherent accessory cells in this system, rather than exerting a direct effect on the lymphocytes. It has been reported that some carrageenans selectively destroy phagocytic cells (Allison et al, 1966; Catanzaro et al, 1971), and death of the accessory population during the early part of this culture would prevent generation of cytotoxic activity. However, viability studies indicate that murine adherent cells survive exposure to carrageenan (section 2.4.5.1.), and adherent cells recovered from carrageenan-treated MLCs have been found capable of ingesting opsonised yeast particles. Indirect quantitation of adherent cells showed no difference in survival between control and carrageenan-treated populations (section 2.4.5.2.), and it therefore appears that the immunosuppressive effects of this material, while mediated by the accessory population, do not result from the death of these cells.

2.4.3. ZYMOSAN

2.4.3.1. Effects of zymosan on the development of cell-mediated cytotoxicity in vitro

Suspensions of zymosan particles were prepared as described

Figure 2.4.10.

Effect of pre-exposing responder and/or stimulator cells to ι-carrageenan before the MLC

Responder and/or stimulator suspensions exposed individually to 20 μg ι-carrageenan/ml were washed after 24 hours and set up in MLC in the presence of adherent cells of the responder strain. No further carrageenan was added to the cultures.

Line 1: control; untreated cells cultured undisturbed for 6 days

2: cells cultured undisturbed for 6 days in the presence of carrageenan

3: control; untreated responder and stimulator cells cultured separately for 24 hours, then mixed and cultured for a further 5 days

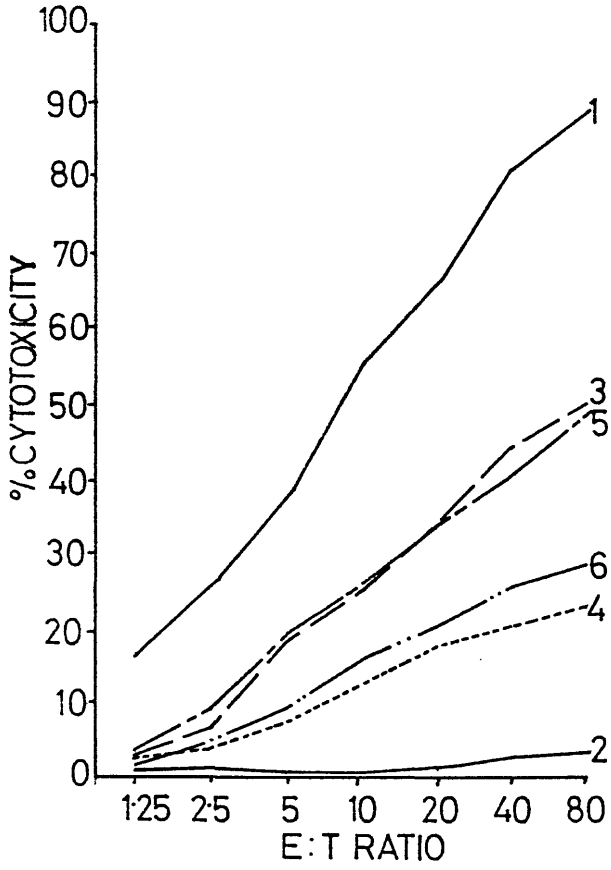
4: as for line 3, with responder cells exposed to carrageenan

5: as for line 3, with stimulator cells exposed to carrageenan

6: as for line 3, with both responder and stimulator cells exposed to carrageenan

CULTURE	1	2	3	4	5	6
% recovery	111	103	94	97	101	98
% viability	91	88	89	93	91	86

TARGET CELLS	P815Y *in vitro* cell line
Spontaneous release	8%


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
3
5
6
4
2
1·25
2·5
5
10
20
40
80
E:T RATIO

in section 2.2.1.9.3. A range of concentrations of zymosan (10 µg to 500 µg/ml culture fluid) was added to cell cultures at the start of the 5-day culture period, and a dose-response relationship for this agent was constructed from the results, as shown in Figure 2.4.11. These data clearly reflect a dose-dependent suppressive effect of zymosan in this system, with complete abrogation of cytotoxicity at higher concentrations. There was no evidence of lymphocyte toxicity at any of the doses tested, and subsequent experiments were performed with zymosan at a final concentration of 200 µg/ml culture fluid.

2.4.3.2. Time-course of zymosan-induced suppression of the cytotoxic response

To determine the time-course of zymosan-induced suppression, this agent was added to MLCs at the initiation of culture or at 24-hour intervals subsequent to this; the same dose (200 µg/ml) was also added to previously untreated cells for the duration of the cytotoxicity assay on day 5.

The results of such an experiment are presented in Figure 2.4.12. Zymosan added at the start of the culture period caused full suppression of the cytotoxic response, with no apparent effect on lymphocyte viability. However, if zymosan was added to cultures 24 hours after initiation, this suppression was less pronounced, and later additions had no effect on cytotoxicity.

These results suggest that zymosan exerts its suppressive effects on the initial stages of the MLC, the most sensitive phase occurring during the first 24 hours. The decreasing influence of subsequent zymosan treatment, together with its apparent lack of toxicity towards non-adherent cells, indicates that this agent does not directly affect cytotoxic T lymphocytes in this system.

If zymosan is only influencing the initial stages of the MLC,

Figure 2.4.11.

Effect of zymosan on the generation of CTL in the MLC

Zymosan was added to MLCs at initiation to give the following range of final concentrations.

Line 1: control; no zymosan added to culture

2: 10 µg zymosan/ml

3: 20 µg zymosan/ml

4: 50 µg zymosan/ml

5: 100 µg zymosan/ml

6: 200 µg zymosan/ml

7: 500 µg zymosan/ml

CULTURE	1	2	3	4	5	6	7
% recovery	117	122	111	120	104	114	119
% viability	84	81	87	90	92	86	87

TARGET CELLS	TGC-induced peritoneal cells
Spontaneous release	11%

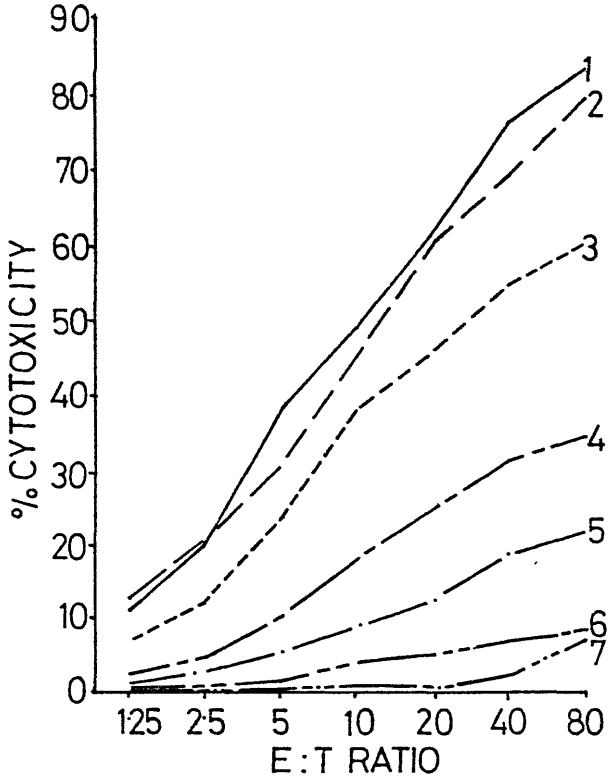

90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
2
3
4
5
6
7
125
2·5
5
10
20
40
80
E : T RATIO

Figure 2.4.12.

Time-course of zymosan-induced suppression of the cytotoxic response in vitro

Zymosan was added to MLCs at initiation or at 24-hour intervals after this, to give a final concentration of 200 μg/ml. Zymosan was also added to some previously untreated cells for the duration of the cytotoxicity assay.

Line 1: control; no zymosan added to culture

2: zymosan added on day 0

3: zymosan added on day 1

4: zymosan added on day 2

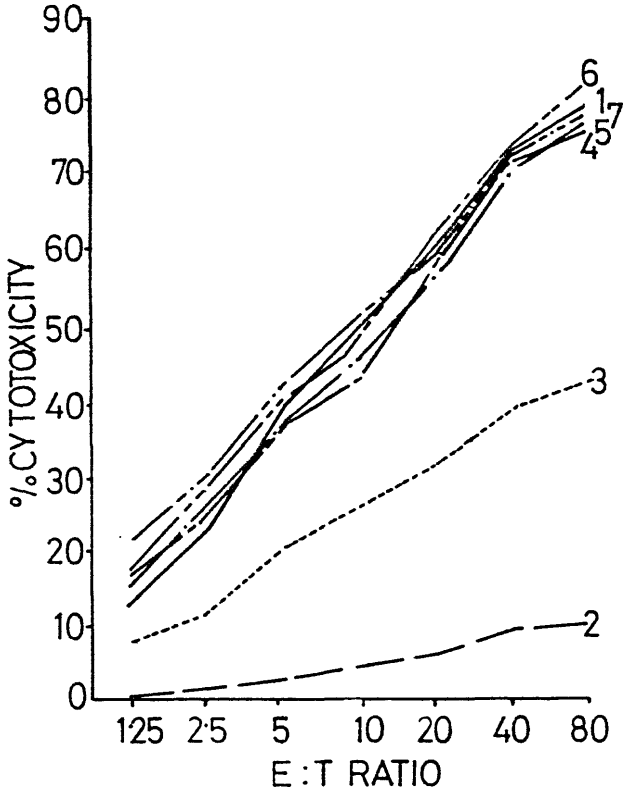
5: zymosan added on day 3

6: zymosan added on day 4

7: zymosan added to cytotoxicity assay on day 5

CULTURE	1	2	3	4	5	6
% recovery	121	118	112	115	122	113
% viability	79	82	80	78	84	79

TARGET CELLS	P815Y in vitro cell line
Spontaneous release	12%


90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
6
1
7
5
4
3
2
125
2·5
5
10
20
40
80
E : T RATIO

then it may still induce suppression if it is removed later in the culture period. To investigate this hypothesis, zymosan was added to MLCs at different stages, and was then removed 24 hours later in the supernatant culture fluid, as described in section 2.4.2.2. In view of the particulate nature of zymosan, it is important to note that this procedure would not eliminate all traces of this material.

If cells were exposed to zymosan for the first 24 hours of culture, the subsequent cytotoxic response was fully suppressed, as shown in Figure 2.4.13. However, if zymosan was added on day 1, and was therefore present from 24-48 hours of culture, this suppressive effect was markedly reduced, while further delay in zymosan treatment resulted in a cytotoxic response of control levels. Control cultures revealed that the washing process did not affect the response.

These results support the previous observation that zymosan primarily affects events occurring within the first 24 hours of the MLC. As mentioned previously, some zymosan particles would remain in the culture medium after the 24-hour exposure period; if one assumes that such particles were evenly distributed, the washing process would dilute these to less than 5% of the original dose, which could not account for the observed suppression of cytotoxicity (see Figure 2.4.11.). It is therefore reasonable to conclude from these data that continuous presence of zymosan in the culture medium is not required for the manifestation of its suppressive effects.

The suppression induced by zymosan during the initial stages of the MLC was further investigated by pre-exposing responder and/or stimulator cells to this agent. The two cell suspensions were cultured separately for 24 hours with or without zymosan, as described in section 2.4.2.2. The non-adherent cells were then resuspended, harvested, and washed to remove as much zymosan as possible. After irradiation of the stimulator population, both suspensions were returned to one of the initial

Figure 2.4.13.

Effect of short-term exposure to zymosan at different stages of the MLC

Zymosan was added to MLCs

a) at initiation

b) after 24 hours

or c) after 48 hours, to give a final concentration of 200 μg/ml

The zymosan was washed off again 24 hours after addition, without disturbing the cells.

Line 1: control; no zymosan added to culture

2: washed control, no zymosan added

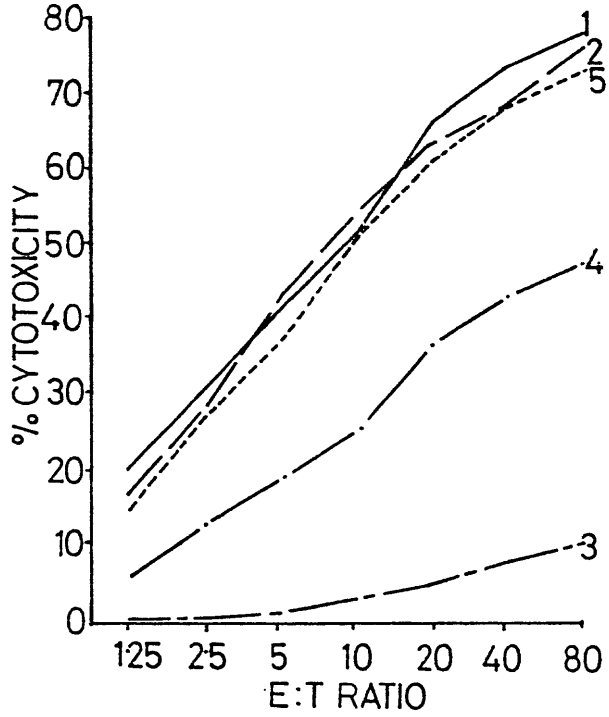
3: zymosan present 0-24 hours of culture

4: zymosan present 24-48 hours

5: zymosan present 48-72 hours

CULTURE	1	2	3	4	5
% recovery	117	119	117	118	114
% viability	87	89	91	88	92

TARGET CELLS	P815Y *in vitro* cell line
Spontaneous release	10%


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
2
5
4
3
1·25
2·5
5
10
20
40
80
E:T RATIO

culture plates, containing adherent cells of either stimulator or responder origin. No further zymosan was added to any of the cultures.

Mixed non-adherent cells were initially returned to plates containing the adherent fraction of the stimulator suspension, as these cells were known to be required for optimal generation of cytotoxicity (section 2.3.2.). As shown in Figure 2.4.14., cells cultured undisturbed for 6 days in the presence of zymosan expressed little cytotoxic activity. Control cultures confirmed that extending the culture period by 24 hours, or culturing responder and stimulator cells separately before mixing, did not affect the response of untreated cells. Pre-exposure of responder cells alone to zymosan also failed to influence cytotoxicity; however, similar treatment of the stimulator population induced considerable suppression of the response. Comparable suppression was obtained if both responder and stimulator cells were pre-exposed to zymosan, indicating that this material was acting solely via the stimulator population.

To determine whether this suppressive mechanism depended on adherent cells present in the culture, a similar experiment was performed in which mixed non-adherent cells were subsequently cultured in the presence of adherent cells of responder origin. In the absence of adherent cells of the stimulator strain, the response of untreated cells cultured separately for the first 24 hours of the MLC was now reduced (see section 2.3.2.), as shown in Figure 2.4.15. Pre-exposure of responder cells alone to zymosan before mixing now resulted in further suppression of the response, while pre-treatment of the stimulator population did not influence cytotoxicity relative to that of pre-cultured control cells.

These results support the hypothesis that zymosan is acting through adherent accessory cell types, with cells of both responder

Figure 2.4.14.

Effect of pre-exposing responder and/or stimulator cells to zymosan before the MLC

Cells from responder and/or stimulator suspensions were exposed to 200 μg zymosan/ml for 24 hours. Non-adherent cells were then resuspended and washed, and were set up in MLC in the presence of adherent cells of the stimulator strain. No further zymosan was added to the cultures.

Line 1: control; untreated cells cultured undisturbed for 6 days

2: cells cultured undisturbed for 6 days in the presence of 200 μg zymosan/ml

3: control; untreated responder and stimulator cells cultured separately for 24 hours, then mixed and cultured for a further 5 days

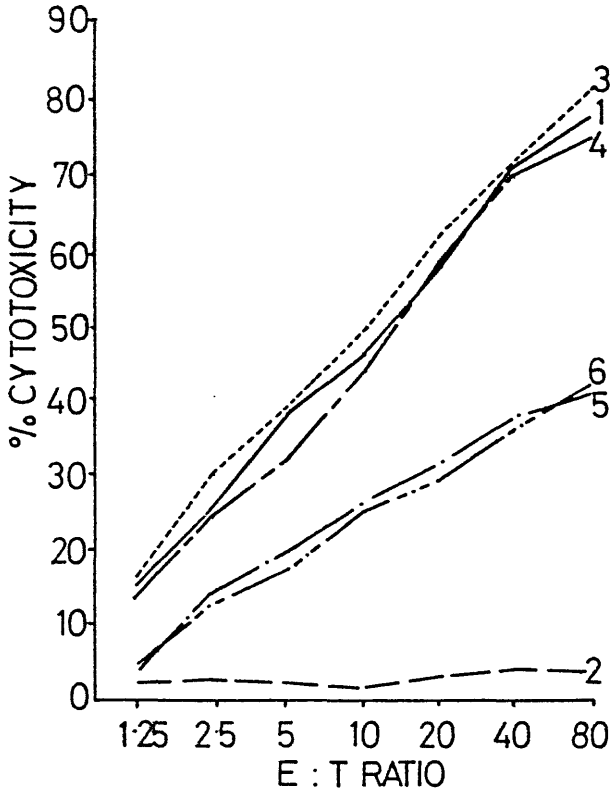
4: as for line 3, with responder cells exposed to zymosan

5: as for line 3, with stimulator cells exposed to zymosan

6: as for line 3, with both responder and stimulator cells exposed to zymosan

CULTURE	1	2	3	4	5	6
% recovery	153	141	139	148	132	109
% viability	82	86	80	78	86	93

TARGET CELLS	P815Y *in vivo* tumour cells
Spontaneous release	13%


90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
3
1
4
6
5
2
1·25
2·5
5
10
20
40
80
E : T RATIO

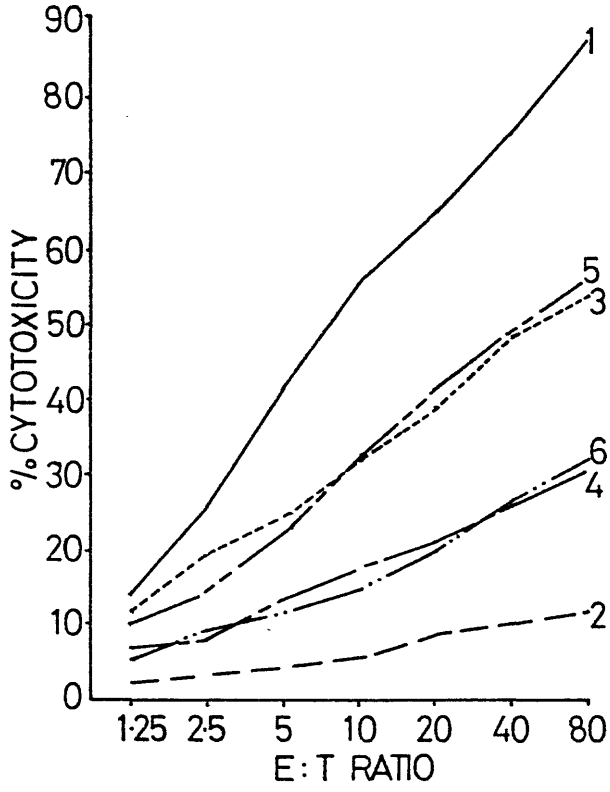

90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
5
3
6
4
2
1·25
2·5
5
10
20
40
80
E : T RATIO

Figure 2.4.15.

Effect of pre-exposing responder and/or stimulator cells to zymosan before the MLC

Responder and/or stimulator suspensions exposed individually to 200 μg zymosan/ml were washed after 24 hours and set up in MLC in the presence of adherent cells of the responder strain. No further zymosan was added to the cultures.

Line 1: control; untreated cells cultured undisturbed for 6 days

2: cells cultured undisturbed for 6 days in the presence of zymosan

3: control; untreated responder and stimulator cells cultured separately for 24 hours, then mixed and cultured for a further 5 days

4: as for line 3, with responder cells exposed to zymosan

5: as for line 3, with stimulator cells exposed to zymosan

6: as for line 3, with both responder and stimulator cells exposed to zymosan

CULTURE	1	2	3	4	5	6
% recovery	122	117	127	130	119	127
% viability	83	84	81	80	87	83

TARGET CELLS	P815Y *in vitro* cell line
Spontaneous release	9%

and stimulator populations capable of mediating suppression. Previous experiments have shown that short-term exposure of cells to zymosan at the initiation of culture is sufficient to induce suppression, and the activity of this agent therefore follows a similar time-course to the adherent cell requirement in this system. Indirect quantitation and viability studies revealed that zymosan has little effect on the survival of murine adherent cells in vitro (section 2.4.5.), and adherent cells recovered from zymosan-treated cultures were able to ingest opsonised yeast particles. It is therefore proposed that the suppressive effects of zymosan on the generation of cytotoxic activity in the MLC are not due to destruction of an essential accessory population, but may reflect an alteration in the function of such cells.

2.4.4. LATEX

2.4.4.1. Effects of latex on the development of cell-mediated cytotoxicity in vitro

Suspensions of latex particles were prepared as described in section 2.2.1.9.4., and were added to MLCs to give a range of final concentrations. The proportion of adherent/phagocytic cells in a spleen cell suspension was estimated to be approximately 25% from earlier cell depletion experiments using carbonyl iron powder (section 2.3.2.1.), and this figure was used to calculate a dose of latex particles per accessory cell.

The results of such an experiment are presented in Figure 2.4.16., and clearly show that the presence of latex particles in the MLC had no effect on the subsequent cytotoxic response, even at the highest dose of 200 particles per adherent/phagocytic cell. However, recovery of viable non-adherent cells was reduced in cultures exposed to high doses of latex particles.

Figure 2.4.16.

Effect of latex particles on the generation of CTL in the MLC

Suspensions of latex particles were added to MLCs at initiation to give a range of final concentrations. As the particles were initially suspended in water, a "solvent" control was included for the highest concentration.

Line 1: control; no latex added to culture

2: water control

3: 25 latex particles per accessory cell

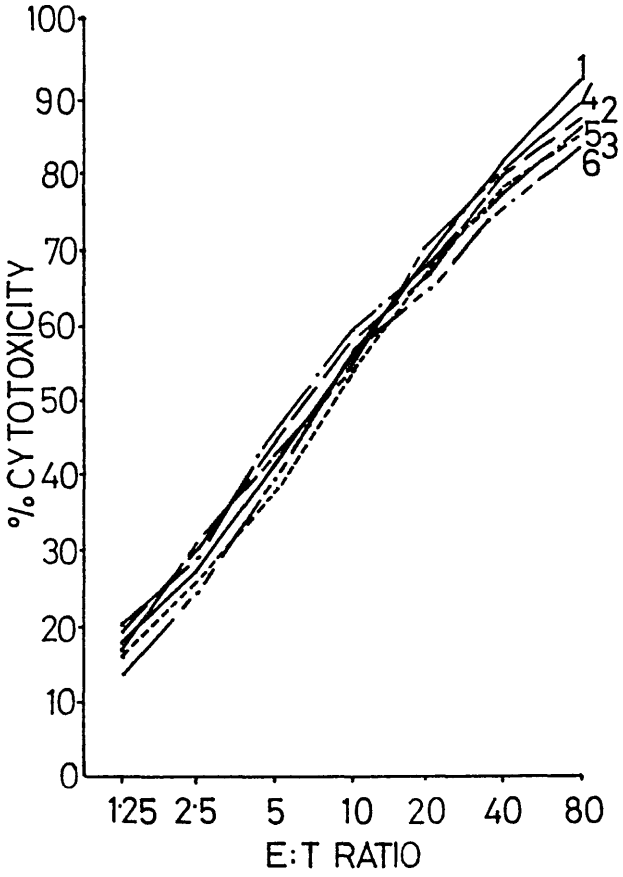
4: 50 latex particles per accessory cell

5: 100 latex particles per accessory cell

6: 200 latex particles per accessory cell

CULTURE	1	2	3	4	5	6
% recovery	129	113	123	98	89	58
% viability	78	74	72	69	69	60

TARGET CELLS	P815Y in vitro cell line
Spontaneous release	11%


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
4
2
5
3
6
1·25
2·5
5
10
20
40
80
E:T RATIO

From these results it was concluded that the presence of a biologically inert particle such as latex in the MLC does not induce suppression of cytotoxicity. The effects of silica, carrageenan and zymosan must therefore be specific for these materials, rather than resulting non-specifically from the ingestive process.

2.4.5. TOXICITY OF SILICA, CARRAGEENAN AND ZYMOSAN TOWARDS ADHERENT CELLS *IN VITRO*

2.4.5.1. *Viability of adherent cells cultured with macrophage-active agents*

Peritoneal cells were taken from untreated mice as described in section 2.2.2.1.1., and were cultured in multiwell plates for 24 or 48 hours. The non-adherent cells were then removed, and adherent cells remaining in the culture wells were washed 3x with culture medium. Fresh medium was then placed in all wells, and silica (100 µg/ml), ι-carrageenan (20 µg/ml) or zymosan (200 µg/ml) was added to some cultures. After a further 24 hours *in vitro*, the adherent cells were again washed 3x, and medium containing 50% FDA-EB stain was added to each well. Cells adhering to the wells were then examined under incident UV illumination, as described in section 2.2.2.2.2.

Over 96% of cells cultured alone or in the presence of carrageenan or zymosan were shown to be viable using this method. Cells exposed to zymosan contained multiple cytoplasmic inclusions, presumably due to the particulate nature of this material. In contrast, only 1-2% of cells cultured with silica were viable. A fall in cell density was also apparent in culture wells containing silica, though this was not quantified.

2.4.5.2. Effect of macrophage-active agents on the number of adherent cells remaining after culture

Direct counting of the number of adherent cells present using a microscope fitted with a graticule was not satisfactory for this purpose, due to the uneven distribution of cells over the culture wells. In particular, high cell densities were found towards the edges of wells, perhaps due to surface tension. Consequently, changes in absolute cell numbers after exposure to different macrophage-active agents were determined by analysis of the protein content of adherent cells remaining at the end of the culture period.

Peritoneal cells were maintained in vitro for 48 hours, at a concentration of 1.7×10^6 cells/well. The non-adherent cells were then resuspended and removed, and the adherent population was cultured for a further 24 hours with or without macrophage-active agents, as described in section 2.4.5.1. The adherent cells were then washed 3x with saline, and were digested with sodium hydroxide. The amount of cell protein present was then determined as described in section 2.2.2.15.

As shown in Table 2.4.2., the amount of cell protein recovered from wells containing ~~ζ~~-carrageenan or zymosan was not significantly different from that of control wells. In contrast, the amount of protein in cultures treated with silica was reduced by over 50% compared to control cultures, indicating that 24-hour exposure to this material caused the loss of a considerable proportion of adherent cells. Taken together with the results obtained in section 2.4.5.1., these results strongly suggest that silica is highly toxic towards the adherent population, while carrageenan and zymosan have no adverse effects on the recovery and viability of such cells in culture. These findings are compatible with results obtained in the MLC.

Table 2.4.2. Amount of cell protein in cultures of peritoneal adherent cells exposed to macrophage-active agents for 24 hours

Macrophage-active agent	µg protein per well[a]	Mean ± S.D.	P value[b]
None	29.30	28.92 ± 0.76	-
	29.42		
	28.05		
Silica 100 µg/ml	13.07	13.75 ± 0.74	< 0.001
	13.64		
	14.54		
ι-carrageenan 20 µg/ml	31.23	29.61 ± 3.53	> 0.7
	32.03		
	25.56		
Zymosan 200 µg/ml	21.02	25.97 ± 4.37	> 0.3
	27.60		
	29.30		

[a] Calculated from a standard curve for BSA, using the regression equation

[b] Calculated using a Two-Tailed Student's t-test

2.5. INVOLVEMENT OF POLYUNSATURATED FATTY ACID METABOLITES IN THE MURINE MIXED LEUCOCYTE CULTURE

2.5.1. INDOMETHACIN

2.5.1.1. Effects of indomethacin on the development of cell-mediated cytotoxicity in vitro

2.5.1.2. Combined effects of indomethacin and macrophage-active agents on the development of cell-mediated cytotoxicity in vitro

2.5.2. FLURBIPROFEN

2.5.2.1. Effects of flurbiprofen on the development of cell-mediated cytotoxicity in vitro

2.5.2.2. Combined effects of flurbiprofen and macrophage-active agents on the development of cell-mediated cytotoxicity in vitro

2.5.3. PROSTAGLANDIN E_1 AND ANTI-PGE ANTISERUM

2.5.3.1. Effects of exogenous PGE_1 on the development of cell-mediated cytotoxicity in vitro

2.5.3.2. Time-course of PGE_1-induced suppression of the cytotoxic response

2.5.3.3. Effects of exogenous PGE_1 and anti-PGE antiserum on the development of cell-mediated cytotoxicity in vitro

2.5.3.4. Effects of anti-PGE antiserum and macrophage-active agents on the development of cell-mediated cytotoxicity in vitro

2.5.4. PROSTACYCLIN, CARBACYCLIN AND ANTI-PGI_2 ANTISERUM

2.5.4.1. Effects of exogenous PGI_2 and carbacyclin on the development of cell-mediated cytotoxicity *in vitro*

2.5.4.2. Effects of exogenous PGE_1 and anti-PGI_2 antiserum on the development of cell-mediated cytotoxicity *in vitro*

2.5.4.3. Effects of anti-PGI_2 antiserum and macrophage-active agents on the development of cell-mediated cytotoxicity *in vitro*

2.5.4.4. Combined effects of anti-PGE and anti-PGI_2 antisera on carrageenan- and zymosan-treated cultures

2.5.1. INDOMETHACIN

2.5.1.1. Effects of indomethacin on the development of cell-mediated cytotoxicity in vitro

Solutions of indomethacin were prepared as described in section 2.2.1.10.1., and a range of concentrations was added to cells at the initiation of culture to find an optimal working dose for this material. As shown in Figure 2.5.1., low doses of indomethacin (0.1 µg/ml to 10 µg/ml) had no apparent effect on the cytotoxic response; however, concentrations in excess of 10 µg/ml proved highly toxic, and such cultures yielded too few cells to assay. Further experiments incorporating carrageenan or zymosan were therefore performed with indomethacin at final concentrations of 1 µg/ml and 10 µg/ml.

2.5.1.2. Combined effects of indomethacin and macrophage-active agents on the development of cell-mediated cytotoxicity in vitro

Cells were cultured in the presence of known suppressive doses of carrageenan or zymosan (sections 2.4.2.1., 2.4.3.1.), and the effects of simultaneous exposure to indomethacin were observed. Neither dose of indomethacin was able to prevent carrageenan-induced suppression of cytotoxic activity. However, this drug did afford some protection against the suppressive effects of zymosan, as shown in Figure 2.5.2. In zymosan-treated cultures, the same degree of restoration of cytotoxicity was obtained with both concentrations of indomethacin; there was no evidence of a dose-dependent effect of this drug.

Further experiments using indomethacin were inconclusive, partly because of the toxicity of high doses of this drug. However, the results obtained here do suggest that some product of the indomethacin-sensitive cyclo-oxygenase pathway may be involved in mediating zymosan-induced suppression of cytotoxicity.

Figure 2.5.1.

Effect of indomethacin on the generation of CTL in the MLC

Indomethacin was added to MLCs at initiation, to give the following range of final concentrations.

Line 1: control; no indomethacin added to culture

2: 0.1 µg indomethacin/ml

3: 1.0 µg indomethacin/ml

4: 2.0 µg indomethacin/ml

5: 5.0 µg indomethacin/ml

6: 10.0 µg indomethacin/ml

7: 20.0 µg indomethacin/ml

8: 50.0 µg indomethacin/ml

CULTURE	1	2	3	4	5	6	7	8
% recovery	123	122	129	118	124	120	59	63
% viability	74	69	67	72	70	68	11	16

TARGET CELLS	TGC-induced peritoneal cells
Spontaneous release	13%

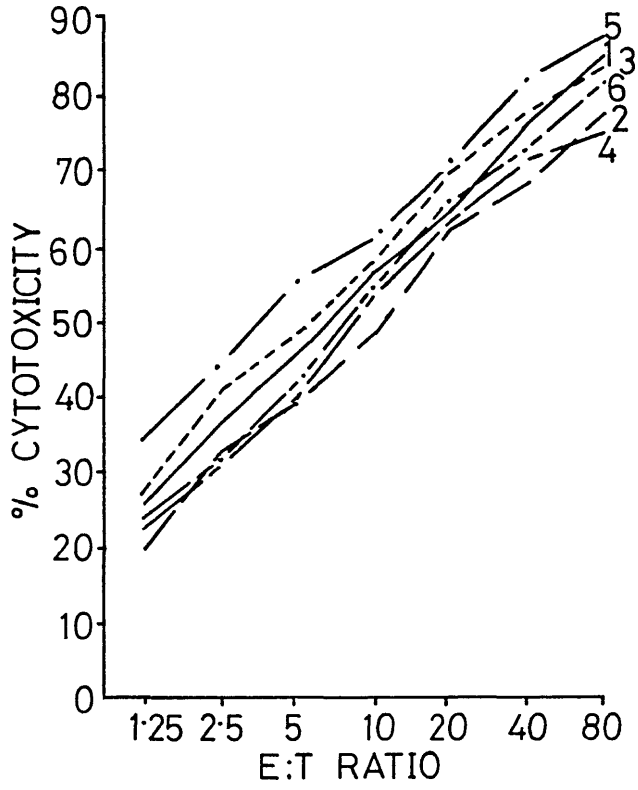

90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25
2·5
5
10
20
40
80
E:T RATIO
5
13
6
2
4

Figure 2.5.2.

Combined effects of indomethacin and macrophage-active agents on the generation of CTL in the MLC

Two concentrations of indomethacin were tested for their ability to restore the cytotoxic activity of cultures treated with suppressive doses of ι-carrageenan or zymosan. Both materials were added at the initiation of culture.

Line 1: control; untreated culture

2: 20 μg ι-carrageenan/ml

3: 200 μg zymosan/ml

4: 1 μg indomethacin/ml

5: 10 μg indomethacin/ml

6: 1 μg indomethacin/ml + 20 μg ι-carrageenan/ml

7: 10 μg indomethacin/ml + 20 μg ι-carrageenan/ml

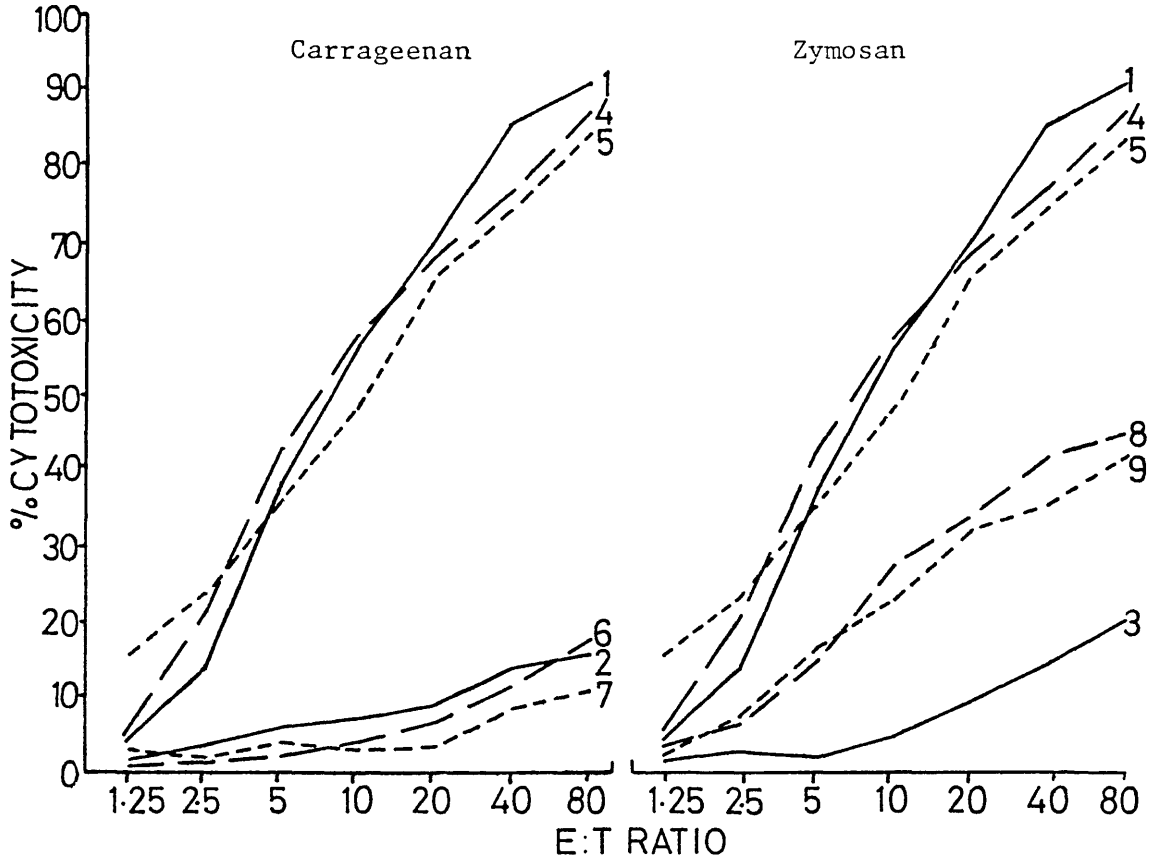
8: 1 μg indomethacin/ml + 200 μg zymosan/ml

9: 10 μg indomethacin/ml + 200 μg zymosan/ml

CULTURE	1	2	3	4	5	6	7	8	9
% recovery	118	123	119	109	102	96	89	99	88
% viability	73	71	77	79	71	71	68	70	72

TARGET CELLS P815Y *in vitro* cell line

Spontaneous release 14%


Carrageenan
Zymosan
% CYTOTOXICITY
E:T RATIO
100
90
80
70
60
50
40
30
20
10
0
1·25
2·5
5
10
20
40
80
1
4
5
6
2
7
8
9
3

2.5.2. FLURBIPROFEN

2.5.2.1. Effects of flubiprofen on the development of cell-mediated cytotoxicity in vitro

Flurbiprofen was dissolved as described in section 2.2.1.10.2., and was added to MLCs to give a range of final concentrations. As shown in Figure 2.5.3., flurbiprofen alone had little effect on the generation of cytotoxic activity in vitro, and recovery of viable cells from treated cultures was comparable to that of controls over this dose range. It was not possible to test flurbiprofen at concentrations in excess of 20 μg/ml, as this drug is poorly soluble in culture medium. Subsequent experiments involving carrageenan or zymosan were performed with flurbiprofen at final concentrations of 1 μg/ml, 10 μg/ml, and 20 μg/ml.

2.5.2.2. Combined effects of flurbiprofen and macrophage-active agents on the development of cell-mediated cytotoxicity in vitro

The possible protective effect of flurbiprofen against carrageenan- and zymosan-induced suppression in this system was examined by adding macrophage-active agents and this drug to MLCs at the start of the 5-day culture period. The results of such an experiment are presented in Figure 2.5.4. Addition of flurbiprofen to cultures treated with a suppressive dose of carrageenan or zymosan resulted in some restoration of the response; however, this effect was limited, and apparently independent of drug dose. Repeat experiments confirmed these observations, suggesting that cyclo-oxygenase products may be partly responsible for the effects of carrageenan and zymosan in this system. The results obtained using flurbiprofen therefore confirm and extend those found with indomethacin. However, further work with this drug was limited by technical considerations, and consequently insufficient data were obtained using this approach to firmly establish a role for

Figure 2.5.3.

Effect of flurbiprofen on the generation of CTL in the MLC

Flurbiprofen was added to MLCs at initiation, to give the following range of final concentrations.

Line 1: control; no flurbiprofen added to culture

2: 0.1 μg flurbiprofen/ml

3: 1.0 μg flurbiprofen/ml

4: 5.0 μg flurbiprofen/ml

5: 10.0 μg flurbiprofen/ml

6: 20.0 μg flurbiprofen/ml

CULTURE	1	2	3	4	5	6
% recovery	132	137	119	122	125	119
% viability	70	68	74	71	69	70

TARGET CELLS P815Y *in vivo* tumour cells

Spontaneous release 11%

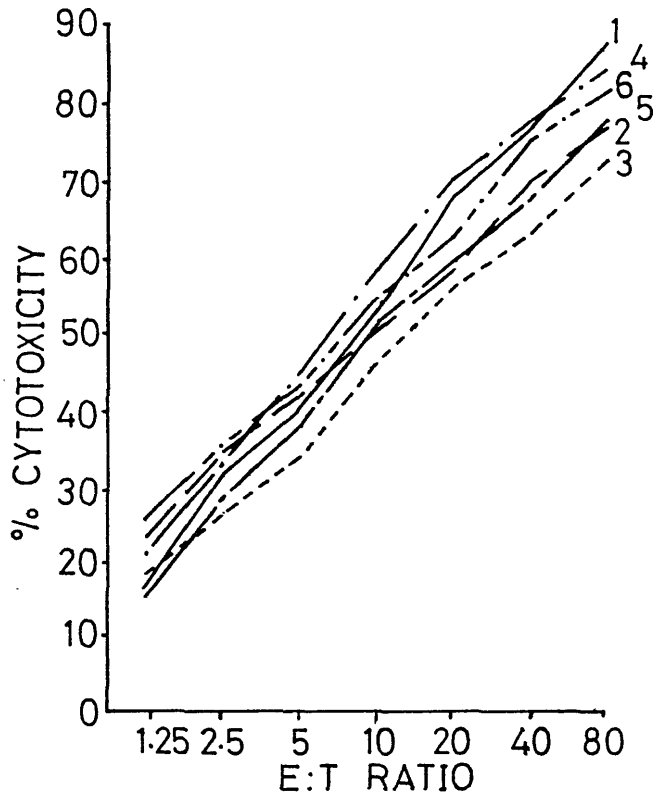

90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25 2·5 5 10 20 40 80
E:T RATIO
1
4
6
5
2
3

Figure 2.5.4.

Combined effects of flurbiprofen and macrophage-active agents on the generation of CTL in the MLC

Three concentrations of flurbiprofen were tested for their ability to restore the cytotoxic activity of cultures treated with suppressive doses of ι-carrageenan or zymosan. Both materials were added at the initiation of culture.

Line 1: control; untreated culture

2: 20 μg ι-carrageenan/ml

3: 200 μg zymosan/ml

4: 1 μg flurbiprofen/ml

5: 10 μg flurbiprofen/ml

6: 20 μg flurbiprofen/ml

7: 1 μg flurbiprofen/ml + 20 μg ι-carrageenan/ml

8: 10 μg flurbiprofen/ml + 20 μg ι-carrageenan/ml

9: 20 μg flurbiprofen/ml + 20 μg ι-carrageenan/ml

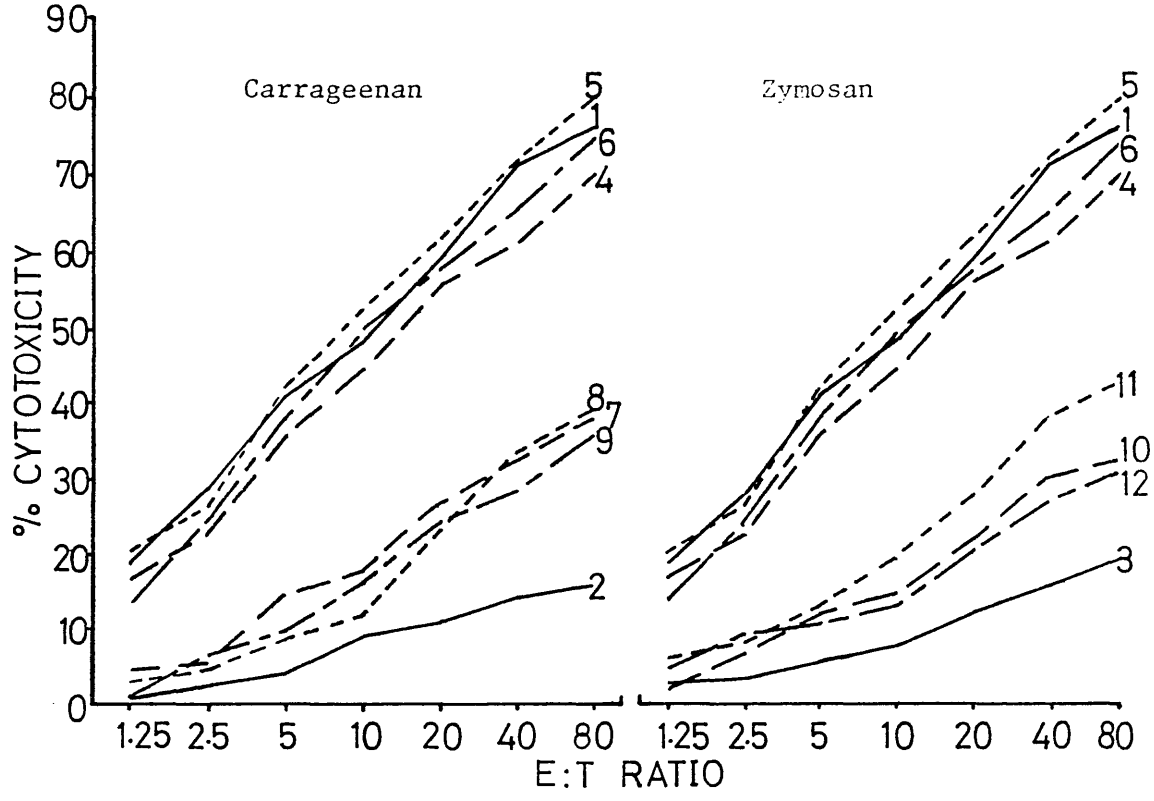
10: 1 μg flurbiprofen/ml + 200 μg zymosan/ml

11: 10 μg flurbiprofen/ml + 200 μg zymosan/ml

12: 20 μg flurbiprofen/ml + 200 μg zymosan/ml

CULTURE	1	2	3	4	5	6	7	8	9	10	11	12
% recovery	140	131	137	113	121	108	111	117	104	101	112	110
% viability	66	62	68	71	66	67	69	70	65	67	66	68

TARGET CELLS	P815Y *in vitro* cell line
Spontaneous release	12%


Carrageenan
Zymosan
% CYTOTOXICITY
E:T RATIO
0
10
20
30
40
50
60
70
80
90
1·25
2·5
5
10
20
40
80
1
2
3
4
5
6
7
8
9
10
11
12

cyclo-oxygenase products in carrageenan- and zymosan-induced suppression.

2.5.3. PROSTAGLANDIN E_1 AND ANTI-PGE ANTISERUM

2.5.3.1. Effects of exogenous PGE_1 on the development of cell-mediated cytotoxicity in vitro

Solutions of PGE_1 were prepared as described in section 2.2.1.11.1., and a range of concentrations was added to MLCs to determine the dose-effect relationship of this material.

As shown in Figure 2.5.5., full suppression of the cytotoxic response was obtained with the highest dose of 1×10^{-6} molar PGE_1 (0.25 µg/ml). Some suppression was still obtained with 1×10^{-7} molar PGE_1; however, further dilution resulted in a response of control levels. In view of the considerable ethanol content of the PGE_1 solutions (0.1% v/v maximum), ethanol controls were included in the experiment; these cultures yielded a cytotoxic response comparable to that of untreated control cells, showing that the observed suppression was entirely due to PGE_1 activity. Repeat experiments confirmed the suppressive effects of PGE_1 in this culture system, and further work was performed using PGE_1 at a final concentration of 1×10^{-6} molar.

2.5.3.2. Time-course of PGE_1-induced suppression of the cytotoxic response

To determine the time-course of PGE_1-induced suppression, this material was added to MLCs at the start of the culture period or at 24-hour intervals subsequent to this. The same dose of PGE_1 (1×10^{-6} molar) was also added to some previously untreated cells for the duration of the cytotoxicity assay on day 5.

As shown in Figure 2.5.6., PGE_1 added at the initiation of culture fully suppressed the cytotoxic response. A similar degree of suppression was observed after treatment with PGE_1 on day 1, 24 hours

Figure 2.5.5.

Effect of exogenous PGE_1 on the generation of CTL in the MLC

PGE_1 was added to MLCs at initiation to give a range of final concentrations. Solvent controls were included in the experiment, as PGE_1 was initially dissolved in ethanol.

Line 1: control; untreated culture

2: ethanol control for 1×10^{-6} molar PGE_1

3: ethanol control for 1×10^{-7} molar PGE_1

4: 1×10^{-6} molar PGE_1

5: 1×10^{-7} molar PGE_1

6: 1×10^{-8} molar PGE_1

7: 1×10^{-9} molar PGE_1

CULTURE	1	2	3	4	5	6	7
% recovery	146	137	132	130	137	129	123
% viability	69	71	73	68	71	67	70

TARGET CELLS	P815Y *in vitro* cell line
Spontaneous release	12%

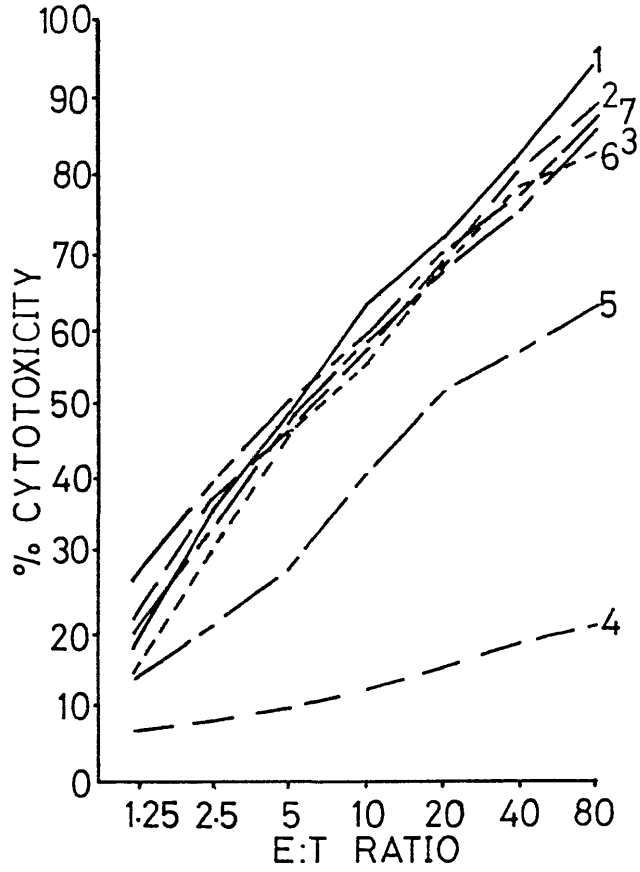

100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25 2·5 5 10 20 40 80
E:T RATIO
1
2
7
3
6
5
4

Figure 2.5.6.

Time-course of PGE_1-induced suppression of cytotoxicity in vitro

PGE_1 was added to MLCs at initiation or at 24-hour intervals after this, to give a final concentration of 1×10^{-6} molar. PGE_1 was also added to some previously untreated cells for the duration of the cytotoxicity assay.

Line 1: control; no PGE_1 added to culture

2: PGE_1 added on day 0

3: PGE_1 added on day 1

4: PGE_1 added on day 2

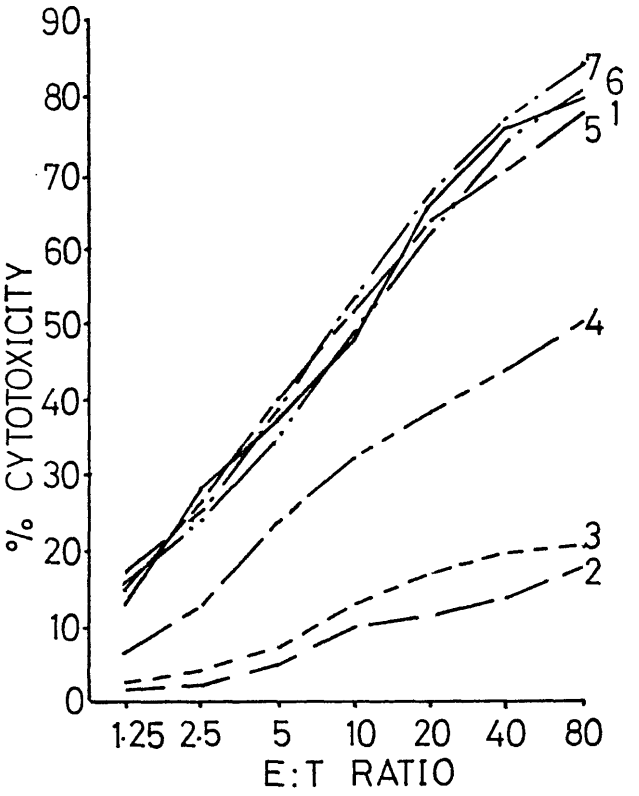
5: PGE_1 added on day 3

6: PGE_1 added on day 4

7: PGE_1 added to cytotoxicity assay on day 5

CULTURE	1	2	3	4	5	6
% recovery	139	134	138	142	136	140
& viability	72	70	67	68	66	71

TARGET CELLS	P815Y in vivo tumour cells
Spontaneous release	12%


90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25
2·5
5
10
20
40
80
E:T RATIO
7
6
1
5
4
3
2

after initiation; however, further delay in adding this material resulted in progressively weaker suppression, and PGE_1 did not influence cytotoxicity if added to cultures on or after day 3.

These results indicate that PGE_1 is affecting an early stage in the MLC, before the appearance of mature cytotoxic cells on day 4. The decreasing suppressive potency of this agent as the culture progresses confirms that PGE_1 is not directly influencing the effector T lymphocytes in this system. The time-course of this effect shows some parallels with the observed time-course of carrageenan- and zymosan-induced suppression of cytotoxicity (sections 2.4.2.2., 2.4.3.2.), and is compatible with the hypothesis that PGE_1 may be involved in mediating the effects of these macrophage-active agents.

2.5.3.3. Effects of exogenous PGE_1 and anti-PGE antiserum on the development of cell-mediated cytotoxicity in vitro

The possible role of E-series prostaglandins in carrageenan- and zymosan-induced suppression was further investigated using a rabbit anti-PGE antibody. To test the efficacy with which this antibody could prevent the effects of exogenous PGE_1, a range of final dilutions of antibody and a known suppressive dose of PGE_1 were simultaneously added to cultures at initiation.

As shown in Figure 2.5.7., the antiserum provided protection against PGE_1-induced suppression in a dose-dependent manner, with cytotoxicity restored almost to control levels at the lowest dilution of antibody (1 in 20 v/v). Control serum from the same source had no protective effect in this system, as shown in Figure 2.5.8. These results confirm that this anti-PGE antiserum can be effectively used in vitro to inhibit PGE_1. The antiserum is believed to be equally potent against PGE_2 (J. Mertin, personal communication), and it was therefore used to determine the involvement of E-series prostaglandins

Figure 2.5.7.

Effect of anti-PGE antiserum on PGE_1-induced suppression of cytotoxicity in vitro

Anti-PGE antiserum was added to PGE_1-treated MLCs to give the following final dilutions.

Line 1: control; untreated culture

2: 1×10^{-6} molar PGE_1

3: ethanol control

4: antiserum control, 1 in 20 dilution

5: antiserum control, 1 in 50 dilution

6: antiserum control, 1 in 100 dilution

7: antiserum control, 1 in 200 dilution

8: PGE_1 + antiserum at 1 in 20

9: PGE_1 + antiserum at 1 in 50

10: PGE_1 + antiserum at 1 in 100

11: PGE_1 + antiserum at 1 in 200

CULTURE	1	2	3	4	5	6	7	8	9	10	11
% recovery	129	132	129	133	127	131	124	133	126	121	130
% viability	70	69	68	71	67	72	66	68	69	71	67

TARGET CELLS	TGC-induced peritoneal cells
Spontaneous release	18%

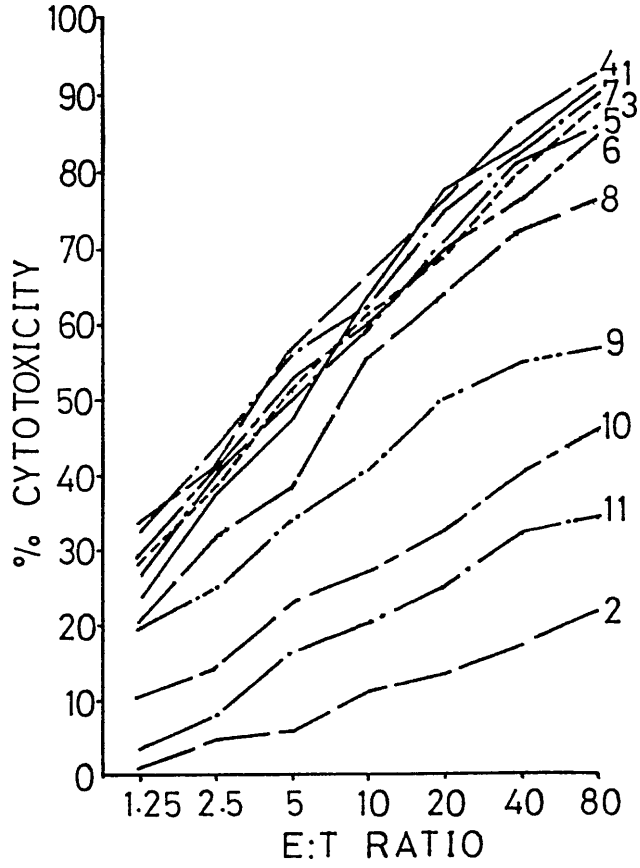

100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25 2·5 5 10 20 40 80
E:T RATIO
4
1
7
3
5
6
8
9
10
11
2

Figure 2.5.8.

Effect of control serum on PGE_1-induced suppression of cytotoxicity in vitro

Control rabbit serum was added to PGE_1-treated MLCs to give the following final dilutions.

Line 1: control; untreated culture

2: 1×10^{-6} molar PGE_1

3: ethanol control

4: serum control, 1 in 20 dilution

5: serum control, 1 in 50 dilution

6: serum control, 1 in 100 dilution

7: serum control, 1 in 200 dilution

8: PGE_1 + serum at 1 in 20

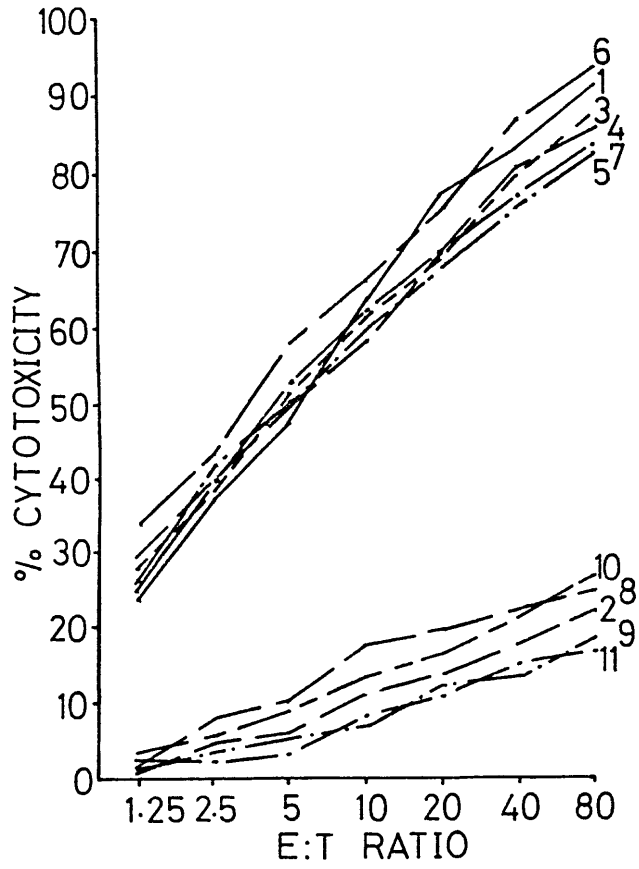
9: PGE_1 + serum at 1 in 50

10: PGE_1 + serum at 1 in 100

11: PGE_1 + serum at 1 in 200

CULTURE	1	2	3	4	5	6	7	8	9	10	11
% recovery	129	132	129	126	117	138	129	119	127	122	131
% viability	70	69	68	69	73	63	66	73	67	74	65

TARGET CELLS	TGC-induced peritoneal cells
Spontaneous release	18%


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
6
1
3
4
5
7
10
8
2
9
11
1·25 2·5 5 10 20 40 80
E:T RATIO

in carrageenan- and zymosan-induced effects in this culture system.

2.5.3.4. Effects of anti-PGE antiserum and macrophage-active agents on the development of cell-mediated cytotoxicity in vitro

Two dilutions of anti-PGE antiserum (1 in 20 and 1 in 50 v/v) were added to MLCs at initiation, together with suppressive doses of carrageenan or zymosan. As shown in Figure 2.5.9., both dilutions of antiserum effected some restoration of cytotoxicity, while control rabbit serum did not influence the lytic activity generated in carrageenan- and zymosan-treated cultures. The suppressive effect of each macrophage-active agent appeared to be equally sensitive to the antiserum, suggesting that PGE_1 and/or PGE_2 may be involved in mediating the effects of carrageenan and zymosan in this system. However, some suppression of cytotoxicity was still obtained with the antiserum at a final dilution of 1 in 20 v/v, a dose previously shown to provide almost complete protection against the effects of 1×10^{-6} molar exogenous PGE_1 in the MLC (Figure 2.5.7.). This may be due to an excess of PGE in these cultures, such that insufficient antibody was present to fully inhibit this material. However, it seems unlikely that endogenous PGE production would exceed 1×10^{-6} molar in this system, and it is therefore suggested that the incomplete restoration obtained with the antiserum may reflect the presence of other inhibitory mediators, perhaps originating from the same metabolic pathway.

2.5.4. PROSTACYCLIN, CARBACYCLIN AND ANTI-PGI_2 ANTISERUM

2.5.4.1. Effects of exogenous PGI_2 and carbacyclin on the development of cell-mediated cytotoxicity in vitro

In view of the short half-life of prostacyclin (PGI_2) under culture conditions, the stable analogue carbacyclin was tested in parallel with the natural product.

Figure 2.5.9.

Effect of anti-PGE antiserum on carrageenan- and zymosan-induced suppression of cytotoxicity *in vitro*

CULTURE	% recovery	% viability
1	162	73
2	148	75
3	150	71
4	147	72
5	139	74
6	132	76
7	154	69
8	151	71
9	146	74
10	155	71
11	157	72
12	139	73
13	142	70
14	146	72
15	130	76

TARGET CELLS	P815Y *in vitro* cell line
Spontaneous release	12%

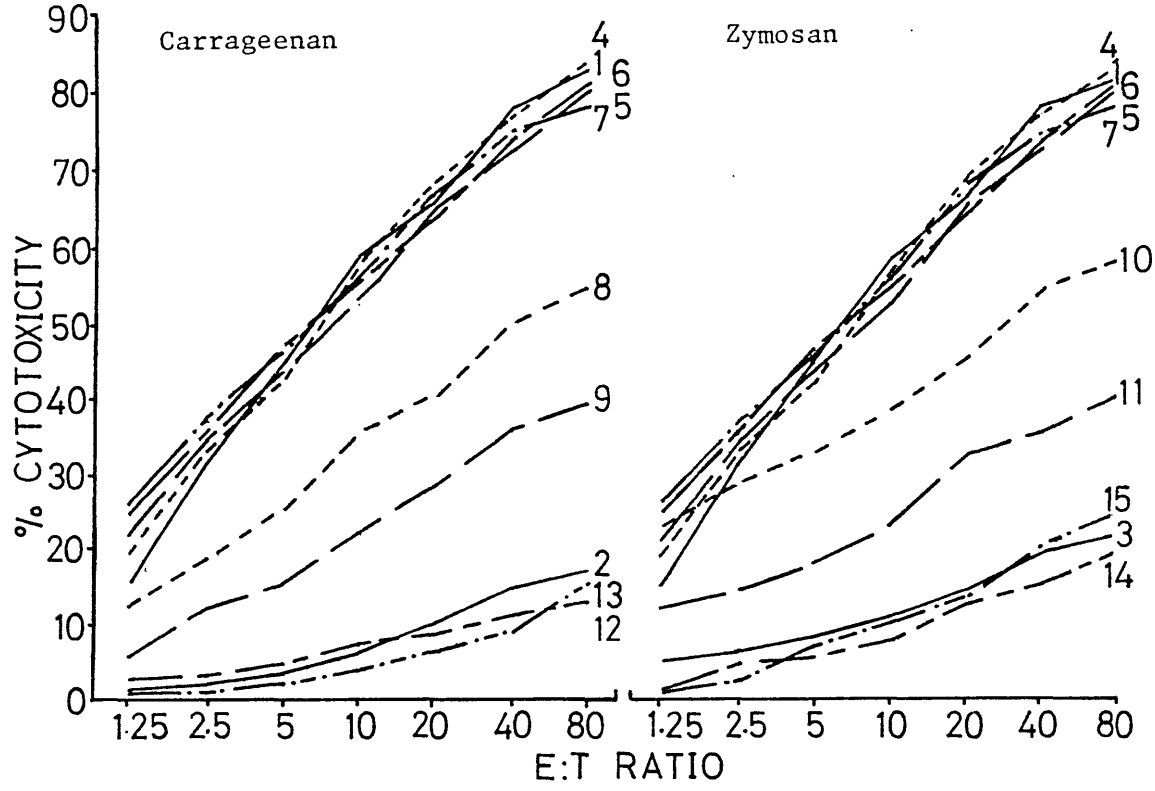


Figure 2.5.9.

Effect of anti-PGE antiserum on carrageenan- and zymosan-induced suppression of cytotoxicity in vitro

Two dilutions of anti-PGE antiserum and control rabbit serum were added to carrageenan- and zymosan-treated MLCs.

Line 1: control; untreated culture
2: 20 µg ι-carrageenan/ml
3: 200 µg zymosan/ml
4: anti-PGE antiserum, 1 in 20 dilution
5: anti-PGE antiserum, 1 in 50 dilution
6: control serum, 1 in 20 dilution
7: control serum, 1 in 50 dilution
8: carrageenan + antiserum at 1 in 20
9: carrageenan + antiserum at 1 in 50
10: zymosan + antiserum at 1 in 20
11: zymosan + antiserum at 1 in 50
12: carrageenan + control serum at 1 in 20
13: carrageenan + control serum at 1 in 50
14: zymosan + control serum at 1 in 20
15: zymosan + control serum at 1 in 50

Solutions of PGI_2 and carbacyclin were prepared as described in section 2.2.1.11.2., and were added to MLCs to give a range of final concentrations. As exogenous PGE_1 induced good suppression of cytotoxicity at a dose of 1×10^{-6} molar, PGI_2 was tested from this dose down to 1×10^{-8} molar. Carbacyclin is reported to be considerably less active than PGI_2 (G. Higgs, personal communication), so this compound was tested from 1×10^{-5} molar down to 1×10^{-7} molar.

As shown in Figure 2.5.10., neither PGI_2 or carbacyclin had any effect on cytotoxicity at the doses tested, and neither these compounds or the Tris solvent control affected cell recovery or viability. The apparent inactivity of PGI_2 could be attributed to the instability of this compound, although the equal inertness of the stable analogue suggests that exogenous PGI_2 does not influence the development of cytotoxicity *in vitro*. However, these results do not rule out the possibility that endogenous PGI_2 is involved in this system. Continuous secretion of PGI_2 by a population of cells in the MLC may balance the degradative process, and permit local accumulation of a low but effective concentration, in contrast to rapid breakdown of a single large dose of this material after exogenous treatment. Prostacyclin is difficult to detect *in vitro* due to its instability; however, it can be effectively neutralised using a specific antiserum. Subsequent experiments were therefore performed to determine the involvement of endogenous PGI_2 in carrageenan- and zymosan-induced suppression of cytotoxicity.

2.5.4.2. Effects of exogenous PGE_1 and anti-PGI_2 antiserum on the development of cell-mediated cytotoxicity *in vitro*

As mentioned in section 2.2.1.12.2., the anti-PGI_2 antiserum used in this study also binds to PGE_1 (Bunting *et al*, 1978). Consequently, before this antiserum could be used to examine the involvement of PGI_2 in carrageenan- and zymosan-induced effects on cytotoxicity, it was

Figure 2.5.10.

Effect of exogenous prostacyclin and carbacyclin on the generation of CTL in the MLC

Prostacyclin and carbacyclin were added to MLCs at initiation, to give a range of final concentrations. As these compounds were initially dissolved in Tris buffer, a solvent control was included in the experiment.

Line 1: control; untreated culture

2: Tris buffer control

3: 1×10^{-6} molar PGI_2

4: 1×10^{-7} molar PGE_2

5: 1×10^{-8} molar PGI_2

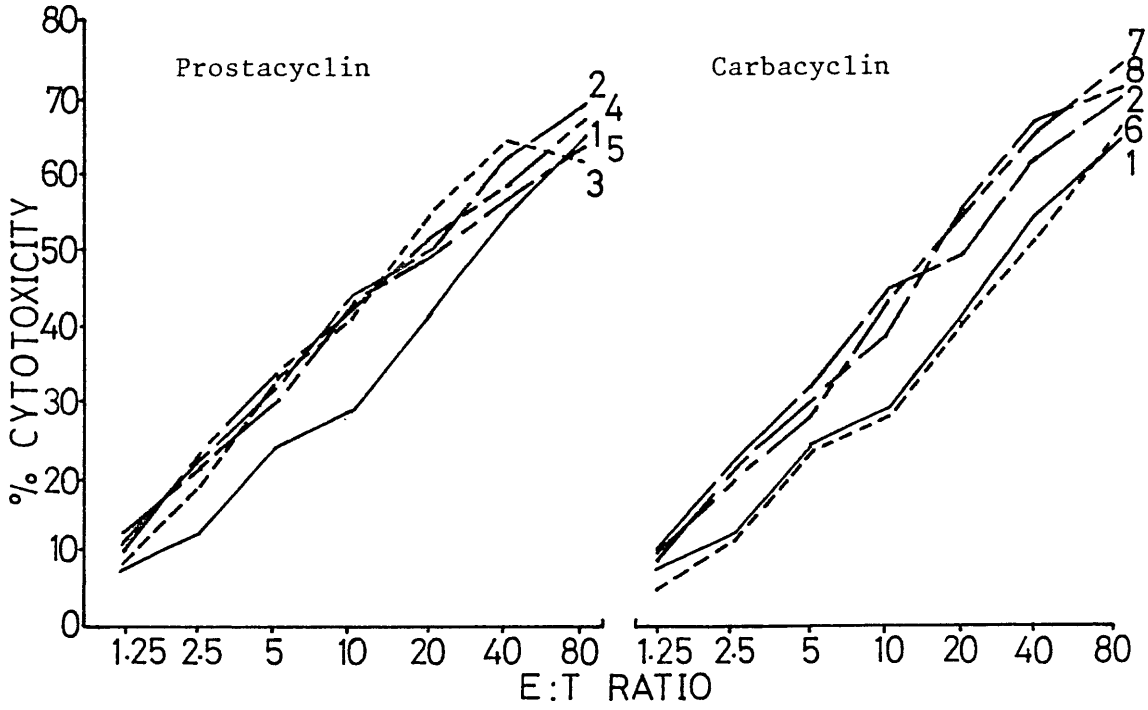
6: 1×10^{-5} molar carbacyclin

7: 1×10^{-6} molar carbacyclin

8: 1×10^{-7} molar carbacyclin

CULTURE	1	2	3	4	5	6	7	8
% recovery	195	193	168	151	180	152	145	151
% viability	69	66	69	66	61	66	59	63

TARGET CELLS	P815Y *in vitro* cell line
Spontaneous release	13%


Prostacyclin
Carbacyclin
% CYTOTOXICITY
E:T RATIO
0
10
20
30
40
50
60
70
80
1·25
2·5
5
10
20
40
80
2
4
1
5
3
7
8
2
6
1

necessary to determine its functional specificity in this system. Cultures exposed to 1 x 10^{-6} molar exogenous PGE_1 were therefore treated with anti-PGI_2 antiserum, to assess the ability of the antiserum to neutralise this material.

As shown in Figure 2.5.11., anti-PGI_2 antiserum at a final dilution of 1 in 50 v/v afforded no protection against the suppressive effects of exogenous PGE_1, while the same dilution of an antiserum raised against E-series prostaglandins permitted considerable restoration of the response. In a previous experiment using a lower dilution of the latter antiserum, a response approaching control levels was obtained (Figure 2.5.7.); however, insufficient anti-PGI_2 antiserum was available to test at dilutions below 1 in 50 v/v.

These results show that this dilution of anti-PGI_2 antiserum is unable to neutralise 1 x 10^{-6} molar exogenous PGE_1 in this system. However, it should be noted that lower concentrations of endogenous E-series prostaglandins may be generated locally in the MLC, and such small amounts may be effectively neutralised by cross-reaction with antiserum raised against PGI_2.

2.5.4.3. Effects of anti-PGI_2 antiserum and macrophage-active agents on the development of cell-mediated cytotoxicity in vitro

Suppressive doses of carrageenan or zymosan were added to MLCs at the start of the 5-day culture period, together with anti-PGI_2 antiserum at a final dilution of 1 in 50 v/v. As shown in Figure 2.5.12., the antiserum produced some restoration of cytotoxicity, while control rabbit serum did not affect the lytic activity of carrageenan- or zymosan-treated cultures. However, as with anti-PGE antiserum (section 2.5.3.4.), full restoration of the response was not obtained.

The results of this experiment suggest that PGI_2 may be involved in mediating carrageenan- and zymosan-induced suppression of cytotoxicity.

Figure 2.5.11.

Specificity of anti-PGI_2 antiserum in the MLC

Control rabbit serum and antisera raised against PGI_2 and PGE_1 were compared with respect to their ability to neutralise the suppression effects of exogenous PGE_1. Each serum was tested at a final dilution of 1 in 50.

Line 1: control; untreated culture

2: 1×10^{-6} molar PGE_1

3: control serum

4: anti-PGI_2 antiserum

5: anti-PGE antiserum

6: PGE_1 + control serum

7: PGE_1 anti-PGI_2 antiserum

8: PGE_1 + anti-PGE antiserum

CULTURE	1	2	3	4	5	6	7	8
% recovery	153	146	144	149	152	147	149	145
% viability	69	71	70	75	71	68	72	73

TARGET CELLS	P815Y in vitro cell line
Spontaneous release	10%

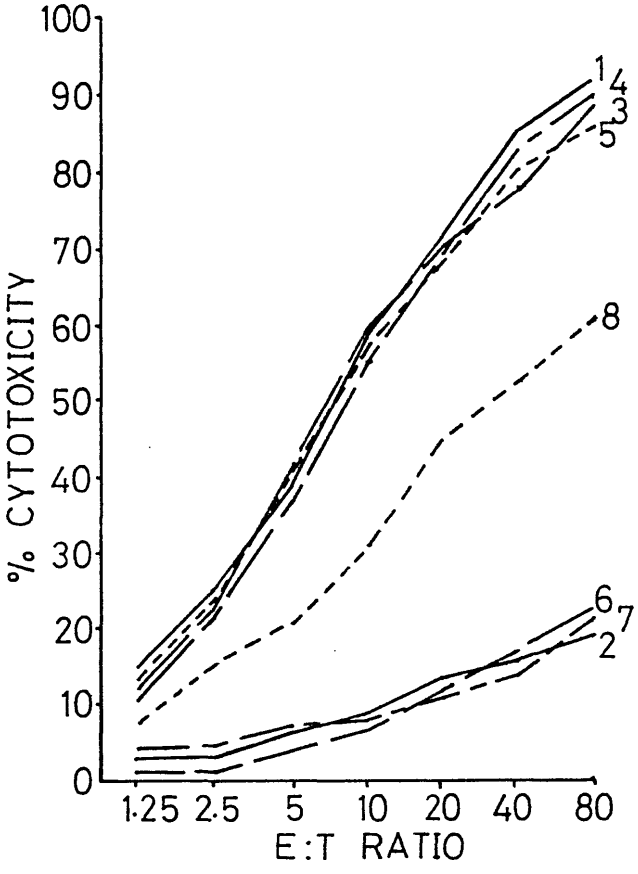

100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
4
3
5
8
6
7
2
1·25 2·5 5 10 20 40 80
E:T RATIO

Figure 2.5.12.

Effect of anti-PGI_2 antiserum on carrageenan- and zymosan-induced suppression of cytotoxicity *in vitro*

Anti-PGI_2 antiserum and control serum were added to carrageenan- and zymosan-treated MLCs, to give a final dilution of 1 in 50.

Line 1: control; untreated culture

2: 20 µg ι-carrageenan/ml

3: 200 µg zymosan/ml

4: control serum

5: anti-PGI_2 antiserum

6: carrageenan + control serum

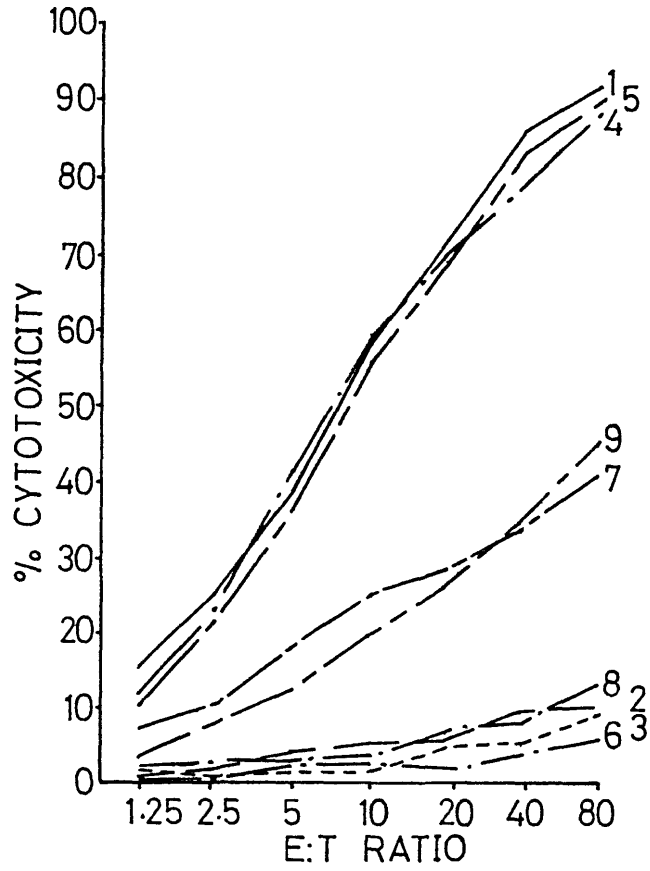
7: carrageenan + anti-PGI_2 antiserum

8: zymosan + control serum

9: zymosan + anti-PGI_2 antiserum

CULTURE	1	2	3	4	5	6	7	8	9
% recovery	153	157	149	144	149	147	151	149	153
% viability	69	72	76	70	75	73	70	77	72

TARGET CELLS	P815Y *in vitro* cell line
Spontaneous release	10%


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25 2·5 5 10 20 40 80
E:T RATIO
1
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9
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3
6

However, such observations should be interpreted with caution, in view of the possibility of cross-reaction between anti-PGI_2 antiserum and endogenous E-series prostaglandins, as mentioned in section 2.5.4.2.

2.5.4.4. Combined effects of anti-PGE and anti-PGI_2 antisera on carrageenan- and zymosan-treated cultures

In view of the failure of antisera to either PGE or PGI_2 to fully restore the cytotoxic response of cells exposed to carrageenan or zymosan, it was suggested that both these prostanoids may be involved in mediating suppression. Consequently, neutralisation of only one using a specific antiserum still allowed the other to exert some suppressive influence. To test this hypothesis, both anti-PGE and anti-PGI_2 antisera were added to carrageenan- and zymosan-treated cultures, in an attempt to fully inhibit the suppressive effects of these agents. However, such treatment failed to improve restoration of the cytotoxic response, and there was little evidence of an additive effect of the two antisera, as shown in Figure 2.5.13. These results appear to support the suggestion that the protective effects of anti-PGI_2 antiserum in this system are due solely to cross-reaction with E-series prostaglandins. However, involvement of PGI_2 *per se* should not be dismissed, particularly in view of the reports that the major E-series prostaglandin released by macrophages is PGE_2 (Glatt *et al*, 1977; Wahl, 1981), for which the anti-PGI_2 antiserum used here is believed to have low affinity (S. Bunting, personal communication).

Further experiments using anti-PGE and anti-PGI_2 antisera were limited by the availability of these preparations. The data presented in sections 2.5.3. and 2.5.4. of this chapter also suggest that E- and I-series prostaglandins may not be solely responsible for mediating the suppressive effects of carrageenan and zymosan in the MLC. Accessory cells are known to release a wide range of biologically

Figure 2.5.13.

Combined effects of anti-PGE and anti-PGI_2 antisera on carrageenan- and zymosan-induced suppression of cytotoxicity in vitro

CULTURE	% recovery	% viability
1	129	78
2	122	79
3	134	76
4	119	80
5	125	78
6	120	79
7	127	76
8	130	74
9	128	73
10	118	81
11	131	75
12	127	77

TARGET CELLS	P815Y in vitro cell line
Spontaneous release	11%

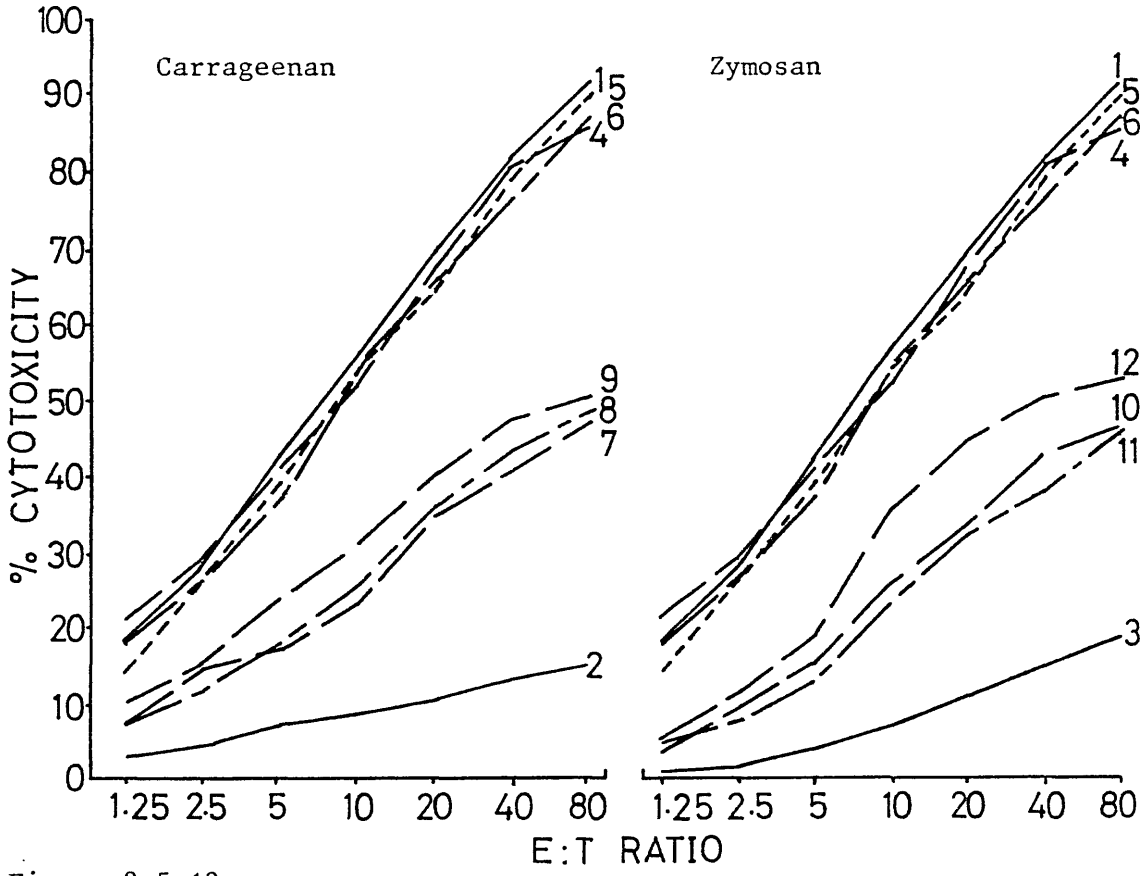


Figure 2.5.13

Combined effects of anti-PGE and anti-PGI_2 antisera on carrageenan- and zymosan-induced suppression of cytotoxicity in vitro

Anti-PGE and anti-PGI_2 antisera were added to carrageenan- and zymosan-treated MLCs, alone or in combination, to give final dilutions of 1 in 50.

Line 1: control; untreated culture

2: 20 µg ι-carrageenan/ml

3: 200 µg zymosan/ml

4: anti-PGE antiserum

5: anti-PGI_2 antiserum

6: anti-PGE + anti-PGI_2 antisera

7: carrageenan + anti-PGE antiserum

8: carrageenan + anti-PGI_2 antiserum

9: carrageenan + both antisera

10: zymosan + anti-PGE antiserum

11: zymosan + anti-PGI_2 antiserum

12: zymosan + both antisera

active substances which may be involved in this system (discussed more fully in section 1.3.); however, no study of their possible role was undertaken here.

2.6. DISCUSSION

The experiments described in previous sections of this chapter were designed to examine the role of adherent macrophage-like cells in specific cell-mediated immune responses in vitro, using the generation of alloreactive T lymphocytes in a one-way MLC as the test system. In subsequent sections, the results obtained in these experiments will be discussed in more detail.

2.6.1. PRELIMINARY WORK ON THE CULTURE SYSTEM

Several conclusions may be drawn from the results presented in section 2.3.1.. The murine one-way MLC has been shown to generate specific cytotoxic effector cells in multiwell plates, and the optimal conditions for this system have been determined. Although the principle behind the generation of such effector lymphocytes in the MLC is straightforward, considerable care is needed to obtain optimal results. The correct culture medium must be selected to ensure maximal cell viability, and this may entail using different media for preparing cell suspensions and for maintaining cultures. Two such media were used in this study; a balanced salt solution proved preferable for handling the cells in air at room temperature, while bicarbonate-buffered RPMI was used when the cells were exposed to higher levels of CO_2. Similarly, the means by which cells are released into suspension must be rapid, with minimal trauma. Spleen and lymph node cell suspensions are frequently prepared by sieving; however, this method proved inferior to teasing the organ apart in a dish of medium, as shown in Table 2.3.1.. Data presented in the same table indicate that removal of erythrocytes from mixed cell suspensions is also unnecessary; this step is frequently included in

routine spleen cell preparation. With respect to the culture vessels used, multiwell plates have several advantages over the more conventional flasks. Fewer cells are required, economising on both animals and culture reagents, and plates are less susceptible to contamination by micro-organisms; if contamination does occur, it can be contained within the original well. In addition, the yield of viable cells is greater following culture in plates (see Table 2.3.2.); it is possible that the smaller volumes per well result in more efficient gas exchange.

Cell numbers and responder to stimulator (R:S) ratios are critical for optimal development of the cytotoxic response. In this study, 2 x 10^6 responder cells mixed with an equal number of X-irradiated stimulator cells in a final volume of 2·0 ml yielded the best results.

Cell survival *in vitro* over the 5-day culture period, and the subsequent development of cytotoxicity, are shown to be absolutely dependent on the presence of an optimal concentration of 2-mercaptoethanol (2-ME) in the culture medium. This substance is believed to exert its enhancing effect on immune responses *in vitro* during the first 24 hours of culture (Click *et al*, 1972a); however, the mechanism of this effect is not clear. The sulphydryl group of 2-ME has been shown to be the active moiety, and it has been suggested that this may serve to activate essential SH-requiring enzyme systems, or to protect important membrane thiol groups from oxidation (Click *et al*, 1972b).It has ben reported that 2-ME can substitute for macrophages in some *in vitro* lymphocyte responses, for example the murine primary antibody response to sheep erythrocytes (Chen and Hirsch, 1972a). In such systems, 2-ME may mimic the effects of macrophage-derived soluble factors, perhaps via

direct action on the reactive lymphocytes, and functional replacement of IL-1 has been suggested (Rosenstreich and Oppenheim, 1976). Enhancement of T cell responses has also been reported, for example the murine MLC (Heber-Katz and Click, 1972). However, 2-ME does not appear able to fully replace macrophages in cell-mediated responses (Rode and Gordon, 1974; Yamashita and Hamaoka, 1979), and it has been suggested that several macrophage factors may be required for T cell activation, one or more of which may be replaced by 2-ME (Broome and Jeng, 1973; Rosenstreich and Oppenheim, 1976). Results presented in section 2.3.2. of this study and discussed more fully in section 2.6.2. support the opinion that 2-ME cannot substitute for macrophages in the MLC. In view of the absolute requirement for this substance, even when adherent macrophage-like cells are also present, this may suggest that the function of 2-ME in this culture system is unrelated to that of the accessory population.

2.6.2. REQUIREMENT FOR ADHERENT CELLS

The generation of cytotoxic T lymphocytes in this culture system has been shown to depend on the presence of a population of macrophage-like accessory cells. These cells may be removed from the responder and stimulator cell suspensions by various methods relying on their adherent properties, and the results of such experiments are presented in section 2.3.2.. Three procedures for depletion of the adherent fraction have been studied. The adherent cells could not be recovered following depletion using carbonyl iron powder and magnetism (section 2.3.2.1.); however, nylon wool (section 2.3.2.2.) and plastic adherence (section 2.3.2.3.) yielded enriched adherent and non-adherent populations, which could then be further

manipulated. The results obtained using each procedure were essentially similar, although considerable non-specific cell loss was apparent following depletion on nylon wool columns. The results presented in Figure 2.3.16. indicate that plastic adherence is the least efficient depletion technique, as some cytotoxic activity still developed after treatment of both responder and stimulator populations in this manner.

Removal of adherent cells from both cell suspensions prior to culture revealed an absolute requirement for such cells; however, recovery and viability of non-adherent cells at the end of the culture period were unaffected by this procedure, indicating that the adherent fraction is not essential for survival of the responding lymphocytes in vitro. It has been suggested that macrophage-like accessory cells may function non-specifically in some culture systems, perhaps conditioning the culture medium and providing optimal conditions for cell growth and replication (Chen and Hirsch, 1972a); reports that the macrophage requirement may be fulfilled in some humoral in vitro systems by the addition of 2-ME (Chen and Hirsch, 1972a, b; Lemke and Opitz, 1976), or macrophage culture supernatants (Hoffman and Dutton, 1971) support this view. Bach et al (1970) observed similar restoration of the human MLC using cell-free macrophage supernatants. However, the results obtained in this study indicate that the adherent population plays a more specific role in this culture system, and it is suggested that such cells may be directly involved in lymphocyte activation. This is consistent with Wagner et al (1972, 1973), who demonstrated an absolute requirement for macrophages in immune induction, and agrees with the suggestion of Persson et al (1978) that adherent cells and their products play an important role in the activation process.

It should be noted that the cell depletion techniques used in this study would not yield pure adherent and non-adherent cell populations, but rather selectively enriched suspensions. The cells comprising the adherent population studied here have not been precisely identified. They are known to be actively phagocytic, as shown using latex beads and stained yeast particles, and their adherent properties have already been described. In preliminary experiments to this study, the majority of the cells (>95%) were found to stain for cytoplasmic non-specific esterase (NSE) (data not shown). These features place the cells in the MPS; they probably belong to the monocyte-macrophage lineage described in section 1.1.1.. However, use of the term "macrophage" here and by others can be misleading; it should be noted that other cell types may be present in an adherent preparation, and that these may be responsible for the observed effects. In particular, the dendritic cell is currently receiving attention as an important constituent of adherent spleen cell preparations, and there is increasing evidence that this cell is responsible for some of the properties previously attributed to classical macrophages. Such cells are present in fresh unfractionated spleen cell suspensions, and would be removed by the adherence techniques used here. However, this cell type is reported to be negative for NSE, and is only weakly phagocytic (Steinman and Nussenzweig, 1980). It is also interesting to note that the dendritic cell is reported to lose its adherent properties after 12-16 hours <u>in vitro</u> (Steinman and Cohn, 1975); in this case, the restorative capacity of 24-hour adherent cells shown in Figure 2.3.19. may indicate that other more strongly adherent cell types are involved.

The precise function of accessory cells in the MLC is not clear.

The design of the experiments reported here indicates that such cells fulfill their role during the initial 24 hours of culture, after which they appear to have no further influence on the subsequent response of the effector population. This time-course is in agreement with observations for the macrophage requirement in the MLC (Davidson, 1977), while Benacerraf and Pierce (1969) and Cosenza and Leserman (1971) found a similar early requirement for adherent cells in the in vitro antibody response to sheep erythrocytes.

Following the initial requirement for adherent cells, the subsequent independent development of the in vitro cytotoxic response may be taken as evidence against a purely nutritive role for these cells, and suggests that the adherent population plays a vital role in the early or inductive phase of the response. Experiments in which either responder or stimulator cell suspensions were depleted of the adherent fraction (Figures 2.3.6., 2.3.11., 2.3.16.) suggest that these cells have a dual role in this culture system; an allostimulatory function which is served by adherent cells of the stimulator strain, and an accessory function, which does not appear to be governed by histocompatibility restrictions. Consequently, adherent cell depletion of the responder cells alone has little effect on the cytotoxic response, as the accessory cell requirement can still be met by adherent cells in the unfractionated stimulator suspension (line 2, Figures 2.3.6., 2.3.11., 2.3.16.). However, if adherent cells of the responder strain are still present, but the stimulator population is depleted of this fraction, a weaker cytotoxic response results (line 3, Figures 2.3.6., 2.3.11., 2.3.16.). It is suggested that this reflects removal of a major part of the allogeneic stimulus, although some stimulation is still provided by the non-adherent lymphoid cells of the stimulator

suspension. This weaker stimulaton is not due to the change in cell numbers (Figure 2.3.7., line 4; Figure 2.3.12., line 2), indicating that non-adherent cells are less efficient allostimulators than their adherent counterparts. This is compatible with the route I - route II model of T cell activation proposed by Lechler and Batchelor (1982); adapted to the current in vitro system, this hypothesis states that, in the absence of direct stimulation by adherent cells of the stimulator strain (route I), indirect stimulation may be obtained after phagocytosis and presentation of lymphocyte-associated alloantigen by accessory cells present in the responder population (route II). Helper T cells are then able to respond to MHC alloantigens presented on syngeneic accessory cells; though less efficient than route I activation, this pathway still enables co-operation between activated helper and cytotoxic T cells, resulting in the development of mature cytolytic effector cells.

It therefore appears that the adherent cells in this culture system are serving to present antigen to the responding T lymphocytes in a manner comparable to that found in other T and B cell responses. This is in agreement with Rode and Gordon (1974), who reported that the stimulatory substance must be presented by a macrophage to achieve T cell activation in the MLC, and suggested that the stimulatory efficiency and accessory role of the macrophage may therefore represent different aspects of this same phenomenon.

Cytotoxic precursor lymphocytes generally respond to class I MHC alloantigens, while activation of helper T cells requires class II antigens. Although the latter MHC products show a restricted tissue distribution, they are not confined to cells of the MPS (see section 1.1.2.). This then raises the question as to why there is an absolute requirement for macrophage-like cells in T cell activation.

Minami et al (1980) observed the failure of B lymphocytes bearing both class I and II MHC antigens to induce an MLC, and suggested that a second signal is required to complete the inductive stimulus; this signal could only be provided by accessory cells in the adherent population. This second signal is now believed to be interleukin 1 (IL-1) (Farrar et al, 1980; Mizel, 1982), which is understood to act on precursors of helper and cytotoxic T cells, serving as a maturational signal and driving these cells into a primed state in which they may respond to antigen. This maturation step is likely to be very rapid, resulting in a minimal lag period before antigen responsiveness is attained. In response to stimulation with class II alloantigens, primed helper T cells then release the mitogenic lymphokine interleukin 2 (IL-2), previously known as thymocyte mitogenic/stimulating factor (TMF/TSF) (Aarden et al, 1979; Wagner and Rollinghoff, 1978). Binding of this lymphokine to its specific receptor on the lymphocyte surface induces proliferation and maturation of the cell, leading to production of clones of cytolytic effector lymphocytes (Farrar et al, 1982). Wagner et al (1982) have suggested that a further signal is required to complete this maturation step, and have proposed a cytotoxic T cell differentiation factor (CTDF), which functions to convert IL-2-driven immature cytotoxic cells into mature effector lymphocytes. However, the cellular origin of this putative factor is as yet unknown.

The results presented in Figure 2.3.10. show that third party adherent cells can function as efficiently as adherent cells of the responder strain in this culture system. This suggests that allogeneic accessory cells are equally capable of presenting antigen to responding T lymphocytes via route II, and it has already been

demonstrated that the genetically unrestricted second signal (IL-1) may be delivered by allogeneic cells. It is therefore suggested that a mechanism of T cell activation approximating that represented in Figure 2.6.1. is operating in this culture system, with the adherent cells providing a stimulatory signal via route I or II, and a second signal in the form of IL-1. In a culture where adherent cells of both responder and stimulator strains are present, both route I and II will probably occur. This mechanism is in agreement with the two-signal hypothesis described by Wagner and Rollinghoff (1978) and Mizel (1982).

No experiments were performed to further characterise the nature of the cells and soluble factors involved in the MLC, or the mechanisms of their interaction. However, it is interesting to note that in the case of third party adherent cells, two populations of helper and cytotoxic T cells would presumably be activated, one specific for antigen of the stimulator strain presented via route II, and another activated by third party alloantigen presented by route I. Although induced by a specific antigenic stimulus, IL-2 is believed to act non-specifically, and is not genetically restricted (Farrar et al, 1982); it is therefore possible that helper T cells activated by one haplotype may co-operate with cytotoxic cells specific for a different haplotype, and this may provide an interesting theme for further investigation.

2.6.3. EFFECTS OF MACROPHAGE-ACTIVE AGENTS ON THE MLC

The role of adherent macrophage-like cells in cell-mediated cytotoxic responses in vitro was further investigated using substances believed to act on the phagocytic population. The results of such experiments are given in section 2.4., and show that the

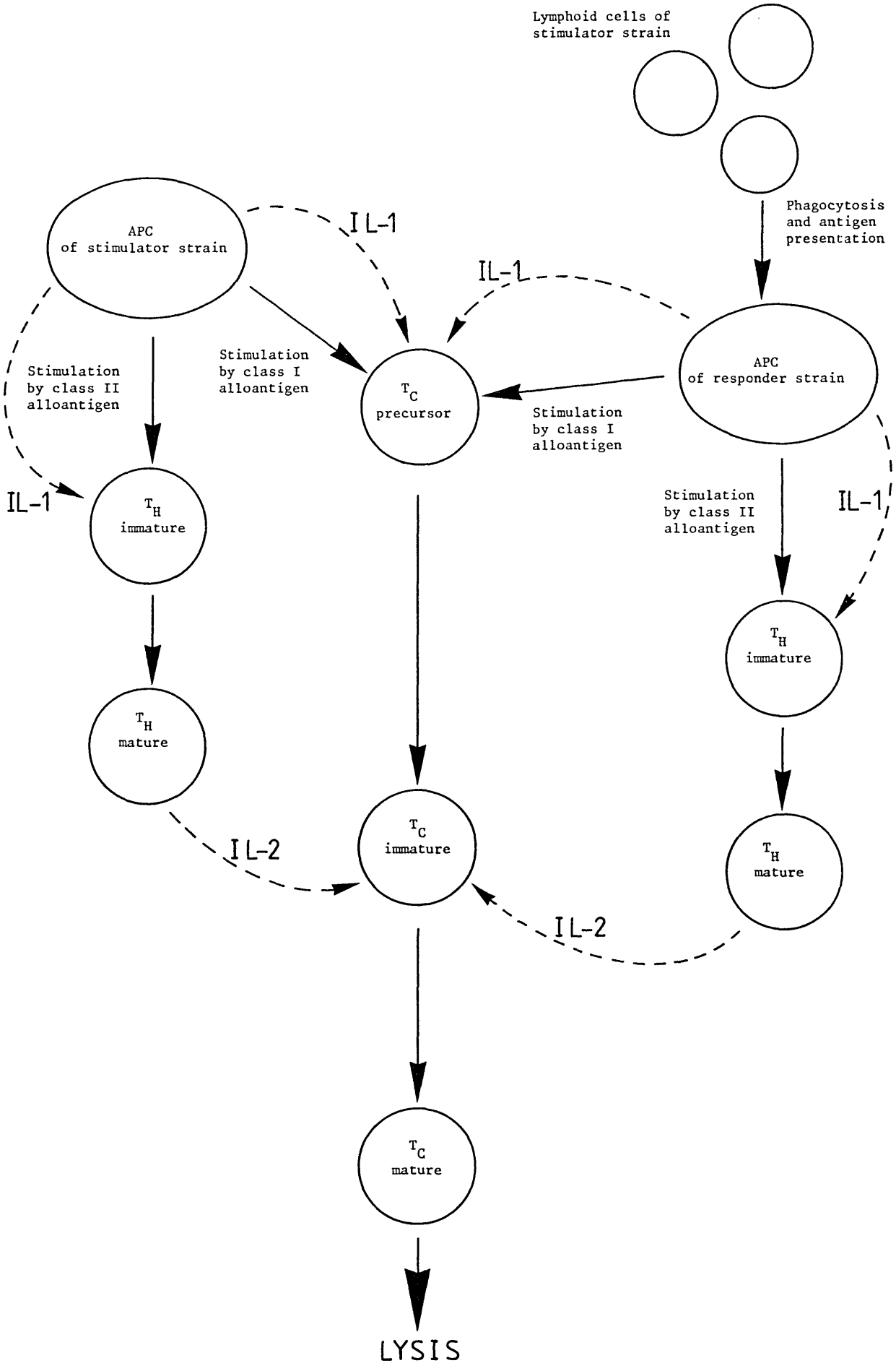
Figure 2.6.1.

Mechanism of T cell activation in the generation of alloreactive cytotoxic T lymphocytes in vitro

In the presence of an unfractionated stimulator population, both the allostimulatory signal (Class I and Class II alloantigens) and the accessory signal (Interleukin 1) can be provided by adherent cells allogeneic to the responding lymphocytes. If the only adherent cells present are of responder or 3rd party strain, then the accessory signal is still supplied; however, the allogeneic stimulus is now confined to the less efficient Route II, resulting in a weaker cytotoxic response. In practice, if neither the responder or stimulator cells are depleted of the adherent fraction, both routes of T cell activation probably operate.

Figure 2.6.1.

Mechanism of T cell activation in the generation of alloreactive cytotoxic T lymphocytes in vitro



generation of cytotoxic activity in this culture system is subject to suppression by silica, carrageenan and zymosan. However, latex particles failed to influence the cytotoxic response, as shown in Figure 2.4.16., although adherent cells recovered at the end of the culture period had clearly ingested this material. It was therefore concluded that the phagocytic process per se was not responsible for the suppression induced by the other agents. This is consistent with work by Schnyder and Baggiolini (1978), who reported activation of mouse peritoneal macrophages by zymosan but not by latex beads, as measured by enzyme activity. It has been suggested that the attachment of adherent cells to a substratum represents the initial stages of the phagocytic process (North, 1968); as this event clearly does not cause suppression of cell-mediated immune responses, it is perhaps not surprising that ingestion of a biologically inert particle such as latex also failed to induce suppression in this culture system.

Having determined that the observed suppression was specific for these agents, further experiments were performed using silica, carrageenan and zymosan to identify their mechanism(s) of action. There was no evidence for a lymphocyte-toxic effect of any of these materials at the doses used in these experiments; consequently their suppressive effects cannot be attributed to selective destruction of the effector cells.

Time-course studies using each agent suggested interference with the early stages of the cytotoxic response. The suppressive effects of silica were only observed if this material was added to cultures during the first 24 hours (Figure 2.4.2.), the silica-sensitive period therefore corresponding to the adherent cell requirement in this system (Figure 2.3.18.), as shown in Figure 2.6.2.. Few viable

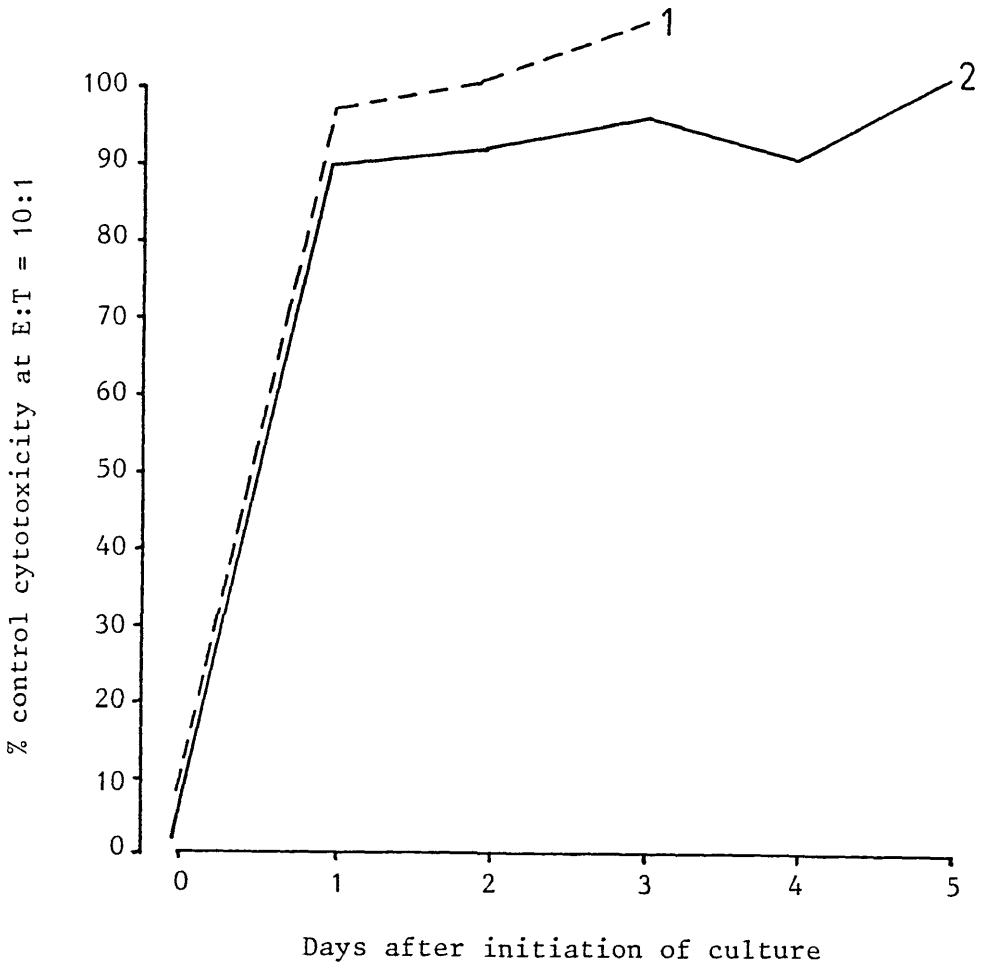


Figure 2.6.2.

Comparison of adherent cell requirement and time-course of silica-induced suppression in the in vitro cytotoxic response

Data on adherent cell requirement taken from experiment presented in Figure 2.3.18. Data on time-course of silica-induced suppression taken from experiment presented in Figure 2.4.2.

Values for per cent specific lysis were corrected against the control value for untreated cultures, which was set at 100%. Using regression analysis of the experimental data, values were calculated at an E:T ratio of 10:1, as this ratio falls in the linear portion of the curve.

Line 1: time-course of adherent cell requirement

2: time-course of silica-induced suppression

adherent cells could be recovered from silica-treated cultures (section 2.4.5.), and those present were incapable of phagocytosis, suggesting an anti-macrophage activity for this material. This is in agreement with Allison et al (1966), who observed rapid uptake of protein-coated silica particles by macrophages, followed by death of the phagocytic cell. A similar process was reported by O'Rourke et al (1978) and Sanderson and Taylor (1976), who confirmed the macrophage-specific toxicity of protein-coated silica particles. Accordingly, the silica used in this study was first coated with serum protein. Silica-induced destruction of the phagocytic/adherent population during the first 24 hours of the culture period studied here would remove essential accessory cells. In the absence of such cells, no cytotoxic response is generated, and it is therefore suggested that the suppressive effects of silica observed here reflect specific depletion of macrophage-like cells. The failure of silica to influence cytotoxicity when added after the critical macrophage-dependent period at initiation supports this conclusion, and confirms the lack of toxicity towards the non-adherent effector population. A similar mechanism for silica-induced suppression has been proposed by Lotzova and Cudkowicz (1974) using a murine allograft model, and by Pawelec and Brons (1978) working on the primary MLC of the pig.

The results obtained using silica indicate that this material may be a useful tool for selective removal of phagocytic cells, and in some cases it may prove more specific than physical depletion methods. Using the culture system employed here, further experiments with silica may serve to define more precisely the duration of the adherent cell requirement.

In the case of carrageenan and zymosan, the period during which

cultures were subject to suppression extended beyond the first 24 hours. Of the three purified forms tested, only lambda and iota carrageenan proved suppressive in this culture system, each of these showing a similar pattern of activity. Purified kappa carrageenan failed to influence the generation of cytotoxic activity in vitro (Figure 2.4.4.). A similar order of carrageenan activity has been reported by Yung and Cudkowicz (1977, 1978a). Both lambda and iota carrageenan suppressed the cytotoxic response when added to cultures 24 hours after initiation, and some suppression was still obtained after addition on day 2 of culture (Figures 2.4.6., 2.4.7.). The time-course of zymosan activity was somewhat shorter, with only partial suppression being obtained after treatment on day 1 of culture (Figure 2.4.12.); however, both carrageenan and zymosan were still able to suppress the cytotoxic response after the macrophage-dependent period had expired. This relationship is shown in Figure 2.6.3..

These results suggest that carrageenan and zymosan do not exert their suppressive effects by destroying essential accessory cells, as suppression was still obtained after the accessory function had been fulfilled. This conclusion is supported by the high viability and phagocytic activity of adherent cells recovered from carrageenan- and zymosan-treated cultures, as described in section 2.4.. With respect to carrageenan, such findings disagree with reports describing the macrophage-toxic effects of this material in vitro; lysosomal disruption and subsequent death of the phagocytic cell after ingestion of carrageenan have been reported, in a manner comparable to that observed using silica (Allison et al, 1966; Catanzaro et al, 1971; Thomson et al, 1976). Macrophage-toxicity has also been proposed to explain the immunosuppression induced by

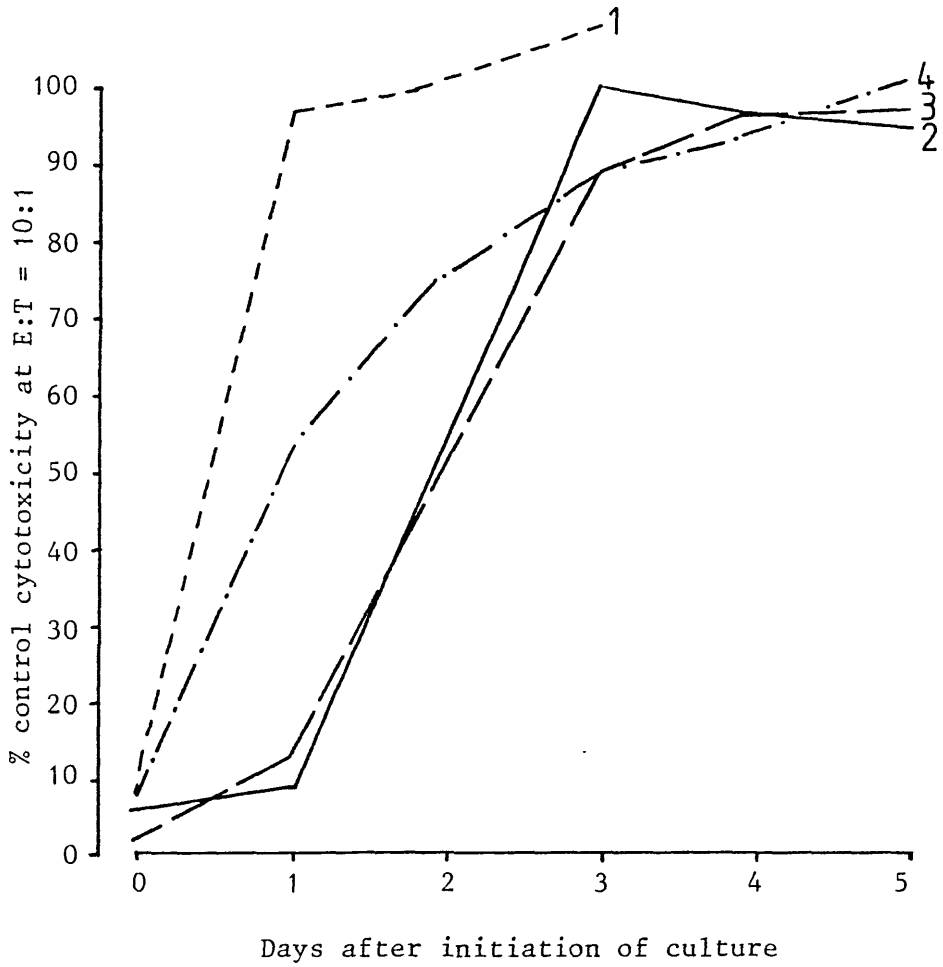


Figure 2.6.3.

Comparison of adherent cell requirement and time-course of carrageenan- and zymosan-induced suppression in the in vitro cytotoxic response

Data on adherent cell requirement taken from experiment presented in Figure 2.3.18. Data on time-course of carrageenan-induced suppression taken from experiments presented in Figures 2.4.6. and 2.4.7. Data on time-course of zymosan-induced suppression taken from experiment presented in Figure 2.4.12.

Per cent control cytotoxicity was calculated as described for Figure 2.6.2.

Line 1: time-course of adherent cell requirement

2: time-course of ι-carrageenan-induced suppression

3: time-course of λ-carrageenan-induced suppression

4: time-course of zymosan-induced suppression

carrageenan *in vivo* (Fowler and Thomson, 1978; Rios and Simmons, 1972; Thomson *et al*, 1976). However, other workers have failed to demonstrate such a toxic effect (Aschheim and Raffel, 1972; Sawicki and Catanzaro, 1975; Simon and Jones, 1979), and it has been suggested that only certain macrophage sub-populations may be killed by carrageenan (Bash and Cochran, 1980; Sawicki and Catanzaro, 1975). Anti-lymphocyte activity has also been reported for this material (Yung and Cudkowicz, 1978a), though this was only apparent at high concentrations relative to those used in this study. The reason for this variation in the literature is unknown; however, differences in the source and type of carrageenan used, or in its purification and preparation may be responsible.

Carrageenan and zymosan are clearly acting during the early part of the response, as evidenced by their decreasing potency as the culture progresses. The results presented in Figures 2.4.8. and 2.4.13. show that these agents need not be present for the entire duration of culture to exert their suppressive effects; an exposure period of 24 hours at the start of culture proved sufficient, supporting the conclusion that carrageenan and zymosan are primarily influencing the inductive phase of the cytotoxic response. Similar observations regarding the continuous presence of carrageenan were reported by Yung and Cudkowicz (1978a), working on a comparable *in vitro* system.

As with the earlier time-course experiments, zymosan was found to be active over a shorter period of culture than carrageenan (Figures 2.4.8. and 2.4.13.). The reason for this difference between the two materials is unknown, and must await further elucidation of their mechanisms of action. A similar time-course for carrageenan-induced suppression has been reported by Lake *et al* (1971) using antigen-

induced lymphocyte transformation, by Thomson et al (1976) working on the antibody response to sheep erythrocytes, and by Yung and Cudkowicz (1978a) in a study of F_1 anti-parent and anti-allogeneic cell-mediated cytotoxicity in vitro. A direct effect on the mature effector cells in this system is therefore unlikely, although the precursors of this population may form a target for the suppressive effect. However, as both carrageenan and zymosan are known to be ingested by phagocytic cells (Allison et al, 1966; Catanzaro et al, 1971; Riggi and DiLuzio, 1961; Schnyder and Baggiolini, 1978), it is suggested that this population may mediate their suppressive effects in vitro. This hypothesis is supported by the results obtained following pre-treatment of the responder and stimulator cell suspensions with carrageenan or zymosan before the MLC. The design of such experiments dictates that one of the cell suspensions will be depleted of the adherent fraction before culture, and the subsequent development of a cytotoxic response has been shown to depend on the state of the one adherent population present. Thus, if the adherent cells remaining in the MLC have been exposed to carrageenan (Figures 2.4.9. and 2.4.10.) or zymosan (Figures 2.4.14. and 2.4.15.), the cytotoxic response is suppressed relative to that obtained in the presence of untreated adherent cells.

The mechanism(s) by which carrageenan and zymosan exert their suppressive effects in this system are not clear. Interference with the antigen-presenting or accessory role of adherent cells is unlikely in view of the time-course of carrageenan- and zymosan-sensitivity, and it is therefore suggested that these agents may induce a secondary accessory cell function resulting in suppression of cytotoxicity. The nature of this suppression is still controversial; induction of macrophage-like suppressor cells has

been suggested (Yung and Cudkowicz, 1978b), and preliminary data not presented here are compatible with this theory. As mentioned in sections 1.2. and 1.3., both carrageenan and zymosan have been reported to induce secretion of a number of bioactive macrophage products, many of which may affect lymphoid cells. In particular, cyclo-oxygenase products of fatty acid metabolism have been proposed as mediators of immunosuppression, and further experiments to determine the involvement of such materials in this system are discussed in section 2.6.4..

2.6.4. INVOLVEMENT OF CYCLO-OXYGENASE PRODUCTS OF FATTY ACID METABOLISM

Cyclo-oxygenase metabolism of polyunsaturated fatty acids (PUFA) generates a range of potent bioactive compounds including the prostaglandins (PG); this metabolic pathway is described in section 1.3.3.. Prostaglandins have been detected in carrageenan-induced inflammatory exudates (Velo et al, 1973; Willis, 1969), and Gemsa et al (1978) observed PG release from rat peritoneal macrophages exposed to carrageenan in vitro. Similarly, Bonney et al (1980) and Schnyder and Baggiolini (1978) demonstrated PG secretion following ingestion of zymosan particles. However, ingestion of latex beads failed to induce PG release (Bonney et al, 1980; Humes et al, 1977; Schnyder and Baggiolini, 1978), indicating that the phagocytic process per se is not responsible for this effect. Thus a parallel may be drawn between the effects of carrageenan, zymosan and latex in this study and their ability to induce PG release.

The experiments presented in section 2.5. were performed in order to assess the involvement of PG, particularly of the E and I series, in carrageenan- and zymosan-induced suppression of cell-mediated

cytotoxicity. Prostaglandins are not stored intracellularly in large quantities (Metcalfe and Kaliner, 1981; Samuelsson et al, 1971), and their synthesis and secretion may therefore be regarded as one process for the purposes of this study. Consequently, a valid approach to determining their role is to inhibit the synthetic pathway. Several of the drugs used clinically to relieve inflammatory symptoms are believed to act via such a mechanism (Vane, 1971), and two of these were studied here. Both indomethacin and flurbiprofen are non-steroidal anti-inflammatory drugs, and have been shown to interfere with PG production by inhibiting cyclo-oxygenase (Higgs et al, 1981; Rome and Lands, 1975; Vane, 1971, 1978).

As shown in Figures 2.5.1. and 2.5.3., indomethacin or flurbiprofen alone had no effect on the generation of cytotoxicity in the MLC. However, at concentrations in excess of 10ug/ml, indomethacin proved toxic to the cells, and few viable lymphocytes were recovered at the end of the culture period. A similar dose of indomethacin has been reported to cause non-specific inhibition of cell-mediated immune responses in vitro (Darrow and Tomar, 1980), and it has been suggested that the specificity of this drug for cyclo-oxygenase may be lost at higher concentrations, resulting in non-selective paralysis of other enzyme systems (Kantor and Hampton, 1978; Stenson and Parker, 1980). In view of this toxic effect, further work using indomethacin was limited to concentrations not exceeding 10ug/ml.

Indomethacin and flurbiprofen were then added to cultures in combination with carrageenan or zymosan, in an attempt to protect against the suppressive effects of these agents. As shown in Figure 2.5.2., indomethacin was unable to restore the cytotoxic response of cultures treated with a suppressive dose of carrageenan; however,

cultures exposed to zymosan attained a partial cytotoxic response in the presence of this drug. Using flurbiprofen this restorative effect was extended to include carrageenan, as shown in Figure 2.5.4.. However, at the doses used, neither drug was able to effect full restoration of the cytotoxic response. Similar doses of indomethacin have been shown to inhibit PG release from phagocytic cells (Bonney et al, 1978; Brune et al, 1978; Gemsa et al, 1978); together with the apparent absence of a dose-dependent restorative effect for the two drugs, this may suggest that cyclo-oxygenase products are only partly responsible for the observed suppression of cytotoxicity. It was clearly desirable to repeat these experiments using higher doses of indomethacin and flurbiprofen; however, this proved impractical due to the non-specific toxicity of indomethacin and the poor solubility of flurbiprofen. This pharmacological approach to investigating PG involvement was therefore abandoned, and the study was continued using exogenous PG and antisera specific for these compounds.

By adding exogenous PGE_1 to the MLC, it was possible to mimic the suppressive effects of carrageenan and zymosan, as shown in Figure 2.5.5.. This suppression did not result from toxicity towards the responding cells. Further experiments revealed that PGE_1 was only effective during the first 2-3 days of culture (Figure 2.5.6.), thus following a similar time-course to carrageenan, and being effective over a slightly longer period than zymosan; the time-course of each material is shown in Figure 2.6.4.. It is therefore reasonable to conclude that PGE_1 does not exert a direct effect on mature cytolytic effector cells, but is acting during the induction and development of the cytotoxic response. This is compatible with work by Darrow and Tomar (1980), who observed a suppressive regulatory

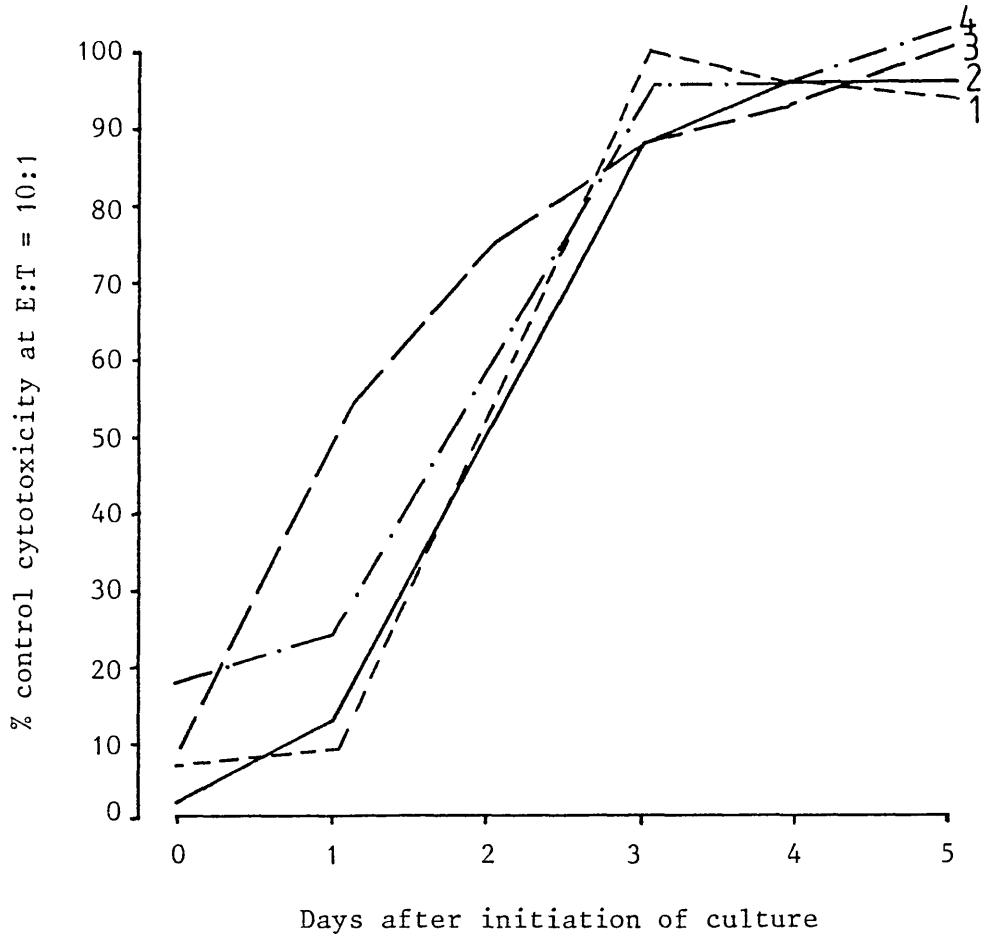


Figure 2.6.4.

Time-course of carrageenan-, zymosan- and PGE_1-induced suppression of the in vitro cytotoxic response

Data on time-course of carrageenan-induced suppression taken from experiments presented in Figures 2.4.6. and 2.4.7. Data on time-course of zymosan-induced suppression taken from experiment presented in Figure 2.4.12. Data on time-course of PGE_1-induced suppression taken from experiment presented in Figure 2.5.6.

Line 1: time-course of ι-carrageenan-induced suppression

2: time-course of λ-carrageenan-induced suppression

3: time-course of zymosan-induced suppression

4: time-course of PGE_1-induced suppression

Per cent control cytotoxicity was calculated as described for Figure 2.6.2.

role for endogenous PG during *in vitro* induction of alloreactive cytotoxic lymphocytes. Using the same culture system, Leung and Mihich (1980) demonstrated a suppressive effect of exogenous E-series PG during the initial stages of culture, while mature effector cells were apparently insensitive to these compounds. Similarly, Webb and Nowowiejski (1978) reported that exogenous PGE_2 could only effect suppression of PHA-induced DNA synthesis if added within 24 hours of the mitogenic stimulus. However, Henney *et al* (1972) have described a direct suppressive effect on mature cytolytic effector cells generated *in vivo*, and challenged *in vitro* in the presence of E-series PG; although this effect was most pronounced at doses of PG higher than those used in the previous publications, the possibility of differential PG-sensitivity between cytotoxic cells generated *in vitro* and *in vivo* should not be dismissed (Plaut, 1979). Darrow and Tomar (1980) have shown that while freshly explanted cells were sensitive to PGE-mediated suppression during the course of a cytotoxic assay, cultured cells were not suppressed, and Goodwin *et al* (1979) have suggested that lymphocyte receptors for PG may be lost from the cell surface during overnight incubation, rendering cultured cells non-responsive to suppressive doses of these mediators.

In this study, maximal suppression of the cytotoxic response was obtained following addition of $1x10^{-6}$ molar PGE_1, and concentrations of $1x10^{-8}$ molar and below were ineffective (Figure 2.5.5.). Darrow and Tomar (1980) reported generation of $1x10^{-8}$ molar endogenous PG as part of an autoregulatory mechanism in the MLC; however, at this dose PG served only to modulate the response, and abrogation of cytotoxic activity was not observed. In the same study, addition of exogenous PGE_2 at concentrations from $1x10^{-8}$ to $1x10^{-5}$ molar

suppressed the proliferative response in a dose-dependent manner. Similarly, Stockman and Mumford (1974) demonstrated a suppressive effect on PHA-induced mitogenesis of human peripheral blood mononuclear leucocytes following addition of PGE_2 from $1x10^{-7}$ to $1x10^{-5}$ molar, and Fulton and Levy (1981) were able to induce murine suppressor activity using $1x10^{-7}$ molar exogenous PGE_1. Anggard (1971) has reported that PG may be present in the tissues and body fluids from $1x10^{-9}$ to $1x10^{-7}$ molar under normal conditions, and higher concentrations may be generated in response to stimulation (Willis, 1969). The concentrations of PGE_1 used in this study are therefore comparable to those employed by other workers, and are likely to fall within the physiological range.

In view of the ability of exogenous PGE_1 to mimic the suppression induced by carrageenan and zymosan, it was suggested that this prostanoid may mediate their effects in the MLC. This possibility was investigated using an antiserum raised against PGE_1, which has been shown to react with PGE_1 and PGE_2 (J. Mertin, personal communication), and which was found to oppose the effects of E-series PG <u>in vivo</u> (Mertin and Stackpoole, 1981). As shown in Figure 2.5.7., anti-PGE antiserum neutralised the suppressive effects of $1x10^{-6}$ molar exogenous PGE_1, while control serum from the same source was ineffective (Figure 2.5.8.). The efficacy of this antiserum <u>in vitro</u> was thus confirmed. When added to cultures in combination with carrageenan or zymosan, the antiserum effected partial restoration of the cytotoxic response in a dose-dependent manner (Figure 2.5.9.), indicating that E-series PG may indeed play a role in mediating the suppressive effects of these materials. However, even at the lowest final dilution of antibody (1 in 20 v/v), a response of control levels could not be attained. As this

dilution of antiserum had already been shown to provide almost complete protection against the suppressive effects of $1x10^{-6}$ molar exogenous PGE_1 (Figure 2.5.7., line 8), its failure to fully restore the response of carrageenan- and zymosan-treated cultures may indicate that higher concentrations of E-series PG were being produced. However, generation of PG in excess of $1x10^{-6}$ molar in this culture system seems unlikely; a more plausible explanation for this effect is to invoke additional suppressive materials. If ingestion of carrageenan or zymosan stimulates cyclo-oxygenase, then several other PUFA metabolites will also be produced, many of which have potent biological activity and may influence lymphocyte responses. Accordingly, another cyclo-oxygenase product was chosen for study in this culture system, namely prostacyclin (PGI_2). This material is known to be more active than E-series PG in some systems (Moncada et al, 1976); however, few experiments have been reported concerning the effects of PGI_2 on immune responses. In view of its short half-life under culture conditions, this compound is difficult to detect in vitro; however, its stable breakdown product 6-keto-PGF_1 has been identified in macrophage preparations (Bonney et al, 1978, 1980; Hsueh et al, 1980; Humes et al, 1977; Scott et al, 1980), indicating that such cells are capable of synthesising PGI_2.

In the culture system studied here, neither PGI_2 or the stable synthetic analogue carbacyclin influenced cytotoxicity (Figure 2.5.10.), suggesting that this material does not modulate the in vitro cytotoxic response to alloantigen at the doses tested. Leung and Mihich (1980) also noted the failure of PGI_2 (from $3x10^{-10}$ to $3x10^{-6}$ molar) to modify development of cell-mediated immunity in vitro; however, they observed inhibition of the effector activity of cytotoxic cells generated in vivo, supporting the previous

suggestion that effector cells induced *in vitro* may differ from those generated *in vivo*.

The apparent failure of exogenous PGI_2 to influence cytotoxicity in these experiments does not preclude a role for low endogenous concentrations of this material in carrageenan- and zymosan-induced immunosuppression. This possibility was investigated using an antiserum reactive against PGI_2. This antiserum is known to cross-react slightly with PGE_1 (S. Bunting, personal communication; Bunting *et al*, 1978); although experiments to confirm its specificity in this culture system proved it unable to neutralise $1x10^{-6}$ molar exogenous PGE_1 (Figure 2.5.11.), it should be noted that smaller local concentrations of endogenous PGE_1 may be effectively neutralised. Consequently, the partial restoration of the response of carrageenan-and zymosan-treated cultures observed in the presence of this antiserum (Figure 2.5.12.), while indicating that PGI_2 may be involved, does not confirm this material as a mediator of the immunosuppressive effect.

As only one dilution of anti-PGI_2 antiserum was tested, no significance can be attached to the failure of this material to fully restore a suppressed cytotoxic response. However, when both anti-PGE and anti-PGI_2 antisera were used in combination, the restoration observed was little different to that provided individually (Figure 2.5.13.). The absence of an additive effect for the two antisera may support the suggestion that both preparations are acting against E-series PG, although this then indicates an antibody excess, and so implicates additional suppressive mediators. Alternatively, if the antisera are more specific and are neutralising different materials, then full restoration of the response may be attained using lower dilutions, particularly of the

anti-PGI_2 preparation. Unfortunately, such experiments were not possible here due to the limited supply of antisera. This latter interpretation is compatible with reports that the major endogenous PG released by macrophages is PGE_2 (Glatt et al, 1977; Wahl, 1981); the anti-PGI_2 antiserum used here has low affinity for PGE_2 (S. Bunting, personal communication), and the involvement of PGI_2 per se in mediating the effects of carrageenan and zymosan should not therefore be dismissed.

Further experiments are clearly necessary to define the role of PUFA metabolites in this culture system. From the current results, it is reasonable to conclude that PG of the E and possibly I series are involved in mediating the effects of carrageenan and zymosan in vitro; however, it should be noted that other substances may also play a role (see section 1.3.), in particular some of the reactive metabolites of molecular oxygen, and the suppression observed here probably results from the combined effects of these different materials.

CHAPTER THREE

The role of accessory cells in the spontaneous cytotoxic activity of human peripheral blood mononuclear cells in vitro

3.1. INTRODUCTION

Following the initial observation of cell-mediated target cell destruction, it became apparent that several types of leucocyte possessed lytic ability. Such activity has been demonstrated for macrophages, granulocytes and a variety of lymphoid populations. Specific MHC-restricted cytotoxic activity mediated by T lymphocytes following antigenic challenge has been described in chapter 2; however, it was subsequently shown that cells isolated from the lymphoid compartment of non-immunised normal individuals could display non-MHC-restricted cytotoxic activity directed against a range of target cells. Such activity has been variably described as natural cytotoxicity, natural killer activity or spontaneous cytotoxicity (Perlmann et al, 1979), and has since been extensively studied and reviewed using both human and rodent systems (Herberman, 1980; Moller, 1979; Normann and Sorkin, 1982; Riethmuller et al, 1979).

3.1.1. SPONTANEOUS CYTOTOXICITY IN VIVO

During the early stages of viral infection, natural killer (NK) cells become activated in response to elevated levels of gamma-IFN, and this has been interpreted as an anti-viral function for these cells (Welsh and Zinkernagel, 1977). However, the absence of immunological memory suggests that such activity would be of limited usefulness, although it may form an initial defence mechanism prior to development of specific CTL (Ortaldo and Herberman, 1984).

Detailed study has been made of the role of NK cells in host resistance to malignant disease (Roder and Haliotis, 1980). Neoplastic transformation renders cells more susceptible to NK-

mediated lysis, and such effectors have been postulated as a first line of defence against tumour growth. This hypothesis is supported by various clinical observations, though much of the evidence remains equivocal. Natural killer cells have also been implicated as homeostatic regulators of normal cell growth and differentiation, particularly of haemopoietic lineages (Burns et al, 1985); the ability of these cells to both lyse syngeneic targets and produce cytokines (growth factors) such as the interferons, IL-1 and IL-2 supports such a role (Ortaldo and Herberman, 1984). It is not yet clear whether both lytic and secretory activity may be simultaneously expressed by a single NK cell.

Natural killer activity is subject to regulation by both adherent and non-adherent suppressor cells, and by soluble products (reviewed by Ortaldo and Herberman, 1984). Cytotoxic activity is enhanced by both IL-2 and gamma-IFN, with an increase in cell number and efficiency. In contrast, spontaneous cytotoxicity appears to be down-regulated by cyclic nucleotides, prostaglandins and some sugars; there is evidence that this represents interference with the effector function of the cell. Immunosuppressive drugs such as the corticosteroids, and some carcinogens and tumour promoters also depress natural killing, supporting the involvement of these cells in tumour surveillance (Ortaldo and Herberman, 1984).

3.1.2. CELLS MEDIATING SPONTANEOUS CYTOTOXICITY

Spontaneously cytotoxic effector cells are functionally defined by their ability to lyse a range of susceptible target cells during short-term cytotoxicity assays in vitro. Such effector cells derive from foetal liver and bone marrow, and are widely distributed throughout the mammalian lymphoid system; they are believed to

reside within the large granular lymphocyte (LGL) fraction of mononuclear cells, which may form up to five per cent of PBMC (Ortaldo and Herberman, 1984). Frequently referred to as natural killer or NK cells, in man these lymphocytes are generally non-adherent and non-phagocytic (Ortaldo and Herberman, 1984). In view of the paucity of conventional lymphoid cell markers, it has proved difficult to define a surface phenotype both specific for and embracing all NK cells, and this population has been shown to have features in common with both T lymphocyte and monocyte-macrophage lineages. Natural killer cells do not carry surface immunoglobulin (Ig), and although they usually express receptors for the Fc fragment of IgG, receptors for the third component of complement are absent. Heterogeneous expression of T lymphocyte antigens (some NK cells are T8+, T11+, while most are T3-, T4- [Ortaldo and Herberman, 1984]) may indicate subsets of effector cells, or may reflect different stages of differentiation or activation. In common with thymus-derived T lymphocytes, NK cells form rosettes with sheep erythrocytes; together with their responsiveness to some mitogens and to interleukin 2 (IL-2) (Burns et al, 1985), this supports their proposed relationship with T cells. However, NK cell development is independent of thymic influences, as evidenced by their normal function in athymic individuals (Ortaldo and Herberman, 1984), and it has been suggested that these cells may represent a primitive undifferentiated form of pre-thymic T lymphocyte (Saksela and Timonen, 1980).

Although spontaneous cytotoxicity is characteristically non-specific, NK cells do exhibit selective activity; some sub-populations of effectors show very broad patterns of reactivity, while others can be isolated on the basis of target cell preference

(Burton, 1980; Ortaldo and Herberman, 1984). A wide range of targets is susceptible to lysis, including syngeneic, allogeneic and xenogeneic carcinomas and lymphomas, virus-infected cells, and some normal cells, particularly of haemopoietic lineages. The target cell surface components recognised by NK cells have not yet been determined. Undifferentiated cells appear to be more susceptible to lysis (Ortaldo and Herberman, 1984), suggesting that differentiation antigens may form the target molecules. Alternatively, cytotoxicity may be directed against surface protein or carbohydrate structures, which may be common to all susceptible targets (Herberman et al, 1986). The identity of the receptor on the effector cell remains similarly elusive. Interaction of the NK cell with its target does not demand prior antigenic sensitisation, nor is it genetically restricted in the manner of CTL-mediated lysis, although the overall level of NK activity may be genetically controlled. It therefore seems unlikely that NK-target cell conjugation employs a conventional T cell receptor, composed of an alpha-beta heterodimer in association with T3 (Herberman et al, 1986; Reynolds and Ortaldo, 1987). In some lymphoid subsets, an unusual form of T cell receptor comprising gamma and delta chains has been discovered; this dimer may be a candidate for the NK cell receptor, as it shows less heterogeneity than the alpha-beta equivalent and may therefore confer broader target specificity on the effector cell (Hersey and Bolhuis, 1987). However, such a T cell-like receptor is unlikely to be involved in recognition by T3-negative LGL.

There is no evidence that NK cells recognise target antigen via Ig molecules, although the sheep erythrocyte receptor has been proposed as a recognition molecule based on its ability to bind the widespread lymphocyte function-associated antigen LFA-3 (Hersey and

Bolhuis, 1987). Possession of surface receptors for IgG enables LGL to attack opsonised target cells via a mechanism referred to as antibody-dependent cell-mediated cytotoxicity (ADCC). While some LGL can display both NK and ADCC activity, suggesting that multiple receptors for different target structures may be present on the same effector cell, others appear to be restricted to only one cytotoxic mechanism, underlining the heterogeneity apparent within this population (Ortaldo and Herberman, 1984). Following binding of the NK cell to its target, cytolysis is believed to proceed in a similar manner to that observed for CTL (section 2.1.2.) (Young et al, 1986); however, NK cells have also been reported to release reactive oxygen intermediates at this stage, in parallel with the secretory activity of activated mononuclear phagocytes (section 1.3.2.) (Roder et al, 1982). In addition, there is evidence for a soluble cytotoxic factor secreted by NK cells which may interact with target cells and initiate their destruction (Farram and Targan, 1983), and it has been suggested that lysis may result from the combined effects of this factor and cell-cell conjugation (Hiserodt et al, 1983).

It has recently been proposed that the term "natural killing" is reserved to describe non-MHC-restricted cytotoxic functions and not the effector cells per se, in recognition of the diversity of species which may mediate this phenomenon (Reynolds and Ortaldo, 1987). This proposal should be welcomed in view of the confusing terminology which has arisen following in vitro studies of spontaneous cytotoxicity. Cells activated in vitro in a mixed leucocyte culture, or by mitogens, interleukin 2 or foetal calf serum, have been shown to acquire non-specific lytic activity, which has been variously described as NK-like cytotoxicity, anomalous killing or lymphokine-activated killing (Hersey and Bolhuis, 1987;

Ortaldo and Herberman, 1984). The relationship of the different cells described in these systems is not clear. Lymphokine-activated killer (LAK) cells arising from non-T non-B lymphoid cells after 3-5 days culture in the presence of IL-2 show some similarities with NK cells freshly isolated from peripheral blood, but are able to lyse NK-resistant tumour targets (Rosenberg and Lotze, 1986), and have been shown to express the T cell antigens T3 and T8 (Grimm and Rosenberg, 1984). Activated lymphocyte killing (ALK) has been proposed as a general description for this type of cytotoxic activity, but does not differentiate between NK cells and T lymphocytes which have acquired broad lytic specificity (Hersey and Bolhuis, 1987). In an attempt to resolve this confusion, Lanier and Phillips (1986) have suggested that all human cytotoxic effector lymphocytes should be divided into three types: conventional MHC-restricted CTL, non-MHC restricted CTL which use a similar T cell receptor, and non-MHC restricted NK cells which recognise targets via an as yet undefined receptor. Cells mediating antibody-independent cytotoxicity as described in this section probably fall into the third category; however, the nomenclature of cells mediating spontaneous cytotoxicity remains controversial, and accordingly the cells described later in this chapter are referred to as spontaneous cytotoxic effector cells. Experiments presented in subsequent sections employ freshly isolated cells and those which have been cultured in foetal calf serum, and it is noted that these may not be identical.

3.2. MATERIALS AND METHODS

3.2.1. MATERIALS

3.2.1.1. Peripheral blood mononuclear cells

3.2.1.2. Heparin

3.2.1.3. Ficoll Hypaque

3.2.1.4. Complement

3.2.1.5. Target cells

All other materials used were as described in section 2.2.1.

3.2.2. METHODS

3.2.2.1. Preparation of cell suspensions

3.2.2.2. Mononuclear cell cultures

3.2.2.3. Phagocytic assay

3.2.2.4. Maintaining cell lines

3.2.2.5. Spontaneous cell-mediated cytotoxicity assay

All other methods used were as described in section 2.2.2.

3.2.1. MATERIALS

3.2.1.1. Peripheral blood mononuclear cells

Peripheral blood mononuclear cells were prepared from freshly drawn venous blood, donated by healthy volunteers.

3.2.1.2. Heparin

Stock solutions of preservative-free heparin at 5000 I.U./ml were obtained from the Pharmacy at St. Mary's Hospital. These were diluted in sterile saline to 1000 I.U./ml, and were stored at 4°C.

3.2.1.3. Ficoll Hypaque

Ficoll mononuclear cell separation medium was obtained as sterile Lymphoprep from Nyegaard and Company, Oslo, Norway, and was stored in the dark at 4°C.

3.2.1.4. Complement

Human AB serum was used as a source of complement to opsonise yeast for phagocytosis; this was stored at -80°C.

3.2.1.5. Target cells

K562 target cells were maintained *in vitro*. Stocks were also kept frozen in liquid nitrogen. The K562 line was originally derived from a patient with chronic myeloid leukaemia, and is believed to be a largely undifferentiated granulocytic cell (Klein *et al*, 1976).

Molt 4 target cells were also maintained *in vitro*, and frozen stocks of these cells were kept in liquid nitrogen. The Molt 4 cell line was originally isolated from a patient with acute lymphoblastic leukaemia, and is believed to be a leukaemic lymphocyte of T cell origin (Minowada *et al*, 1972).

3.2.2. METHODS

3.2.2.1. Preparation of cell suspensions

Fresh venous blood was drawn into heparin, 0.1 ml heparin being required for each 10 ml blood. The blood was mixed thoroughly to prevent clotting, and was then diluted 1:1 in HBSS + 5% FCS.

Mononuclear cells were isolated by layering this diluted blood on Ficoll; optimal recovery was obtained by layering 15 ml diluted blood onto 5 ml Ficoll in a 30 ml Universal container. The Ficoll layers were then spun at 1200 x g for 15 min, and the mononuclear cells were harvested from the plasma-Ficoll interface. These cells were topped up with culture medium and spun again (750 x g, 10 min), and were then resuspended at the required concentration in RPMI + 5% FCS + G.

3.2.2.2. Mononuclear cell cultures

A suspension of peripheral blood mononuclear cells was prepared as described in section 3.2.2.1., and was adjusted to a concentration of 2×10^6 cells/ml in RPMI + 5% FCS + G. The cells were then aliquoted into multiwell culture plates, 1.0 ml being placed in each well. 2-mercaptoethanol was not included in these cultures. Any macrophage-active agents, prostanoids, or antisera which were required were now added as described in sections 2.2.2.7. and 2.2.2.8., and the plates were placed in a CO_2 incubator. The length of the culture period varied from 24 hr to several days.

3.2.2.3. Phagocytic assay

Glass coverslips were placed in some culture wells, to enable recovery of the adherent cells after the non-adherent population had been harvested. The adherent cells were then exposed to a suspension

of yeast opsonised with human serum, as described in section 2.2.2.9., and their morphology and phagocytic capacity were assessed.

3.2.2.4. Maintaining cell lines

Target cells for the cytotoxicity assay were maintained in vitro in RPMI + 5% FCS + G. Sub-culturing once a week was usually sufficient to maintain optimal conditions for cell growth; overgrown cultures frequently contained large numbers of dead cells, which could be removed by spinning the cells through neat FCS.

Frozen cells were stored in liquid nitrogen, as described in section 2.2.2.11. After thawing, these cells were cultured in medium containing 10% or 20% FCS for the first week, and were spun through neat FCS when they were first sub-cultured. Freshly thawed cells were not suitable for use as targets until they had been cultured in vitro for at least 10 days.

3.2.2.5. Spontaneous cell-mediated cytotoxicity assay

The spontaneous cytotoxic activity of cultured mononuclear cells was assessed as described in section 2.2.2.13. Most cultures were assayed in duplicate, although triplicates were set up if sufficient cells were recovered.

Results obtained from the cytotoxicity assay were calculated as described in section 2.2.2.14.

3.3. PRELIMINARY WORK ON THE SPONTANEOUS CYTOTOXIC ACTIVITY OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS *IN VITRO*

3.3.1. CULTURE CONDITIONS

3.3.1.1. Mononuclear cell recovery from different donors

3.3.1.2. Choice of mononuclear cell donor

3.3.1.3. Effect of 2-mercaptoethanol on mononuclear cells *in vitro*

3.3.1.4. Choice of target cells

3.3.1.5. Duration of culture

3.3.2. ROLE OF ACCESSORY CELLS

3.3.2.1. Cell depletion using carbonyl iron powder

3.3.2.2. Cell separation by adherence to plastic

3.3.2.3. Co-operation between precursor-effector cells and allogeneic accessory cells

3.3.1. CULTURE CONDITIONS

3.3.1.1. Mononuclear cell recovery from different donors

Peripheral blood mononuclear cells (PBMC) were isolated from fresh venous blood, as described in section 3.2.2.1. Blood was drawn from several donors, and in repeat experiments cells from different individuals were found to express the same general pattern of reactivity against K562 and Molt 4 tumour target cells. However, cell recovery per unit volume of blood varied considerably, both between donors and with time, as shown in Table 3.3.1. Generally speaking, it was assumed that 1.0 ml of venous blood would yield 1 x 10^6 mononuclear cells, and this approximation was used to estimate the volume of blood required when planning an experiment.

Mononuclear cells were adjusted to 2 x 10^6 cells/ml and were cultured in 1.0 ml volumes in multiwell plates, as described for murine cells (section 2.3.1.5.). These conditions yielded a large proportion of viable cells at the end of the culture period.

3.3.1.2. Choice of mononuclear cell donor

The spontaneous cytolytic activity expressed by human PBMC has been reported to vary according to the sex and sometimes the age of the donor, being higher in males (Santoli et al, 1976; Saksela et al, 1979; Trinchieri et al, 1977), and sometimes being found to increase with advancing age (Saksela et al, 1979). However, other workers have found no correlation between cytotoxic activity and age of the donor (Oldham et al, 1975; Takasugi et al, 1973). Such effects were not observed in the experiments reported here, although this may be due to the small number of individuals studied (9 males, 4 females). Consequently the sex and age of donors were not taken into account when planning experiments.

Table 3.3.1. Recovery of mononuclear cells per unit volume of peripheral blood

Venous blood was drawn into heparin and the mononuclear cells were isolated on Ficoll. After washing, cell numbers were assessed in white cell counting fluid.

Data shown are mean cell counts from a number of separate experiments; where more than one sample is indicated, this represents repeat experiments using blood from the same donor.

Table 3.3.1. Recovery of mononuclear cells per unit volume of peripheral blood

	Donor	Sample	Mononuclear cells per 1.0 ml blood (x 10^{-6})
a) Male donors	VS	1	1.7
		2	1.4
		3	1.1
		4	1.8
		5	2.1
	LR	1	1.1
		2	1.2
	DD	1	0.8
		2	0.6
		3	0.9
	RB	1	1.3
		2	1.5
	AB	1	2.2
		2	1.7
		3	1.8
	DB	1	1.3
	GH	1	1.1
	LB	1	1.2
	MW	1	0.9
b) Female donors	FL	1	1.6
		2	2.1
		3	1.9
		4	1.7
	KS	1	1.3
		2	1.5
	CB	1	1.4
		2	1.6
	HB	1	1.6

3.3.1.3. Effect of 2-mercaptoethanol on mononuclear cells in vitro

In early experiments to determine the optimal conditions for culturing spontaneously cytotoxic human PBMC, 2-mercaptoethanol (2-ME) was added to the culture medium to give a final concentration of 5×10^{-5} molar, as in the murine system (section 2.3.1.6.). However, this agent proved highly toxic to the reactive cells, inhibiting both their survival in vitro over a 24-hour culture period and their cytotoxic activity, as shown in Figure 3.3.1. This toxic effect was observed using cells from two different donors, and 2-ME was therefore omitted from future cultures.

3.3.1.4. Choice of target cells

Two target cell lines were available for use in the spontaneous cytotoxicity assay, the myeloid K562 line and the leukaemic Molt 4 line. Wherever possible, effector cells were assayed against both targets; however, if relatively few effector cells were available, only one target was used. In most experiments, little difference was observed in the level of cytotoxic activity directed against each target by a given effector population, and the same general pattern of reactivity was observed against each. No donor was found to react against only one of the target cells.

In a few early experiments the effector cells occasionally showed considerably greater reactivity towards one target; in all cases this could be attributed to the condition of the target cells. If these cells are taken from a culture which has passed the logarithmic phase of its growth, the cells are somewhat overcrowded and consequently less healthy. As a result of this, they show a high level of spontaneous release of radioactivity, which tends to mask the specific release obtained on lysis of the target by an effector cell. This difficulty may be due to failure of such target cells to take up

Figure 3.3.1.

Effect of 2-mercaptoethanol on PBMC in vitro

PBMC were cultured for 24 hours with or without 2-mercaptoethanol at a final concentration of 5×10^{-5} molar. In view of the poor recovery of viable cells after exposure to 2-ME, the cytotoxic activity of these cultures was titrated out from an E:T ratio of 40:1.

Line 1: no 2-ME, cells assayed against K562 target cells

2: 5×10^{-5} molar 2-ME, K562 target cells

3: no 2-ME, Molt 4 target cells

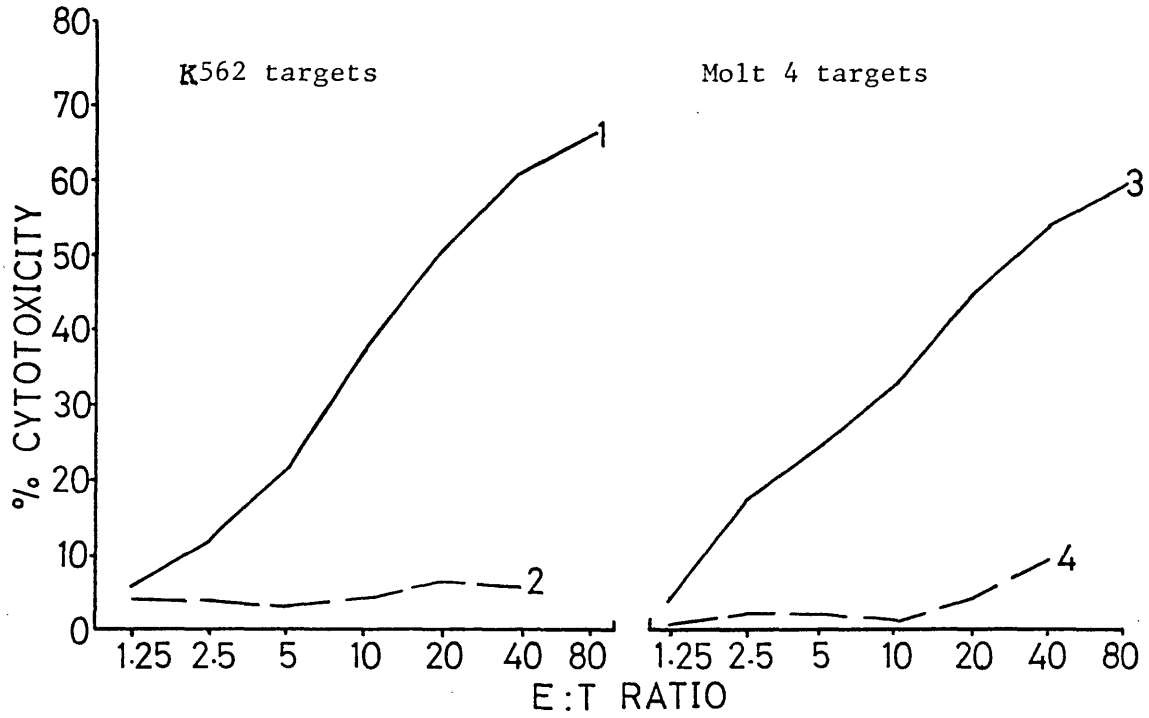
4: 5×10^{-5} molar 2-ME, Molt 4 target cells

CULTURE	1	2	3	4
% recovery	98	42	89	37
% viability	89	49	91	51

TARGET CELLS	K562	Molt 4
Spontaneous release	16%	19%

DONOR VS

DURATION OF CULTURE 24 hours


K562 targets
Molt 4 targets
% CYTOTOXICITY
80
70
60
50
40
30
20
10
0
1
2
3
4
1·25 2·5 5 10 20 40 80
1·25 2·5 5 10 20 40 80
E:T RATIO

^{51}Cr-sodium chromate efficiently during the labelling process, and to erratic release of the radiolabel during the cytotoxicity assay. It is therefore preferable to take target cells from a recent subculture, and this principle was adhered to wherever possible in subsequent experiments, particularly if only one target cell was being used in the assay.

3.3.1.5. Duration of culture

Time-course experiments were set up to determine the optimal culture period for this system. Mononuclear cells were taken from the same donor on consecutive days, and their cytotoxic activities were assessed together after varying periods of culture. Fresh cells from the same donor were included in the assay. As shown in Figure 3.3.2., freshly drawn cells showed a good response against both K562 and Molt 4 targets. Cells which had been maintained in vitro for one day were equally effective, but cells cultured for 2 days showed very little cytotoxic activity. After 3 days in culture, the level of killing was restored to that of fresh cells, and an equal or greater response was obtained from cultures assayed on day 4. Similar results were obtained in repeat experiments using different donors, as shown in Figure 3.3.3.

In a separate experiment, the cytotoxic response of cells cultured for 5 or 6 days was compared to that of fresh cells. This extended culture period did not affect the level of the response, as demonstrated in Figure 3.3.4.

These results indicate that the effector cells responsible for spontaneous cytotoxicity in this system can be maintained in vitro, for at least 24 hours. The fall in cytotoxic activity on day 2 suggests that either these effector cells have died, or they have lost their lytic capacity. The former hypothesis seems unlikely, as recovery of viable cells from cultures harvested on day 2 was equivalent to recovery and

Figure 3.3.2.

Length of culture period and subsequent cytotoxic activity of PBMC

Cultures were set up on consecutive days and assayed together against the same labelled target cells. Fresh cells from the same donor were included in the assay.

Line 1: fresh uncultured cells

2: 1-day culture

3: 2-day culture

4: 3-day culture

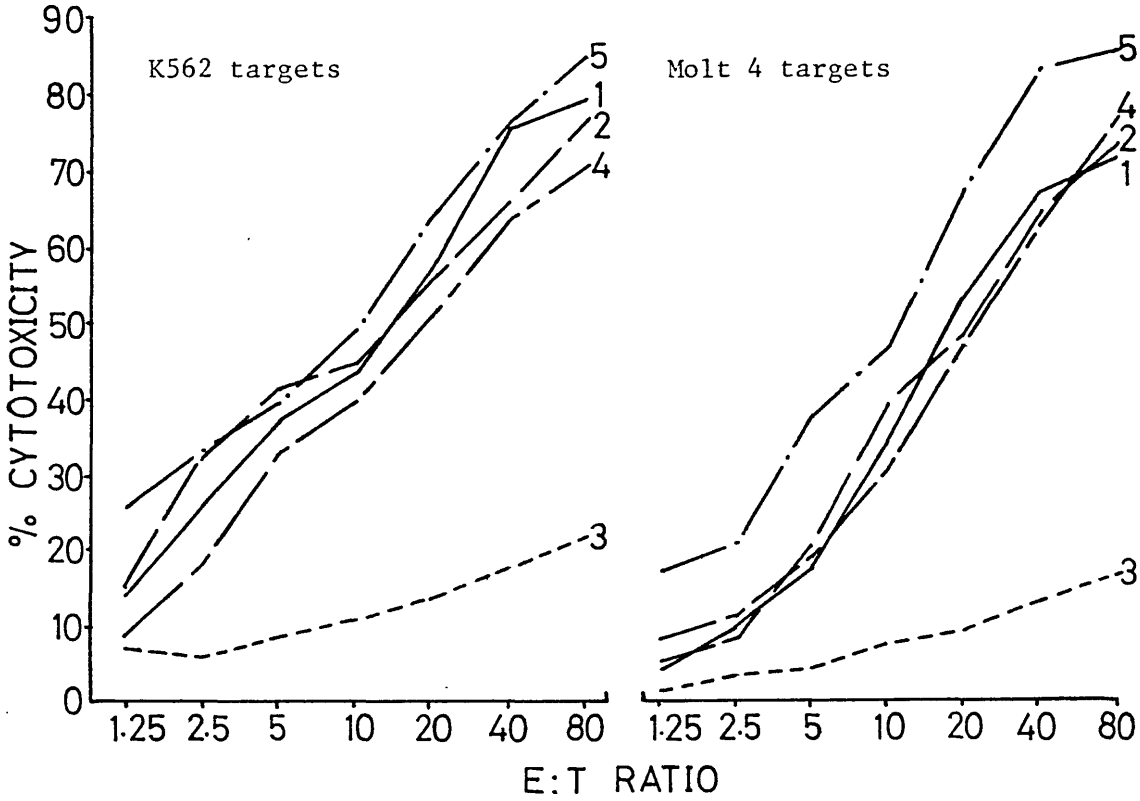
5: 4-day culture

CULTURE	1	2	3	4	5
% recovery	-	98	89	93	86
% viability	-	87	71	84	94

TARGET CELLS	K562	Molt 4
Spontaneous release	9%	6%

DONOR VS

DURATION OF CULTURE 0-4 days


K562 targets
Molt 4 targets
% CYTOTOXICITY
E:T RATIO
0
10
20
30
40
50
60
70
80
90
1·25
2·5
5
10
20
40
80
1
2
3
4
5

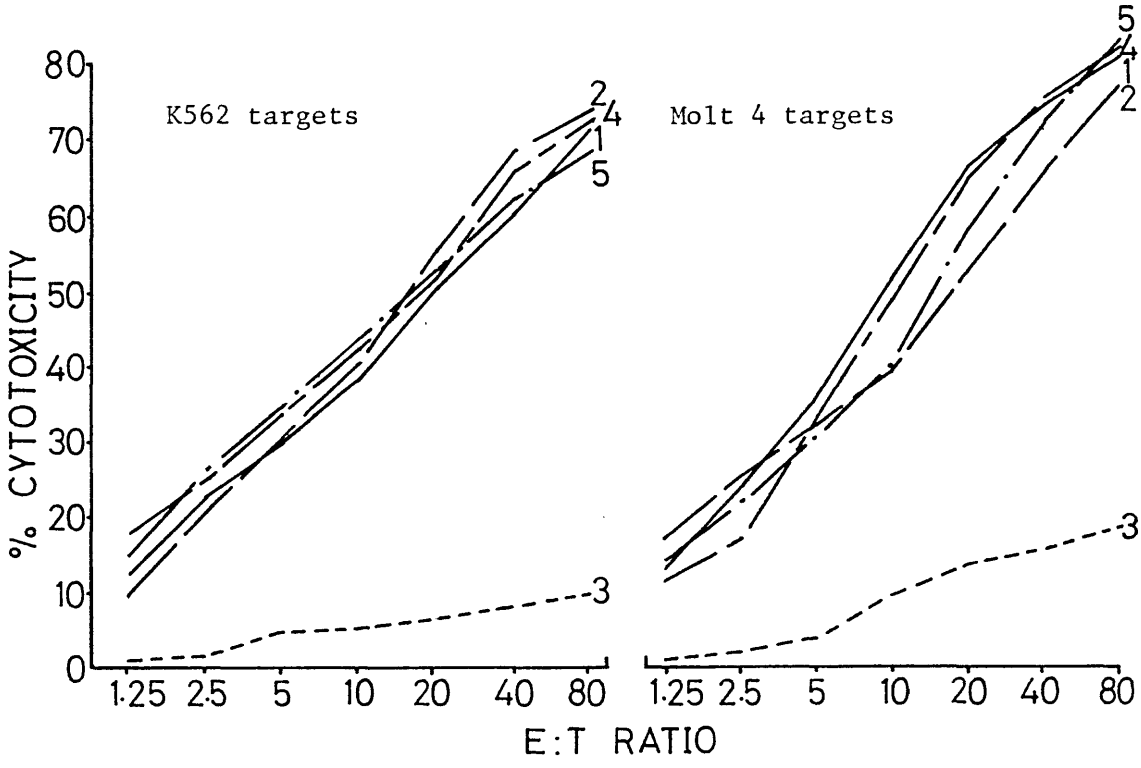


Figure 3.3.3.

Length of culture period and subsequent cytotoxic activity of PBMC

Line 1: fresh uncultured cells

2: 1-day culture

3: 2-day culture

4: 3-day culture

5: 4-day culture

CULTURE	1	2	3	4	5
% recovery	-	97	96	94	98
% viability	-	89	91	90	87

TARGET CELLS	K562	Molt 4
Spontaneous release	8%	11%

DONOR FL

DURATION OF CULTURE 0-4 days



Figure 3.3.4.

Length of culture period and subsequent cytotoxic activity of PBMC

Line 1: fresh uncultured cells

2: 5-day culture

3: 6-day culture

CULTURE	1	2	3
% recovery	-	87	83
% viability	-	91	89

TARGET CELLS	K562	Molt 4
Spontaneous release	9%	10%

DONOR VS

DURATION OF CULTURE 0, 5, 6 days

viability of cells cultured for only one day, or for 3 or more days. Hence it appears that the cytotoxic cells found in peripheral blood lose their activity after short periods in culture. From these experiments, it is not possible to say whether this reflects a short half-life for the activity of this cell in vivo, or is merely an in vitro artefact. The subsequent recovery of cytotoxic activity in cultures exceeding 2 days is discussed elsewhere in this chapter (sections 3.3.2.1., 3.3.2.2., 3.6.1., 3.6.2.). In view of the results obtained here, further experiments on this system were performed using a culture period of 24 hours or 3 or more days.

3.3.2. ROLE OF ACCESSORY CELLS

3.3.2.1. Cell depletion using carbonyl iron powder

Suspensions of human PBMC may be depleted of their adherent macrophage-like accessory cells using carbonyl iron powder, under the same conditions as those described for the treatment of murine cells (section 2.2.2.3.). This procedure was found to remove up to 15% of the original cell number in some cases; however, depletion usually ranged from 5-10%. In most experiments the depleted cell suspension was then diluted 10x by volume, to give a concentration of non-adherent cells equivalent to that found in an undepleted suspension at 2×10^6 cells/ml. However, in a few experiments the concentration of the depleted suspension was adjusted to 2×10^6 cells/ml to replenish the deficit in absolute cell numbers. Depleted and undepleted cell suspensions were then cultured in parallel for varying periods of time, after which the cells were harvested and assayed against radiolabelled target cells.

The results of a typical 24-hour culture are presented in Figure 3.3.5. Carbonyl iron treatment of the suspension before culture had no effect on either the level of cytotoxicity or the recovery of viable cells. Similar results were obtained from fresh undepleted and depleted cells, drawn from the same donor on the day of assay, indicating that the adherent cells removed by carbonyl iron powder are not required in this system, either for survival of the cells *in vitro* or for maintenance of their cytolytic activity. Repeat experiments using cells from different donors confirmed these observations.

As mentioned in section 3.3.1.5., it is probable that the cells mediating spontaneous cytotoxicity after 24 hours *in vitro* are effector cells present in peripheral blood which have survived the culture period, without requiring adherent accessory cells. However, cytotoxic activity

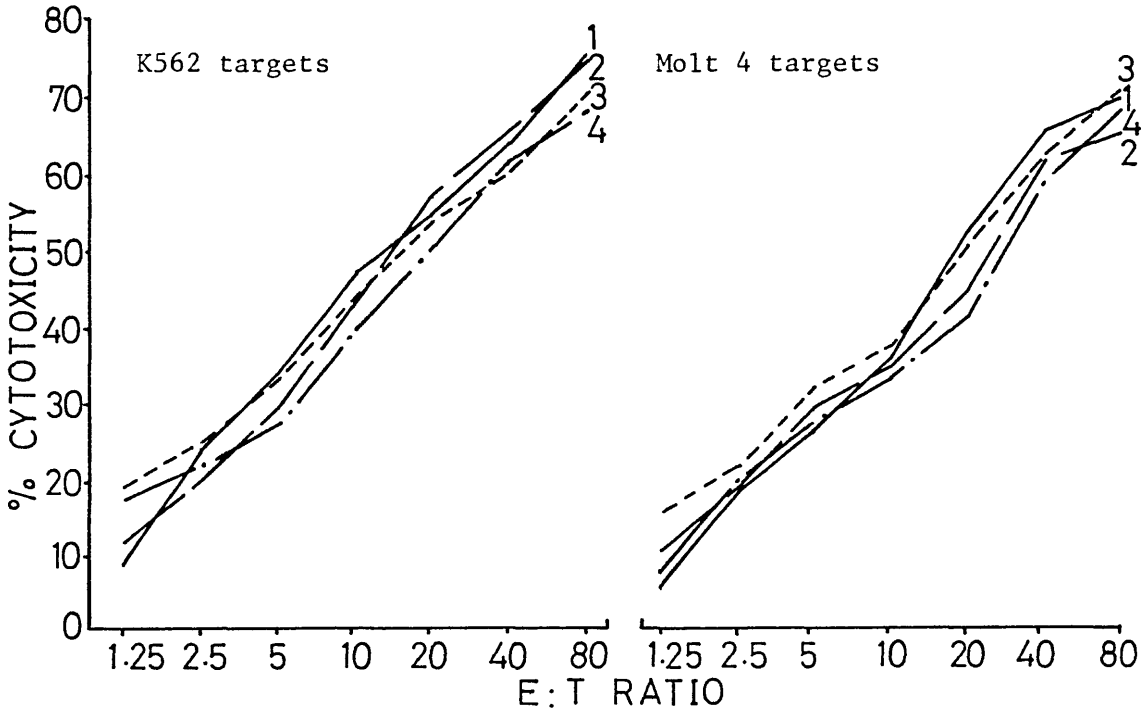


Figure 3.3.5.

Spontaneous cytotoxic activity of carbonyl iron-treated PBMC

Line 1: fresh undepleted cells

2: fresh carbonyl iron-treated cells

3: undepleted cells cultured for 24 hours

4: carbonyl iron-treated cells cultured for 24 hours

CULTURE	1	2	3	4
% recovery	-	-	89	94
% viability	-	-	93	90

TARGET CELLS	K562	Molt 4
Spontaneous release	12%	15%

DONOR LR

DURATION OF CULTURE 0, 24 hours

present on or after day 3 of culture may have arisen by the maturation of a precursor cell into a cytolytic effector cell. To investigate the possible role of accessory cells in such a maturation step, carbonyl iron-treated cell suspensions were cultured for periods of 3 and 4 days, and their cytotoxic activity was compared with that of undepleted cultures of the same duration. As shown in Figure 3.3.6., removal of the adherent cells prior to this extended culture period had a marked effect on the level of cytotoxicity; however, in contrast to the suppression expected on the basis of murine experiments (section 2.3.2.1.), adherent cell-depleted cultures showed enhanced cytolytic activity. Cell recovery and viability did not differ greatly between depleted and undepleted cultures, and as the cytotoxicity assay is set up using the number of viable effector cells, the observed enhancement could not be attributed to poor survival of cells in the control cultures. Repeat experiments established the reproducibility of this effect, which was observed in all six donors tested, while comparison of 24-hour and 3-day control and carbonyl iron-treated cultures from the same individual confirmed that enhancement of the response resulted from in vitro maintenance of the cells, as shown in Figure 3.3.7.

As the non-adherent cells recovered after carbonyl iron treatment were not adjusted to 2×10^6 cells/ml in these experiments, the increase in cytotoxic activity could not be due to an increased number of effector or precursor cells in the depleted suspension. It was suggested that enhancement of the lytic response may have resulted from fewer cells being present, perhaps reducing deleterious overcrowding. In view of the small number of cells removed by carbonyl iron, this seems rather unlikely; however, to test this hypothesis similar cultures were set up in which absolute numbers of cells in carbonyl iron-treated suspensions were restored to 2×10^6 cells/ml. The results of this experiment are presented in Figure 3.3.8.; as expected, this procedure

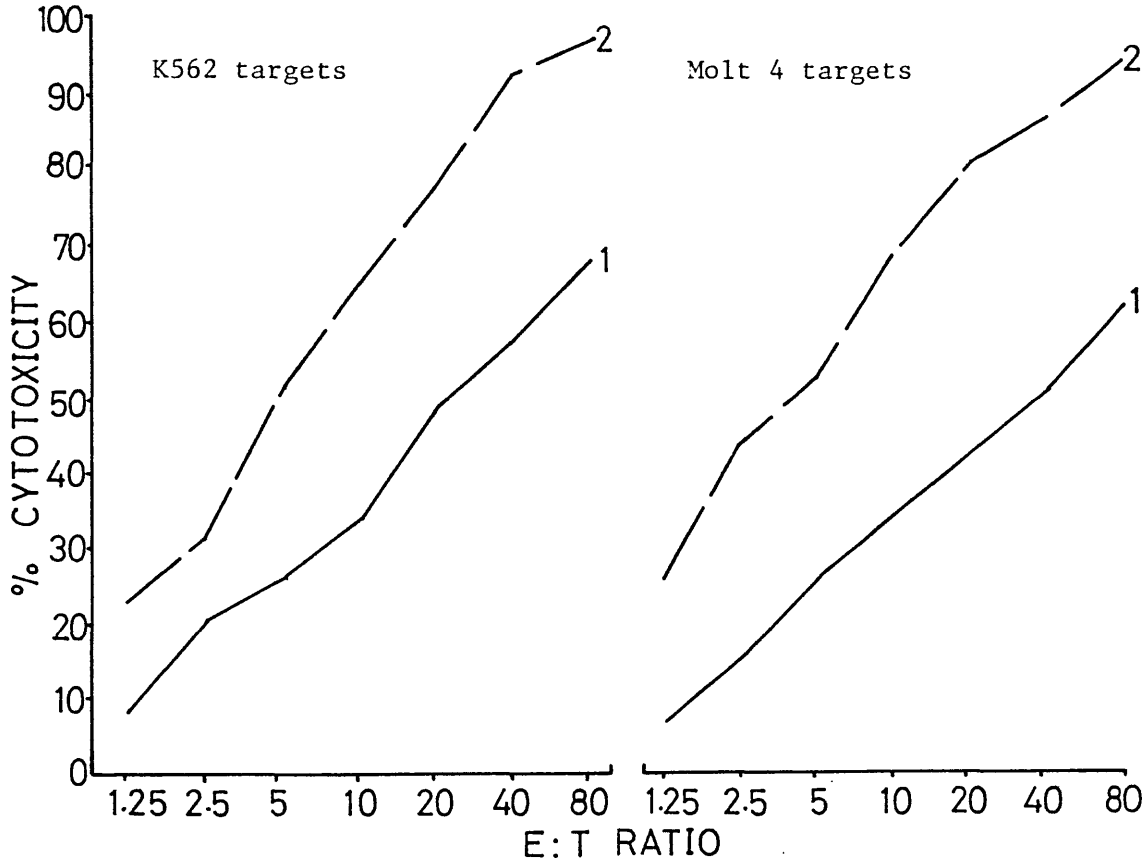


Figure 3.3.6.

Spontaneous cytotoxic activity of carbonyl iron-treated PBMC

Line 1: undepleted cells

2: carbonyl iron-treated cells

CULTURE	1	2
% recovery	89	91
% viability	92	87

TARGET CELLS	K562	Molt 4
Spontaneous release	13%	9%

DONOR KS

DURATION OF CULTURE 3 days

Figure 3.3.7.

Spontaneous cytotoxic activity of carbonyl iron-treated PBMC

PBMC from the same donor were cultured for 24 hours or 3 days, and were then assayed together against the same labelled target cells.

Line 1: undepleted cells cultured for 24 hours

2: carbonyl iron-treated cells cultured for 24 hours

3: undepleted cells cultured for 3 days

4: carbonyl iron-treated cells cultured for 3 days

CULTURE	1	2	3	4
% recovery	86	93	90	87
% viability	88	89	92	95

TARGET CELLS	Molt 4
Spontaneous release	14%
DONOR	MW
DURATION OF CULTURE	24 hours, 3 days


24-hour culture
3-day culture
% CYTOTOXICITY
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
1·25 2·5 5 10 20 40 80
1·25 2·5 5 10 20 40 80
E:T RATIO

Figure 3.3.8.

Spontaneous cytotoxic activity of carbonyl iron-treated PBMC

Line 1: undepleted cells cultured for 24 hours

2: carbonyl iron-treated cells cultured for 24 hours

3: carbonyl iron-treated cells adjusted to 2×10^6 cells/ml, and cultured for 24 hours

4: undepleted cells cultured for 4 days

5: carbonyl iron-treated cells cultured for 4 days

6: carbonyl iron-treated cells adjusted to 2×10^6 cells/ml, and cultured for 4 days

CULTURE	1	2	3	4	5	6
% recovery	91	87	87	90	93	91
% viability	84	90	88	93	96	89

TARGET CELLS	K562
Spontaneous release	11%
DONOR	LR
DURATION OF CULTURE	24 hours, 4 days


24-hour culture
4-day culture
% CYTOTOXICITY
E:T RATIO
0
10
20
30
40
50
60
70
80
90
100
1·25
2·5
5
10
20
40
80
1
2
3
4
5
6

did not affect the enhancement of the response, indicating that this effect is not due to changes in the number or concentration of non-adherent cells in this culture system.

3.3.2.2. Cell separation by adherence to plastic

Further investigation of the role of accessory cells in the generation of spontaneous cytotoxic activity in vitro required the use of a cell separation procedure which yielded the adherent population as well as the non-adherent cells. Cell separation using nylon wool requires a large number of cells, ideally several millilitres of a suspension at 50×10^6 cells/ml, and was not practicable in this case due to the limited availability of mononuclear cells. Further experiments were therefore performed using the property of plastic adherence to isolate the two cell types (section 2.2.2.5.).

Human PBMC were allowed to adhere to culture wells for periods of 3 hours, 24 hours, or 3 days, and the non-adherent cells were then transferred to fresh plates and cultured for varying periods of time. As shown in Figure 3.3.9., removal of the 3-hour adherent population did not affect the subsequent response of depleted cells cultured for a further 24 hours. However, if these depleted cells were cultured for 3 days after removal of the adherent population, they displayed enhanced cytotoxic activity. This is in agreement with observations made using carbonyl iron powder to deplete the cells, indicating that the adherent population is not required for maintenance of cytotoxicity in short-term cultures, but plays a suppressive and possibly regulatory role in the development of cytotoxic activity in cultures of 3 days or longer.

In a repeat experiment, PBMC were allowed to adhere for 24 hours before transferring the non-adherent cells, which were then cultured for a further 3 days. Such treatment again resulted in enhanced cytotoxicity, as shown in Figure 3.3.10.

Figure 3.3.9.

Role of plastic-adherent cells in spontaneous cytotoxic activity in vitro

PBMC were depleted of the adherent fraction by allowing these cells to attach to plastic culture wells over a 3-hour period. Undepleted and depleted PBMC were then cultured for a further 24 hours or 3 days.

Line 1: undepleted cells cultured for 24 hours

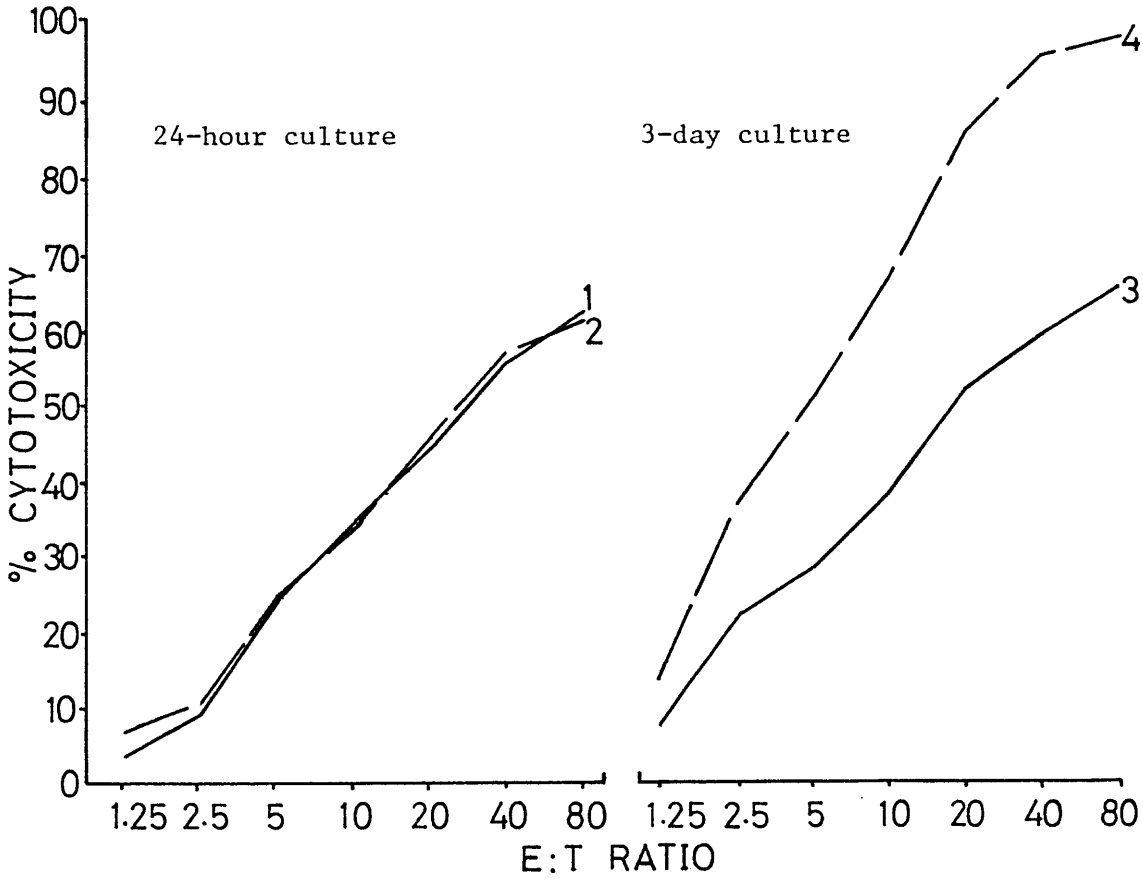
2: depleted cells cultured for 24 hours

3: undepleted cells cultured for 3 days

4: depleted cells cultured for 3 days

CULTURE	1	2	3	4
% recovery	91	88	86	85
% viability	86	89	91	90

TARGET CELLS	K562
Spontaneous release	10%
DONOR	LR
DURATION OF CULTURE	24 hours, 3 days


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
24-hour culture
3-day culture
1
2
3
4
1.25 2.5 5 10 20 40 80
1.25 2.5 5 10 20 40 80
E:T RATIO

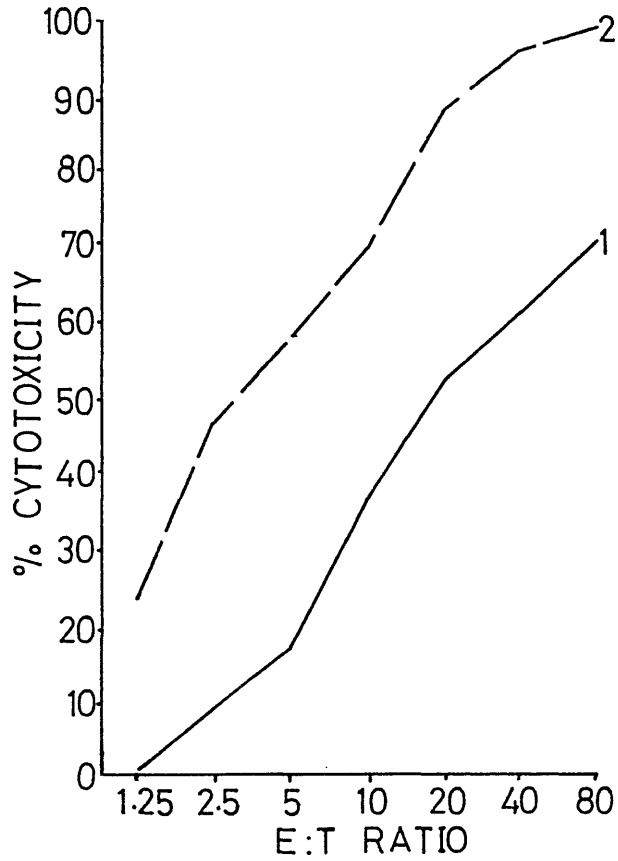


Figure 3.3.10.

Role of plastic-adherent cells in spontaneous cytotoxic activity in vitro

Adherent cells were removed by attachment to plastic over a 24-hour period. Undepleted and depleted PBMC were then cultured for a further 3 days.

Line 1: undepleted cells cultured for 4 days

2: depleted cells cultured for 3 days after removal of the adherent fraction (4 days total)

CULTURE	1	2
% recovery	92	88
% viability	88	85

TARGET CELLS	Molt 4
Spontaneous release	9%

DONOR	VS
DURATION OF CULTURE	4 days

Work presented earlier in this section has indicated that the accessory cells present in human peripheral blood do not play an important role in the expression of cytotoxic activity in short-term cultures. If their suppressive effect is exerted during the maturation of a cytotoxic precursor cell during the first 2-3 days of cultures, then removal of the adherent cells after this time should not lead to enhanced cytotoxicity, as the inhibitory function will already have been performed. To test this hypothesis, PBMC were allowed to adhere for 3 days before removal of the non-adherent fraction. These cells were then transferred to fresh wells and cultured for a further 24 hours. As shown in Figure 3.3.11., such cells did not display enhanced cytotoxic activity. However, if these non-adherent cells were cultured for 3 days after removal of the adherent population, their cytotoxicity was again enhanced, suggesting that the effector population here had not been subjected to suppressive influences from the adherent cells. These cytolytic cells must therefore have passed through their sensitive maturation phase during the 3 days after removal of the adherent population. Such observations imply that there is a second cycle of precursor maturation later in the culture, producing effector cells after 6 days in vitro. Preliminary studies (data not shown) have indicated that spontaneous cytotoxic activity can be maintained in vitro for periods in excess of 10 days, after the initial fall in activity on day 2, and it is suggested that this may reflect a continuous process of effector cell maturation from non-lytic precursors found in the non-adherent fraction.

After transfer of the non-adherent population, adherent cells remain attached to the wells of the culture plate. These cells may be used to enrich cultures for the adherent population, and this technique can be used for further investigation of the accessory function of such cells in this system.

Figure 3.3.11.

Role of plastic-adherent cells in spontaneous cytotoxic activity in vitro

Adherent cells were removed by attachment to plastic over a 3-day period. Undepleted and depleted PBMC were then cultured for a further 24 hours or 3 days.

Line 1: undepleted cells cultured for 4 days

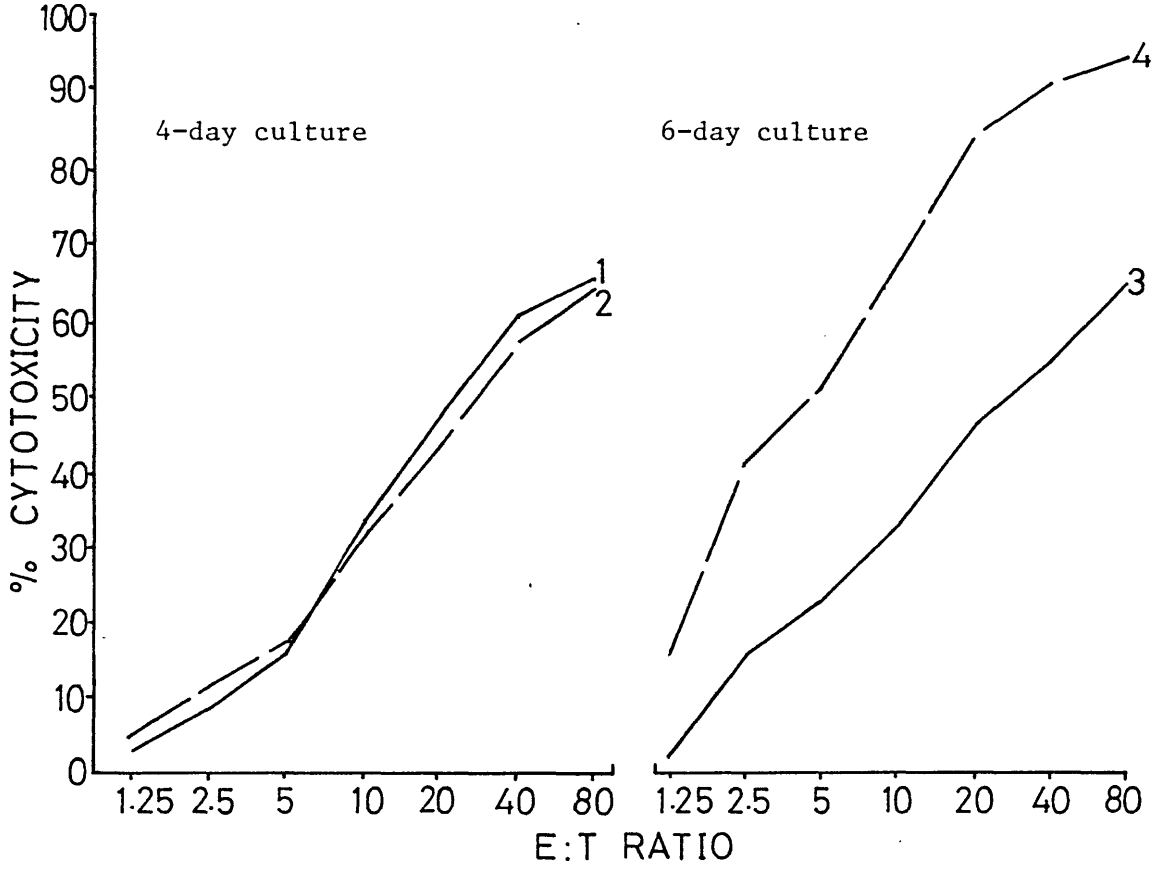
2: depleted cells cultured for 24 hours after removal of the adherent fraction (4 days total)

3: undepleted cells cultured for 6 days

4: depleted cells cultured for 3 days after removal of the adherent fraction (6 days total)

CULTURE	1	2	3	4
% recovery	92	88	90	93
% viability	88	87	91	91

TARGET CELLS	K562
Spontaneous release	13%
DONOR	AB
DURATION OF CULTURE	4 days, 6 days


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
4-day culture
6-day culture
1
2
3
4
1·25 2·5 5 10 20 40 80
1·25 2·5 5 10 20 40 80
E:T RATIO

In a typical experiment, freshly drawn PBMC were allowed to adhere to culture wells over a period of 3 days. The non-adherent cells were then resuspended and transferred to a fresh culture plate, forming an adherent cell-depleted culture. A fresh aliquot of blood was drawn from the same donor; part of this was cultured as a control, while the remainder was added to the adherent cells already attached to the culture plate, to form an adherent cell-enriched culture. All the cultures were then maintained in vitro for a further 24 hours or 3 days. As shown in Figure 3.3.12., 24-hour cultures gave similar results to the control, with neither depletion or enrichment of the adherent fraction having any effect on cytotoxicity. However, cells cultured for a further 3 days were markedly affected by this manipulation of the proportion of adherent cells present. Cells depleted of the adherent population showed enhanced cytotoxic activity, as demonstrated previously, while effector cells harvested from adherent cell-enriched cultures were suppressed. However, the cytotoxic activity of these cells was not totally abrogated. All the cultures appeared to be in good condition on harvesting, so this reduced cytotoxicity could not be attributed to poor culture conditions, or to release of toxic products from dying cells. Repeat experiments confirmed the reproducibility of this suppression in the presence of excess adherent cells.

These results provide further evidence for a suppressive regulatory role of adherent cells in the development of spontaneous cytotoxic activity in vitro. Adherent cells present in excess had no apparent effect on the cytotoxic activity of 24-hour cultures, confirming the previous hypothesis that adherent cells do not regulate the activity of mature effector cells, and supporting the suggestion that the accessory population exerts its effects on the process of maturation of such cells from their precursors.

Figure 3.3.12.

Role of plastic-adherent cells in spontaneous cytotoxic activity in vitro

PBMC were separated into adherent and non-adherent fractions over a 3-day period. Fresh undepleted PBMC from the same donor were then added to the adherent fraction, and the different culture combinations were maintained for a further 24 hours or 3 days.

Line 1: undepleted cells cultured for 4 days

2: depleted cells cultured for 24 hours after removal of the adherent fraction (4 days total)

3: PBMC enriched for adherent cells cultured for a further 24 hours

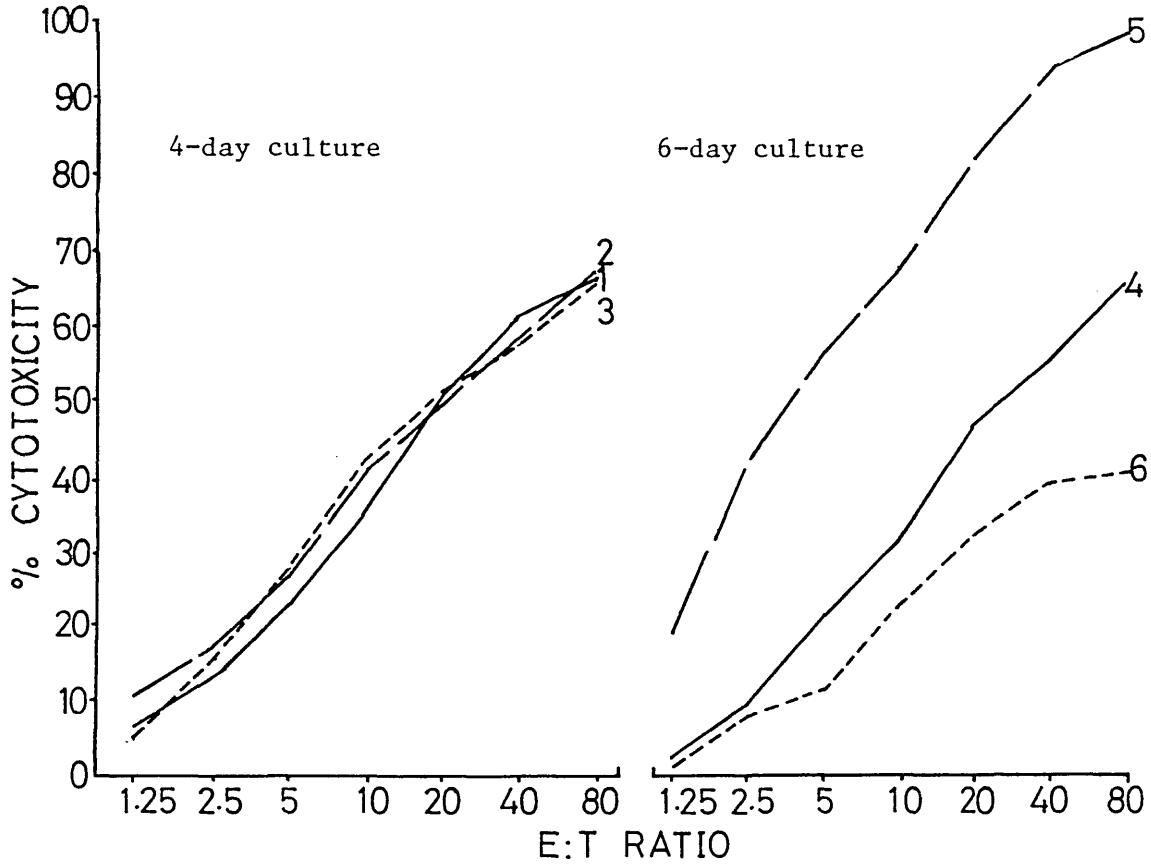
4: undepleted cells cultured for 6 days

5: depleted cells cultured for 3 days after removal of the adherent fraction (6 days total)

6: PBMC enriched for adherent cells cultured for a further 3 days

CULTURE	1	2	3	4	5	6
% recovery	92	91	92	89	92	91
% viability	93	86	88	93	94	90

TARGET CELLS	K562
Spontaneous release	14%, 16%
DONOR	FL
DURATION OF CULTURE	4 days, 6 days


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
4-day culture
6-day culture
1
2
3
4
5
6
1·25 2·5 5 10 20 40 80
1·25 2·5 5 10 20 40 80
E:T RATIO

3.3.2.3. Co-operation between precursor-effector cells and allogeneic accessory cells

Simultaneous culture of cells from two unrelated donors, followed by transferring the non-adherent population and taking fresh blood samples from each donor, yielded cultures enriched with allogeneic adherent cells.

In a typical experiment, adherent cells were separated by allowing them to attach to culture wells over a 3-day period. The non-adherent cells were then removed, and fresh unseparated cells from the original donor or from an unrelated donor were added to the adherent population. Cultures were then maintained for a further 3 days before assay. As shown in Figure 3.3.13., enrichment of cultures with adherent cells from either donor resulted in suppressed cytotoxic activity; allogeneic adherent cells were as effective as syngeneic cells in this respect, suggesting that there is no genetic restriction on interaction between the adherent accessory population and the non-adherent precursor-effector population in this system.

Figure 3.3.13.

Effect of syngeneic and allogeneic plastic-adherent cells on spontaneous cytotoxicity

Parallel cultures using PBMC from two unrelated donors were separated into adherent and non-adherent fractions over a 3-day period. Using fresh undepleted PBMC from the same donors, cultures enriched for either syngeneic or allogeneic adherent cells were obtained.

Line 1: undepleted cells cultured for 6 days

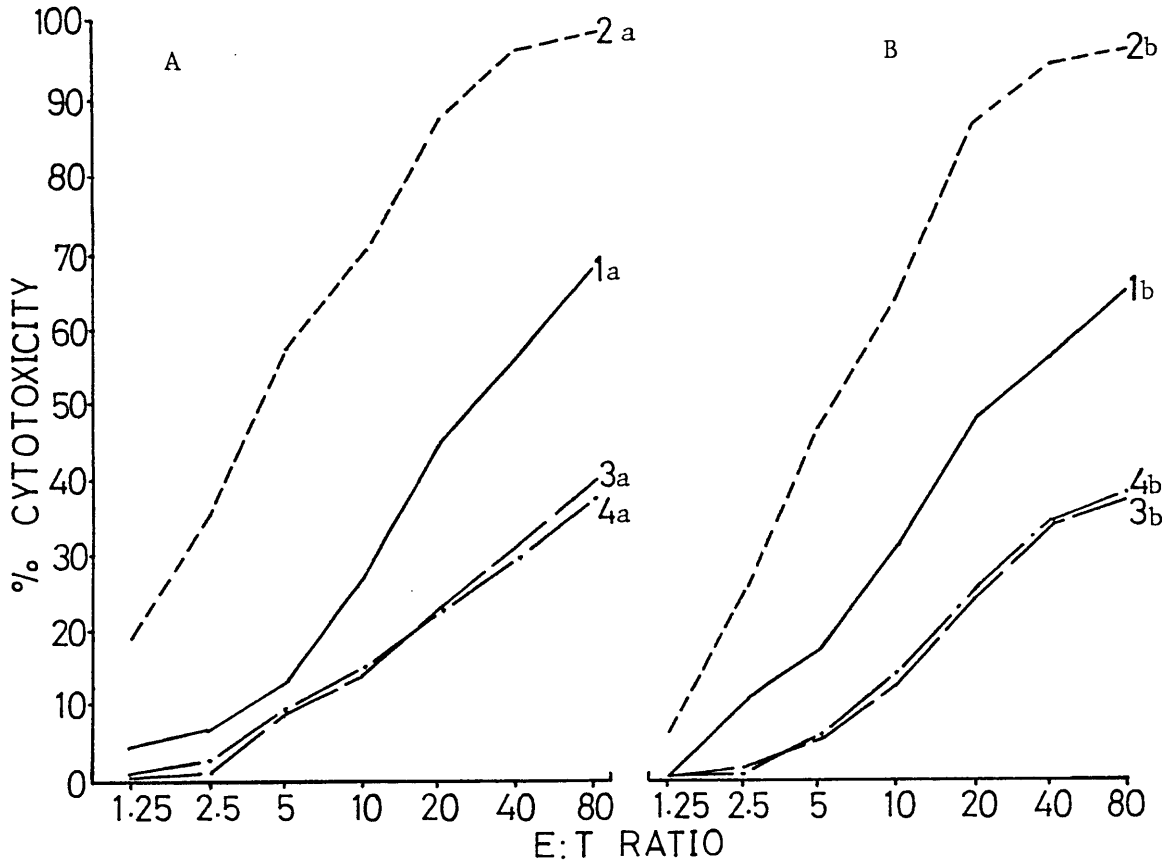
2: depleted cells cultured for 3 days after removal of the adherent fraction (6 days total)

3: PBMC enriched for syngeneic adherent cells, cultured for a further 3 days

4: PBMC enriched for allogeneic adherent cells, cultured for a further 3 days

CULTURE	1a	2a	3a	4a	1b	2b	3b	4b
% recovery	94	96	95	90	95	89	94	89
% viability	89	93	93	96	97	94	96	97

TARGET CELLS	K562
Spontaneous release	17%
DONOR	VS (A), FL (B)
DURATION OF CULTURE	6 days


A
B
2a
2b
1a
1b
3a
4a
4b
3b
% CYTOTOXICITY
100
90
80
70
60
50
40
30
20
10
0
1·25 2·5 5 10 20 40 80
1·25 2·5 5 10 20 40 80
E:T RATIO

3.4. EFFECTS OF MACROPHAGE-ACTIVE AGENTS ON THE SPONTANEOUS CYTOTOXIC ACTIVITY OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS *IN VITRO*

3.4.1. SILICA

3.4.1.1. Effects of silica on the spontaneous cytotoxic activity of PBMC *in vitro*

3.4.2. CARRAGEENAN

3.4.2.1. Effects of ι-carrageenan on spontaneous cytotoxicity *in vitro*

3.4.2.2. Comparison of different types of carrageenan

3.4.2.3. Time-course of carrageenan-induced suppression of spontaneous cytotoxicity

3.4.2.4. Role of adherent cells in carrageenan-induced suppression of spontaneous cytotoxicity

3.4.3. ZYMOSAN

3.4.3.1. Effects of zymosan on spontaneous cytotoxic activity *in vitro*

3.4.3.2. Time-course of zymosan-induced suppression of spontaneous cytotoxicity

3.4.3.3. Role of adherent cells in zymosan-induced suppression of spontaneous cytotoxicity

3.4.4. LATEX

3.4.4.1. Effects of latex on spontaneous cytotoxic activity *in vitro*

3.4.1. SILICA

3.4.1.1. Effects of silica on the spontaneous cytotoxic activity of PBMC in vitro

Different concentrations of silica were added to PBMC at the start of a 24-hour culture, to determine the dose-response relationship for this agent. However, of the five silica-treated cultures subsequently assayed, none showed any change in cytotoxic activity against either target, as shown in Figure 3.4.1. An additional culture in this experiment was exposed to a higher dose of silica, but yielded insufficient viable cells to assay; this may indicate non-selective toxicity of this agent at high concentrations.

Adherent cells remaining attached to a plate after culture can be recovered, as described in section 2.2.2.5. In these experiments, no adherent cells could be recovered from cultures treated with the three highest doses of silica, while cells exposed to lower doses were less numerous than in control cultures, and appeared to be in poor health, confirming the toxicity of silica towards macrophage-like cells. If this agent is acting solely via the destruction of such cells, then its failure to affect cytotoxicity after 24-hour culture is not surprising, as the adherent population has already been shown to have little influence on the level of killing over this period. However, an adherent cell involvement has been demonstrated in longer-term cultures, and to investigate this further, different doses of silica were added to cultures lasting for 3 or more days.

As shown in Figure 3.4.2., results obtained from longer-term cultures exposed to silica were comparable to those found using 24-hour cultures; silica again failed to suppress cytotoxicity, and there was evidence of toxicity towards the non-adherent cells at the highest dose of 500 μg silica/ml. Examination of the adherent cells after culture

Figure 3.4.1.

Effect of silica on spontaneous cytotoxicity after short-term culture

PBMC were cultured for 24 hours with varying concentrations of silica particles. Cells exposed to the highest concentration of silica were not assayed for cytotoxic activity, as insufficient viable cells were recovered.

Line 1: control; no silica added to culture

2: 10 µg silica/ml

3: 20 µg silica/ml

4: 50 µg silica/ml

5: 100 µg silica/ml

6: 200 µg silica/ml

Culture 7: 500 µg silica/ml (not assayed)

CULTURE	1	2	3	4	5	6	7
% recovery	91	89	88	90	93	87	81
% viability	94	97	95	91	90	86	11

TARGET CELLS	K562	Molt 4
Spontaneous release	8%	8%

DONOR FL

DURATION OF CULTURE 24 hours

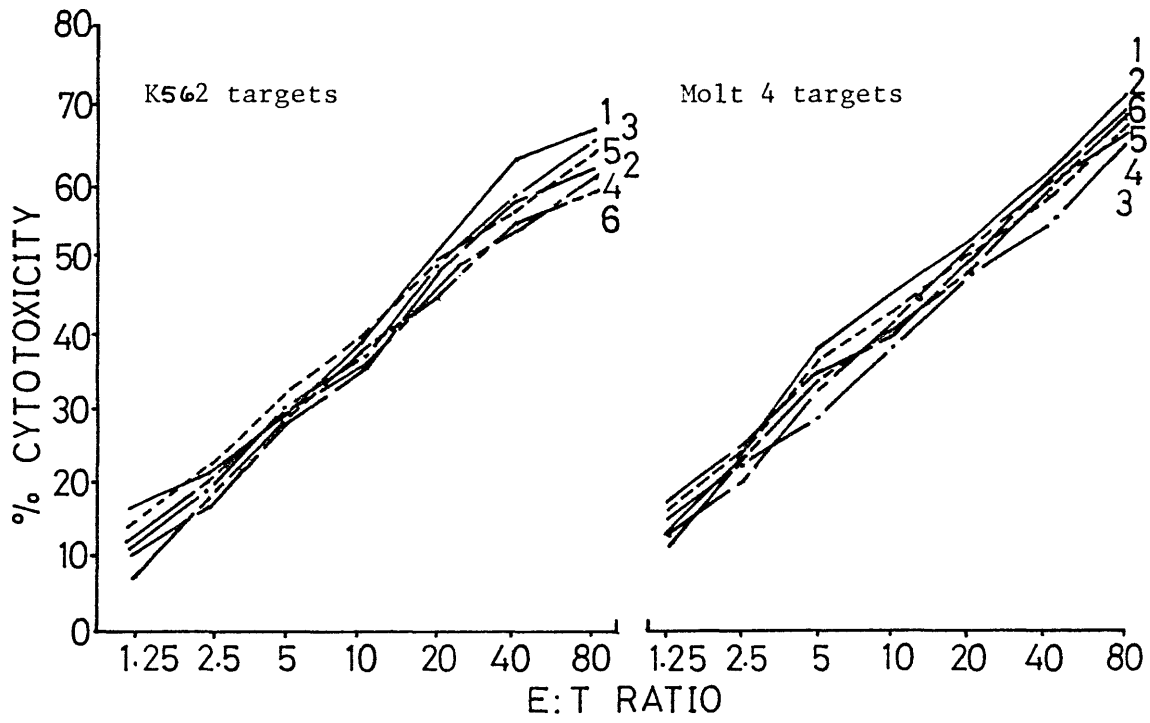

K562 targets
Molt 4 targets
% CYTOTOXICITY
E:T RATIO
0
10
20
30
40
50
60
70
80
1·25
2·5
5
10
20
40
80
1
3
5
2
4
6
1
2
6
5
4
3

Figure 3.4.2.

Effect of silica on spontaneous cytotoxicity after 3-day culture

Silica was added to 3-day cultures of PBMC at initiation, to give a range of final concentrations. Cells exposed to the highest concentration of silica were not assayed in view of their low viability.

Line 1: control; no silica added to culture

2: 20 µg silica/ml

3: 50 µg silica/ml

4: 100 µg silica/ml

5: 200 µg silica/ml

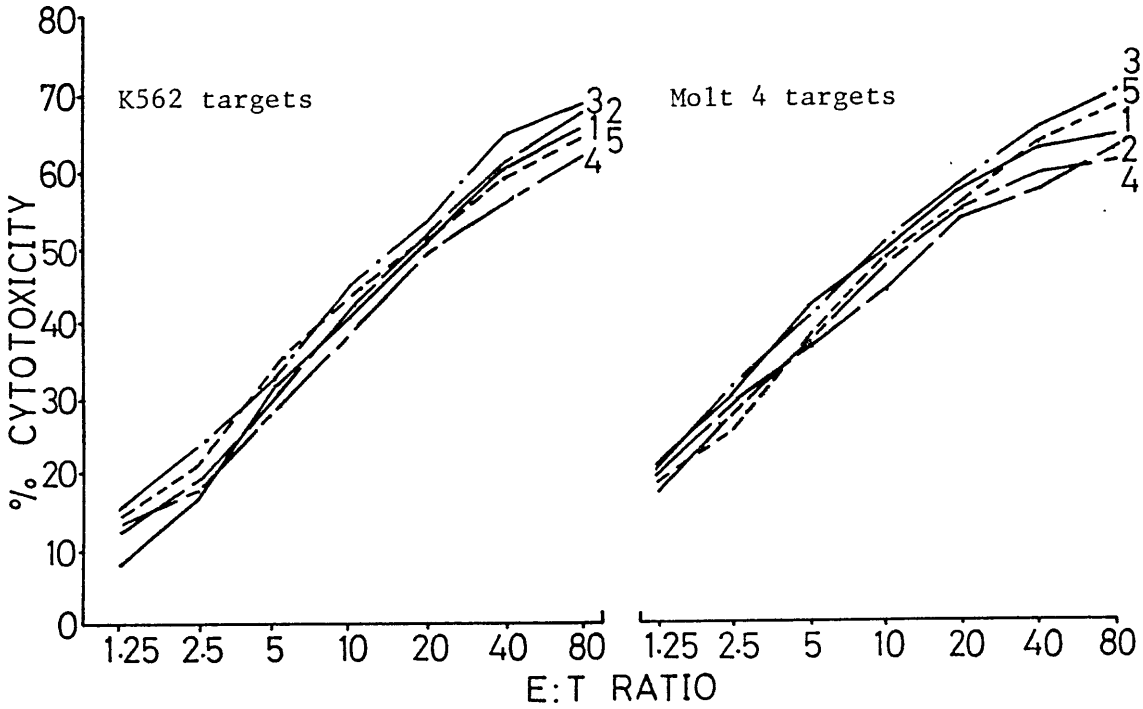
Culture 6: 500 µg silica/ml (not assayed)

CULTURE	1	2	3	4	5	6
% recovery	86	89	90	87	82	79
% viability	91	89	90	93	84	9

TARGET CELLS	K562	Molt 4
Spontaneous release	8%	10%

DONOR AB

DURATION OF CULTURE 3 days


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
K562 targets
3
2
1
5
4
Molt 4 targets
3
5
1
2
4
1·25
2·5
5
10
20
40
80
E:T RATIO

again revealed depletion of this population, particularly at higher doses of silica.

Preliminary work on this culture system demonstrated that removal of adherent macrophage-like cells prior to 3-day culture results in enhanced target cell lysis (sections 3.3.2.1., 3.3.2.2.), suggesting that these cells may exert a regulatory effect on cytotoxicity. Removal of a similar population of cells by the toxic effects of silica may therefore be expected to result in a comparable degree of enhancement. However, if adherent cells do influence the level of cytotoxicity expressed by the effector cells, this regulation may be mediated by soluble factors secreted by the accessory population. Lysis of such cells after ingestion of silica, with concomitant loss of intracellular materials into the culture medium, may permit release of these regulatory mediators and so maintain control over the level of killing. Such soluble mediators could presumably be removed from the culture by replacing the culture medium. Experiments were therefore performed in which unfractionated mononuclear cells were exposed to silica for 24 hours; 0.8 ml of culture fluid (total volume 1.1 ml) was then removed and replaced by fresh medium, without disturbing the cells, and the cultures were maintained in vitro for a further 3 days.

As shown in Figure 3.4.3., silica-treated cells which were washed after the first 24 hours of a 4-day culture subsequently displayed enhanced cytotoxic activity, comparable to that of cells depleted of the adherent fraction. Non-adherent cells transferred from silica-treated cultures to fresh wells at this stage also showed increased cytotoxic activity, indicating that silica was not directly affecting the non-adherent population. It is therefore reasonable to conclude that ingestion of silica particles by mononuclear phagocytes, and subsequent destruction of these cells, results in release of regulatory and/or inhibitory materials into the culture medium, which may then limit the

Figure 3.4.3.

Effect of silica on spontaneous cytotoxicity following short-term exposure at start of 4-day culture

PBMC were cultured for 24 hours with 100 µg silica/ml. The culture medium was then replaced, and the cells were cultured undisturbed for a further 3 days, either in the original culture wells or in a fresh plate.

Line 1: control; untreated cells cultured undisturbed for 4 days

2: cells cultured undisturbed for 4 days in the presence of silica

3: untreated cells washed after 24 hours and cultured for a further 3 days

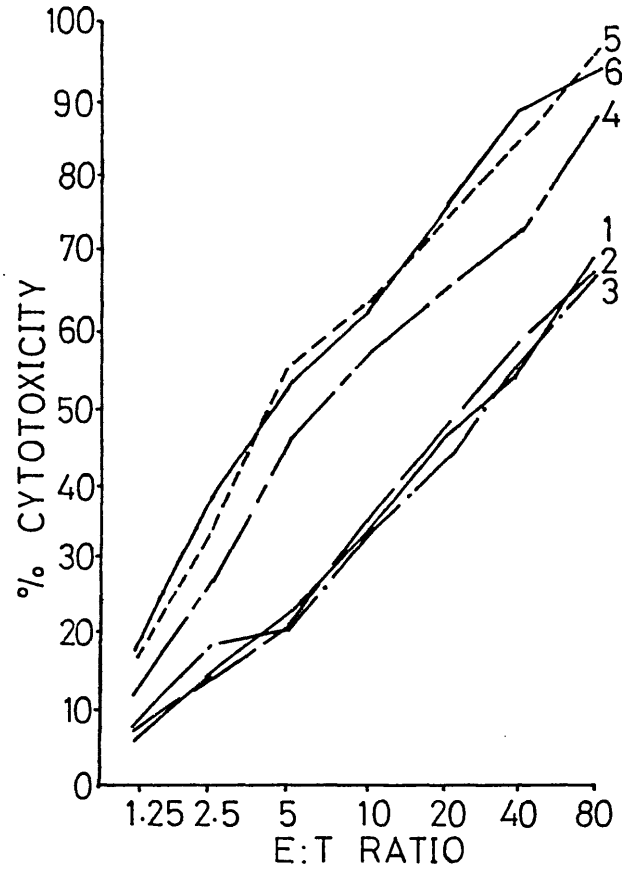
4: silica-treated cells washed after 24 hours and cultured for a further 3 days

5: untreated cells washed after 24 hours; non-adherent cells transferred to fresh wells and cultured for a further 3 days

6: silica-treated cells washed after 24 hours; non-adherent cells transferred to fresh wells and cultured for a further 3 days

CULTURE	1	2	3	4	5	6
% recovery	79	75	76	78	73	80
% viability	95	91	98	98	88	84

TARGET CELLS	K562
Spontaneous release	12%
DONOR	VS
DURATION OF CULTURE	4 days


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25 2·5 5 10 20 40 80
E:T RATIO
1
2
3
4
5
6

development of cytotoxic activity even in the absence of viable phagocytic cells.

In both short-term and long-term cultures, apparently non-selective toxicity of high doses of silica was observed. This may reflect a direct effect on the non-adherent population, or may result indirectly from lysis of the phagocytic cells, with concomitant release of potentially toxic intracellular materials into the culture medium. To distinguish between these two possibilities, cultures were set up in which unfractionated cells, and suspensions depleted of the adherent fraction using carbonyl iron powder (as described in section 2.2.2.3.), were exposed to silica for the duration of a 3-day culture period. As shown in Figure 3.4.4., silica had no toxic effect on the non-adherent cells in adherent cell-depleted cultures, and non-adherent cells harvested from such cultures gave an enhanced level of target cell killing, typical of cells maintained in vitro for 3 or more days after removal of the adherent fraction. These data support the hypothesis that the toxicity of silica at high concentrations results indirectly from damage to the phagocytic population.

3.4.2. CARRAGEENAN

3.4.2.1. Effects of ι-carrageenan on spontaneous cytotoxicity in vitro

Previous work on the murine MLC showed that the purified λ and ι forms of carrageenan were most active (section 2.4.2.1.). Preliminary experiments on human cells were therefore performed using ι-carrageenan.

A range of concentrations of ι-carrageenan was added to 24-hour cultures of PBMC. At the end of the culture period, the non-adherent cells were harvested and their cytotoxic activity was compared. As shown in Figure 3.4.5., exposure to carrageenan for 24 hours had little

Figure 3.4.4.

Effect of silica on spontaneous cytotoxic activity of carbonyl iron-treated PBMC

Undepleted PBMC and cells depleted of the adherent fraction using carbonyl iron powder were cultured for 3 days with varying concentrations of silica.

Line 1: control; undepleted cells, no silica added to culture

2: undepleted cells, 100 µg silica/ml

3: undepleted cells, 200 µg silica/ml

Culture 4: undepleted cells, 500 µg silica/ml (not assayed)

Line 5: carbonyl iron-treated cells, no silica added

6: carbonyl iron-treated cells, 100 µg silica/ml

7: carbonyl iron-treated cells, 200 µg silica/ml

8: carbonyl iron-treated cells, 500 µg silica/ml

CULTURE	1	2	3	4	5	6	7	8
% recovery	91	90	88	84	81	83	79	80
% viability	89	92	85	16	92	97	93	91

TARGET CELLS Molt 4

Spontaneous release 8%

DONOR VS

DURATION OF CULTURE 3 days

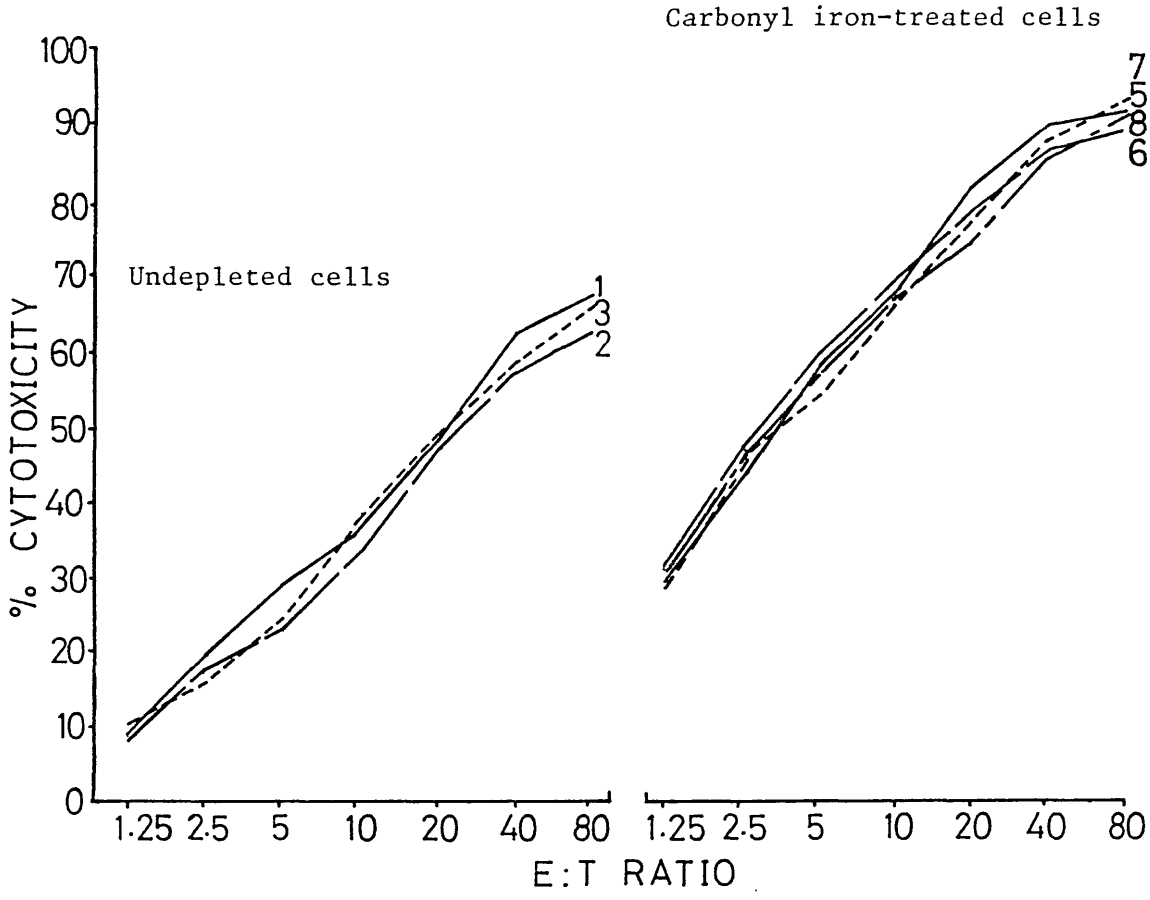

Carbonyl iron-treated cells
Undepleted cells
% CYTOTOXICITY
100
90
80
70
60
50
40
30
20
10
0
1
3
2
7
5
8
6
1·25 2·5 5 10 20 40 80
1·25 2·5 5 10 20 40 80
E:T RATIO

Figure 3.4.5.

Effect of ι-carrageenan on spontaneous cytotoxicity after short-term culture

PBMC were cultured for 24 hours with varying concentrations of ι-carrageenan.

Line 1: control; no carrageenan added to culture

2: 10 µg carrageenan/ml

3: 20 µg carrageenan/ml

4: 50 µg carrageenan/ml

5: 100 µg carrageenan/ml

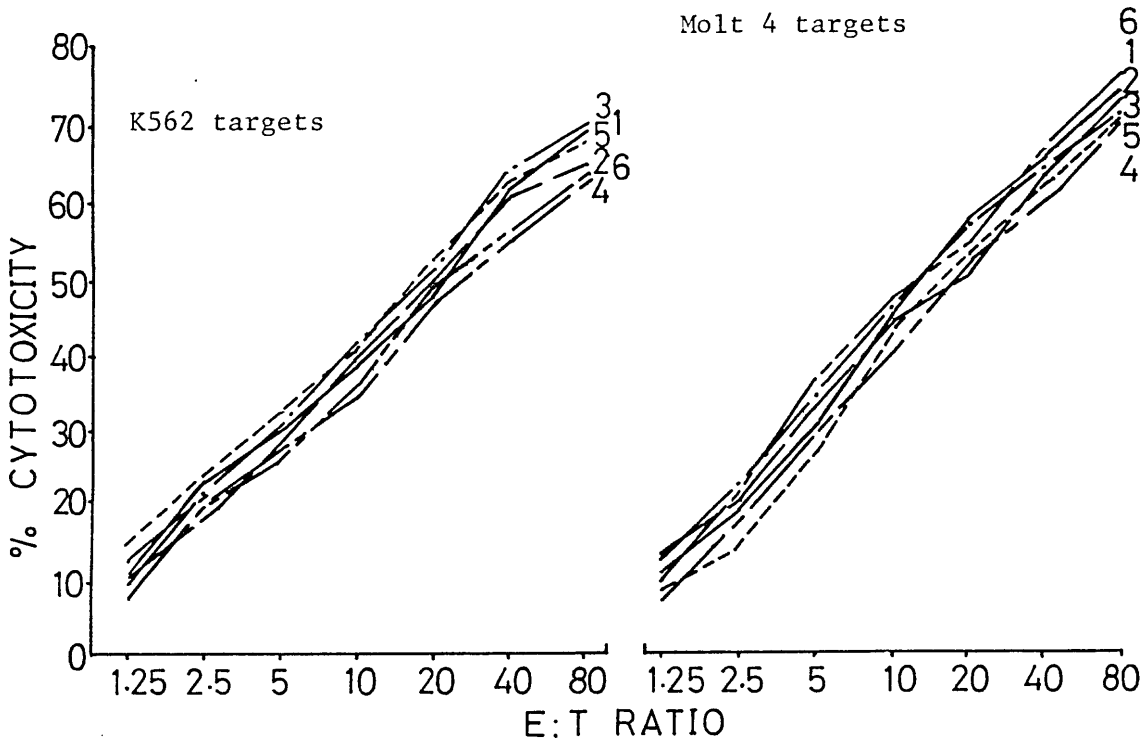
6: 200 µg carrageenan/ml

CULTURE	1	2	3	4	5	6
% recovery	88	89	86	92	89	90
% viability	91	93	90	88	89	88

TARGET CELLS	K562	Molt 4
Spontaneous release	8%	9%

DONOR DD

DURATION OF CULTURE 24 hours


K562 targets
Molt 4 targets
% CYTOTOXICITY
E:T RATIO
0
10
20
30
40
50
60
70
80
1·25
2·5
5
10
20
40
80
3
5
1
2
6
4
6
1
2
3
5
4

effect on either the level of killing or the recovery of viable cells, even at the highest dose tested. These observations were confirmed in later experiments, and similar results were obtained when carrageenan was added to fresh uncultured cells for the duration of the cytotoxicity assay, as shown in Figure 3.4.6.

Selective toxicity of carrageenans towards macrophage-like phagocytic cells has been reported in other systems (Allison et al, 1966; Catanzaro et al, 1971), and such an effect would be compatible with the results obtained here, as physical removal of such cells prior to short-term culture has already been shown to have no effect on cytotoxicity (sections 3.3.2.1., 3.3.2.2.). However, examination of adherent cells recovered after 24-hour culture with carrageenan revealed no evidence of damage; similar numbers of cells were obtained from treated or control cultures, and these cells seemed healthy and were able to ingest opsonised yeast particles. It therefore appears that carrageenan does not exert a toxic effect on adherent cells in this culture system.

Previous experiments have shown that adherent macrophage-like cells may be involved in regulating the cytotoxic activity expressed by PBMC in longer-term cultures (sections 3.3.2.1., 3.3.2.2.). If carrageenan is acting on cells of this lineage, then its effects may become apparent over an extended culture period of 3 days. To test this hypothesis, cells were exposed to different concentrations of ι-carrageenan for 3 days, then assayed together with fresh cells from the same donor. As shown in Figure 3.4.7., carrageenan suppressed the cytotoxic activity of cultured cells in a dose-dependent manner, while fresh cells were again unaffected. Comparable numbers of viable cells were recovered from all cultures, indicating that the observed suppression did not result from direct toxicity towards the effector cells. Similar results were obtained in repeat experiments using cells

Figure 3.4.6.

Effect of ι-carrageenan on spontaneous cytotoxicity after short-term culture

Line 1: control; no carrageenan added to culture

2: 20 µg carrageenan/ml

3: 50 µg carrageenan/ml

4: 100 µg carrageenan/ml

5: 200 µg carrageenan/ml

6: 100 µg carrageenan/ml added to fresh uncultured cells for the duration of the cytotoxicity assay

CULTURE	1	2	3	4	5
% recovery	84	86	82	85	86
% viability	87	91	92	89	93

TARGET CELLS	Molt 4
Spontaneous release	9%
DONOR	AB
DURATION OF CULTURE	24 hours

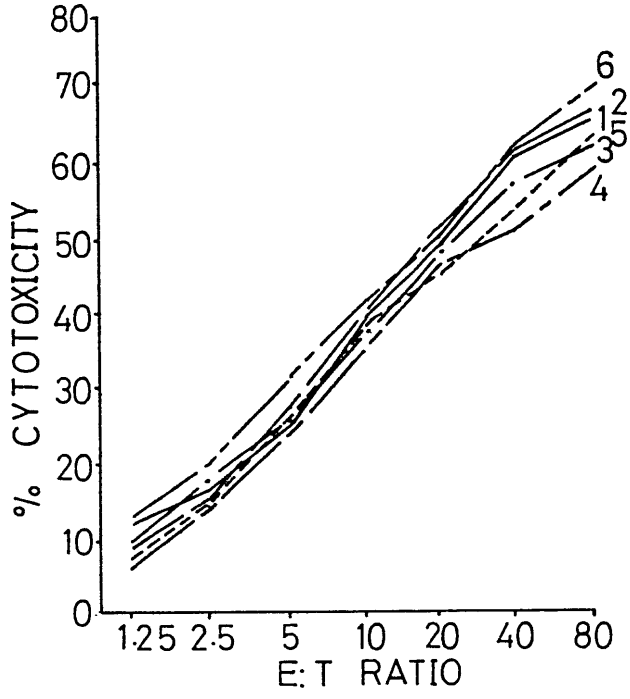

80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25 2·5 5 10 20 40 80
E:T RATIO
6
2
1
5
3
4

Figure 3.4.7.

Effect of ι-carrageenan on spontaneous cytotoxicity after 3-day culture

ι-carrageenan was added to 3-day cultures of PBMC at initiation, to give the following range of final concentrations.

Line 1: control; no carrageenan added to culture

2: 20 µg carrageenan/ml

3: 50 µg carrageenan/ml

4: 100 µg carrageenan/ml

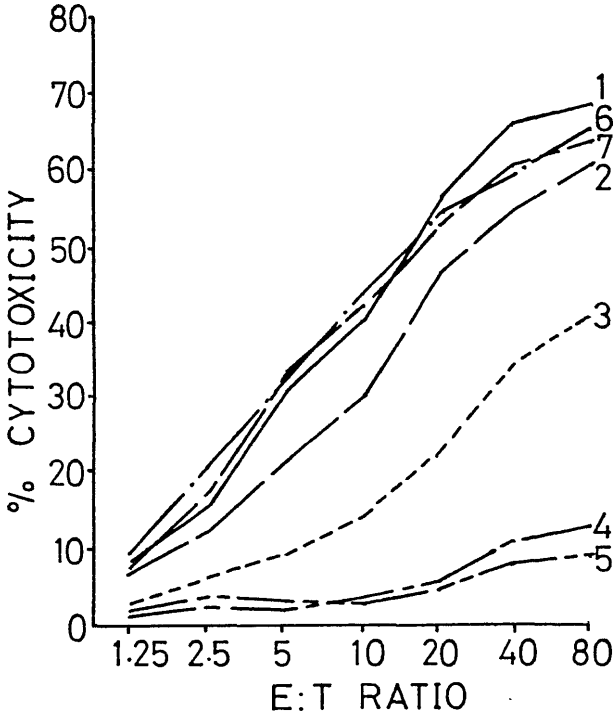
5: 200 µg carrageenan/ml

6: fresh uncultured cells, no carrageenan added

7: 100 µg carrageenan/ml added to fresh uncultured cells for the duration of the cytotoxicity assay

CULTURE	1	2	3	4	5
% recovery	91	90	88	87	89
% viability	91	87	92	88	90

TARGET CELLS	K562
Spontaneous release	13%
DONOR	LR
DURATION OF CULTURE	3 days


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
6
7
2
3
4
5
1·25 2·5 5 10 20 40 80
E:T RATIO

from different donors, and from these data a dose of carrageenan was selected for further work, most subsequent experiments being performed using κ-, λ- or ι-carrageenan at a final concentration of 100 μg/ml, over a culture period of at least 3 days.

3.4.2.2. Comparison of different types of carrageenan

The immunosuppressive effects of different purified carrageenans in this system were compared, as shown in Figure 3.4.8. Lambda carrageenan was found to display similar properties to the ι form, and was active over the same dose range. In contrast, purified κ-carrageenan had little effect on cytotoxicity, even at the highest dose tested. These results are in agreement with those obtained in the murine system (section 2.4.2.1.), and consequently the rest of the experiments in this section were performed using λ- or ι-carrageenan.

3.4.2.3. Time-course of carrageenan-induced suppression of spontaneous cytotoxicity

To determine the time-course of carrageenan-induced suppression in this system, a single dose of carrageenan (100 μg/ml) was added to 3-day cultures of PBMC at initiation or at 24-hour intervals subsequent to this. The results of such an experiment are presented in Figure 3.4.9. When added to cells at the start of the culture period, both λ- and ι-carrageenan caused full suppression of cytotoxicity; however, this suppressive effect was markedly reduced if treatment was delayed for 24 hours, and further delay resulted in activity comparable to that of untreated cells. These results support the earlier conclusion that carrageenan does not act directly on the effector cells in this system, and indicate that this agent must be present during the early part of culture to exert its suppressive effects.

In a repeat experiment, a slightly longer culture period of 5 days was employed. As shown in Figure 3.4.10., ι-carrageenan was

Figure 3.4.8.

Effect of different types of purified carrageenan on spontaneous cytotoxicity in vitro

Purified κ-, λ-, and ɩ-carrageenans were added to 3-day cultures of PBMC at initiation. Each type was tested at three concentrations.

Line 1: control; no carrageenan added to culture

2: 50 µg carrageenan/ml

3: 100 µg carrageenan/ml

4: 150 µg carrageenan/ml

TREATMENT	-	κ-carrageenan			λ-carrageenan			ɩ-carrageenan		
CULTURE	1	2a	3a	4a	2b	3b	4b	2c	3c	4c
% recovery	78	79	76	80	81	79	81	78	82	80
% viability	86	86	89	83	86	83	85	82	87	84

TARGET CELLS Molt 4

Spontaneous release 9%

DONOR FL

DURATION OF CULTURE 3 days

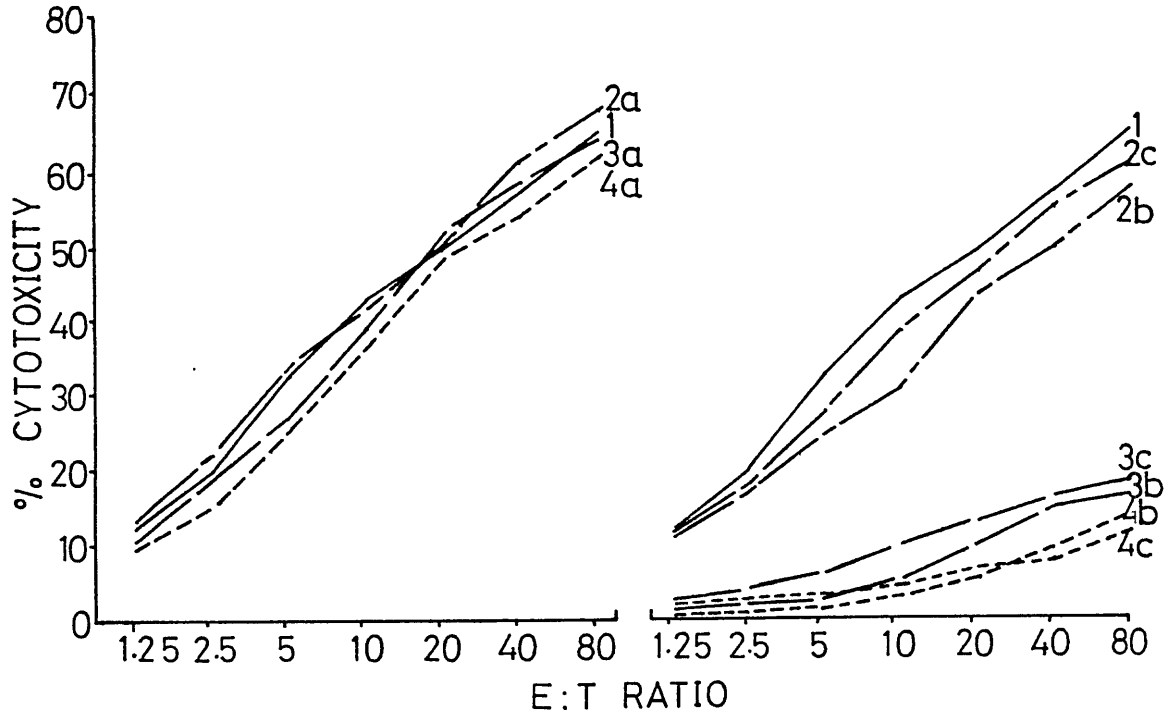

80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
2a
1
3a
4a
1
2c
2b
3c
3b
4b
4c
1·25 2·5 5 10 20 40 80
1·25 2·5 5 10 20 40 80
E:T RATIO

Figure 3.4.9.

Time-course of carrageenan-induced suppression of spontaneous cytotoxicity in vitro

λ- and ι-carrageenans were added to cultures of PBMC at initiation or at 24-hour intervals after this, to give a final concentration of 100 μg/ml. Carrageenan was also added to some previously untreated cells for the duration of the cytotoxicity assay.

Line 1: control; no carrageenan added to culture

2: carrageenan added on day 0

3: carrageenan added on day 1

4: carrageenan added on day 2

5: carrageenan added to cytotoxicity assay on day 3

TREATMENT	-	λ-carrageenan			ι-carrageenan		
CULTURE	1	2a	3a	4a	2b	3b	4b
% recovery	87	84	89	82	85	81	83
% viability	87	87	83	85	83	86	84

TARGET CELLS Molt 4

Spontaneous release 10%

DONOR LR

DURATION OF CULTURE 3 days

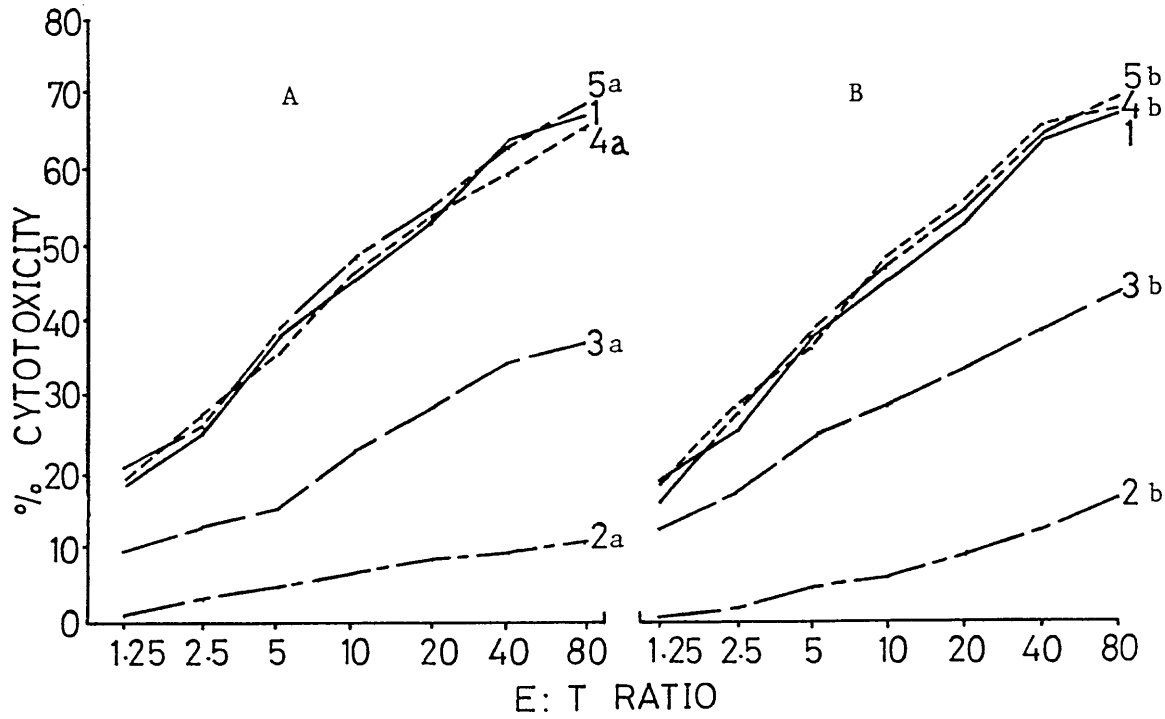

80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
A
B
5a
1
4a
3a
2a
5b
4b
1
3b
2b
1·25 2·5 5 10 20 40 80
1·25 2·5 5 10 20 40 80
E: T RATIO

Figure 3.4.10.

Time-course of ι-carrageenan-induced suppression of spontaneous cytotoxicity after 5-day culture

Line 1: control; no carrageenan added to culture

2: carrageenan added on day 0

3: carrageenan added on day 1

4: carrageenan added on day 2

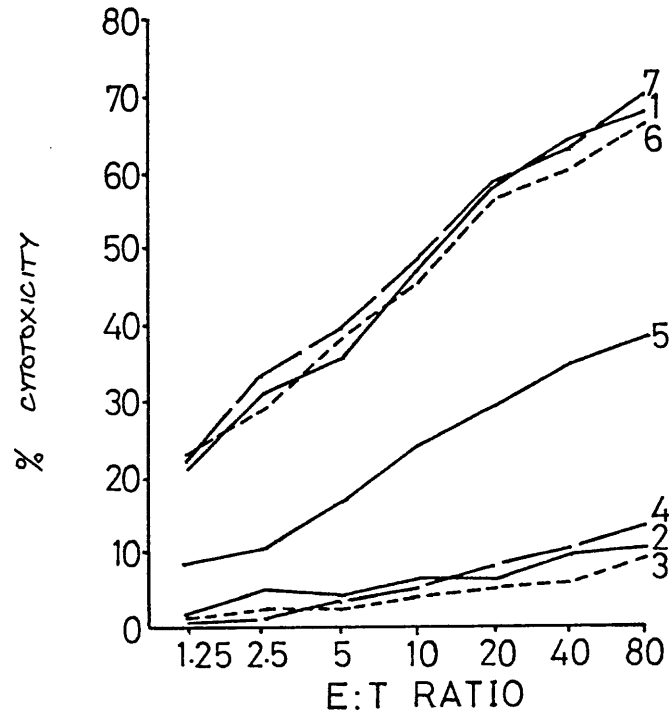
5: carrageenan added on day 3

6: carrageenan added on day 4

7: carrageenan added to cytotoxicity assay on day 5

CULTURE	1	2	3	4	5	6
% recovery	86	87	82	88	85	81
% viability	91	90	91	88	92	91

TARGET CELLS	Molt 4
Spontaneous release	10%
DONOR	VS
DURATION OF CULTURE	5 days


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
7
1
6
5
4
2
3
1·25 2·5 5 10 20 40 80
E:T RATIO

now still fully suppressive when added to cultures 48 hours after initiation, after which the suppressive effect rapidly diminished.

The results of these time-course experiments suggest that the important factor in determining carrageenan-induced suppression is not the length of time between initiation of culture and addition of the active agent, but rather the period between carrageenan treatment and assay of the cells for cytotoxic activity; it appears that carrageenan must be present for 2-3 days before the assay in order to exert its effects. Previous experiments described in section 3.3.2.2. suggested maturation of a population of effector cells in vitro over a similar time period, and the persistance of cytotoxic activity in long-term cultures after an initial fall on day 2 (section 3.3.1.5.) may indicate a continuous maturation process in this system. The apparent carrageenan-sensitive period in longer-term cultures is compatible with this hypothesis, as suppression is only obtained if this agent is present during the proposed maturation period of the effector cells.

3.4.2.4. Role of adherent cells in carrageenan-induced suppression of spontaneous cytotoxicity

The sensitivity of spontaneous cytotoxic activity to carrageenan in vitro follows a similar time-course to the adherent cell involvement in this system, suggesting that this agent may exert its suppressive effects through the adherent population. To test this hypothesis, PBMC were depleted of the adherent fraction using carbonyl iron powder, before culturing for 3 days with or without ι-carrageenan.

As shown in Figure 3.4.11., removal of the adherent cells from control cultures resulted in enhanced cytotoxic activity, in agreement with the results obtained in section 3.3.2.1. Addition of carrageenan to undepleted cultures caused full suppression of cytotoxicity; however, carrageenan appeared to have little effect on cultures depleted of the

Figure 3.4.11.

Role of adherent cells in carrageenan-induced suppression of spontaneous cytotoxicity

Undepleted PBMC and cells depleted of the adherent fraction using carbonyl iron powder were cultured for 3 days in the presence of 100 μg ℓ-carrageenan/ml.

Line 1: control; undepleted cells, no carrageenan added to culture

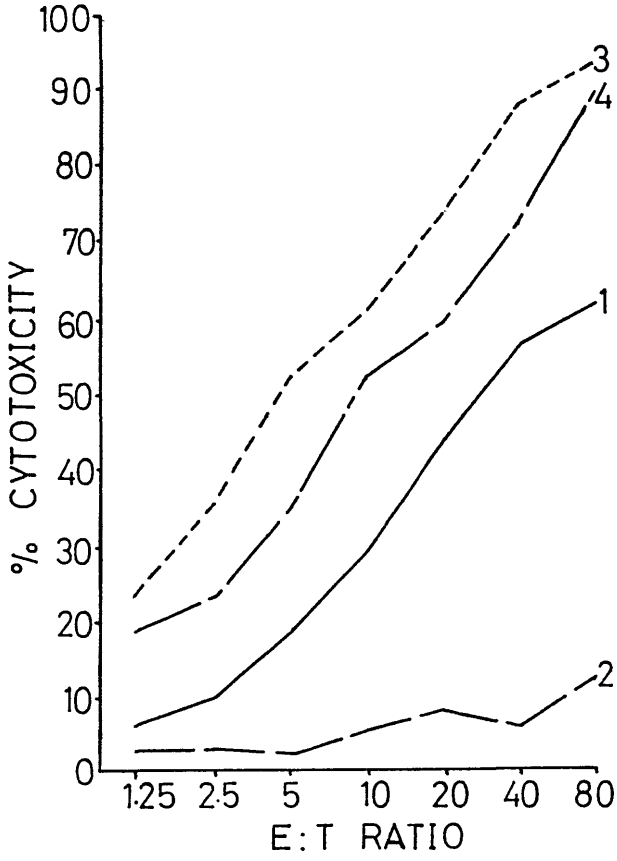
2: undepleted cells + carrageenan

3: carbonyl iron-treated cells, no carrageenan added

4: carbonyl iron-treated cells + carrageenan

CULTURE	1	2	3	4
% recovery	83	85	87	85
% viability	87	88	90	89

TARGET CELLS	K562
Spontaneous release	11%
DONOR	HB
DURATION OF CULTURE	3 days


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25
2·5
5
10
20
40
80
E:T RATIO
1
2
3
4

adherent fraction, indicating that such cells are required for expression of carrageenan-induced suppression. Such a role for adherent cells was further investigated after separating the adherent and non-adherent populations by adherence to plastic, as described in section 2.2.2.5. Using this method, cultures either depleted or enriched for adherent cells were obtained.

The results of a typical experiment are shown in Figure 3.4.12.; adherent cells were separated over a period of 3 days, then a fresh blood sample was taken from the same donor, and the various culture combinations were maintained in vitro for a further 3 days, with or without ι-carrageenan. Cells depleted of the adherent fraction in this manner yielded results similar to those obtained after carbonyl iron treatment, the enhanced cytotoxic activity of depleted cultures being unaffected by the addition of carrageenan. However, cultures enriched for adherent cells showed suppressed cytotoxicity (as described in sections 3.3.2.2., 3.3.2.3.), and this limited activity was abrogated in the presence of carrageenan.

These results support the hypothesis that adherent cells mediate the suppressive effects of carrageenan in this system; however, such suppression does not appear to result from destruction of the adherent population. Previous experiments have shown that silica can be used to selectively destroy phagocytic mononuclear cells in vitro (section 3.4.1.1.), and further experiments were therefore set up to study the combined effects of silica and carrageenan on spontaneous cytotoxicity in this culture system. Unfractionated PBMC were exposed to silica and carrageenan, alone or in combination, for the duration of a 3-day culture. Results obtained from such an experiment are presented in Figure 3.4.13., and show that addition of silica to the culture medium may afford some protection against the suppressive effects of carrageenan. However, some carrageenan-induced suppression was still

Figure 3.4.12.

Role of adherent cells in carrageenan-induced suppression of spontaneous cytotoxicity

PBMC were separated into adherent and non-adherent fractions over a 3-day period. Fresh undepleted PBMC from the same donor were used to establish cultures enriched for adherent cells, and the different culture combinations were maintained for a further 3 days with or without 100 μg λ-carrageenan/ml.

Line 1: control; undepleted cells cultured for 6 days, no carrageenan added

2: undepleted cells cultured for 6 days, carrageenan present for final 3 days

3: depleted cells cultured for 3 days after removal of the adherent fraction (6 days total), no carrageenan added

4: as for line 3, with carrageenan present for final 3 days

5: PBMC enriched for adherent cells, no carrageenan added

6: PBMC enriched for adherent cells, carrageenan present for final 3 days of culture

CULTURE	1	2	3	4	5	6
% recovery	89	91	92	87	91	87
% viability	93	89	94	91	90	94

TARGET CELLS	K562
Spontaneous release	16%
DONOR	FL
DURATION OF CULTURE	6 days

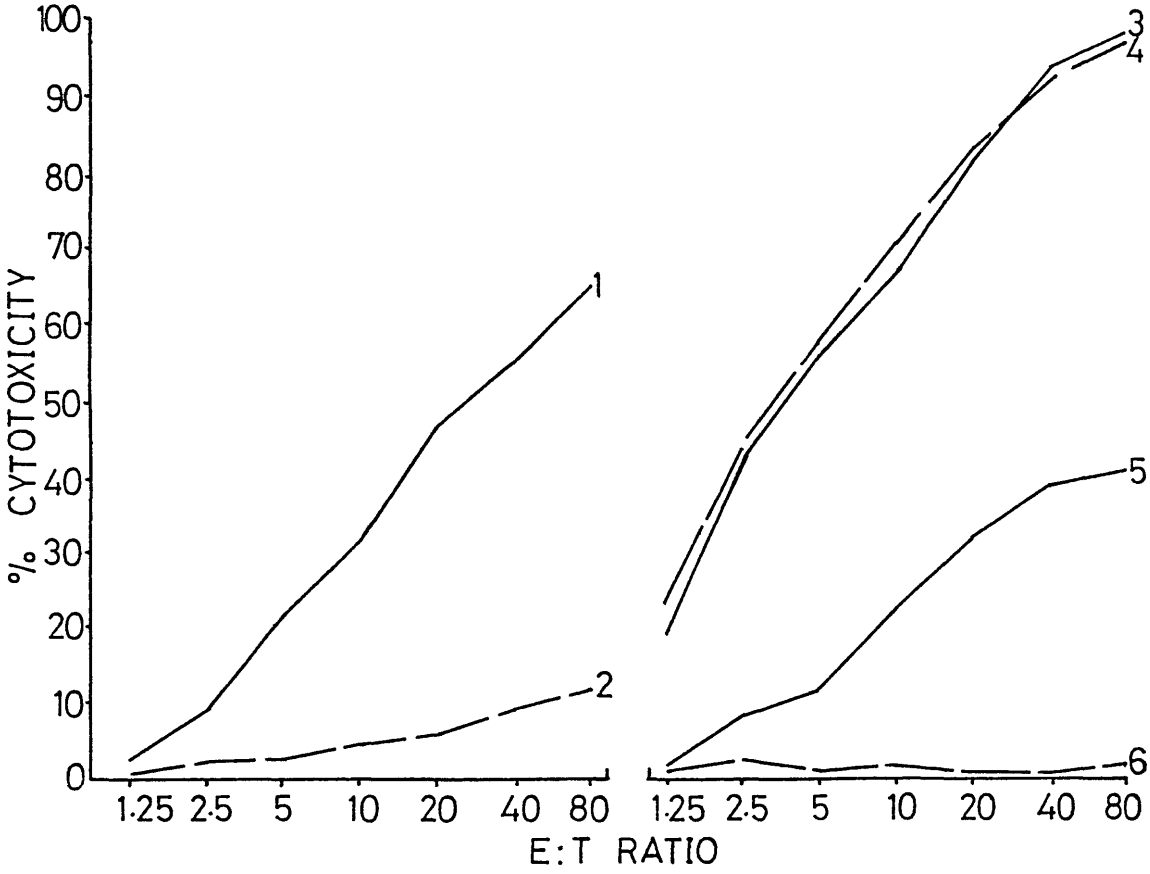

100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
2
3
4
5
6
1·25 2·5 5 10 20 40 80
1·25 2·5 5 10 20 40 80
E:T RATIO

Figure 3.4.13.

Combined effects of silica and ↺-carrageenan on spontaneous cytotoxicity in vitro

Silica (100 µg/ml) and ↺-carrageenan (100 µg/ml) were added to 3-day cultures of undepleted PBMC, alone or in combination.

Line 1: control; untreated cells

2: carrageenan added to culture

3: silica added to culture

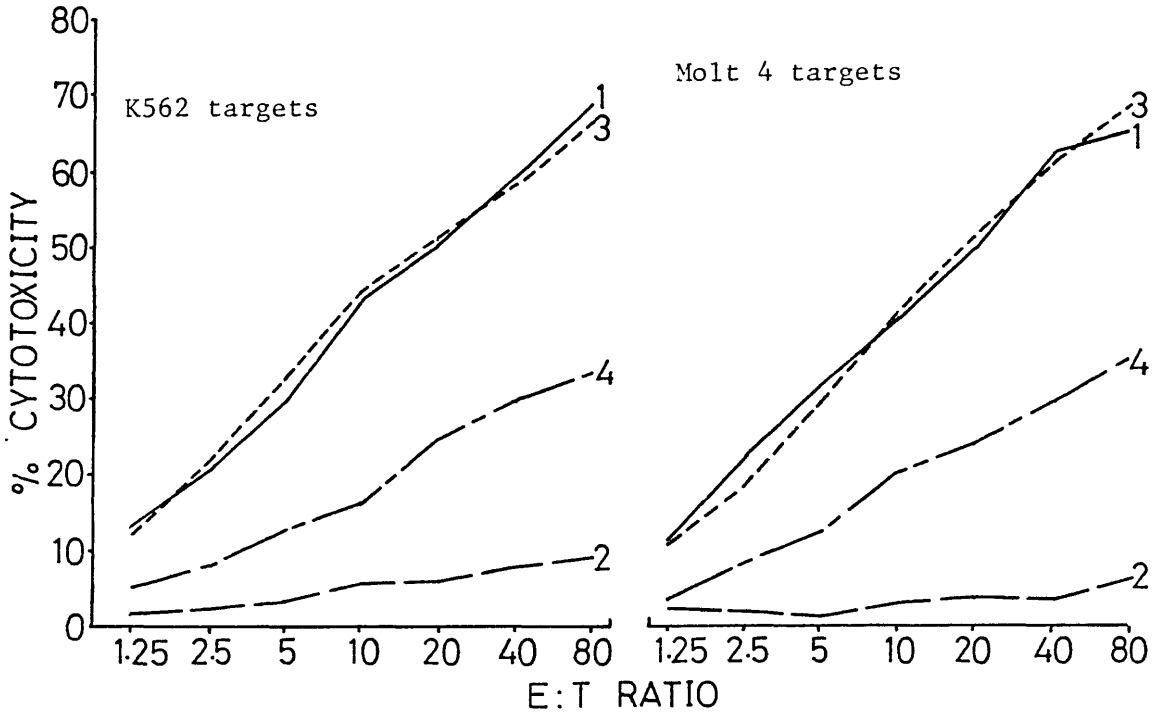
4: silica and carrageenan added to culture simultaneously

CULTURE	1	2	3	4
% recovery	92	90	87	85
% viability	90	89	90	91

TARGET CELLS	K562	Molt 4
Spontaneous release	11%	10%

DONOR AB

DURATION OF CULTURE 3 days


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
K562 targets
Molt 4 targets
1
3
4
2
1·25 2·5 5 10 20 40 80
E:T RATIO

obtained. This study was extended by pre-exposing cells to silica for 24 hours prior to treatment with carrageenan, then culturing for a further 3 days after addition of this agent. As shown in Figure 3.4.14., such pre-exposure resulted in improvement in the protective effect of silica. It is suggested that this may reflect destruction of a large proportion of the carrageenan-sensitive accessory population, insufficient cells remaining to mediate full suppression of cytotoxicity after addition of this agent. Such an effect is compatible with other results obtained in this system, and supports the earlier hypothesis that carrageenan is acting through marcophage-like accessory cells present in suspensions of PBMC, without destroying this population.

3.4.3. ZYMOSAN

3.4.3.1. Effects of zymosan on spontaneous cytotoxic activity in vitro

Different concentrations of zymosan were added to 24-hour cultures of PBMC to establish the dose-effect relationship for this material. However, zymosan had little effect on viable cell recovery or cytotoxic activity over this culture period, as shown in Figure 3.4.15. Fresh uncultured cells were also unaffected by addition of zymosan to the cytotoxicity assay, indicating that this agent does not influence mature effector cells in this system.

Zymosan particles have been used to stimulate murine phagocytic cells in vitro (Schnyder and Baggiolini, 1978). Previous work using this culture system indicated that cells of the monocyte-macrophage lineage are not involved in the spontaneous cytotoxic activity expressed by PBMC after short-term culture, so it is perhaps not surprising that zymosan failed to influence cytotoxicity over this time period. However,

Figure 3.4.14

Effect of pre-exposing PBMC to silica before treatment with ι-carrageenan

Undepleted PBMC were cultured with silica (100 µg/ml) for 24 hours, then exposed to ι-carrageenan (100 µg/ml) for the remaining 3 days of culture.

Line 1: control; untreated cells cultured for 4 days

2: carrageenan added on day 0

3: silica added on day 0

4: carrageenan added on day 1

5: silica added on day 1

6: silica and carrageenan both added on day 0

7: silica and carrageenan both added on day 1

8: silica added on day 0, then carrageenan added on day 1

CULTURE	1	2	3	4	5	6	7	8
% recovery	88	86	90	87	91	87	89	86
% viability	91	89	88	91	89	90	90	86

TARGET CELLS	Molt 4
Spontaneous release	11%
DONOR	VS
DURATION OF CULTURE	4 days

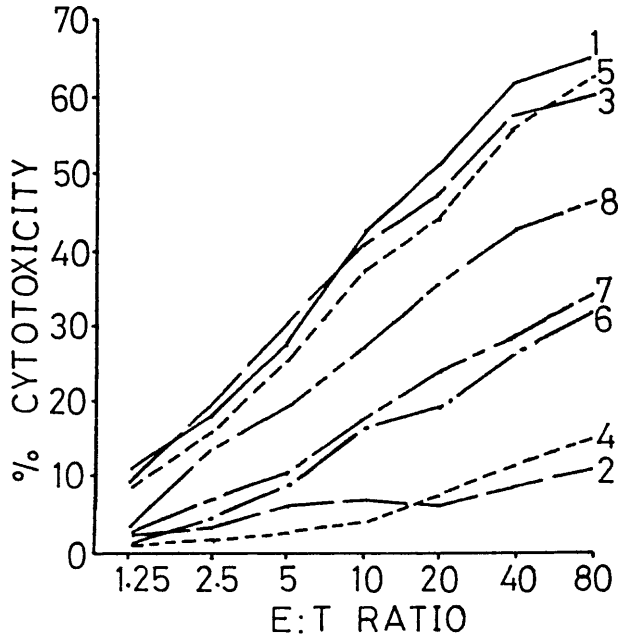

70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25
2·5
5
10
20
40
80
E:T RATIO
1
5
3
8
7
6
4
2

Figure 3.4.15.

Effect of zymosan on spontaneous cytotoxicity after short-term culture

PBMC were cultured for 24 hours with varying concentrations of zymosan.

Line 1: control; no zymosan added to culture

2: 100 µg zymosan/ml

3: 200 µg zymosan/ml

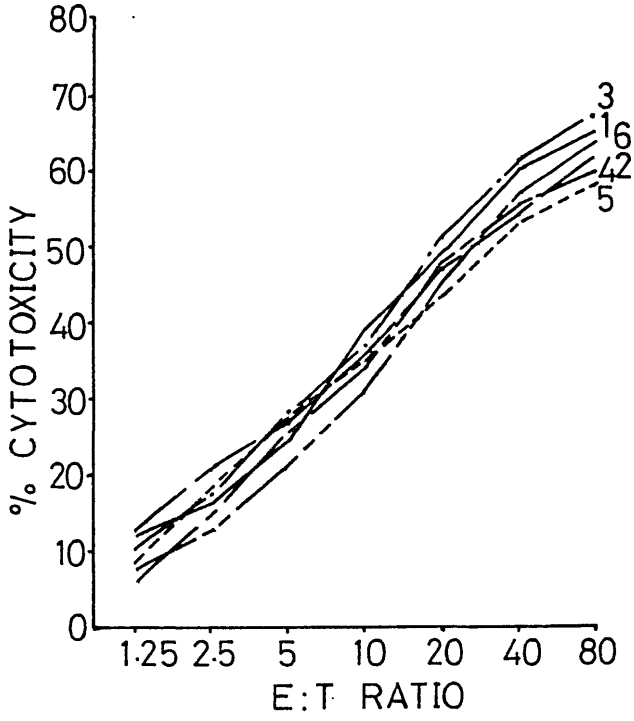
4: 400 µg zymosan/ml

5: 800 µg zymosan/ml

6: 400 µg zymosan/ml added to fresh uncultured cells for the duration of the cytotoxicity assay

CULTURE	1	2	3	4	5
% recovery	84	89	83	87	86
% viability	87	90	85	91	88

TARGET CELLS	Molt 4
Spontaneous release	9%
DONOR	AB
DURATION OF CULTURE	24 hours


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25 2·5 5 10 20 40 80
E:T RATIO
3
1
6
4
2
5

experiments presented in sections 3.3.2.1. and 3.3.2.2. indicated that such cells may participate in the generation and maintenance of cytotoxic activity after longer periods in vitro, and it was therefore suggested that an extended culture period may be required for manifestation of any zymosan-induced effects in this system. A range of concentrations of zymosan was therefore added to cells at the start of a 3-day culture, after which their cytotoxic activity was compared with that of fresh cells from the same donor.

Results obtained in such an experiment are presented in Figure 3.4.16. Zymosan was found to exert a dose-dependent suppressive effect on cytotoxicity over this prolonged culture period, while fresh uncultured cells remained unaffected. There was no evidence of a toxic effect of zymosan on the non-adherent cells, or on adherent cells recovered at the end of the culture period; the observed suppression cannot therefore be attributed to destruction of the effector or accessory population.

Repeat experiments using cells from different donors confirmed these results, and an optimal concentration of zymosan was selected for further work. Subsequent experiments presented in this section were therefore performed using zymosan at a final concentration of 400 μg/ml culture fluid.

3.4.3.2. Time-course of zymosan-induced suppression of spontaneous cytotoxicity

The time-course of zymosan-induced suppression in this system was investigated by adding a single dose of this agent to culture at initiation, or at 24-hour intervals after this. The results of such an experiment are shown in Figure 3.4.17. When added at the start of the culture period, zymosan caused full suppression of cytotoxicity, without affecting viable cell recovery. However, if zymosan was added to cultures

Figure 3.4.16.

Effect of zymosan on spontaneous cytotoxicity after 3-day culture

Zymosan was added to 3-day cultures of PBMC at initiation, to give the following range of final concentrations.

Line 1: control; no zymosan added to culture

2: 100 µg zymosan/ml

3: 200 µg zymosan/ml

4: 400 µg zymosan/ml

5: 800 µg zymosan/ml

6: fresh uncultured cells, no zymosan added

7: 400 µg zymosan/ml added to fresh uncultured cells for the duration of the cytotoxicity assay

CULTURE	1	2	3	4	5
% recovery	91	89	84	86	88
% viability	91	91	92	89	90

TARGET CELLS	K562
Spontaneous release	13%
DONOR	LR
DURATION OF CULTURE	3 days

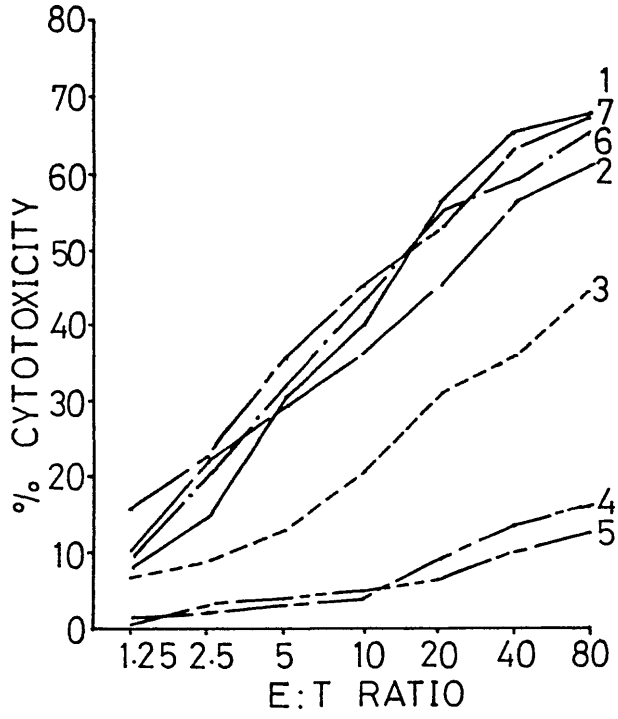

80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
7
6
2
3
4
5
1·25 2·5 5 10 20 40 80
E:T RATIO

Figure 3.4.17.

Time-course of zymosan-induced suppression of spontaneous cytotoxicity in vitro

Zymosan was added to cultures of PBMC at initiation or at 24-hour intervals after this, to give a final concentration of 400 µg/ml. Zymosan was also added to some previously untreated cells for the duration of the cytotoxicity assay.

Line 1: control; no zymosan added to culture

2: zymosan added on day 0

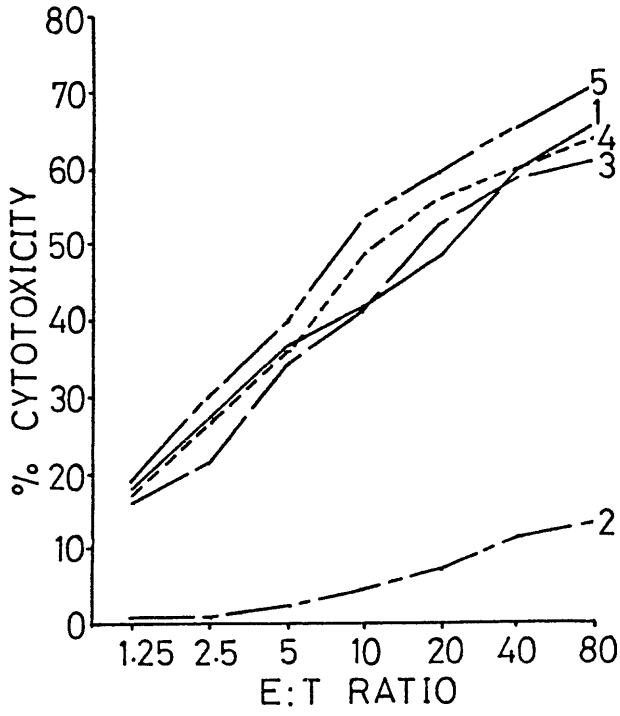
3: zymosan added on day 1

4: zymosan added on day 2

5: zymosan added to cytotoxicity assay on day 3

CULTURE	1	2	3	4
% recovery	90	88	85	87
% viability	87	90	93	92

TARGET CELLS	K562
Spontaneous release	10%
DONOR	VS
DURATION OF CULTURE	3 days


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25
2·5
5
10
20
40
80
E:T RATIO
5
1
4
3
2

on day 1, 24 hours after initiation, no suppression was observed. Further delay in treatment also failed to influence cytotoxic activity. These results suggest the existence of a zymosan-sensitive period during the early stages of culture, and provide further evidence that this agent does not act directly on the cytotoxic effector cells in this system. A repeat experiment was set up with an extended culture period of 5 days. As shown in Figure 3.4.18., full suppression of cytotoxicity was again obtained when zymosan was added at initiation. However, comparable suppression was also found after addition of zymosan on day 1 or day 2 of culture, and only when treatment was delayed until day 3 (72 hours after initiation) was activity of control levels obtained.

The results of these time-course experiments are comparable to those obtained using carrageenan (section 3.4.2.3.), and suggest that the zymosan-sensitive phase does not occur specifically at the start of the culture period, but rather is determined by the interval between administration of this agent and assay of the cells for cytotoxic activity, suppression being obtained if zymosan is present for the final 3 days of culture. This time-course coincides with the 3-day maturation period of effector cells in vitro proposed in section 3.3.2.2., in which adherent cells may play a regulatory role, and is thus compatible with the hypothesis that the suppressive effects of zymosan are mediated by such an accessory population.

3.4.3.3. Role of adherent cells in zymosan-induced suppression of spontaneous cytotoxicity

Carbonyl iron powder was used to deplete PBMC of the adherent fraction, prior to 3-day culture with or without zymosan. Removal of the adherent fraction from control cultures resulted in enhanced cytotoxic activity, as shown in Figure 3.4.19; this is in accordance with results presented in section 3.3.2.1. Addition of zymosan to unfractionated

Figure 3.4.18.

Time-course of zymosan-induced suppression of spontaneous cytotoxicity after 5-day culture

Line 1: control; no zymosan added to culture

2: zymosan added on day 0

3: zymosan added on day 1

4: zymosan added on day 2

5: zymosan added on day 3

6: zymosan added on day 4

7: zymosan added to cytotoxicity assay on day 5

CULTURE	1	2	3	4	5	6
% recovery	89	88	88	87	89	90
% viability	87	91	90	91	88	89

TARGET CELLS	K562
Spontaneous release	16%
DONOR	FL
DURATION OF CULTURE	5 days

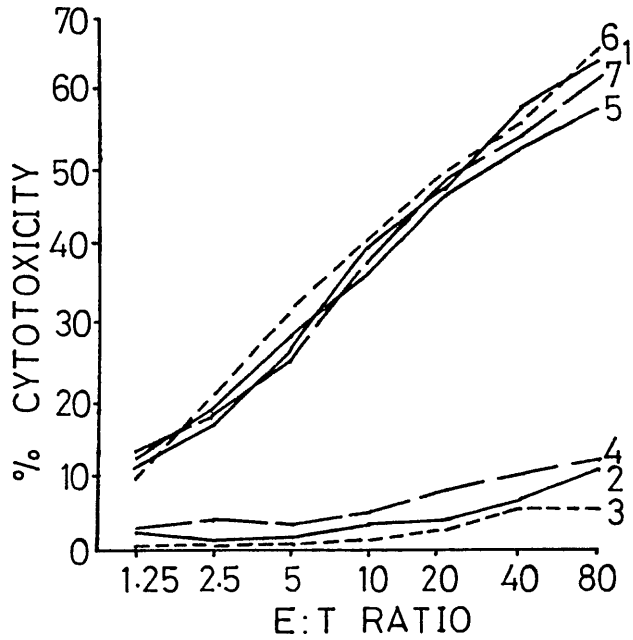

70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25
2·5
5
10
20
40
80
E:T RATIO
6
1
7
5
4
2
3

Figure 3.4.19.

Role of adherent cells in zymosan-induced suppression of spontaneous cytotoxicity

Undepleted PBMC and cells depleted of the adherent fraction using carbonyl iron powder were cultured for 3 days in the presence of 400 µg zymosan/ml.

Line 1: control; undepleted cells, no zymosan added to culture

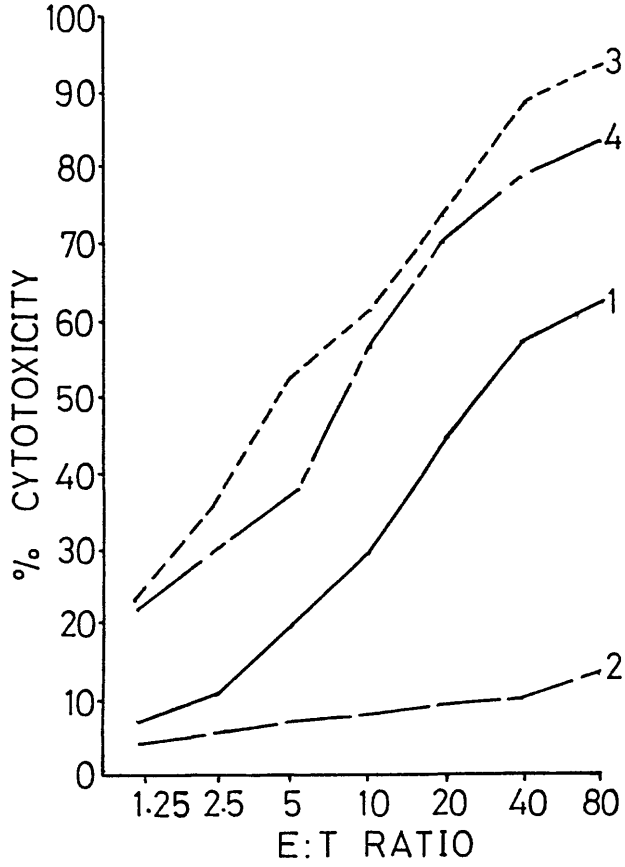
2: undepleted cells + zymosan

3: carbonyl iron-treated cells, no zymosan added

4: carbonyl iron-treated cells + zymosan

CULTURE	1	2	3	4
% recovery	83	81	87	88
% viability	87	82	90	91

TARGET CELLS	K562
Spontaneous release	11%
DONOR	HB
DURATION OF CULTURE	3 days


100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25 2·5 5 10 20 40 80
E:T RATIO
1
2
3
4

cultures caused full suppression of cytotoxicity, while this agent had little effect on cultures depleted of the adherent fraction, suggesting that such cells are essential for expression of zymosan-induced effects.

The involvement of adherent cells in zymosan-induced suppression was further investigated after separating this population by adherence to plastic (section 2.2.2.5.). This procedure yielded cultures either depleted or enriched for adherent cells, and these were maintained for a further 3 days with or without zymosan. As shown in Figure 3.4.20., results obtained from such cultures were comparable to those found using carbonyl iron powder, zymosan having little effect on cytotoxicity in the absence of adherent cells. The activity of untreated cultures enriched for adherent cells was suppressed in comparison to unfractionated controls (as described in sections 3.3.2.2., 3.3.2.3.), and addition of zymosan to such cultures resulted in complete abrogation of cytotoxicity.

From these results, it was concluded that the suppressive effects of zymosan in this system are mediated by adherent cells; however, suppression does not reflect destruction of this population. Experiments presented in section 3.4.1.1. revealed a selective toxic effect of silica particles towards phagocytic cells in this culture system, and this effect was therefore exploited to further investigate the role of adherent cells in zymosan-induced suppression. Unfractionated PBMC were cultured for 3 days in the presence of silica and/or zymosan. As shown in Figure 3.4.21., the suppressive effect of zymosan over this time period was reduced in the presence of silica, presumably due to destruction of the zymosan-sensitive phagocytic cells. However, some suppression was still obtained. It was suggested that simultaneous addition of the two agents may enable zymosan to interact with some of the sensitive cells prior to their destruction by silica, and a further experiment was therefore set up in which cells were pre-exposed to silica for 24 hours before addition

Figure 3.4.20.

Role of adherent cells in zymosan-induced suppression of spontaneous cytotoxicity

PBMC were separated into adherent and non-adherent fractions over a 3-day period. Fresh undepleted PBMC from the same donor were used to establish cultures enriched for adherent cells, and the different culture combinations were maintained for a further 3 days with or without 400 μg zymosan/ml.

Line 1: control; undepleted cells cultured for 6 days, no zymosan added

2: undepleted cells cultured for 6 days, zymosan present for final 3 days

3: depleted cells cultured for 3 days after removal of the adherent fraction (6 days total), no zymosan added

4: as for line 3, with zymosan present for final 3 days

5: PBMC enriched for adherent cells, no zymosan added

6: PBMC enriched for adherent cells, zymosan present for final 3 days of culture

CULTURE	1	2	3	4	5	6
% recovery	89	88	92	90	91	90
% viability	93	92	94	88	90	93

TARGET CELLS	K562
Spontaneous release	16%
DONOR	FL
DURATION OF CULTURE	6 days

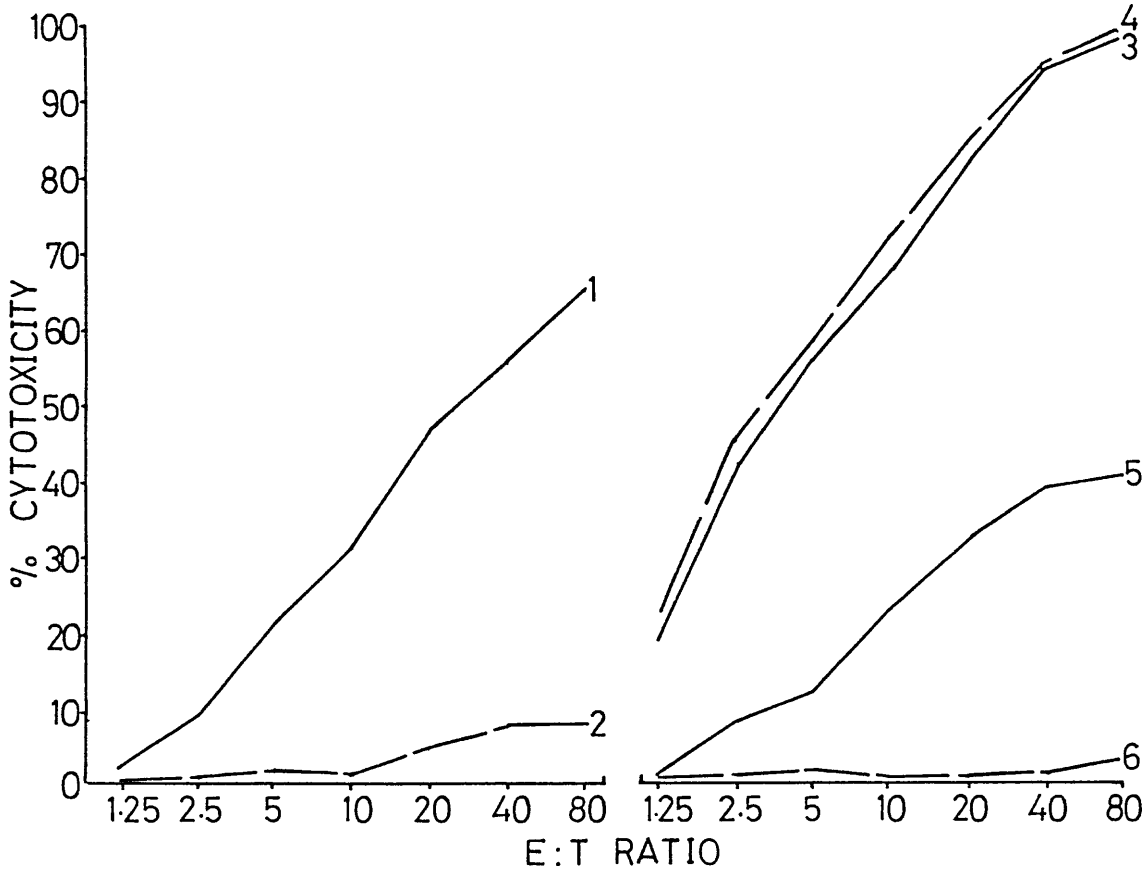

100
90
80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
2
3
4
5
6
1·25 2·5 5 10 20 40 80
1·25 2·5 5 10 20 40 80
E:T RATIO

Figure 3.4.21.

Combined effects of silica and zymosan on spontaneous cytotoxicity in vitro

Silica (100 µg/ml) and zymosan (400 µg/ml) were added to 3-day cultures of undepleted PBMC, alone or in combination.

Line 1: control; untreated cells

2: zymosan added to culture

3: silica added to culture

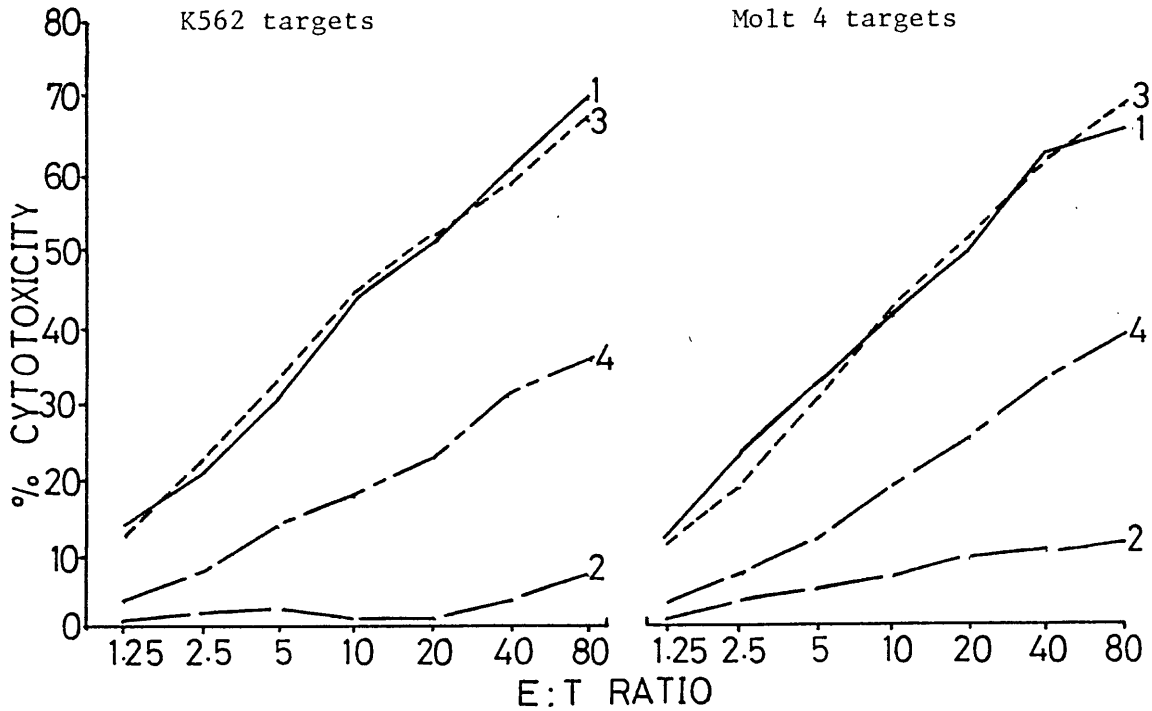
4: silica and zymosan added to culture simultaneously

CULTURE	1	2	3	4
% recovery	92	88	87	86
% viability	90	92	90	89

TARGET CELLS	K562	Molt 4
Spontaneous release	11%	10%

DONOR AB

DURATION OF CULTURE 3 days


K562 targets
Molt 4 targets
% CYTOTOXICITY
80
70
60
50
40
30
20
10
0
1
2
3
4
1·25 2·5 5 10 20 40 80
E:T RATIO

of zymosan, after which they were cultured for a further 3 days. Such delay between addition of the two agents resulted in an improved protective effect of silica, as shown in Figure 3.4.22., supporting the proposed mechanism of interaction of these materials.

The results presented in this section are comparable to those obtained using carrageenan. Both agents appear to act through an adherent cell displaying some of the properties of the monocyte-macrophage lineage, without destroying such cells. It is therefore suggested that the suppressed cytotoxic activity observed in the presence of these materials reflects an alteration of accessory cell function.

3.4.4. LATEX

3.4.4.1. Effects of latex on spontaneous cytotoxic activity in vitro

Latex particles were added to 3-day cultures of PBMC to give a range of final concentrations. From the results of depletion experiments using carbonyl iron powder (section 3.3.2.1.), it was estimated that approximately 10% of the PBMC suspension displayed adherent/phagocytic properties, and this figure was used to calculate a dose of latex particles per accessory cell.

As shown in Figure 3.4.23., latex particles had no effect on cytotoxicity, even at the highest dose of 500 particles per accessory cell. However, there was some evidence of non-selective toxicity at this dose. From these results it was concluded that ingestion of an inert particle does not result in suppression of cytotoxic activity, and no further experiments were performed with this material.

Figure 3.4.22.

Effect of pre-exposing PBMC to silica before treatment with zymosan

Undepleted PBMC were cultured with silica (100 µg/ml) for 24 hours, then exposed to zymosan (400 µg/ml) for the remaining 3 days of culture.

Line 1: control; untreated cells cultured for 4 days

2: zymosan added on day 0

3: silica added on day 0

4: zymosan added on day 1

5: silica added on day 1

6: silica and zymosan both added on day 0

7: silica and zymosan both added on day 1

8: silica added on day 0, then zymosan added on day 1

CULTURE	1	2	3	4	5	6	7	8
% recovery	88	87	90	88	91	88	88	90
% viability	91	90	88	89	89	89	87	87

TARGET CELLS	Molt 4
Spontaneous release	11%
DONOR	VS
DURATION OF CULTURE	4 days

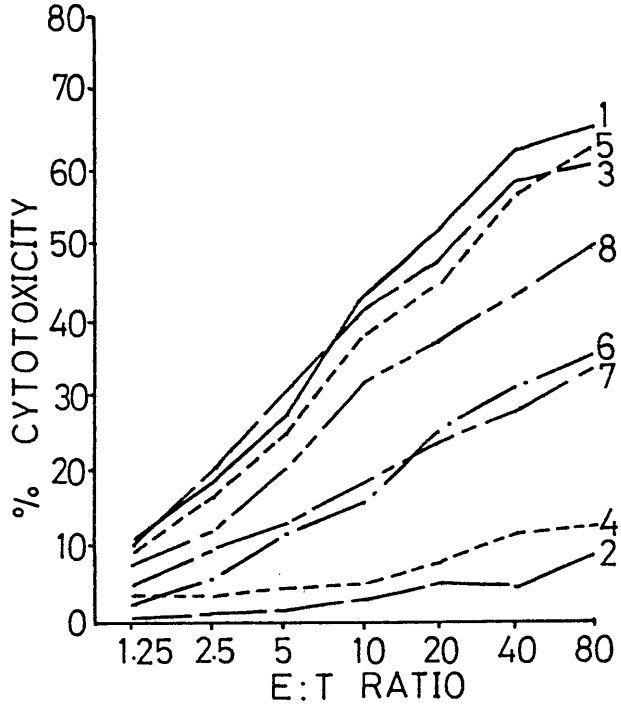

80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
5
3
8
6
7
4
2
1.25
2.5
5
10
20
40
80
E:T RATIO

Figure 3.4.23.

Effect of latex particles on spontaneous cytotoxicity in vitro

Latex particles were added to 3-day cultures of PBMC to give a range of final concentrations. As the particles were initially suspended in water, a "solvent" control was included for the highest concentration.

Line 1: control; no latex added to culture

2: water control

3: 25 latex particles per accessory cell

4: 50 latex particles per accessory cell

5: 100 latex particles per accessory cell

6: 200 latex particles per accessory cell

7: 500 latex particles per accessory cell

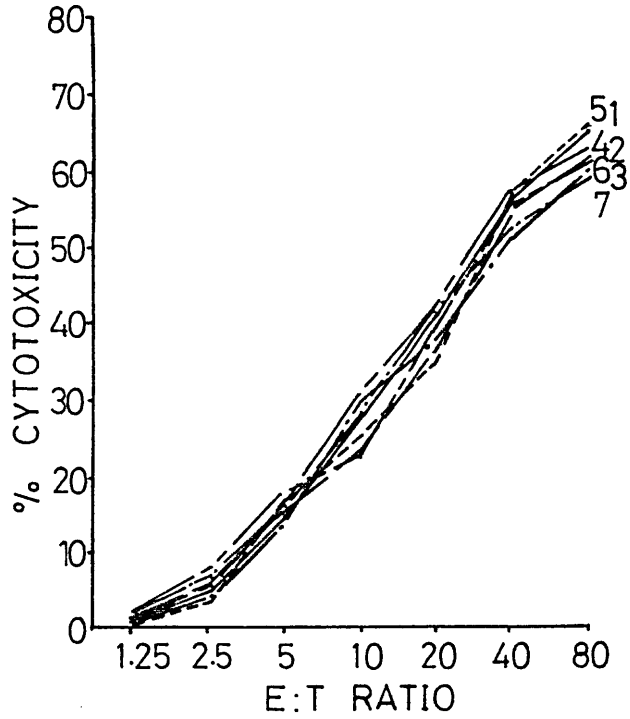
CULTURE	1	2	3	4	5	6	7
% recovery	85	75	74	75	71	72	69
% viability	92	95	94	92	87	85	80

TARGET CELLS K562

Spontaneous release 8%

DONOR VS

DURATION OF CULTURE 3 days


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1.25
2.5
5
10
20
40
80
E:T RATIO
51
42
63
7

3.5. INVOLVEMENT OF POLYUNSATURATED FATTY ACID METABOLITES IN THE SPONTANEOUS CYTOTOXIC ACTIVITY OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS *IN VITRO*

3.5.1. PROSTAGLANDIN E_1 AND ANTI-PGE ANTISERUM

3.5.1.1. Effects of exogenous PGE_1 on spontaneous cytotoxic activity *in vitro*

3.5.1.2. Time-course of PGE_1-induced suppression of spontaneous cytotoxic activity

3.5.1.3. Effects of anti-PGE antiserum and macrophage-active agents on spontaneous cytotoxic activity *in vitro*

3.5.2. PROSTACYCLIN, CARBACYCLIN AND ANTI-PGI_2 ANTISERUM

3.5.2.1. Effects of exogenous PGI_2 and carbacyclin on spontaneous cytotoxic activity *in vitro*

3.5.2.2. Effects of exogenous PGI_2 and anti-PGI_2 antiserum on spontaneous cytotoxic activity *in vitro*

3.5.2.3. Effects of anti-PGI_2 antiserum and macrophage-active agents on spontaneous cytotoxic activity *in vitro*

3.5.2.4. Combined effects of anti-PGE and anti-PGI_2 antisera on carrageenan- and zymosan-treated cultures

3.5.1. PROSTAGLANDIN E_1 AND ANTI-PGE ANTISERUM

3.5.1.1. Effects of exogenous PGE_1 on spontaneous cytotoxic activity in vitro

Solutions of PGE_1 were prepared as described in section 2.2.1.11.1., and were added to 3-day cultures of PBMC to give a range of final concentrations. The results of such an experiment are shown in Figure 3.5.1. Exogenous PGE_1 suppressed cytolytic activity in a dose-dependent manner, with complete abrogation of cytotoxicity at the highest dose (1×10^{-6} molar). Viable cell recovery was unaffected by PGE_1, and ethanol controls indicated that the observed suppression was entirely due to the prostaglandin. Further experiments were therefore performed with PGE_1 at a final concentration of 1×10^{-6} molar.

3.5.1.2. Time-course of PGE_1-induced suppression of spontaneous cytotoxic activity

The time-course of PGE_1-induced suppression was investigated by adding exogenous PGE_1 to cells at the initiation of culture or at 24-hour intervals after this. As shown in Figure 3.5.2., PGE_1 added at the start of a 3-day culture caused full suppression of cytotoxicity. If PGE_1 treatment was delayed for 24 hours, partial suppression was still obtained; however, PGE_1 added on or after day 2 of culture failed to influence cytotoxic activity. These results suggest that PGE_1 must be present during the early part of the culture to exert its suppressive effects, and imply that this material does not act directly on mature cytolytic cells in this system.

Previous experiments using carrageenan and zymosan (sections 3.4.2.3., 3.4.3.2.) indicated that the period of culture sensitive to suppressive influences is related to the subsequent interval between treatment and assay, rather than the delay between initiation of culture and addition of the active agent. A repeat experiment was therefore

Figure 3.5.1.

Effect of exogenous PGE_1 on spontaneous cytotoxicity in vitro

PGE_1 was added to PBMC at the start of a 3-day culture, to give a range of final concentrations. Ethanol controls were included in the experiment.

Line 1: control; untreated culture

2: ethanol control for 1×10^{-7} molar PGE_1

3: ethanol control for 1×10^{-6} molar PGE_1

4: 1×10^{-8} molar PGE_1

5: 1×10^{-7} molar PGE_1

6: 1×10^{-6} molar PGE_1

CULTURE	1	2	3	4	5	6
% recovery	82	85	81	90	91	95
% viability	89	88	92	89	92	87

TARGET CELLS	K562	Molt 4
Spontaneous release	19%	8%

DONOR FL

DURATION OF CULTURE 3 days

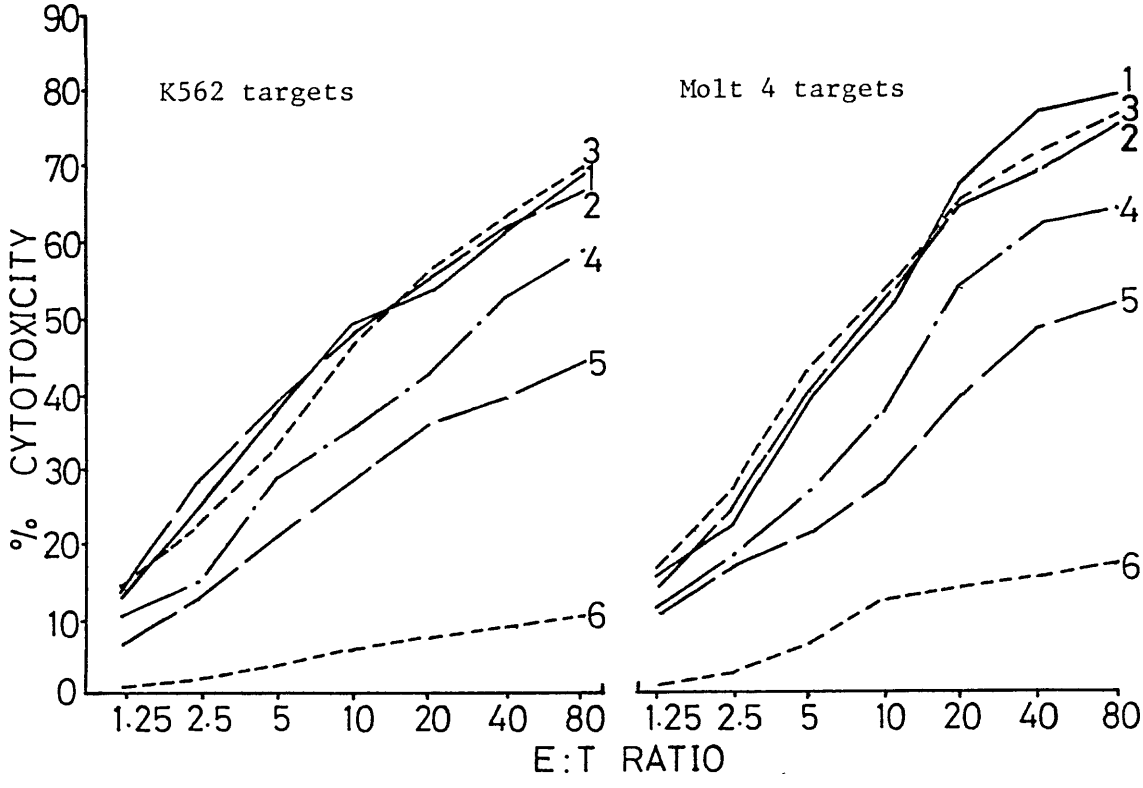

K562 targets
Molt 4 targets
% CYTOTOXICITY
E:T RATIO
0
10
20
30
40
50
60
70
80
90
1·25
2·5
5
10
20
40
80
1
2
3
4
5
6

Figure 3.5.2.

Time-course of PGE_1-induced suppression of spontaneous cytotoxicity in 3-day cultures

PGE_1 was added to PBMC at the initiation of culture or at 24-hour intervals after this, to give a final concentration of 1×10^{-6} molar. The same dose of PGE_1 was also added to some previously untreated cells for the duration of the cytotoxicity assay.

Line 1: control; untreated culture

2: PGE_1 added on day 0

3: PGE_1 added on day 1

4: PGE_1 added on day 2

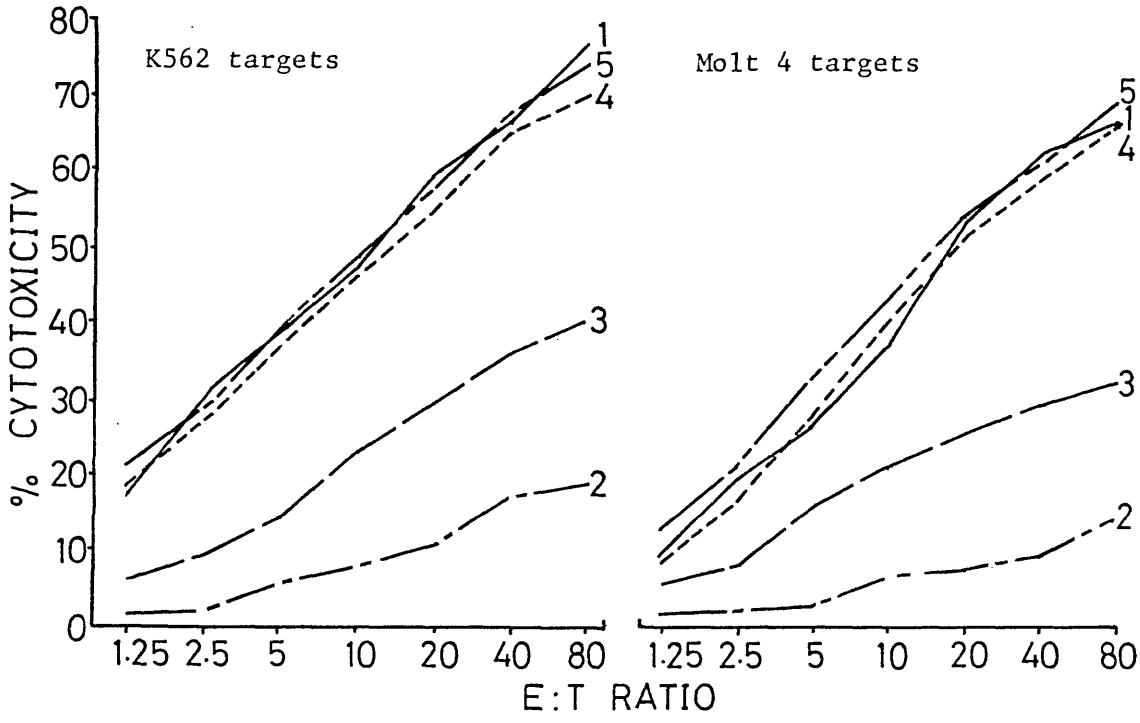
5: PGE_1 added to cytotoxicity assay on day 3

CULTURE	1	2	3	4
% recovery	91	89	87	89
% viability	87	90	88	87

TARGET CELLS	K562	Molt 4
Spontaneous release	7%	14%

DONOR FL

DURATION OF CULTURE 3 days


K562 targets
Molt 4 targets
% CYTOTOXICITY
E:T RATIO
0
10
20
30
40
50
60
70
80
1·25
2·5
5
10
20
40
80
1
2
3
4
5

set up, in which PGE_1 was added to cells at 24-hour intervals throughout a 5-day culture. As shown in Figure 3.5.3., the time-course of PGE_1-induced suppression over this extended culture period supports the above hypothesis; full suppression was now obtained if PGE_1 was added on day 2, and only when this material was added on or after day 4 did it fail to exert such an effect.

3.5.1.3. Effects of anti-PGE antiserum and macrophage-active agents on spontaneous cytotoxic activity *in vitro*

The possible involvement of PGE_1 in mediating the effects of carrageenan and zymosan was investigated using an anti-PGE antiserum, which had been shown to neutralise exogenous PGE_1 *in vitro* using murine cells (section 2.5.3.3.). In the experiments described here, a single dilution of antiserum was added to PBMC, together with doses of carrageenan or zymosan previously found to be suppressive. As shown in Figure 3.5.4., a 1 in 50 (v/v) dilution of anti-PGE antiserum afforded some protection against the suppressive effects of carrageenan and zymosan, while control rabbit serum had no restorative effect. With both macrophage-active agents, cytotoxicity at an effector to target ratio of 80:1 was restored to approximately 50% of the control level in the presence of antiserum. Insufficient antiserum was available to extend this study using a lower dilution; however, previous experiments on the murine MLC suggest that full restoration of cytotoxic activity cannot be attained, even with a dilution of antiserum capable of neutralising 1×10^{-6} molar exogenous PGE_1 (section 2.5.3.4.). While supporting the involvement of E-series prostaglandins in carrageenan- and zymosan-induced suppression, this incomplete protection may reflect the presence of other inhibitory mediators, released by the phagocytic population after ingestion of these agents.

Figure 3.5.3.

Time-course of PGE_1-induced suppression of spontaneous cytotoxicity in 5-day cultures

PGE_1 was added to PBMC at initiation or at 24-hour intervals throughout a 5-day culture, to give a final concentration of 1×10^{-6} molar. The same dose of PGE_1 was also added to some previously untreated cells for the duration of the cytotoxicity assay.

Line 1: control; untreated culture

2: PGE_1 added on day 0

3: PGE_1 added on day 1

4: PGE_1 added on day 2

5: PGE_1 added on day 3

6: PGE_1 added on day 4

7: PGE_1 added to cytotoxicity assay on day 5

CULTURE	1	2	3	4	5	6
% recovery	88	87	87	90	86	88
% viability	91	89	88	86	92	88

TARGET CELLS	Molt 4
Spontaneous release	15%
DONOR	FL
DURATION OF CULTURE	5 days

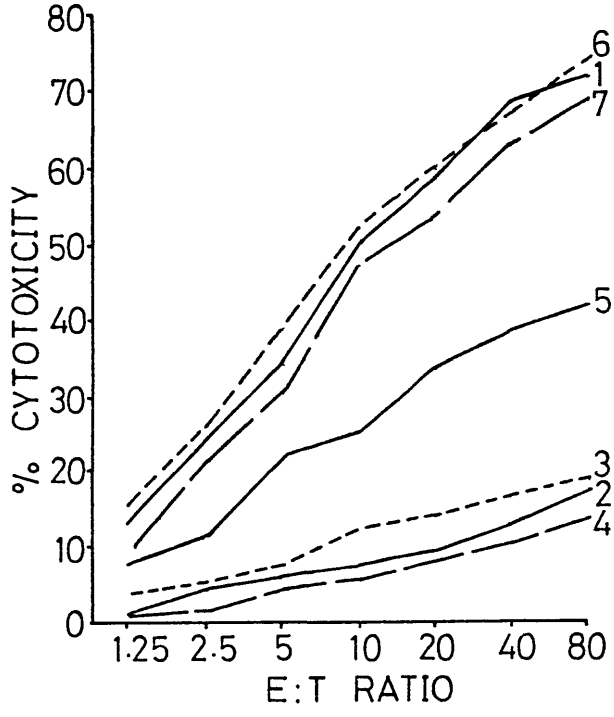

80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25
2·5
5
10
20
40
80
E:T RATIO
6
1
7
5
3
2
4

Figure 3.5.4.

Effect of anti-PGE antiserum on carrageenan- and zymosan-induced suppression of spontaneous cytotoxicity

Anti-PGE antiserum and control rabbit serum were added to carrageenan- and zymosan-treated PBMC, to give a final dilution of 1 in 50.

Line 1: control; untreated culture

2: 100 μg λ-carrageenan/ml

3: 400 μg zymosan/ml

4: anti-PGE antiserum

5: control serum

6: carrageenan + anti-PGE antiserum

7: zymosan + anti-PGE antiserum

8: carrageenan + control serum

9: zymosan + control serum

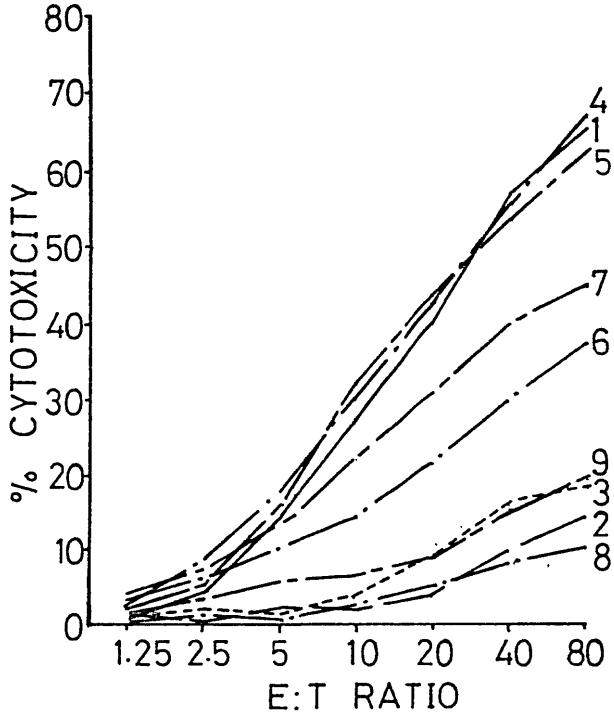
CULTURE	1	2	3	4	5	6	7	8	9
% recovery	85	92	87	89	84	83	86	85	82
% viability	92	93	91	97	88	92	93	90	87

TARGET CELLS K562

Spontaneous release 8%

DONOR VS

DURATION OF CULTURE 3 days


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1.25 2.5 5 10 20 40 80
E:T RATIO
4
1
5
7
6
9
3
2
8

3.5.2. PROSTACYCLIN, CARBACYCLIN AND ANTI-PGI_2 ANTISERUM

3.5.2.1. Effects of exogenous PGI_2 and carbacyclin on spontaneous cytotoxic activity in vitro

Solutions of PGI_2 and the synthetic analogue carbacyclin were prepared as described in section 2.2.1.11.2., and were added to 3-day cultures of PBMC to give a range of final concentrations. Previous experiments using exogenous PGE_1 revealed that a dose of 1×10^{-6} molar was sufficient to abrogate cytotoxic activity; however, in the murine system, PGI_2 failed to suppress cytotoxicity at this dose (section 2.5.4.1.). Consequently, PGI_2 was added to PBMC to give a maximum final concentration of 1×10^{-5} molar, while the less active analogue was tested from 1×10^{-4} molar down to 1×10^{-6} molar.

As shown in Figure 3.5.5., carbacyclin again failed to influence cytotoxic activity. However, some suppression was obtained with the highest dose of PGI_2, cytotoxicity being reduced to approximately 50% of the control value at an effector to target ratio of 80:1. From these results, it was concluded that PGI_2 can suppress the cytotoxic activity of PBMC *in vitro*, though this effect was limited, and only observed with relatively high concentrations. In view of reports comparing the relative activities of PGI_2 and E-series prostaglandins in other systems (reviewed by Moncada and Vane, 1979; Higgs *et al*, 1981), this low activity of PGI_2 is somewhat surprising, and may reflect cross-reaction between this material and receptors for E-series prostaglandins, rather than an effect of PGI_2 *per se*. However, the instability of PGI_2 under culture conditions should be borne in mind, as rapid breakdown of this material (within a few minutes at 37°C) could easily prevent full expression of any suppressive activity. Additional experiments to investigate the role of PGI_2 in carrageenan- and zymosan-induced suppression of spontaneous cytotoxicity *in vitro* were therefore performed using an antiserum against this material.

Figure 3.5.5.

Effect of exogenous prostacyclin and carbacyclin on spontaneous cytotoxicity in vitro

Prostacyclin and carbacyclin were added to 3-day cultures of PBMC, to give a range of final concentrations. As these compounds were initially dissolved in Tris buffer, a solvent control was included in the experiment.

Line 1: control; untreated culture

2: Tris buffer control

3: 1×10^{-5} molar PGI_2

4: 1×10^{-6} molar PGI_2

5: 1×10^{-7} molar PGI_2

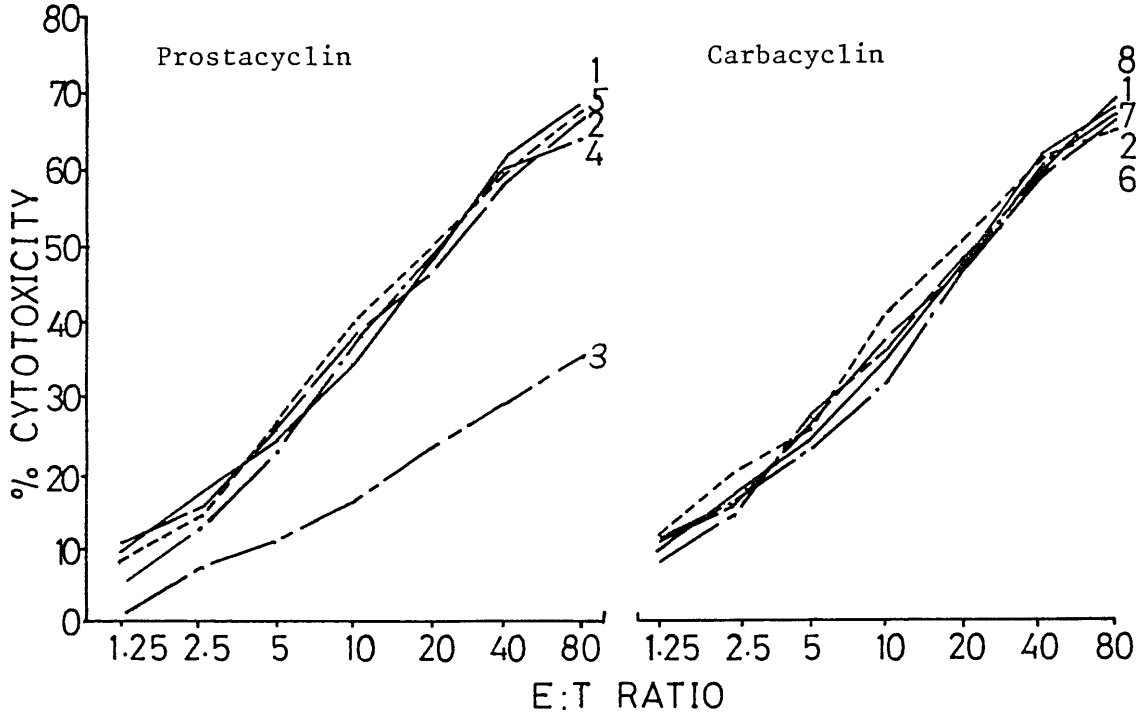
6: 1×10^{-4} molar carbacyclin

7: 1×10^{-5} molar carbacyclin

8: 1×10^{-6} molar carbacyclin

CULTURE	1	2	3	4	5	6	7	8
% recovery	83	85	90	89	86	89	90	88
% viability	92	90	88	87	88	91	90	89

TARGET CELLS	Molt 4
Spontaneous	13%
DONOR	VS
DURATION OF CULTURE	3 days


Prostacyclin
Carbacyclin
% CYTOTOXICITY
E:T RATIO
0
10
20
30
40
50
60
70
80
1.25
2.5
5
10
20
40
80
1
5
3
2
4
8
1
7
2
6

3.5.2.2. Effects of exogenous PGI_2 and anti-PGI_2 antiserum on spontaneous cytotoxic activity *in vitro*

To determine the efficacy with which the anti-PGI_2 antiserum described in section 2.2.1.12.2. could neutralise exogenous PGI_2 in this culture system, the antiserum was added to PBMC at the start of a 3-day culture, together with a suppressive dose of PGI_2. In view of the limited supply of antiserum, only one dilution was tested.

As shown in Figure 3.5.6., anti-PGI_2 antiserum at a final dilution of 1 in 50 (v/v) restored the cytotoxic activity of PGI_2-treated cells to control levels, while control rabbit serum had no effect. It was therefore concluded that this antiserum can be used to neutralise the suppressive effects of PGI_2 *in vitro*. Subsequent experiments were performed to study the combined effects of anti-PGI_2 antiserum and macrophage-active agents in this culture system.

3.5.2.3. Effects of anti-PGI_2 antiserum and macrophage-active agents on spontaneous cytotoxic activity *in vitro*

Anti-PGI_2 antiserum and suppressive doses of carrageenan or zymosan were added to PBMC at the start of the 3-day culture period, to determine the role of endogenous PGI_2 in mediating the effects of these agents *in vitro*.

As shown in Figure 3.5.7., the antiserum provided considerable protection against the suppressive effects of carrageenan and zymosan, while control rabbit serum failed to influence the cytotoxic activity of cells exposed to these agents. However, cytotoxicity was not fully restored, in agreement with results obtained using anti-PGE antiserum in this system, and using both antisera in the murine system. It is therefore suggested that a lower dilution of anti-PGI_2 antiserum would be unlikely to increase the restoration observed here; however, insufficient antiserum was available to test this hypothesis.

Figure 3.5.6.

Effect of anti-PGI_2 antiserum on PGI_2-induced suppression of spontaneous cytotoxicity

Anti-PGI_2 antiserum and control rabbit serum were added to PGI_2-treated PBMC at the initiation of culture, to give a final dilution of 1 in 50.

Line 1: control; untreated culture

2: 1×10^{-5} molar PGI_2

3: Tris buffer control

4: anti-PGI_2 antiserum

5: control serum

6: PGI_2 + anti-PGI_2 antiserum

7: PGI_2 + control serum

CULTURE	1	2	3	4	5	6	7
% recovery	88	91	90	89	86	88	91
% viability	92	89	90	88	90	89	90

TARGET CELLS	Molt 4
Spontaneous release	9%
DONOR	FL
DURATION OF CULTURE	3 days

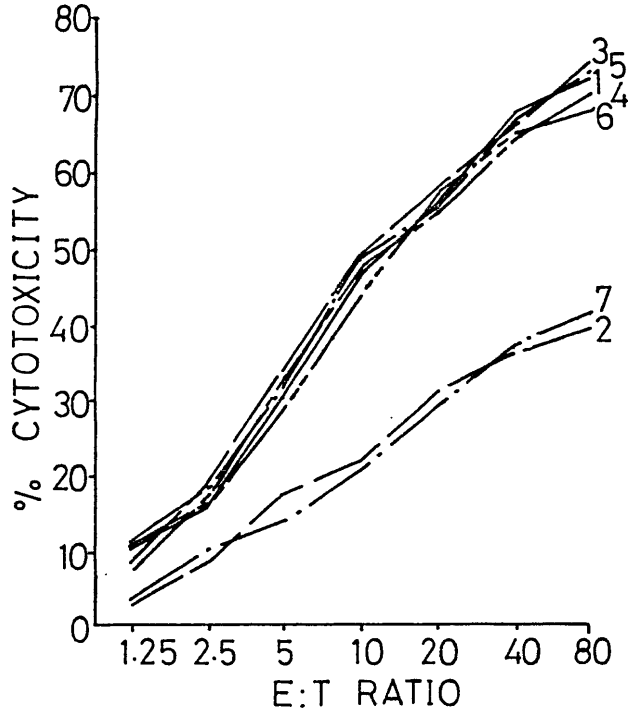

80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1·25 2·5 5 10 20 40 80
E:T RATIO
3
5
1
4
6
7
2

Figure 3.5.7.

Effect of anti-PGI_2 antiserum on carrageenan- and zymosan-induced suppression of spontaneous cytotoxicity

Anti-PGI_2 antiserum and control rabbit serum were added to carrageenan- and zymosan-treated PBMC, to give a final dilution of 1 in 50.

Line 1: control; untreated culture

2: 100 µg ι-carrageenan/ml

3: 400 µg zymosan/ml

4: anti-PGI_2 antiserum

5: control serum

6: carrageenan + anti-PGI_2 antiserum

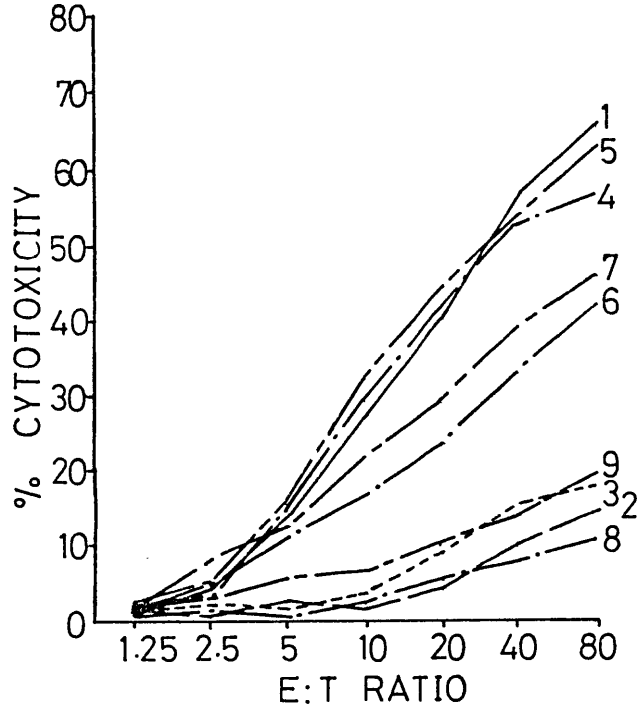
7: zymosan + anti-PGI_2 antiserum

8: carrageenan + control serum

9: zymosan + control serum

CULTURE	1	2	3	4	5	6	7	8	9
% recovery	85	92	87	85	84	82	81	85	82
% viability	92	93	91	90	88	90	94	90	87

TARGET CELLS	K562
Spontaneous release	8%
DONOR	VS
DURATION OF CULTURE	3 days


80
70
60
50
40
30
20
10
0
% CYTOTOXICITY
1
5
4
7
6
9
3
2
8
1.25 2.5 5 10 20 40 80
E:T RATIO

3.5.2.4. Combined effects of anti-PGE and anti-PGI_2 antisera on carrageenan- and zymosan-treated cultures

Results presented in this section indicate that both E- and I-series prostaglandins may play a role in mediating the suppressive effects of carrageenan and zymosan on spontaneous cytotoxic activity. However, in the presence of these macrophage-active agents, antisera binding to either PGE or PGI_2 were unable to effect a response of control levels. It was therefore suggested that both prostanoids may be involved in the suppressive mechanism. To test this hypothesis, an experiment was set up in which cells exposed to carrageenan or zymosan were treated with a combination of the two antisera, both at a final dilution of 1 in 50 (v/v).

As shown in Figure 3.5.8., simultaneous addition of both antisera at the initiation of culture had no further restorative effect on carrageenan- and zymosan-treated cells, the cytotoxic activity obtained being equivalent to that found using only one antiserum. This is in agreement with results obtained using the murine culture system (Figure 2.5.13.). In view of the limited supply of antisera, it was not possible to repeat this experiment using lower dilutions; however, previous results indicate that this would not produce complete restoration of cytotoxicity. It is therefore suggested that E- and I-series prostaglandins, while clearly involved in mediating the suppression induced by carrageenan and zymosan, are not solely responsible for this effect. Several other soluble factors derived from accessory cell types may be proposed as immuno-suppressive agents (discussed in more detail in section 1.3.); however, their involvement in this culture system is beyond the scope of this study.

Figure 3.5.8.

Combined effects of anti-PGE and anti-PGI_2 antisera on carrageenan- and zymosan-induced suppression of spontaneous cytotoxicity

Culture	% recovery	% viability
1	97	91
2	92	87
3	95	89
4	90	92
5	96	89
6	95	90
7	93	91
8	96	90
9	91	94
10	95	88
11	89	93
12	93	90

TARGET CELLS	K562
Spontaneous release	13%
DONOR	FL
DURATION OF CULTURE	3 days

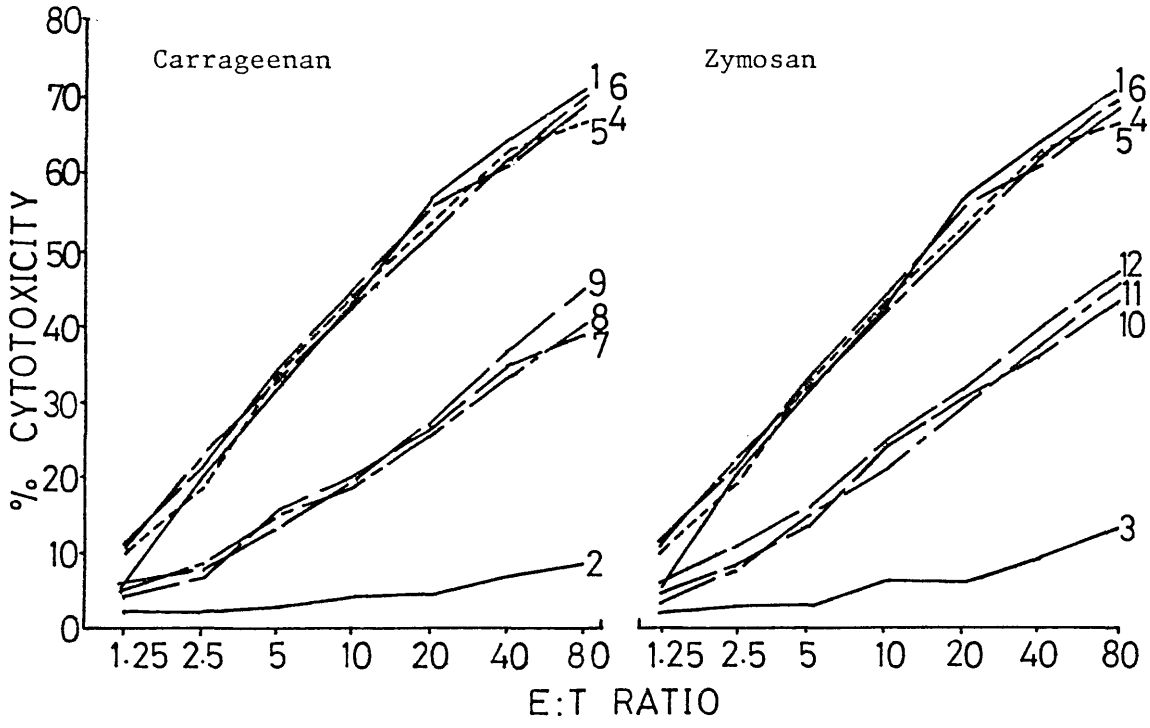


Figure 3.5.8.

Combined effects of anti-PGE and anti-PGI_2 antisera on carrageenan- and zymosan-induced suppression of spontaneous cytotoxicity

Anti-PGE and anti-PGI_2 antisera were added to carrageenan- and zymosan-treated PBMC, alone or in combination, to give final dilutions of 1 in 50.

Line 1: control; untreated culture

2: 100 µg λ-carrageenan/ml

3: 400 µg zymosan/ml

4: anti-PGE antiserum

5: anti-PGI_2 antiserum

6: anti-PGE + anti-PGI_2 antisera

7: carrageenan + anti-PGE antiserum

8: carrageenan + anti-PGI_2 antiserum

9: carrageenan + both antisera

10: zymosan + anti-PGE antiserum

11: zymosan + anti-PGI_2 antiserum

12: zymosan + both antisera

3.6. DISCUSSION

The results presented earlier in this chapter suggest a regulatory role for adherent cells in the non-specific spontaneous cytotoxicity expressed by normal human peripheral blood mononuclear cells (PBMC) *in vitro*. The significance and possible mechanism of such regulatory activity will now be considered.

3.6.1. PRELIMINARY WORK ON THE CULTURE SYSTEM

Optimal culture conditions for maintaining normal human PBMC *in vitro* were determined as described in section 3.3.1.. Mononuclear cells separated on Ficoll were cultured under conditions similar to those found satisfactory using murine leucocytes, the cells being adjusted to a final concentration of $2x10^6$/ml in medium supplemented with 5% FCS. However, 2-ME proved highly toxic to human cells even over a short culture period (Figure 3.3.1.), and was therefore omitted from the experimental protocol. This is compatible with other studies of human leucocytes, which are rarely cultured in the presence of 2-ME (Kendall and Targan, 1980; Hofman *et al*, 1981; Jondal *et al*, 1981; Lopez-Botet *et al*, 1982; Opremcak *et al*, 1981). Wherever possible, cultured cells were assayed for cytotoxic activity against both Molt 4 and K562 target cells, taken from recent sub-cultures. In most cases, comparable values for specific lysis were obtained against each target.

Using this assay system, it was shown that the spontaneous cytotoxicity expressed by normal human PBMC can be maintained *in vitro*. By assaying cytotoxicity on a daily basis, such activity was found to follow an interesting time-course, declining sharply after

24 hours of culture and then recovering to its original level after 3 days in vitro (Figures 3.3.2., 3.3.3.); this level of cytotoxicity was then maintained throughout the remainder of the culture period (Figure 3.3.4.). These results may be summarised as shown in Figure 3.6.1.. Comparison of cell recovery and viability after different periods of culture revealed that the fall in cytotoxic activity on day 2 was not associated with extensive cell death; it may therefore reflect loss of lytic capacity rather than death of the effector population. A similar fall in the spontaneous cytotoxic activity of normal human PBMC during days 1-2 of culture has been reported by Jondal et al (1981), followed by recovery of this activity beyond the original level. Working on the spontaneous cytotoxicity exhibited by murine splenocytes in vitro, Cudkowicz and Hochman (1980) also observed a slight drop in activity during the initial 24 hours of culture, while Bartlett and Burton (1982) have described two types of spontaneously cytotoxic effector cell in mouse spleen (termed NK_A and NK_B cells), one of which (NK_A) loses over 80% of its lytic activity during 48-hour culture.

The recovery of cytotoxic activity after 3 or more days in culture may represent development of a new population of killer cells, perhaps via maturation or proliferation of immature or precursor cells during the first 3 days in vitro. This is compatible with a report by Ortaldo and Herberman (1980), demonstrating generation of a spontaneously cytotoxic effector cell in vitro from a precursor in the null cell population; the cultured cells resembled effector cells from fresh peripheral blood in their target cell specificity and surface markers. Jondal et al (1981) observed a similar recovery of the cytotoxic activity of human PBMC on days 3-7 of culture, and have attributed this effect to proliferation and/or activation of a

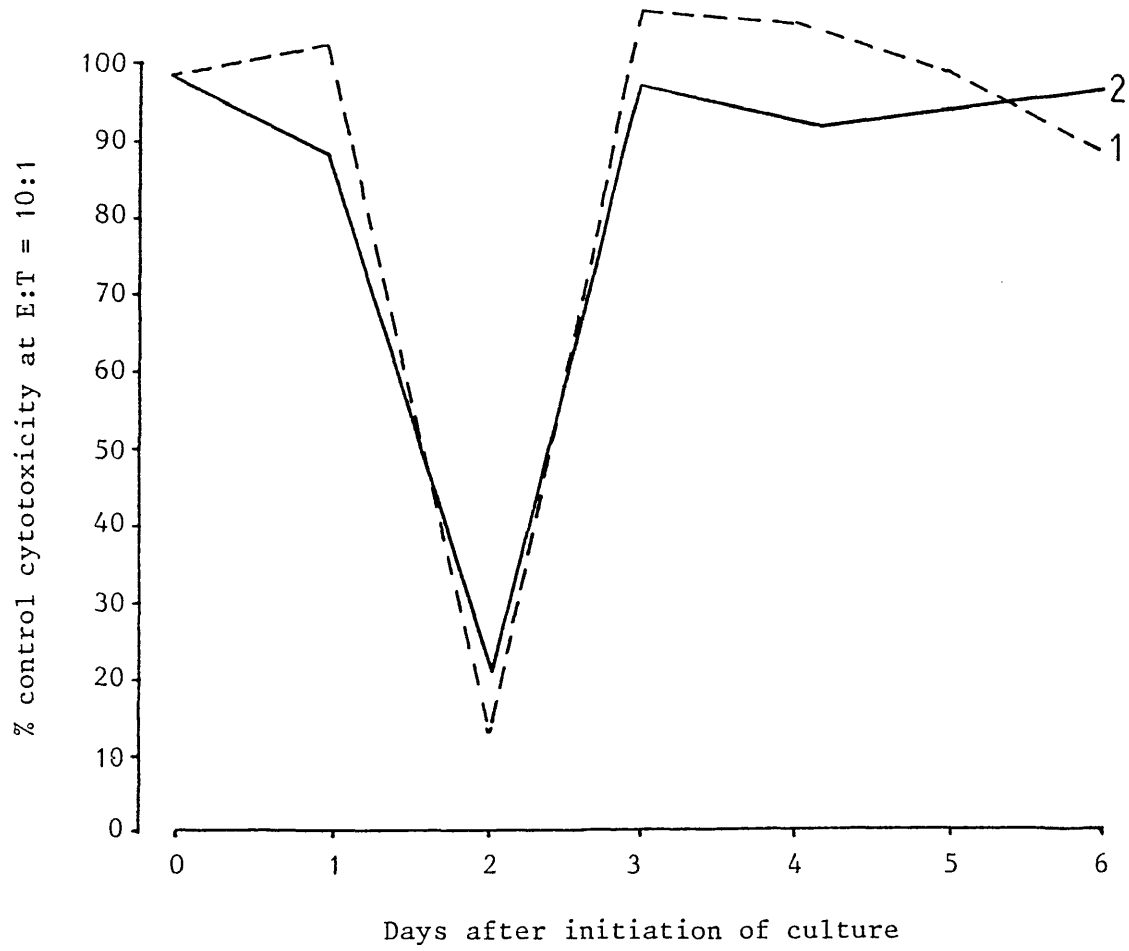


Figure 3.6.1.

Time-course of spontaneous cytotoxic activity of normal human PBMC maintained *in vitro*

Data taken from Figures 3.3.3. and 3.3.4.

Values for per cent specific lysis were calculated from regression analysis of the experimental data at an E:T ratio of 10:1; these values were then corrected against the control value of uncultured cells, which was set at 100%.

Line 1: PBMC assayed against K562 target cells

2: PBMC assayed against Molt 4 target cells

precursor population, while Bartlett and Burton (1982) demonstrated an increase in the cytotoxic activity of their murine NK_B cells as the culture progressed. Similarly, using effector cells enriched by binding to Molt 4 targets, Opremcak et al (1981) reported recovery of augmented spontaneous cytotoxicity following 18-hour culture of a population previously exhibiting a low level of killing. However, reports on the time-course of spontaneous cytotoxic activity in vitro are still limited as most investigators do not assay at daily intervals, and there is clearly scope for further work in this area.

It is possible that the cytotoxic cells arising in this culture system are not identical to the original effector cells, even though they are active against the same targets. Heterogeneity of cells mediating spontaneous cytotoxicity has been suggested by several workers for both human (Herberman et al, 1979; Saksela et al, 1979) and murine systems (Bartlett and Burton, 1982; Lust et al, 1981; Minato et al, 1981), and may reflect different stages of development of the cytotoxic cells or the presence of distinct sub-populations. Additional experiments to determine the precise nature of the cells involved here, for example with respect to their surface markers and their differentiation and maturation, may therefore provide an interesting basis for further study. In particular, the possible role of the lymphokine-activated killer (LAK) cells described in section 3.1. should be explored.

3.6.2. ROLE OF ADHERENT CELLS

The role of an adherent accessory population in the spontaneous cell-mediated cytotoxicity expressed by normal human PBMC in vitro was investigated as described in section 3.3.2.. Adherent cells were removed from cell suspensions either by incubation with carbonyl

iron powder, in which case they could not be recovered, or by separation in plastic culture plates; the latter method yielded enriched adherent and non-adherent fractions, which could then be further manipulated.

Freshly drawn cells and those cultured for 24 hours showed comparable levels of cytotoxic activity regardless of the presence or absence of adherent cells (Figures 3.3.5., 3.3.9.), confirming that the effector cells in this system reside in the non-adherent fraction. These results also indicate that adherent cells are not required for the survival of effector cells or maintenance of their cytotoxic activity in short-term cultures. However, the activity of cells harvested after 3 or more days in vitro may result from maturation or activation of a precursor population, as mentioned in section 3.6.1.. Such a developmental process is likely to be regulated in some manner, and it is suggested that this accessory function may be served by cells in the adherent fraction. Investigation of this hypothesis by carbonyl iron treatment of cell suspensions prior to 3- or 4-day culture revealed that such cells did indeed influence the level of cytotoxicity ultimately expressed by the effector cells (Figures 3.3.6., 3.3.7., 3.3.8.), though not in the manner expected in view of previous work using murine spleen cells. In the latter experiments, an absolute requirement for adherent cells was demonstrated (see section 2.6.2.); however, in the case of human PBMC removal of such cells prior to culture produced enhanced cytotoxic activity. This effect did not result from differences in cell survival, nor could it be attributed to enrichment of the effector or effector-precursor population in carbonyl iron-treated cultures (Figure 3.3.8.).

Similar results were obtained after separation of the adherent

fraction by plastic adherence over a 3-hour or 24-hour period (Figures 3.3.9., 3.3.10.). Using this separation technique, it was also possible to isolate the non-adherent cells at a later stage of culture, and subsequently it was shown that the suppressive effect of the adherent population did not depend on the length of the culture period per se, but rather was determined by the stage of culture at which these cells were removed. The results presented in Figure 3.3.11. indicate that adherent cell-mediated suppression of cytotoxic activity is not apparent if such cells are absent for a period of 3 days prior to assay, suggesting that effector cells harvested from such cultures have not been exposed to suppressive influences from the adherent population, and therefore must have undergone their sensitive "maturation" phase after removal of these cells. The time-course of this effect supports the hypothesis that effector cells are developing from non-lytic precursors during a period of approximately 3 days in vitro (Figure 3.6.1.), and indicates that there is a second maturation cycle in longer-term cultures, generating effector cells on day 6. Preliminary data which are not presented here show that comparable levels of cytotoxic activity may be detected after 10 or 11 days culture, and it is suggested that a continuous process of effector cell maturation may be occurring in vitro. However, further experiments to investigate this phenomenon are required. In particular, $^{(3H)}$thymidine-uptake studies and the use of agents which eliminate proliferating cells (for example hydroxyurea, or bromodeoxyuridine followed by exposure to light) may yield useful information with respect to any proliferative processes involved. Using a similar culture system, Herberman et al (1979) demonstrated a requirement for protein synthesis and cell division in the differentiation of precursor

cells to lytic effector cells, although they also found that macrophages could enhance the level of killing, in direct contrast to the results obtained here. However, it should be pointed out that this positive modulation of cytotoxic activity was attributed to production of interferon in the presence of specific interferon inducers, and it may therefore not be directly comparable to the present study.

Further evidence for a suppressive role of adherent cells in this system was obtained from cultures which were enriched for the adherent fraction. Effector cells maintained in the presence of excess adherent cells exhibited reduced cytotoxic activity compared to controls (Figure 3.3.12.); this effect was apparent over the same time-course as before, with an interval of 3 days being required between adherent cell enrichment and assay. A similar effect was observed when cultures were enriched with adherent cells from a genetically dissimilar donor (Figure 3.3.13.), indicating that the suppressive action of these cells can operate across histocompatibility barriers.

It remains to be determined whether the adherent cell-mediated suppression of spontaneous cytotoxic activity observed in this culture system reflects a regulatory process occurring in vivo, or is confined to the in vitro situation. Hochman and Cudkowicz (1979) and Cudkowicz and Hochman (1980) reported generation of macrophage-like murine suppressor cells both in vivo and in vitro after exposure to hydrocortisone and iota carrageenan; these cells developed over a period of approximately 48 hours, and inhibited the spontaneous cytotoxicity of normal murine spleen cells when added to the cytotoxicity assay. Studies on human PBMC cultured in autologous serum have implicated the monocytic fraction as mediators of

spontaneous suppression of cytotoxic activity (Jondal et al, 1981), and removal of the MHC class II positive population from PBMC suspensions before assay has been shown to result in enhanced levels of killing (Breard et al, 1981). Similarly, Zarling et al (1975) reported increased spontaneous cytotoxic activity of mouse splenocytes after removal of carbonyl iron-adherent cells; these workers suggested that the adherent population may inhibit lysis by competing for target cell antigens. Seaman et al (1982) demonstrated that monocytes (and polymorphonuclear leucocytes) can suppress the spontaneous cytotoxic activity of human PBMC in vitro when stimulated to release reactive metabolites of molecular oxygen (ROI) (see section 1.3.2.); their confirmation of findings by Metzger et al (1980) that some ROI can induce oxygenation of polyunsaturated fatty acids (PUFA) is interesting in the light of experiments presented in sections 3.4. and 3.5. of this chapter, and discussed more fully in section 3.6.4.. The apparent absence of histocompatibility restrictions governing the interaction of effector cells and the adherent population in this system favours a mechanism involving secreted products as mediators of the suppressive effect, rather than cell-cell contact, and this hypothesis will be discussed in more detail in the remainder of this chapter.

3.6.3. EFFECTS OF MACROPHAGE-ACTIVE AGENTS

The involvement of adherent cells in the spontaneous cytotoxic activity of human PBMC in vitro was further explored by adding selective macrophage-active agents to cell cultures, as in the murine system (section 2.4.). In view of the apparent time-course of spontaneous cytotoxic activity in vitro (discussed in section

3.6.2.), these materials were added to cultures of 24 hours or 3 or more days. The results obtained from such experiments are presented in section 3.4..

Neither silica, carrageenan or zymosan had any effect on the cytotoxic activity of cells cultured for 24 hours. However, there was evidence of a non-selective toxic effect of silica at high doses (Figure 3.4.1.), and this material appeared to be toxic towards the adherent population at all doses tested. Subsequent experiments revealed that release of intracellular contents from damaged phagocytic cells was responsible for the toxicity observed towards the non-adherent fraction (Figure 3.4.4.). Such anti-macrophage activity is in agreement with results obtained using silica in the murine MLC, and concurs with the findings of other workers, as discussed in section 2.6.3.. Neither carrageenan or zymosan appeared to exert such a toxic effect on either the cytotoxic effector cells or the adherent population at the doses tested.

If these agents are indeed acting solely through the adherent population, then their failure to influence cytotoxicity during a 24-hour culture is not unexpected, and is compatible with the previous observation that macrophage-like cells do not influence such activity in short-term cultures. However, data presented in section 3.3.2. have demonstrated a role for adherent cells in cultures of 3 or more days; thus any effects of these agents mediated by the adherent population should become apparent during such longer-term cultures. Accordingly, subsequent experiments showed that iota and lambda carrageenan and zymosan suppressed cytotoxic activity in a dose-dependent manner when added to 3-day cultures (Figures 3.4.7., 3.4.16.), thus displaying similar behaviour to that observed in the murine system (Figures 2.4.5.,

2.4.11.). This suppression did not result from toxicity towards the effector population. However, silica was again unable to influence the level of killing (Figure 3.4.2.), indicating that ingestion of exogenous material per se was not responsible for the suppression induced by carrageenan and zymosan. A parallel experiment using latex particles as an inert phagocytic stimulus confirmed this observation (Figure 3.4.23.), in accordance with the results obtained using murine cells.

The failure of silica to influence cytotoxicity during a 3-day culture was somewhat surprising, as this experiment provided further evidence of the macrophage-toxic effect of this material. Previous results have demonstrated enhanced cytotoxic activity over this culture period in the absence of adherent cells (Figure 3.3.6.), and removal of this population by treatment with silica might be expected to yield a similar result. Further experiments revealed that cytotoxicity could be increased towards the expected level if the culture medium was replaced 24 hours after silica treatment (Figure 3.4.3.), suggesting that soluble factors present in the culture fluid, presumably derived from damaged phagocytic cells, were still able to limit the development of cytotoxic activity in the absence of an intact accessory population. However, such factors were apparently unable to induce any further suppression of cytotoxicity, as the activity of unwashed silica-treated cells was not suppressed with respect to that of control cultures. This finding contrasts with reports on the in vivo effects of silica in rodents, where suppression of spontaneous cytotoxicity was observed (Cudkowicz and Yung, 1977; Djeu et al, 1979; Kiessling et al, 1977; Oehler and Herberman, 1978). The reason for this disparity is unclear, and must await further studies using both human and rodent

cells; however, experiments by Oehler and Herberman (1978) using a rat system revealed that silica was not acting directly on the cytotoxic effector cells in vivo, but exerted a suppressive influence through a macrophage-like accessory population. The parallel between this observation and the results obtained in the current study supports the hypothesis that a phagocytic population is playing an accessory role in spontaneous cytotoxicity. Such a population may be capable of both positive and negative regulatory activity, depending on the balance of other intra- and extra-cellular factors, and this may account for the differing results obtained in vitro and in vivo, where the investigator may be assaying the sum of promoting and inhibitory influences. In addition, the nature of the effector and accessory cells under study must be carefully defined, as evidence exists that both these cell types belong to heterogeneous populations, the dominant characteristics of which may vary according to tissue source and experimental conditions (see sections 1.1.1., 3.1.).

Relatively few studies have been performed on the effects of carrageenan and zymosan on spontaneous cell-mediated cytotoxicity in vitro. Work using human tissue is restricted by availability and by difficulty in controlling for possibly influential factors between donors, while murine spontaneous cytotoxic activity is labile under normal culture conditions (Cudkowicz and Hochman, 1980). However, experiments on the in vivo effects of carrageenan in mice have revealed a suppressive effect on spontaneous cytotoxicity (Cudkowicz and Hochman, 1979; Cudkowicz and Yung, 1977; Hochman et al, 1981; Kiessling et al, 1977). This has been variously attributed to a direct toxic effect on the lytic cells (Kiessling et al, 1977) or on an essential accessory population (Cudkowicz and Yung, 1977;

Kiessling et al, 1977), or to the generation or activation of suppressive or regulatory cells (Hochman et al, 1981). As mentioned with respect to silica, such differing conclusions may reflect variation between the systems under study. While controlling for the fall in activity of untreated murine cells in culture, Cudkowicz and Hochman (1979, 1980) have demonstrated carrageenan-induced suppression of spontaneous cytotoxicity in vitro, and have ascribed this to the induction of macrophage-like suppressor cells. In this study, the enhanced cytotoxic activity possible following treatment with silica, together with the absence of any toxic effects of carrageenan and zymosan, strongly suggests that the suppression induced by these two agents does not result from damage to the phagocytic population. Although in contrast to much of the published work regarding carrageenan (discussed in section 2.6.3.), such findings are in agreement with results presented earlier using murine cells, where no evidence of macrophage-toxicity was obtained. Using human leucocytes, Quan and Bertin (1978) demonstrated depressed adherent and phagocytic activity by monocytes exposed to carrageenan in vitro, and Catalona et al (1978) reported decreased viability of such cells as measured by trypan blue exclusion. However, both these studies employed considerably higher doses of carrageenan than those used here (200-1000 ug/ml and 200-500 ug/ml respectively), and in view of this their results are perhaps not directly comparable.

Time-course experiments using carrageenan and zymosan indicated that these agents were only suppressive if present during the early stages of culture (Figures 3.4.9., 3.4.17.), supporting the previous observation that the cytotoxic effector cells were not directly influenced. Other workers have reported direct toxicity of iota

carrageenan towards the effector population in vitro (Catalona et al, 1978); however, this effect was only obtained in the presence of high carrageenan concentrations, and was not observed in the current study. The results obtained here using human cells are therefore comparable to those found in the murine MLC. However, with human PBMC the period during which carrageenan and zymosan were suppressive was found to vary according to the total duration of culture, as shown in Figure 3.6.2.. From these results it was concluded that the critical factor determining suppression was the length of time between addition of the agent to the culture and the subsequent cytotoxicity assay, an interval of 2-3 days being required for carrageenan and zymosan to exert their full effects. As in the murine system, the time during which cultures were susceptible to suppression by zymosan was slightly shorter than the carrageenan-sensitive period.

The time-course of carrageenan- and zymosan-induced suppression of cytotoxicity coincides with that of the adherent cell involvement in this culture system (section 3.6.2.), and reflects the period of effector cell maturation proposed in section 3.6.1.. Thus only those cells passing through this maturation phase in the presence of carrageenan or zymosan, and so susceptible to accessory cell influence, were subsequently found to exhibit suppressed cytotoxic activity. These results support the view that carrageenan and zymosan are acting through a macrophage-like accessory population. Further experiments involving depletion or enrichment of the adherent fraction confirmed this hypothesis; removal of adherent cells prior to culture abrogated the suppressive effects of these agents, while their potency was apparently increased in the presence of excess adherent cells (Figures 3.4.11., 3.4.12., 3.4.19.,

Figure 3.6.2.

Time-course of ι-carrageenan- and zymosan-induced suppression of spontaneous cytotoxicity in vitro

Data on time-course of carrageenan-induced suppression taken from Figures 3.4.9., 3.4.10., 3.4.12. and 3.4.14. Data on time-course of zymosan-induced suppression taken from Figures 3.4.17., 3.4.18., 3.4.20. and 3.4.22.

Per cent control cytotoxicity was calculated as described for Figure 3.6.1.

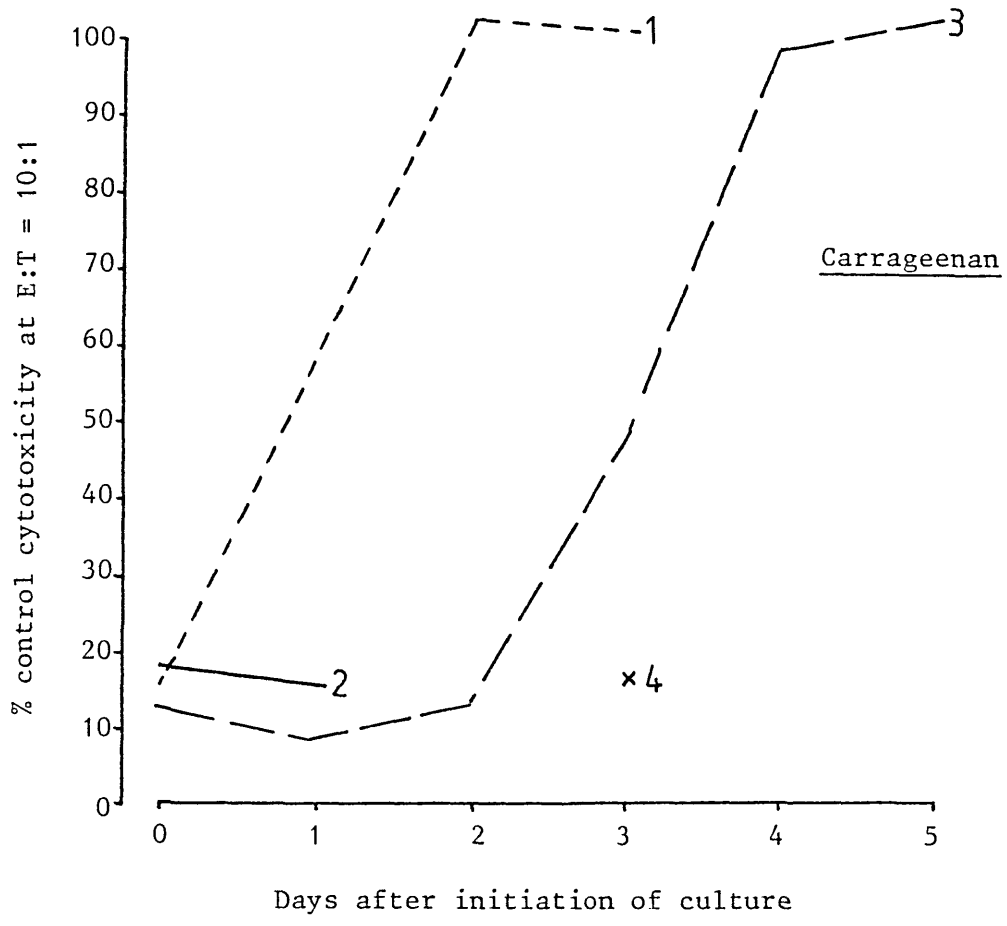
Line 1: 3-day culture

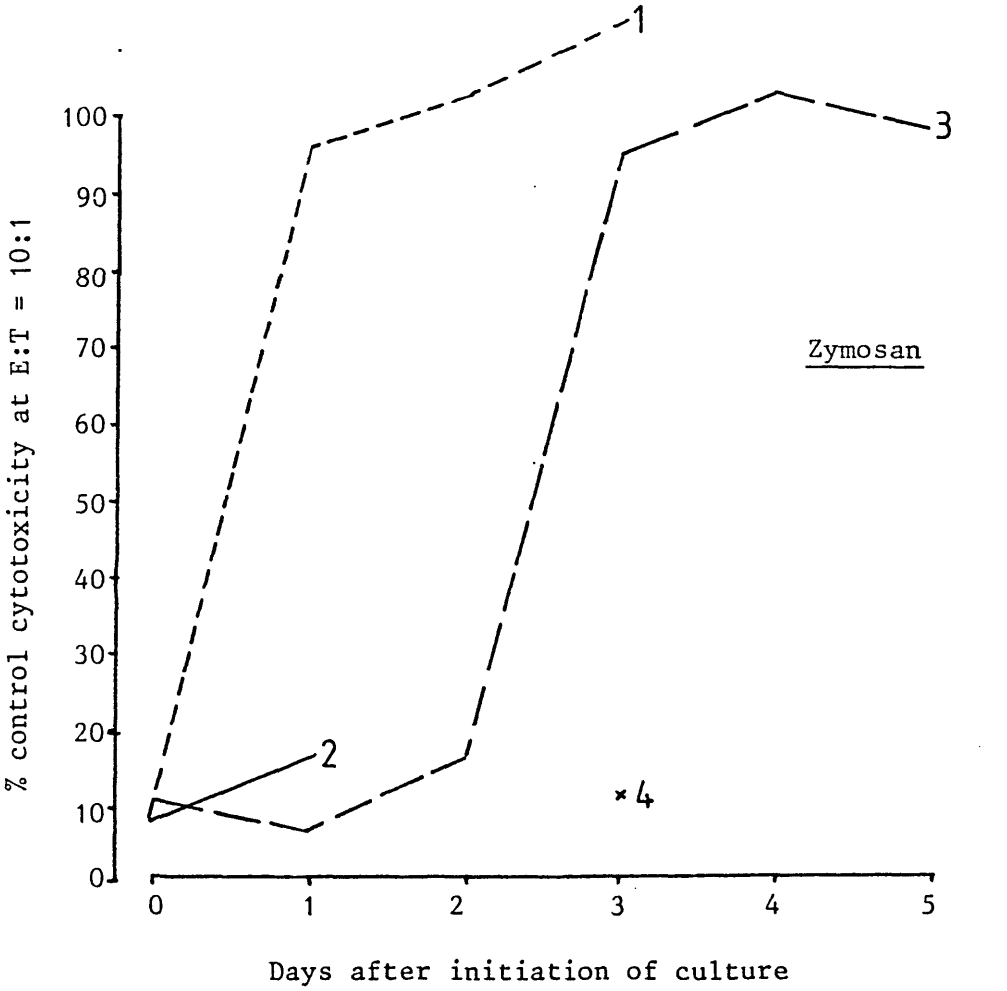
2: 4-day culture (no further additions)

3: 5-day culture

4: 6-day culture (no further additions)

Figure 3.6.2.


% control cytotoxicity at E:T = 10:1
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
1
2
3
×4
Carrageenan
Days after initiation of culture


% control cytotoxicity at E:T = 10:1
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
1
2
3
×4
Zymosan
Days after initiation of culture

3.4.20.). These effects on the level of lytic activity did not result from toxicity towards any of the cell populations involved.

The selective toxicity of silica particles towards the accessory population has already been discussed, and this property was employed to confirm the requirement for such cells in carrageenan- and zymosan-induced suppression of lytic activity. Simultaneous treatment of cultured cells with one of these materials and a macrophage-toxic dose of silica was shown to diminish the suppressive efficacy of the former (Figures 3.4.13., 3.4.21.), and this protective effect was more pronounced if silica treatment preceded addition of carrageenan or zymosan by 24 hours (Figures 3.4.14., 3.4.22.). Such data suggest that carrageenan and zymosan, when added at the same time as silica, were able to affect some phagocytic cells before these were destroyed by interaction with silica particles, leading to partial suppression of cytotoxicity. However, a delay of 24 hours after exposure to silica permitted destruction of a greater proportion of this population, with fewer phagocytes remaining to mediate the effects of carrageenan and zymosan, and under these conditions the suppressive effect was further diminished.

From the results presented in section 3.4., it is not possible to identify the mechanism(s) by which carrageenan and zymosan suppress spontaneous cytotoxicity <u>in vitro</u>. However, these agents have been shown to exert their effects through an adherent accessory population, during a critical period approximately 3 days before assay of the effector cells. Such findings show some parallels with observations made using murine cells (discussed in section 2.6.), where subsequent experiments indicated a role for metabolites of polyunsaturated fatty acids (PUFA). The involvement of such

materials in suppression of human spontaneous cytotoxic activity was therefore explored, and the results of such experiments are discussed in section 3.6.4..

3.6.4. INVOLVEMENT OF CYCLO-OXYGENASE PRODUCTS OF FATTY ACID METABOLISM

Results obtained in the murine culture system implicated prostaglandins (PG) as mediators of carrageenan- and zymosan-induced suppression of cell-mediated cytotoxicity (discussed in section 2.6.4.). These macrophage-active agents have been shown to exert a comparable suppressive influence on the spontaneous cytotoxicity of human PBMC in vitro, and the possibility of a similar mechanism was explored. Practical difficulties precluded use of anti-inflammatory drugs as tools in this investigation, and consequently the results presented in section 3.5. were obtained using exogenous prostanoids and antisera specific for these. In view of previous results concerning the time-course of spontaneous cytotoxic activity in vitro (sections 3.3.1.5., 3.4.2.3., 3.4.3.2.), these experiments were performed over a culture period of at least 3 days.

Both PGE_1 and PGI_2 were found to suppress spontaneous cytotoxicity when added to 3-day cultures of PBMC. This effect was rather limited in the case of PGI_2; only partial suppression was obtained at the relatively high dose of $1x10^{-5}$ molar, while the stable but less active analogue carbacyclin failed to influence cytotoxicity (Figure 3.5.5.). However, exogenous PGE_1 induced dose-dependent suppression with complete abrogation of lytic activity at the highest dose ($1x10^{-6}$ molar, Figure 3.5.1.). Neither prostanoid appeared to affect viable cell recovery at the end of the culture period, so their suppressive activity is unlikely to reflect destruction of the

effector population. Similarly, ethanol and Tris buffer controls confirmed that these solvents were not responsible for the observed effects.

Time-course experiments using PGE_1 revealed that this material was most potent if added during the early part of the culture. However, comparison of the results obtained from 3- and 5-day cultures (Figures 3.5.2., 3.5.3.) demonstrated that the critical factor in determining suppression was the interval between addition of PGE_1 and the cytotoxicity assay, rather than the delay in treatment after initiation of culture. Thus a direct parallel exists between the time-course of suppression due to exogenous PGE_1 and that induced by carrageenan and zymosan, as shown in Figure 3.6.3.. Such findings confirm that PGE_1 does not directly influence the effector cells in this culture system, and support the hypothesis that this prostanoid may be involved in mediating the effects of these macrophage-active agents in vitro. In this respect, the effects of exogenous PGE_1 on human spontaneous cytotoxicity are comparable to those obtained in the murine culture system, where suppression was only induced during the early stages of the MLC (section 2.5.3.2.). However, other workers have reported a direct influence of PG on the effector phase of spontaneous cytotoxicity. Droller et al (1978) observed direct suppression of human spontaneous cytotoxic activity by both PGE_1 and PGE_2 at doses comparable to those used here ($1x10^{-8}$ to $1x10^{-6}$ molar), with brief exposure of the effector cells sufficient to inhibit their activity. Similarly, Lang et al (1982) reported suppression during the course of the cytotoxicity assay, using E-series PG at $1x10^{-5}$ molar. In a comparable study using murine cells, Roder and Klein (1979) demonstrated PGE_1-induced suppression at the level of the effector cell, and suggested a short-lived and easily

Figure 3.6.3.

Time-course of carrageenan- , zymosan- and PGE_1-induced suppression of spontaneous cytotoxicity in vitro

Data on carrageenan-induced suppression taken from Figures 3.4.9. and 3.4.10. Data on zymosan-induced suppression taken from Figures 3.4.17. and 3.4.18. Data on PGE_1-induced suppression taken from Figures 3.5.2. and 3.5.3.

Per cent control cytotoxicity was calculated as described for Figure 3.6.1.

Panel A: 3-day culture

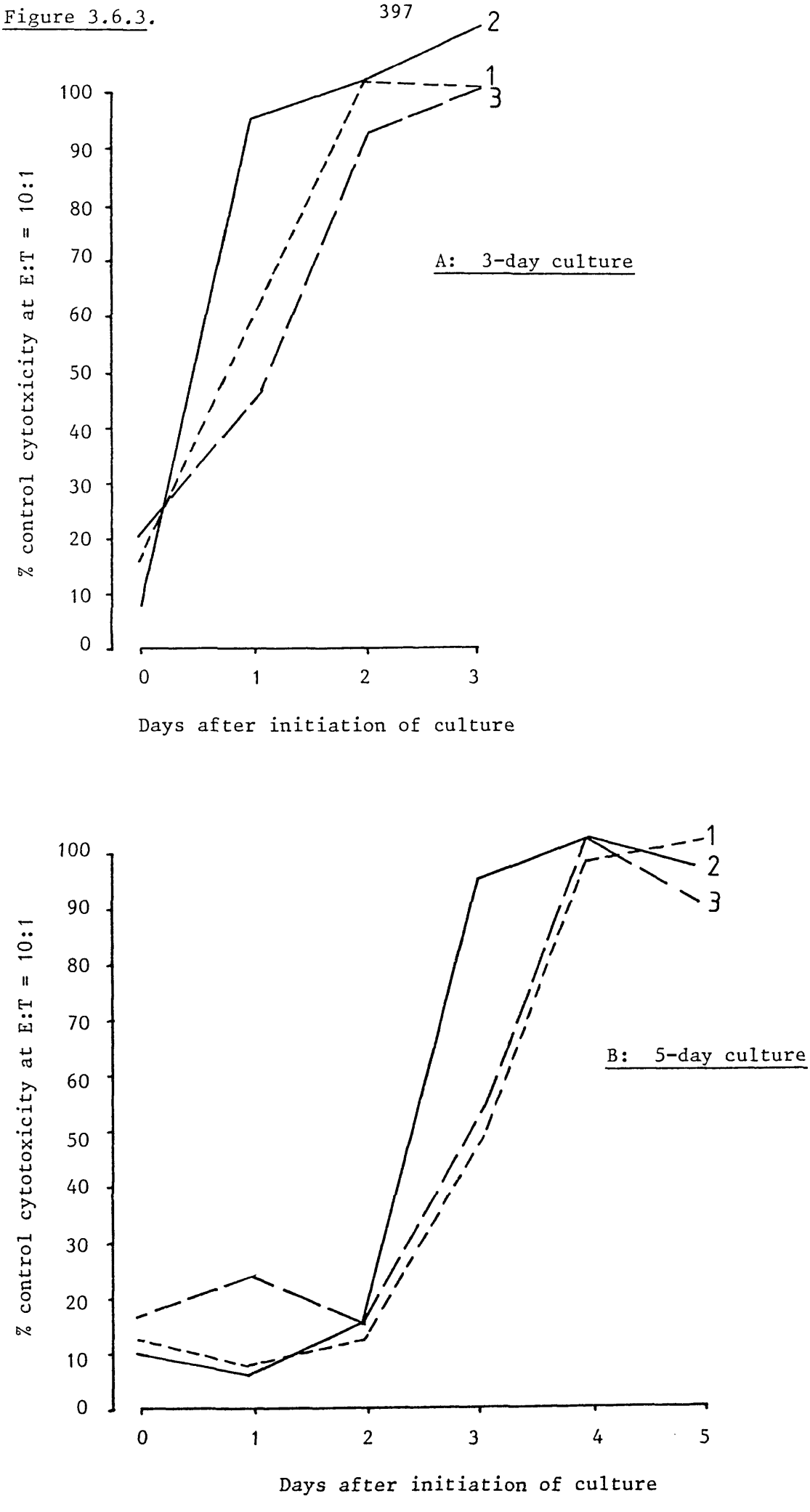
B: 5-day culture

Line 1: time-course of carrageenan-induced suppression

2: time-course of zymosan-induced suppression

3: time-course of PGE_1-induced suppression

Figure 3.6.3.


2
1
3
100
90
80
70
60
50
40
30
20
10
0
% control cytotxicity at E:T = 10:1
A: 3-day culture
0
1
2
3
Days after initiation of culture
1
2
3
100
90
80
70
60
50
40
30
20
10
0
% control cytotoxicity at E:T = 10:1
B: 5-day culture
0
1
2
3
4
5
Days after initiation of culture

reversible inhibitory mechanism, though it should be noted that the concentration of PG used in their experiments was somewhat higher than that employed here ($1x10^{-6}$ to $1x10^{-4}$ molar). However, Brunda et al (1980) found that PG must be present throughout the entire assay period to effect suppression, and demonstrated reduced susceptibility of murine cytotoxic cells to PG after overnight culture. This effect may be attributed to loss of surface receptors for PG during culture, a phenomenon which has been reported using human PBMC (Goodwin et al, 1979). Such a mechanism is compatible with data obtained in the current study. Kendall and Targan (1980) suggested that PG may act as positive or negative modulators of spontaneous cytotoxicity, depending on the assay conditions, and proposed a bell-shaped dose-response curve for the effect of this material on human PBMC; a similar biphasic response of murine cells to PG was reported by Mertin and Stackpoole (1981), using an in vivo model. Clearly, there is wide variation in the literature concerning the effects of PUFA metabolites on spontaneous cytotoxicity. A major cause of such dissent is undoubtedly the heterogeneity of the effector cells in question, and it is hoped that further work to define these will help resolve some of the apparently conflicting reports currently in circulation.

Study of the involvement of PG in carrageenan- and zymosan-induced effects was extended using antisera against both PGI_2 and PGE. The latter preparation was known to neutralise exogenous PGE_1 (section 2.5.3.3.), while anti-PGI_2 antiserum failed to inhibit PGE_1-induced suppression in the murine MLC (section 2.5.4.2.), but provided complete protection against PGI_2-induced suppression of human spontaneous cytotoxicity (Figure 3.5.6.). When added to 3-day cultures of PBMC in combination with carrageenan or zymosan, both

these antisera effected partial restoration of cytotoxicity, as shown in Figures 3.5.4. and 3.5.7.; control sera were inactive in this respect. However, limited availability of the antisera precluded testing of more than one dilution, and consequently little significance may be attached to the level of restoration attained. While lower dilutions of antisera may confer greater protection against carrageenan- and zymosan-induced suppression, results obtained using anti-PGE antiserum in the murine MLC suggest that complete restoration of cytotoxicity cannot be achieved, even with a dilution of antiserum capable of neutralising $1x10^{-6}$ molar exogenous PGE_1. These data prompted the hypothesis that other inhibitory mediators may be involved in the MLC, such that abrogation of the cytotoxic response by carrageenan or zymosan is achieved by the combined suppressive effects of different modulating substances. Similar materials may account in part for the suppression of spontaneous cytotoxicity observed after culturing human PBMC in the presence of carrageenan or zymosan. As in the murine MLC, simultaneous addition of both antisera to carrageenan- or zymosan-treated PBMC failed to improve the restoration obtained (Figure 3.5.8.), suggesting that these prostanoids are not solely responsible for suppression in this system. Additional candidates for this immunosuppressive role have been mentioned in section 1.3., and an investigation into their effects in this culture system may provide an interesting project for future study.

CHAPTER FOUR

General discussion

4. GENERAL DISCUSSION

Several parallels may be drawn between the two *in vitro* cell-mediated cytotoxicity systems studied here. In both human and murine cell cultures, cytotoxic effector cells have been shown to develop in the non-adherent population. In the case of murine alloreactive T lymphocytes, generation of effector cells requires the presence of an adherent accessory population during the first 24 hours of culture, after which the cytotoxic response becomes independent of further accessory signals. In contrast, the development of spontaneous cytotoxic activity in cultures of human peripheral blood mononuclear cells is apparently inhibited by adherent cells; if this population is removed during the maturation period of the effector cells in this system, an enhanced level of cytotoxicity is obtained. In both culture systems, the subsequent level of cytotoxic activity is subject to suppression by carrageenan and zymosan, if these agents are present during the early stages of effector cell maturation. Both these macrophage-active agents appear to influence the adherent population in a non-toxic manner, inducing synthesis of cyclo-oxygenase fatty acid metabolites by these cells; such catabolism does not result from the phagocytic event *per se*, but may reflect the intracellular lysosomal disturbance induced by inflammatory materials. Fatty acid metabolites released from adherent cells, particularly prostaglandins of the E series, exert a strong immunosuppressive effect on the development of lytic activity; in the spontaneous cytotoxicity model a similar effect is obtained with prostacyclin.

Although there are many reports describing the immunosuppressive

properties of prostaglandins both *in vivo* and *in vitro* (reviewed by Goodwin and Webb, 1980; Lewis, 1983), their mechanism of action has not been fully determined. Direct toxicity towards the effector cells is unlikely, as demonstrated in this study, and the prostaglandin molecule is now believed to interact with a specific receptor on the surface of the lymphocyte. Such binding may activate the membrane-associated enzyme adenylate cyclase, catalysing the conversion of adenosine triphosphate (ATP) to cyclic 3'5'adenosine monophosphate (cAMP) (Bourne and Melmon, 1971; Smith *et al*, 1971); this process is comparable to the mechanism by which non-steroidal hormones and neurotransmitters act on their target cells. Consequent changes in the balance of different cyclic nucleotides within the cell govern activation and regulation of various enzyme cascades, and the biological response ultimately obtained will depend on the type of cell under study and its available enzymatic machinery. In accordance with this hypothesis, receptors for E-series prostaglandins have been demonstrated on cells of the immune system (Bourne and Melmon, 1971; Bourne *et al*, 1974; Gemsa, 1981; Goodwin *et al*, 1979; Lewis, 1983; Weinstein *et al*, 1973a,b), and changes in cyclic nucleotide levels have been shown to influence immune responses (Henney and Lichtenstein, 1971; Smith *et al*, 1971; Yamamoto and Webb, 1975). Whitney and Sutherland (1972) observed a biphasic effect of cAMP in the human mixed leucocyte reaction, low concentrations of cAMP present for short periods stimulating the reaction, while extended exposure or high concentrations inhibited the response; this is comparable to the bell-shaped dose-response relationship for the effects of prostaglandins on cell-mediated immunity proposed by Mertin and Stackpoole(1981). A similar biphasic effect of cAMP was demonstrated by Teh and Paetkau (1974),

working on the murine humoral response to sheep erythrocytes. In the case of the *in vitro* cytotoxic activities studied here, interaction of precursor lymphocytes with prostaglandins may result in a differentiative signal, driving the cells into an active suppressor state; such a mechanism for prostaglandin activity has been proposed by Fulton and Levy (1981) and by Webb and Nowowiejski (1978). Alternatively, elevation of intracellular cAMP may influence the function of cells involved in the induction of the cytotoxic response; such a mechanism is compatible with the time-course of carrageenan-, zymosan- and PGE_1-induced suppression observed in this study. Prostaglandins of the E series are potent inhibitors of the expression of class II MHC antigens by mononuclear phagocytes (Snyder *et al*, 1982; Unanue, 1984), and the central role played by such molecules in cell-mediated immune responses has already been described (sections1.1.2., 2.1.). There is evidence that other PUFA metabolites, for example TXB_2, may antagonise this effect of PGE (Snyder *et al*, 1982); the combined influences of eicosanoids and the T cell-derived lymphokine gamma-IFN thus exert fine control over functions of the mononuclear phagocyte which are vital for immune induction (Beller and Ho, 1982). Secretion of opposing mediators by the phagocyte itself suggests that these cells may be subject to autoregulation. Baker *et al* (1981) have reported suppression of lymphokine production by T cells following exposure to E-series prostaglandins, and Rappaport and Dodge (1982) have suggested that these metabolites may inhibit the production of interleukin 2 (IL-2) by T helper cells, thus interfering with the maturation of cytolytic effector cells in the mixed leucocyte culture. Gordon *et al* (1976) and Higgs (1982) demonstrated that phagocyte-derived PGE can inhibit lymphokine release from T cells, revealing potential for negative

feedback regulation of T cell responses. A similar suppressive mechanism may apply to the spontaneous cytotoxic activity mediated by lymphokine-activated killer (LAK) cells (Herberman et al, 1987). Interference with lymphokine activity may also occur at the level of cell surface receptors for these molecules. Specific surface receptors with both high and low affinity for IL-2 have been demonstrated (Dukovich et al, 1987; Smith, 1980; Trowbridge, 1987), and expression of these receptors has been shown to vary according to the state of activation of the cell (reviewed by Trowbridge, 1987). While little evidence is yet available, it is possible that PG-mediated suppression is manifested as quantitative and qualitative changes in receptor expression, rendering the cell less sensitive to activating signals.

Disturbances in eicosanoid-mediated regulation may contribute to the pathogenesis of disease states thought to be associated with defects in immunological control mechanisms. Immunosuppressive prostaglandins have been identified in cases of chronic infection and chronic inflammation (Goodwin and Webb, 1980). Certain types of immunodeficiency may reflect over-production or imbalance of immunosuppressive PUFA metabolites, rather than a lack of effector cells, and decreased immune reactivity in some malignancies has been correlated with suppressive activity mediated by adherent cells (Zighelboim, 1979), coupled with high concentrations of PGE (Goodwin and Webb, 1980). Patients with Hodgkin's lymphoma display depressed cellular immune responsiveness which can be reversed to some extent by the anti-inflammatory drug indomethacin (Stenson and Parker, 1980); a monocytic PGE-producing suppressor cell has been identified in such individuals (Gemsa, 1981; Goodwin and Webb, 1980), and PGE_2 concentrations up to four times control levels have been

demonstrated (Goodwin et al, 1977). In addition, many tumours have been shown to produce high levels of endogenous PGE (Stenson and Parker, 1980); these are usually neoplastic monocyte-macrophage lines rather than T or B lymphomas (Kurland and Bockman, 1978). By suppressing immune reactivity of the host and so inhibiting cytotoxic attack, such tumours may generate a local environment conducive to their survival and growth (Droller et al, 1978; Goodwin and Webb, 1980).

In contrast, break-down of eicosanoid-mediated control may result in an over-active immune system, as observed in hypersensitivity states and autoimmunity. Inappropriate or unlimited immune responses in such individuals may result from reduced PUFA catabolism, or may reflect loss of sensitivity to PG-mediated regulation, perhaps due to changes in PG receptor expression or binding affinity. Variations in PG sensitivity are observed during the course of normal immune or inflammatory reactions, and may serve to limit the extent of the response (Gemsa et al, 1978; Lewis, 1983; Plaut, 1981), while increased PG sensitivity in aged lymphocytes has been suggested to account for the immune hyporesponsiveness of elderly patients (Goodwin and Webb, 1980). Mononuclear cells isolated from the synovial fluid of individuals suffering from rheumatoid arthritis show reduced sensitivity to PGE_1 compared to control cells, binding of this molecule inducing weaker elevation of intracellular cAMP and failing to inhibit lymphokine secretion (Lewis, 1983); T cell reactivity against the auto-antigen is thus freed from negative feed-back control and may be accelerated by positive feed-back signals.

The data obtained in this study demonstrate the crucial role played

by mononuclear phagocytes and related cells in immune responses. Essential elements in the induction of specific cell-mediated immunity, these cells not only present antigen in the context of appropriate MHC molecules, but also provide a soluble lymphocyte activating moiety in the form of interleukin 1. However, with appropriate stimulation accessory cells within the same population may inhibit cell-mediated immune reactivity by release of suppressive materials, including cyclo-oxygenase metabolites of membrane phospholipids. It is suggested that this process represents a physiological control mechanism for regulating both specific and non-specific immunity, ensuring a rapid and self-limiting response to immunological challenge.

Investigation of the role of eicosanoids as immunomodulating agents in vivo is expanding rapidly, under both physiological and pathological conditions. Increased understanding of the positive and negative regulatory mechanisms governing immune responsiveness may contribute to rational protocols for manipulating effector activities in the whole animal, hopefully leading to improved management of allograft rejection, neoplasia and autoimmune responses; from the information currently available, it would seem that control or inhibition of lipid peroxidation may prove a fruitful therapeutic goal.

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