# "Regulation of Anti-GBM Autoantibody Synthesis in Mercuric Chloride Induced Anti-GBM Nephritis in the BN Rat"

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## ABSTRACT

Mercuric chloride ($HgCl_2$) in the Brown Norway (BN) rat induces a biphasic nephritis characterised by linear deposition of IgG autoantibodies and then immune complexes, along the glomerular basement membrane (GBM). Humoral studies showed that anti-GBM antibody synthesis was rapidly self limiting, and consisted of several immunoglobulin classes, but with partial IgG subclass restriction. Anti-GBM antibody was associated with the transient presence of circulating immune complexes. Increases in spleen size, and neutrophilia, were observed, while peripheral blood T suppressor cells, followed by T helper cells, showed temporary decreases, either preceding or at the time of the appearance of circulating antibody, respectively. Tissue injury, as indexed by proteinuria, was highly variable, and did not correlate with levels of circulating antibody or immune complexes.

Examination of autoregulation of anti-GBM antibody production showed that convalescent animals were resistant to rechallenge with $HgCl_2$ even after a primary course of low dose $HgCl_2$ injections, and that the resistance was lost with time. Spleen cell transfer studies demonstrated the presence of an MRC $OX8^+$ T suppressor cell which could transfer antigen specific suppression, and was found in donors during the phase of autoregulation. In addition, a role for anti-idiotypic antibodies, both heterologous and autologous, was found.

To examine autoregulation more closely, an *in vitro* anti-GBM antibody production system was developed using spleen cells from $HgCl_2$ stimulated rats, and used to screen possible regulatory factors and cells. An interesting IgM class reactivity with GBM was discovered in supernatants of unstimulated, cultured normal BN

spleen cells.

The significance of these findings on autoregulation of the autoimmune response in an experimental animal model, was considered with regard to treatment of human autoimmune disease.

To my mother and Richard, my husband.

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## Abbreviations

| | |
|---|---|
| BN | : Brown Norway |
| BSA | : Bovine serum albumin |
| CFA | : Complete Freund's adjuvant |
| CFD | : Complement fixation diluent |
| CIC | : Circulating immune complexes |
| CON A | : Concanvalin A |
| CS | : Calf serum-foetal or newborn |
| DAB | : Dulbecco's A+B salt solution |
| DNA | : Deoxyribonucleic acid |
| DTH | : Delayed type hypersensitivity |
| EAE | : Experimental autoimmune encephalomyelitis |
| EAG | : Experimental allergic glomerulonephritis |
| EAMG | : Experimental autoimmune myasthenia gravis |
| EAT | : Experimental autoimmune thyroiditis |
| ELISA | : Enzyme linked immunosorbent assay |
| EM | : Electron microscopy |
| FITC | : Fluorescein isothiocyanate |
| GAMIg | : Goat anti-mouse Ig antibody |
| GBM | : Glomerular basement membrane |
| G-P | : Goodpasture's syndrome |
| Hb | : Haemoglobin |
| Id | : Idiotype |
| IF | : Immunofluorescence |
| IFA | : Incomplete Freund's adjuvant |
| i.m. | : intramuscular |
| i.p. | : intraperitoneal |
| i.v. | : intravenous |
| KgBA | : conglutinin binding assay |
| KLH | : Keyhole limpet haemocyanin |
| LPS | : Lipopolysaccharide |
| mAb | : monoclonal antibody (ies) |
| MHC | : Major histocompatability complex |
| MIg | : Mouse immunoglobulin |
| MLR | : Mixed lymphocyte reaction |
| MW | : Molecular weight |
| NK | : Natural Killer (cell) |
| NRS | : Normal rat serum |
| N.S. | : Not significant |

NS : Naturally occurring suppressor cells

$OD_{280}$ : Optical density at 280nm

PBS : Phosphate buffered saline

PEG : Polyethylene glycol

PFC : Plaque forming cell

PHA : Phytohaemagglutinin

PWM : Pokeweed mitogen

RaMIg : Rabbit anti-mouse Ig antibody

RBC : Red blood cell

RIA : Radioimmunoassay

RPGN : Rapidly progressive glomerulonephritis

RT : Room temperature

SC : Spleen cell(s)

SD : Standard deviation

SDS-PAGE : Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SIRS : Soluble immune response suppressor

SLE : Systemic lupus erythematosus

SRBC : Sheep red blood cell

TBM : Tubular basement membrane

T20 : Tween 20

$T_h$ : T helper (cell)

TNP : Trinitrophenyl

$T_s$ : T suppressor (cell)

TSH : Thyroid stimulating hormone

WBC : White blood cell

# 1. INTRODUCTION

Autoimmune disease, in which an immune response is mounted to self components, resulting in pathological changes and clinical disease, presents problems in terms of our understanding of the mechanisms involved in its induction and pathology and these have repercussions in the design of a therapeutic approach. Current treatment tends to be non-specific, involving immunosuppression, or stimulation of immunoregulation, or removal of the mediator of damage, where possible, but along with many other useful components. All these treatments may affect the immune response in general, or have other potentially dangerous side effects. More specific forms of immunotherapy are therefore required.

By 1980, Hammersmith Hospital had extensive experience in the treatment of autoantibody mediated nephritis in terms of its basic autoimmune aetiology. However, there was a need for more effective, specific treatment and for this an understanding of the disease process and immunoregulation was required. To this end, I have undertaken a study of an experimental animal model of autoantibody mediated nephritis in the hope that this may provide a basis for such understanding.

In autoantibody mediated nephritis in man, an autoantibody response to the glomerular basement membrane (GBM) results in a rapidly progressive glomerulonephritis. One experimental animal model which bears some features in common with this disease is mercuric chloride ($HgCl_2$) induced nephritis in the Brown Norway (BN) rat (Sapin, Druet and Druet, 1977). The aims of the work described in this thesis were initially to examine this model with regard to disease processes and autoimmune phenomena and then to study regulation of anti-GBM antibody synthesis in the model. This was done with a view to being able in the future to tailor specific

forms of immunotherapy for autoantibody mediated nephritis and other autoimmune diseases.

To provide a background to this work, the following introduction contains brief reviews of recent (since 1975) selected references on Regulation of the Immune Response, Autoimmunity, Autoantibody Mediated Nephritis in Man, and a detailed review of work published on $HgCl_2$ induced autoimmunity in the BN rat.

## 1.1. REGULATION OF THE IMMUNE RESPONSE

Introduction of foreign antigens into the body, either carried on invading organisms or in a soluble form, results in sequestration of various components of the immune system, to produce an immune reponse. This response may be of either a humoral or cellular nature, and may be specific for the antigen, or of a non-specific nature. This initiation of the immune response requires recognition, stimulation, proliferation and frequently cellular cooperation under genetic control. However, once invoked, the immune response must then be regulated to prevent an inordinate proportion of the body's resources sustaining a response which may no longer be required.

Mechanisms by which the body achieves homeostasis by down regulating the immune response may involve feedback mechanisms, whereby the antigen, which is required to drive the response, is catabolised by enzymes and/or neutralised or opsonised by antibody and macrophages, and so lowered in concentration (reviewed in Taylor, 1983), or may involve suppressive components of the immune response: cells, both lymphoid (reviewed in Gershon, 1974), or myeloid (reviewed in Allison, 1978), or their soluble factors (reviewed in Tada, Taniguchi and Takemori, 1975). In addition, as Jerne has described in his network (idiotype) theory, antibody

itself may act as an antigen, leading to formation of anti-antibodies, or 'anti-idiotypic' antibodies which may control the response (Jerne, 1974). These mechanisms may cause suppression either specifically, or non-specifically, on either the humoral or cellular arms of the immune response and may be present in normal individuals, or those undergoing down regulation after an immune response has been invoked, or only demonstrable after *in vitro* stimulation.

### 1.1.1. Cells and Cell Derived Factors

#### T Lymphocytes

The involvement of T cells in regulating the immune response can be seen by early experiments, whereby in certain models, depletion of T cells, by administration of anti-lymphocyte globulin or irradiation, or more specifically anti-θ antiserum or thymectomy caused enhancement of antibody production, while passively transferred thymocytes could terminate or suppress antibody production (reviewed in Katz and Benacerraf, 1972; Tada, Taniguchi and Takemori, 1975; Weigle et al, 1975).

#### Non-specific Suppression

T suppressor cells ($T_S$) may be induced non-specifically *in vitro* using mitogens such as Concanavalin A (Con A (Dutton, 1975; Shou, Schwartz and Good, 1976)) or pokeweed mitogen (PWM (Saxon, Stevens and Ashman, 1977)) and *in vivo* using Con A (Rich and Pierce, 1973) to suppress the humoral immune response. Mitogenic stimulation of cells from non-responders to PWM induced Ig stimulation, greatly enhances their suppressive effect on responders (Tauris, 1983). Even without stimulation, excess T cells from normal individuals may cause non-specific suppression (Librach and

Burns, 1983). In addition to these mitogen induced and naturally present suppressor cells, T cells stimulated by one antigen may suppress the humoral immune response to unrelated antigens in an antigenic competition effect (reviewed in Katz and Benacerraf, 1972). Suppression of delayed type hypersensitivity (DTH) to third party alloantigens by antigen specific $T_S$ has also been shown (Bianchi, Hussaarts-Odijk and Benner, 1983).

Non-specific $T_S$ cells are radiosensitive (Siegal and Siegal, 1977). In murine systems their surface phenotype involves Lyt2,3 antigens (Jandinski et al, 1976); while in humans the T8 antigen is present (Thomas et al, 1980).

Factors in supernatants from Con A stimulated spleen cell cultures, termed Soluble Immune Response Suppressor, 'SIRS' (Tadakuma et al, 1976), in vivo alloantigen activated T cells (Rich and Rich, 1975; Rabourdin-Combe et al, 1979), and 'antigen immune' spleen cell cultures (Thomas, Roberts and Talmage, 1975) may replace the cells in their non-specific suppression of <u>in vitro</u> antibody production to unrelated antigens and also cell mediated responses such as the Mixed Lymphocyte Reaction (MLR-SF).

The mechanism of action of these non-specific T suppressor factors has been analysed and varies with the source of the factor. They may act by limiting cell proliferation (Thomas, Roberts and Talmage, 1975); or may act during initiation via macrophages (Tadakuma and Pierce, 1976) or later in the response directly on B cells (Rabourdin-Combe et al, 1979).

Biochemical characterisation of various non-specific suppressor factors shows some similarities, but many differences in molecular weight (MW), heat stability and pH stability. Many are glycoproteins and molecular weights include the 40K + 18K polypeptide chains for Immunoglobulin binding factor (IBF), which

binds to Ig and around 60K for SIRS, which does not bear Ig determinants (reviewed in Tadakuma et al, 1976; Rabourdin-Combe et al, 1979).

MHC restriction and Ia expression are also variable (reviewed in Rich, David and Rich, 1979; Rabourdin-Combe et al, 1979).

Specific Suppression

Antigen specific suppression of the humoral immune response by T suppressor cells has been studied at length in murine systems, both in vivo and in vitro, and also in human in vitro systems, using responses to haptens, synthesised peptides, proteins or cells (Basten, Miller and Johnson, 1975; Dorf and Benacerraf, 1984). In addition, they may modulate cellular immunity, including DTH (Bach et al, 1978), contact sensitivity (CS (Asherson and Zembala, 1974a)), cytotoxic T cells (Finberg et al, 1979) and T cell proliferation in the MLR (Rich and Rich, 1974).

The source and method of induction of T suppressor cells varies greatly and they may be present during tolerisation, the normal immune response and down regulation, or in non-responders. The first demonstration of T cell mediated antigen specific suppression was in Gershon's 'infectious tolerance', whereby T cells from animals tolerised to one antigen could be transferred to normal, syngeneic recipients, to specifically suppress the antibody response to the same antigen (Gershon and Kondo, 1971). T suppressor cells may be present whether tolerance is induced by low doses (Kölsch, Stumpf and Weber, 1975) or high doses of antigen (Nachtigal, Zan-Bar and Feldman, 1975) and may suppress both T dependent (Nachtigal, Zan-Bar and Feldman, 1975) and T independent (Taylor et al, 1983) antibody responses. In CS, both tolerisation

(Phanuphak, Moorhead and Claman, 1974) and change in route of administration of antigen coupled cells (Greene, Sugimoto and Benacerraf, 1978) cause induction of T suppressor cells. These antigen specific T suppressor cells are cyclophosphamide sensitive (Tagart, Thomas and Asherson, 1978).

T suppressor cells may occur during a normal immune response (Peters and Fauci, 1983) or during down regulation of an immune response (Tada, Taniguchi and Takemori, 1975). Antigen specific T suppressor cells may be present in non-responders immunised with immunogenic doses of antigen (Kapp et al, 1974) or naturally in unstimulated non-responders (Haynes and Fauci, 1978) and will suppress the immune response of cells from responders. Antigen specific suppressor cells have also been cloned (Asano and Hodes, 1983a).

Specificity of the suppression by $T_S$ cells has been shown at various levels and varies with respect to antibody class, allotype and idiotype as well as antigen. Thus suppression may affect the IgG and IgM class of the response unequally (Gershon and Kondo, 1971; Tada, Taniguchi and Takemori, 1975) or may affect only the IgE antibody response (Tada, Taniguchi and Takemori, 1975). Allotype specific suppression by T cells has been extensively reviewed by Herzenberg (Herzenberg, Okumura and Metler, 1975), while a wealth of data is available on antigen specific suppression (reviewed in Weigle et al, 1975; Tada, Taniguchi and Takemori, 1975; Green, Flood and Gershon, 1983; Dorf and Benacerraf, 1984). T cells have also been shown to cause idiotype specific suppression both *in vivo* (Rohrer, Odermatt and Lynch, 1979) and *in vitro* (Abbas, Takaoki and Greene, 1982), such that the cells exhibit surface idiotype and even anti-idiotype (reviewed by Urbain, Wuilmart and Cazenave, 1981). Even T suppressor cells specific for a DTH response may exhibit

surface idiotype (Sugimura et al, 1981). At this point it may be interesting to note that the end suppressive mechanisms may, in many systems be antigen dependent but antigen non-specific (Dorf and Benacerraf, 1984).

Suppression of the immune response by T cells, may act on either the induction phase, such as during B cell differentiation (Rohrer, Odermatt and Lynch, 1979) or during the effector phase of the immune response (Asherson and Zembala, 1974a; Tada, Takemori and Taniguchi, 1975). Further studies on the mechanisms of suppression have shown that T cells may directly suppress B cell production of antibody, as expected for T independent antigens (Braley-Mullen, 1980) or act at the level of the T helper cell (Herzenberg et al, 1976; Sherr et al, 1979). They may even require antigen bridging both with the B cell and T helper cell (Asano and Hodes, 1984). In addition, macrophages have been implicated as target cells for T suppressor cells (Basten, Miller and Johnson, 1975), or such suppression has at least been shown to be macrophage dependent (Yoshinaga, Yoshinaga and Waksman, 1972). Macrophages may also be required, to act as antigen presenting cells to stimulate T suppressor cells (Dorf and Benacerraf, 1984).

The phenotypes of these antigen specific T suppressor cells have been studied extensively in murine systems and their expression of Lyt and Qa markers and I-J gene products shown to vary according to their level or order of action (reviewed by Green, Flood and Gershon, 1983; Dorf and Benacerraf, 1984). First order antigen-reactive $T_{s1}$ or 'inducer' $T_s$, which are involved in induction or recruitment of another $T_s$ population, are Lyt $1^+$, $2^-$, I-$J^+$ and Qa-$1^+$. $T_{s2}$, or 'effector' phase $T_s$ are Lyt $1^-$, $2^+$, but may not necessarily carry the same I-J gene product. In other systems another cell, $T_{s3}$ is required to effect suppression, while in some a

'transducer' cell is necessary. Furthermore, beyond inducer, transducer and effector cells, termed level 1 suppression, are opposing forces called 'contrasuppression' and beyond this, level 2 suppression (Green, Flood and Gershon, 1983).

T suppressor cells may be replaced by antigen specific soluble T suppressor factors present in the supernatants from cultured immune spleen cells, whether disrupted (Tada, Taniguchi and Takemore, 1975; Araneo and Kapp, 1980; Greene et al, 1982), or intact (Evans et al, 1972; Yamauchi et al, 1981a; Dorf and Benacerraf, 1984) and these factors show the same variations and similarities as the cells from which they are derived. Thus they will suppress both humoral (Tada, Taniguchi and Takemore, 1975) and cell mediated responses (Zembala and Asherson, 1974; Greene, Bach and Benacerraf, 1979). $T_S$ factors range in specificity from non-antigen specific but class specific (Suemura et al, 1981) to antigen/hapten specific (Tada, Taniguchi and Takemori, 1975; Webb, Kapp and Pierce, 1983) to idiotype specific (Hirai and Nisonoff, 1980). Their target cells may be primed adherent T cells (Taniguchi and Tokuhisa, 1980) or macrophages (Evans et al, 1972) and they may affect the early phase of the immune response, as for T suppressor cells (Asano and Hodes, 1983b), the late phase (Yamauchi et al, 1981a) or both (Yamauchi et al, 1981b).

T suppressor factors are classified according to the $T_{S1}$, $T_{S2}$, $T_{S3}$, or inducer, transducer or effector $T_S$ cell from which they are derived, and between which they may act (reviewed by Green, Flood and Gershon, 1983; Dorf and Benacerraf, 1984). As for non-specific $T_S$ factors, many have been biochemically/immunochemically characterised. Molecular weight ranges from 19K to 80K and I-J gene products are usually present (reviewed in Webb, Kapp and Pierce, 1983). Genetic restriction is variable and ranges from allotype

(Greene et al, 1982) and H-2 (Taniguchi and Tokuhisa, 1980) to Ig ($V_H$) restricted (Yamauchi et al, 1981a). These factors may bind antigen (Green, Flood and Gershon, 1983), idiotype or anti-idiotype (Hirai and Nisonoff, 1980; Greene et al, 1982) but do not usually bear Ig region determinants themselves (Dorf and Benacerraf, 1984).

B Lymphocytes

Suppression of the immune response by B lymphocytes has been shown, as with T cells, to be either non-specific or specific, and both humoral and cell mediated arms of the immune response are susceptible to regulation by this means. Suppressor B cells, which regulate antibody production non-specifically, have been identified in cultures stimulated by lipopolysaccharide (LPS) or cultures stimulated by Con A supernatants (reviewed by Gilbert and Hoffman, 1983). There is also evidence that they occur naturally and are involved in normal immunoregulation (Petrov and Khaitov, 1977). Antigen non-specific suppressor B cell factors may be released from B cells and suppress B cell responses and/or lymphocyte proliferation of both B and T cells (Persson, Coutinho and Möller, 1977). However, they do not bear Ig determinants, and their MW is less than 70K (reviewed by Gilbert and Hoffman, 1983). Specific suppression of cell mediated immunity by B suppressor cells has been shown for DTH and CS, during normal immunisation schedules (Katz, Parker and Turk, 1974; Zembala et al, 1976) and in CS requires little or no T cell help.

Macrophages

Macrophages have been shown to inhibit lymphocyte responses by a number of different mechanisms (reviewed by Allison, 1978). Non-specifically, they may inhibit PHA, or Con A induced T cell

mitogenesis and lymphoma cell proliferation (Kirchner, Holden and Herbermann, 1975). High density macrophages may cause inhibition of passive transfer of CS (Ptak et al, 1981). However, at low concentrations, macrophages will cause non-specific suppression only after arming by T cell factors (Ptak et al, 1981). Interestingly, in normal rat spleens, an adherent cell with macrophage characteristics has been found to prevent in vitro B cell responses, even when a florid in vivo response occurs (Corvalan and Howard, 1978; Morisaki et al, 1983). Soluble factors capable of depression of blast transformation, cell proliferation or PFC formation, have been found in unstimulated cultures of macrophages from normal or immunised mice (Calderon, Williams and Unanue, 1974) or in macrophages stimulated with T cell derived SIRS (Aune and Pierce, 1981). In addition, a non-specific macrophage suppressor factor, released by macrophages armed with specific T suppressor factors, will suppress CS (Ptak et al, 1981). On a specific level, macrophages are required for T cell regulation of humoral responses (Basten, Miller and Johnson, 1975), myeloma differentiation (Rohrer and Lynch, 1978) and CS (Asherson and Zembala, 1974b; Zembala and Asherson, 1974), although in the latter case, they cause non-specific killing after being specifically triggered and armed.

Other Cells

Natural killer (NK) cells have been reported to terminate the primary humoral immune response both in vivo and in vitro. This is thought to occur via antigen positive accessory cells, and would be 'specific immune regulation by non-specific components' (Abruzzo and Rowley, 1983). Naturally occurring suppressor (NS) cells, which have characteristics of NK cells but not natural killer activity, have been found in neonatal spleens (Oseroff, Okada and Strober,

1984). Similar NS cells have been found transiently in mice spleens after total lymphoid irradiation (reviewed by Strober, 1984).

### 1.1.2. Antibodies/Serum Products

#### Antibodies

##### Non-idiotypic

Antibodies may inhibit the immune response either in an antigen specific or non-specific manner. Non-specifically, antibodies to leukocytic pyrogens inhibit IL1 induced B cell activation, by removing macrophage derived IL1 (Lipsky et al, 1983), while antibodies to IgM and IgD, usually mitogenic themselves, can inhibit mitogen induced IgG, IgA and IgM production (Saiko and Ralph, 1982).

Specific regulation by antibodies is either by feedback inhibition by the antibody itself, or by anti-idiotypic antibodies. Feedback inhibition may be due to blocking, and so preventing access of antigenic determinants to their lymphocyte receptors; or bridging, whereby the antigen-antibody complex forms a bridge between the antigen receptor and the Fc receptor. The latter bridging may either paralyse the cell, inhibiting secretion, or allow destruction of the lymphocyte by macrophages (Taylor, 1982).

Also of interest is Krieger's observation that antibodies to a 78 amino acid fragment of BSA are capable of suppressing the antibody response to all the determinants of BSA (Krieger, Pesce and Michael, 1983). This could not be due to blocking of all determinants, but may mimic earlier studies where anti-carrier antibodies regulate an anti-hapten response, possibly acting in conjunction with antigen on the T cell (Vuagnet, Neveu and Voisin, 1972).

Anti-idiotypes

Jerne's network theory and the recognition that within the diversity of the immune response, antibody molecules (idiotypes, Id) via their variable regions, may act as antigens, to initiate anti-idiotypic antibody production, has led to observations, both theoretically and practically, that these anti-idiotypic antibodies may be involved in regulation of the immune response both by stimulation and suppression (Jerne, 1974; Urbain, Wuilmart and Cazenave, 1981). Since a response to antigen may result in a wide variety of idiotypes to a variety of determinants, and since the spectrum of idiotypes varies from individual to individual, the finding that certain antigens, such as Group A Streptococcus and some haptens, in inbred mice, elicit cross reactive idiotypes (Eichmann, 1974; Tung and Nisonoff, 1975), has allowed a more intensive study of the effect of anti-idiotypic antibodies (reviewed by Urbain, Wuilmart and Cazenave, 1981).

Initially heterologous anti-idiotypic antibodies were used in studies - thus Eichmann showed that heterologous anti-idiotype suppressed the idiotype positive antibody response to Group A Streptococcus in mice; that the heterogeneity of the compensatory idiotype negative responses did not change; that the permanence/transience of the suppression varied depending on whether high or low concentrations of anti-idiotype were used; and that the suppression was mediated by an IgG2 antibody - IgG1 anti-idiotype had an enhancing effect (Eichmann, 1974; Eichmann and Rajewsky, 1975). Iverson has also shown that a heterologous anti-idiotype may inhibit antigen specific T rosette forming cells (Iverson et al, 1983).

The mechanism by which the heterologous anti-idiotype exerted its effect was initially shown to be the result of

elimination of precursors of idiotype-producing cells (Eichmann, 1974), while the prolonged suppression induced by low doses of anti-idiotype was shown to be due to amplification of a T suppressor cell with anti-idiotypic activity (Eichmann, 1975).

At this point it should also be noted that anti-idiotypic antibodies may have specificities against the antigen combining site or other areas in the variable region, and that if they are specific for the combining site, they may be 'internal images' of the antigen (Jerne, 1974) and mimic antigen behaviour, both in a physiological sense, if the antigen is a hormone (Sege and Peterson, 1978), or may act as a stimulant to idiotype production (Jerne, 1974; Urbain, Wuilmart and Cazenave, 1981).

As well as heterologous anti-idiotypic antibodies suppressing an immune response, homologous anti-idiotypes (raised in different strains) have been shown to affect negatively the immune response (Cosenza and Köhler, 1972; Binz and Wigzell, 1975a). However, perhaps the most relevant indicator of a suppressive role for anti-idiotypes in regulation of the immune response has been the observation of the occurrence of auto-anti-idiotypic antibodies, or complexes of Id-anti-id, during the normal immune response to an antigen (Rose and Lambert, 1980), especially associated with a downward regulation of idiotype production, as shown by 'hapten augmentable plaques' (Schrater et al, 1979; Goidl et al, 1979). Anti-idiotype may even be in cyclically opposite phase to idiotype (reviewed in Urbain, Wuilmart and Cazenave, 1981). It is interesting that complexes of idiotype and anti-idiotype, produced after polyclonal (LPS) stimulation in Balb/c mice, may become deposited in the glomeruli, and lead to glomerular lesions and immune complex disease (Goldman et al, 1982).

The possible involvement of auto-anti-idiotypic antibodies

in specific tolerance to MHC/transplantation antigens, has also been linked to idiotypic suppression (Binz, Frischknecht and Wigzell, 1979). Further support for the involvement of anti-idiotypic antibodies or interactions in regulation of the immune response, is the finding of similar idiotypes on B and T cells (Binz and Wigzell, 1975a,b), as is data suggesting the presence of anti-idiotypic cells naturally occurring in the normal animal (Hetzelberger and Eichmann, 1978). Interestingly, the presence of higher levels of auto-anti-idiotypic antibodies in mice, has been proposed as a reason for the decline in immune responsiveness with age (Goidl et al, 1983) and this increase has been shown to be due to the action of peripheral T cells (Tai Kim et al, 1985). Thus both anti-idiotype and cells may act in conjunction with each other in regulating the immune response.

Immune Complexes and Complement

Immune complexes may inhibit the immune response both specifically as has been described above, and non-specifically. It has been found that IgG immune complexes bind to an IgG(Fc)-binding subset of T cells, which then mediate non-specific suppression of PWM induced B cell proliferation (Moretta et al, 1977).

Complement, notably activated fragments of C3, has also been found to suppress the immune response, in addition to its many stimulatory effects (reviewed by Weigle et al, 1983). For example, it has been suggested that the interaction of C3a with monocyte receptors, indirectly activates the Lyt $1^+,2^-$ cell and generates suppressor cell activity (Weigle et al, 1983).

## 1.2. AUTOIMMUNITY

Autoimmunity, or the state in which self components act as

antigens, provoking an immune reponse, mainly humoral but also cellular, has been known for some time, although our understanding of it has evolved from the idea that autoimmune diseases were caused by single agents, to the present concept that the basis of autoimmune disease is essentially multi-factorial, and that the presence of autoantibodies is not necessarily an abnormal occurrence in individuals. It has even been suggested that autoantibodies may have a biological function, in transporting cellular breakdown products (Grabar, 1975).

However, autoimmune diseases are generally classified as those diseases in which autoantibody or autoreactive T cells, are closely associated with the disease, especially in its pathogenesis. This discriminates autoimmune diseases from those situations where tissue damage results in the formation of non-harmful autoantibodies, such as anti-heart antibodies after myocardial infarction. A wider definition of autoimmune diseases, in which immune mediated diseases such as hereditary angioedema are included, has been proposed by Smith and Steinberg (1983).

Autoimmune diseases show a spectrum of variation with respect to many factors including specificity, genetic restriction, mechanisms and pathology.

### 1.2.1. Specificity

Specificity in autoimmune disease exhibits a spectrum in terms of the target organ, antigen and idiotypic restriction of the autoantibody involved. Thus, in the organ specific diseases, typified by Hashimoto's thyroiditis, the target organ incurs a lesion, which is often infiltrated by monocytes and inflammatory cells, and is associated with the presence of circulating antibodies specific for various components from that organ- in the case of

Hashimoto's disease - the thyroid (reviewed in Doniach, Bottazzo and Drexhage, 1982).

Organ specific diseases in which the lesion is only on one target organ, but in which autoantibodies are not organ specific, would include primary biliary cirrhosis, where the target organ is the small bile ductile, but circulating autoantibodies are against mitochondria and are not liver specific (reviewed in Wright, 1982). Non-organ specific diseases include SLE and rheumatoid arthritis. In SLE, target 'organs' are widespread, and range from skin to kidney glomeruli, joints and blood vessels. Autoantibodies are equally wide ranging, including specificities for DNA, nucleoprotein, clotting factors and IgG (reviewed by Glass and Schur, 1977).

The spectrum of self antigens against which autoantibodies are directed varies from intracellular components, including DNA, cytoplasm and mitochondria; through cell membrane receptors such as those for thyroid, acetylcholine and insulin; cell membranes, from red blood cell to epidermal and spermatozoan; to extracellular basement membranes, plasma proteins and hormones (Smith and Steinberg, 1983).

Of interest also, is that certain autoantibodies, notably anti-DNA antibodies, even when obtained as monoclonal antibodies using hybridoma techniques on cells from mice with spontaneous SLE, exhibit a striking polyspecificity, reacting with DNA, polynucleotides, phospholipids, in fact any structure bearing a phosphate ester (Lafer et al, 1981; Shoenfeld and Schwartz, 1984). In contrast, autoantibodies in other diseases show a very narrow specificity. Thus serum autoantibodies in experimental myasthenia gravis usually react primarily with a region in the $\alpha$subunit of the acetylcholine receptor, distinct from the acetylcholine binding site

(Tzartos and Lindstrom, 1980).

The concept of restricted specificity in certain autoantibodies leads to the possibility that the actual idiotypes reacting with these specific epitopes might be highly conserved. In fact, just as cross reactive idiotypes have been found in the antibody response to foreign antigens, recurrent idiotypes may be found in autoantibodies. Such cross reactive idiotypes have been demonstrated in autoantibodies of both a polyspecific as well as specific nature and include rheumatoid factors, and anti-DNA, acetylcholine receptor and thyroglobulin antibodies (Shoenfeld et al, 1983; Shoenfeld and Schwartz, 1984). This has therapeutic implications with respect to agents which may commonly but specifically switch off the autoantibody response.

The presence of one autoimmune disease in an individual is not protective against the development of other autoimmune disorders, and patients with some diseases, such as autoimmune thyroiditis have an even higher incidence than expected, of pernicious anaemia, another 'organ specific' disease. This 'overlap' occurs with a number of autoimmune diseases, and even where additional clinical manifestations are absent, autoantibodies of a different disorder may coexist with those specifically for the autoimmune disease being suffered (Rose et al, 1977; Lippman et al, 1982).

### 1.2.2. Genetic Considerations

The influence of genetic factors in predisposition to autoimmune disease can be seen by the disease incidence in first degree relatives of patients. For example, relatives of patients with autoimmune thyroiditis have a high incidence of thyroid autoantibodies as well as thyroiditis itself (reviewed by Rose et

al, 1977). Of interest here also, is Lippman's study of the combination of one family, where the proband had both autoimmune haemolytic anaemia and hypothyroidism, with another family, whose proband had autoimmune thrombocytopenia, leading to a high incidence in the resulting family, of SLE, autoimmune thrombocytopenia and autoimmune haemolytic anaemia (Lippman et al, 1982).

Further substantiation of a genetic role in predisposition, is seen in concordance rates of autoimmune diseases in identical twins. Rates of 50% or more have been noted for SLE and type I diabetes, together with high concordance rates for Graves' disease and autoimmune thrombocytopenia (reviewed by Shoenfeld and Schwartz, 1984).

Since to some extent the influence of environment cannot be discounted in the above familial studies, an examination of linkage with known genetic loci also might indicate a genetic basis for autoimmune disease. Statistically significant associations have been found between autoimmune disease and certain antigens coded for by the MHC, especially DR3 and HLA-B8 (Woody, Bellanti and Sell, 1985), as well as other antigens. Thus autoimmune thyroiditis is significantly associated with DR3 (Smith and Steinberg, 1983), while rheumatoid arthritis is linked to DR4 (Roitt et al, 1978). On a more detailed examination by molecular analysis, it has been found that patients with type I diabetes mellitus, which has an association with either DR3, DR4 or both, have differences in their DNA encoding for $\beta$ chains of the DR antigen, compared to normal subjects (Owerbach et al, 1983).

As well as DR associations, certain allelic forms of class III antigens (complement) have been shown to be more frequent in autoimmune diseases. The BFf1 allelic form of Factor B is 8 times more common in type 1 diabetes patients than in the general

population (Alper et al, 1982). Polymorphic variants of C4 have also been suggested to be of importance (Whitehead et al, 1984). In addition, phenotypic variants of immunoglobulins, notably Gm, have been shown to correlate with autoimmune diseases, such as Graves' disease and myasthenia gravis (Nakao et al, 1980).

It is of interest to note that there is not only an association with certain DR antigens in autoimmune diseases, but there may also be a connection with their expression. Thus in Graves' and Hashimoto's disease, thyroid epithelium has strong expression of DR antigens, while this occurs only occasionally in non-toxic goitres, and is absent from the normal thyroid (Hanafusa et al, 1983). This may have relevance for recognition of the thyroid antigen as foreign, by T lymphocytes in the presence DR antigens, or may be a result, rather than a cause of the disease.

### 1.2.3. Mechanisms

Mechanisms initiating autoimmune diseases may involve both those causing the production of the autoimmune response and those involved in the pathological manifestations of the disease. The development of autoantibodies may occur either without apparent cause (spontaneously), or as a result of certain drugs or infectious agents. As described above, there is a spectrum of variation in various characteristics of the autoantibodies, whether in their specificity, degree of specificity, target organ, or even their persistence. Since such variation occurs, it might be expected that the immunologic mechanisms responsible may be varied. These may involve either an intact idiotype cascade, where autoantibodies occur due to 'loopholes in the rules' against self immunisation, or impaired immunoregulation (Shoenfeld and Schwartz, 1984).

Mechanisms requiring an intact immunologic network may cause

autoimmunisation by several possible methods. Firstly there are the cases where certain autoantigens are not normally in contact with the immune system, but where damage to a particular organ, for example the testes during vasectomy, may release antigenic components into the circulation, provoking an immune response (Tung, Teuscher and Meng, 1981). However, this process is rare in induction of autoimmunity, and trauma to an organ does not usually result in autoantibody production.

Secondly, modification of the molecule might lead to recognition of the molecule as foreign. This may occur as a result of defects in synthesis of breakdown, such that potentially autoantigenic determinants are presented on apparently new carrier molecules (Roitt, 1977). Alternatively, when self molecules become antigenic on immunisation in Freund's adjuvant, this may be due to the water-oil interface physically altering the shape of the carrier part of the molecule (Roitt, 1977). Chemicals and drugs might also modify a molecule on the cell surface, such as was postulated for alpha methyl dopa induced autoimmune haemolytic anaemia (Allison, Denman and Barnes, 1971; Worlledge, 1973), although more recently methyl dopa has been shown on cause immune dysregulation (Kirtland, Mohler and Horwitz, 1980). Such a mechanism might explain the presence of circulating antibodies to halothane-altered hepatocytes in halothane associated hepatitis (Vergani et al, 1980). In humans infected with Mycoplasma pneumoniae, the resulting red cell autoantibodies have been postulated to be due to Mycoplasma binding to the sialic acid - containing receptor on erythrocytes, which are thus modified and recognised as foreign (Loomes et al, 1984).

Thirdly, autoimmunisation may occur due to antibodies to exogenous antigens crossreacting with autoantigens. These antigens may be present as heterologous tissue in vaccines, or on micro-

organisms themselves, as has been found for Streptococcus induced anti-heart antibodies in rheumatic fever (Zabriskie, 1976), and E. coli 014 induced anti-colon antibodies in ulcerative colitis (reviewed in Doe, 1982). Even where microorganisms are not cross-reacting with the autoantigens, they may cause recognition of self antigens as foreign, such as where virally infected cells act as new carriers for cell surface antigen, and already have MHC expressed on the membrane (Zinkernagel, 1977).

Polyclonal stimulation can also bypass the need for T cell help, and therefore break through T cell tolerance. This may be chemically induced by certain drugs, or due to endotoxins and bacterial glycolipids present in microorganisms (Dziarski, 1982a,b).

Yet another possible mechanism for inducing autoimmunity, yet requiring an intact immunologic network, may be one which results in anti-receptor antibodies, in certain forms of autoimmunity. Shoenfeld has collated experimental evidence suggesting that these pathogenic antibodies are really auto-anti-idiotypes, arising as a result of a non-pathogenic antibody response (idiotypes) to the hormone itself, and replacing the hormone on the receptor surface (Shoenfeld and Schwartz, 1984). This has been cited as an explanation for certain forms of diabetes mellitus, where autoantibodies to insulin receptors exist (Flier et al, 1976). Experimentally, immunisation of mice with insulin, leads to both anti-insulin antibodies and auto-anti-idiotypes, which react with insulin receptors (Shechter et al, 1982). Clinically, a significant proportion of insulin dependent diabetics were shown to have auto-antibodies to insulin (Palmer et al, 1983) and the insulin receptor (Maron et al, 1983) before undergoing insulin treatment. Plotz has described a mechanism for autoantibodies being anti-idiotypes to anti-viral antibodies (Plotz, 1983).

Mechanisms which propose immune dysregulation as initiating autoimmunity are wide ranging. Since lymphocytes from normal individuals may be capable of producing autoantibodies, such as by the action of non-specific mitogens in culture (Koopman and Schrohenloher, 1980), and since polyclonal activators may induce autoantibody secretion in normal mice *in vivo* (Izui et al, 1977), it might be expected that removal or disruption of the regulatory mechanisms normally at work, may result in autoimmunity. As such 'immune surveillance' may decrease with age, it is of interest that the incidence of autoantibodies tends to increase with age (Hooper et al, 1972).

Loss of tolerance and immune dysregulation might occur through somatic mutation or drugs or infectious agents. Thus procainamide may induce SLE (Bluestein et al, 1979), while *Trypanosoma* may also induce autoantibodies (Lindsley, Kysela and Steinberg, 1974). Disturbance of T and B cell regulation has been reviewed by Miller and Schwartz (1982) and Shoenfeld and Schwartz (1984) for a number of diseases: SLE, Graves' disease and myasthenia gravis have all been shown to be associated with depressed T suppressor cell function, while another SLE type syndrome in the MRL-lpr/lpr mouse, and systemic sclerosis, have been linked to increased helper cell function. That certain drugs cause autoimmunity by affecting these regulatory cells has been shown by the depressed T suppressor cell function in methyl dopa induced haemolytic anaemia (Kirtland, Mohler and Horwitz, 1980), and increased T helper cell function in procainamide induced lupus (Miller and Schwartz, 1982). In fact, methyl dopa has been shown to activate cyclic AMP, which results in inhibition of suppressor cells (Kirtland, Mohler and Horwitz, 1980), while *in vitro*, procainamide has been found to inhibit T suppressor cells (Ochi et al, 1983).

Penicillamine, which can induce a number of autoimmune disorders, is also thought to cause immune dysregulation (Jaffe, 1981). It is of interest that the T suppressor defect seen in certain autoimmune diseases, viz. SLE and biliary cirrhosis, is also seen in clinically healthy first degree relatives, indicating a genetic background for the immunodysregulation (Shoenfeld and Schwartz, 1984), with perhaps additional factors causing pathogenesis.

### 1.2.4. Pathology

The finding that autoantibodies exist in low titres in the general population (Smith and Steinberg, 1983), without apparent clinical manifestations, intensifies the need to examine their role in the pathogenesis of autoimmune diseases in order to denote the significance of their presence. Smith and Steinberg (1983) have related the immune mechanisms in autoimmune pathogenesis to the Coombes and Gell classification of the 4 basic types of hypersensitivity: especially type II (cytotoxic), type III (antigen-antibody complexes) and type IV (cell mediated), (Coombs and Gell, 1975). In turn, these mechanisms can be amplified by other non-specific factors, such as complement and lymphokines.

Many autoimmune diseases could be described as Type II. Cytotoxic autoantibody in the circulation reacts with the self-antigen on the cell surface or tissue, and often in conjunction with complement, causes cell lysis or inactivation. This is typified by the anti-erythrocyte autoantibodies in autoimmune haemolytic anaemia. Normal red blood cells coated with antibodies with similar specificities to those eluted from Coombs positive erythrocytes have a decreased half life on reinjection into normals (reviewed by Miescher, 1969). Anti-platelet antibodies from patients with idiopathic thrombocytopenic purpura cause depression of platelet

counts in normals (Shulman, Marder and Weinrach, 1964). Antibodies from patients with Hashimoto's disease are cytotoxic for monolayers of cultured human thyroid cells (Pulvertuft, Doniach and Roitt, 1961). Passive transfer studies into non-human primates have shown that damage to the glomerular basement membrane in Goodpastures's syndrome may also be the result of such complement fixing antibodies (Lerner, Glassock and Dixon, 1967).

Alternatively the circulating autoantibodies may block or stimulate certain functions. Thus autoantibodies to intrinsic factor in pernicious anaemia, if they are present in the lumen of the gastrointestinal tract, may prevent intrinsic factor mediating the absorption of vitamin B12, causing anaemia due to B12 deficiency (reviewed in Doniach, Bottazzo and Drexhage, 1982). In myasthenia gravis, the autoantibodies to the acetylcholine receptor bind to the receptor, blocking the neurotransmitter acetylcholine, accelerating degradation of the receptor, and result in muscle weakness and other clinical manifestations with which their functional activity correlates (Drachman et al, 1982). Transfer experiments have shown that the immunoglobulin from myasthenia patients, when injected into rodents, results in electromyographic manifestations (Toyka et al. 1977). In Graves' disease, an autoantibody to the thyroid stimulating hormone (TSH) receptor, stimulates the receptor, as would the hormone itself, enhancing DNA synthesis by thyroid cells in vitro, and by inference, thyroid growth in vivo (Drexhage et al, 1980). However, anti-TSH receptor antibodies in primary myxoedema block the receptor, preventing its stimulation by TSH (Drexhage et al, 1981). Anti-islet cell surface antibodies in diabetes, have been known to block responsiveness or lead to hypoglycaemia, by acting as insulin on the receptor (Flier et al, 1976).

Autoimmune pathogenesis mediated by immune complexes (Type

III) may occur when antibody complexes with antigen, becomes deposited in tissue, and fixes complement, causing inflammation and destruction. Thus in SLE, complexes of DNA, anti-DNA antibody and complement, have been found as granular deposits in the kidneys of patients with renal complications (Koffler, Schur and Kunkel, 1967). Skin biopsies from patients with discoid lupus erythematosus also indicate the presence of immune complexes, possibly involved in the pathogenesis (reviewed by Hughes, 1982). Many forms of immune complex glomerulonephritis may have an underlying autoimmune aetiology. It has also been suggested that in rheumatoid arthritis, autoantibodies to the Fc portion of IgG form complexes which are deposited in the joints, and cause pathological changes, by generating a local Arthus reaction, fixing complement and attracting polymorphs (Roitt, 1977).

Type IV or cellular hypersensitivity has been implicated in the pathogenesis of autoimmune diseases due to the mononuclear nature of the inflammatory cellular infiltrates present in organ specific autoimmune diseases. It has been suggested that lymphokines can mediate further damage by recruiting extra inflammatory cells to the site of damage in a cascade effect (Smith and Steinberg, 1983).

It should be noted in any examination of the pathogenesis of autoimmune disease by autoantibodies, that titre of the antibody does not necessarily correlate with severity of clinical manifestations. Thus in myasthenia gravis, neither autoantibody specificity, serum titre nor affinity of anti-acetylcholine receptor antibodies correlates with disease severity (Tzartos, Seybold and Lindstrom, 1982; Roses et al, 1981; Bray and Drachman, 1982). In thyroiditis, there is a relationship between levels of serum anti-thyroid antibodies and lymphocytic infiltration of the thyroid gland

(Yoshida et al, 1978), even if not with other clinical indices. This may simply indicate that above a threshold level, other effector systems may be more important with respect to clinical expression of disease, or may indicate that there are further complexities about the specificity, affinity and time course of autoantibody production, which are not yet understood. As such, the same titre of antibody might have minimal effects in one individual, but might be highly damaging in another.

The presence of low titres of autoantibodies in the general population may yet be found to be associated with subclinical pathological changes, since for thyroid and stomach autoantibodies, biopsy has generally shown an association with minor thyroiditis and gastritic lesions (Isokosi et al, 1969; Yoshida et al, 1978). However, relatives of patients with autoimmune disease often produce autoantibodies without clinical abnormalities (Lippman et al, 1982).

### 1.2.5. Treatment

Current therapy for autoimmune diseases involves treatment of the pathological symptoms, as well as numerous methods to control the aberrant humoral and/or cellular responses. Such treatments are based on 2 major principles of (1) immunosuppression, mainly by drugs, and (2) removal of the mediators of damage - in the case of autoantibodies, by plasmaphereses. Application of both of these principles results in broad spectrum effects, and for cell mediated autoimmune responses only immunosuppression is at present possible, due to difficulty in isolating or identifying the pathogenic cells. More specific forms of treatment for both humoral and cell mediated autoimmune diseases are therefore required.

The search for more specific forms of immunotherapy has led to the examination of the possible benefits of anti-idiotypic anti-

bodies. While in one case, administration of anti-idiotype dramatically improved a patient with B cell lymphoma (Miller et al, 1982), care will be necessary due to the possible augmentation of the antibody response by anti-idiotype, as has been described earlier for foreign antigens (Eichmann and Rajewsky, 1975), and has been found experimentally in production of an idiotype of anti-DNA antibody (Teitelbaum et al, 1984).

### 1.2.6. Experimental Models of Autoimmunity

Study of the mechanisms at work in human autoimmune diseases has been greatly aided by the use of experimental models of autoimmunity. Just as do human autoimmune diseases, these models exhibit a spectrum of variation with respect to specificity of antibodies and target organs, as well as genetic restriction and severity. They may be either spontaneous or induced by a number of factors. However, it is not known to what extent those of a spontaneous nature may be induced by common environmental factors which may also be causing 'spontaneous' human autoimmune diseases. These experimental models may be self limiting, as is the case for $HgCl_2$ induced glomerulonephritis in the Brown Norway rat, and in models of myasthenia gravis, or chronic, such as in murine lupus.

#### Spontaneous Models of Autoimmunity

Spontaneous models of autoimmunity have been of great use in the study of autoimmune disease, in that they most closely resemble the apparently spontaneous nature of many human autoimmune diseases. However, their spontaneous nature can place limitations on their study, due to inadequate warning of the onset of the autoimmune response.

The three most widely studied models, spontaneous murine

lupus, spontaneous diabetes in the BB rat and spontaneous thyroiditis in the obese chicken or Buffalo rat, show variation in organ specificity of the diseases, and all these have the disadvantages of not being self limiting, and so are of limited use in studies on autoregulation. Areas in which study of these models may have helped in an understanding of human disease, include genetic restriction/susceptibility, pathology/mechanisms of induction, and treatment. For example, in murine lupus, genetic studies made possible by breeding experiments, show multiple genes to be involved in susceptibility to the disease (Bocchieri et al, 1982), a feature perhaps applicable to human SLE and its wide range of clinical manifestations. Such breeding experiments have even localised the production of the autoantibodies to a certain B cell subset, Lyb $3^+$, $5^+$, coded for by the xid gene (reviewed by Smith and Steinberg, 1983). Development of thyroiditis in the OS chicken has been shown to be under multiple gene control, with at least 3 loci- the MHC B locus, a non B histocompatability locus, and a thyroid locus (Wick, Gundolf and Hala, 1979).

Transfer experiments are possible in these disease models, and have shown, in all strains exhibiting murine lupus, that the disease can be transferred into irradiated recipients by bone marrow stem cells (reviewed by Smith and Steinberg, 1983). Mechanisms of autoimmunity have also been studied by examining the effect of neonatal or adult thymectomy on disease development in thyroiditis (reviewed by Rose et al, 1977), or murine lupus (Smith and Steinberg, 1983). Some of these studies have suggested T suppressor restraint of the disease. Cultures using spleen cells have likewise helped in dissection of defective control in autoimmunity. In diabetes in the BB rat, these have indicated a possible defect at the IL2 receptor or post receptor level (Elder and McLaren, 1983).

The effects of treatment on autoimmune disease can be more easily studied in experimental animals than humans, and immune intervention therapies such as thymectomy, anti-lymphocyte serum, total body irradiation and cyclosporin, have been examined and shown to be beneficial in the BB rat (reviewed by Yale and Marliss, 1984). 'Preventative treatment' by tolerising BB rats with bone marrow transplants from WF donors, has been shown to not only decrease incidence of diabetes, but also normalise T cell function (Naji et al, 1981).

Induced Models of Autoimmunity

Experimental autoimmune disease may be induced in animal models by a variety of methods which would follow from suggested mechanisms of induction of autoimmunity and include the use of: autologous, homologous or heterologous antigen, with or without adjuvant; modified antigen; antigen cultured spleen cells; or a series of other means which may involve polyclonal activators, whether chemical, bacterial or parasitic. Additional methods might include immunisation with an antibody to an organ, which, in the case of nephrotoxic nephritis elicits a heterologous phase of the heterologous antibody binding to kidney, followed by an autologous antibody response. These induced models of autoimmune disease may be organ specific, especially when induced by antigen, or non-organ specific as a result of polyclonal activation. They may be self limiting, and therefore subject to autoregulation, or chronic and relapsing. Study of such models has helped in the elucidation of mechanisms of pathogenesis, genetic restriction and treatment in human disease in a similar way to the study of the spontaneous models, and in addition, reveals methods by which autoimmunity can be induced by environmental factors.

Injection of homologous/autologous antigen in adjuvant, or heterologous antigen with or without adjuvant may induce autoimmunity experimentally by by-passing the requirements for T cell help, as discussed in section 1.2.3. Diseases which may be induced by these means include those which are T cell mediated, such as experimental autoimmune encephalomyelitis, or 'EAE' (reviewed in Weigle, 1977), as well as those which are mediated by autoantibody, such as rat erythrocyte induced anti-erythrocyte autoimmunity in mice (Playfair and Marshall-Clarke, 1973). Other models have 'target organs' as diverse as the acetylcholine receptor in nerve synapses (experimental autoimmune myasthenia gravis or 'EAMG' (reviewed by Seybold, 1983)), joints (collagen induced autoimmune arthritis (reviewed in Stuart, Townes and Kang, 1984)), thyroid (experimental autoimmune thyroiditis or 'EAT' (reviewed in Weigle, 1977)), and kidneys (reviewed in Andres and Brentjens, 1984). Certain disadvantages may exist with these models, however. Where heterologous antigen is used, a response to foreign as well as cross reactive determinants, may ensue, whilst use of adjuvants introduces additional variables due to its non-specific effect on the immune system and its potentiation of pathogenic damage (Moorthy and Abreo, 1983). Human analogues of induction by heterologous antigen might be represented by autoimmune phenomena following vaccine injection.

Antigen expressed in a novel way on spleen or tumour cells may also induce experimental autoimmunity. Spleen cells from normal rats, when cultured with antigen in vitro and passed through a primary host, elicit EAE in Lewis rats (Holda, Silberg and Swanborg, 1983).

Where antigen is modified and escapes self recognition by being conjugated to a hapten, or undergoing denaturation, experimental autoimmunity may result. The former is shown by anti-

erythrocyte autoantibody production in mice after priming with hapten-protein (Yamashita et al, 1976) or rabbit Ig coupled to syngeneic mouse erythrocytes (Naysmith, Ortega-Pierres and Elson, 1981), and may relate to anti-erythrocyte autoantibody production in humans infected with mycoplasma as described earlier (Loomes et al, 1984). Some experimental models of drug induced autoimmunity might also occur by this method.

Other mechanisms of experimentally inducing autoimmunity are by the use of various microorganisms, and chemicals or drugs, which tend to induce a polyclonal response, with a variety of autoantibodies being produced. Autoimmunity during African trypanosomiasis has been shown in a mouse experimental model, to be associated with polyclonal B cell activation, with specific antibodies to DNA, red blood cells and thymocyte antigens being present amongst the large quantities of IgG produced (Kobayakawa et al, 1979). The induction of autoantibodies by gram negative bacteria or their products has been shown in a number of experimental models. Thus, lipopolysaccharide from many bacterial strains has been found to induce anti-DNA antibody production in mice (Fournie, Lambert and Miescher, 1974), and this appears to be a consequence of polyclonal B lymphocyte activation (Izui et al, 1977). Development of antibodies to autoantigens normally under a state of T cell tolerance may be due to such polyclonal activation, since in heterologous systems LPS can terminate T cell mediated tolerance (Chiller and Weigle, 1973).

Autoantibodies have been shown to result in a number of systems, from the effect of chemicals such as methyl dopa (Kirtland, Mohler and Horwitz, 1980), procainamide (Bluestein et al, 1979) and penicillamine, and mercuric chloride. Penicillamine has long been associated with autoantibody production in humans, and its use

results in a proportion of patients, in myasthenia gravis, pemphigus and even Goodpasture's syndrome and SLE (Jaffe, 1981). Whether its mode of action is due to effects on immunoregulation (decreased $T_S$ function or stimulation of T help), or alteration of autoantigens by its highly reactive SH group, is not known. However, a recently described model of penicillamine induced autoimmunity in the rat may help in this area. Donker has shown that prolonged administration of D-penicillamine in BN but not other rat strains, results in anti-nuclear antibodies, anti-GBM antibodies, immune complexes and intravascular coagulation and granulomatous lesions (Donker et al, 1984). It was suggested that D-penicillamine is causing polyclonal activation of both B and T lymphocytes.

Mercury (or mercuric chloride), in small doses, is known to induce an immune complex nephritis in both man (Mandema et al, 1963) and animals (Bariety et al, 1971; Roman-Franco et al, 1978; Weening, Fleuren and Hoedemaeker, 1978). The mechanism whereby $HgCl_2$ induces this group of diseases is not known. Suggestions include modification of autologous antigens, rendering them antigenic (Bariety et al, 1971; Roman-Franco et al, 1978), to immune dysregulation, with suppressor cell activity being targeted (Weening, Hoedemaeker and Bakker, 1981), or polyclonal activation (Hirsch et al, 1982).

Another novel method for inducing autoimmunity experimentally has been described by Zanetti, who has induced anti-thyroglobulin autoantibodies in the Buffalo rat and Balb/c mice, using anti-idiotypic antibodies raised in rabbits against monoclonal anti-thyroglobulin antibodies (Zanetti, Rogers and Katz, 1984). Similar induction methods in nature have been suggested for autoimmunity, whereby antibodies to a foreign protein, which share an epitope found an autoantibodies to self antigens, induce anti-

idiotype according to Jerne's network theory (Jerne, 1974), and these anti-idiotypes induce the formation of that autoantibody.

## Experimental Models of Autoimmune Kidney Diseases

Where kidneys are the target organ, experimental models of autoimmunity have been induced by injection of homologous FX1A kidney antigens, heterologous or homologous GBM or heterologous tubular basement membrane (TBM), usually in complete Freund's adjuvant (CFA), or $HgCl_2$ (reviewed by Andres and Brentjens, 1984).

In Heymann nephritis in rats, injection of a lipoprotein present in the FX1A fraction of renal homogenates results in an immune complex membranous nephropathy and massive proteinuria, associated with autoantibody production to antigens present on proximal tubular cells (Heymann et al, 1959; Edgington, Glassock and Dixon, 1968). The development of the disease is under genetic control, with Brown Norway rats being resistant and the Lewis strain susceptible (Stenglein, Thoenes and Günther, 1978).

Inbred strain 13 guinea pigs, injected with rabbit or bovine TBM, produce anti-TBM antibodies which cross react with their own TBM antigens. This rapidly results in a tubulointerstitial nephritis with an infiltrate of mononuclear cells and multinuclear giant cells in the renal interstitium. Immunofluorescence of renal biopsies shows linear deposits of elutable IgG along the renal cortical TBM (Steblay and Rudofsky, 1971). Cortical involvement correlates with serum anti-TBM antibody levels, and the disease can be transferred by IgG1 or IgG2 anti-TBM autoantibodies (Hall et al, 1977). This disease is also highly genetically restricted (Hyman, Colvin and Steinberg, 1976).

Experimental allergic glomerulonephritis (EAG) may be induced in a number of species as diverse as sheep (Steblay, 1962),

rabbits (Unanue and Dixon, 1967) and rats (Stuffers-Heiman, Günther and Van Es, 1979) by injection of heterologous GBM in CFA. Homologous GBM may also be used in some models, and in others, adjuvant is not an absolute requirement for the production of autoantibodies (Unanue, Dixon and Feldman, 1967). Susceptibility in rats is genetically restricted but not completely controlled by genes linked to the H-1 complex, and BN rats exhibit a susceptibility to anti-GBM antibody production. Linear deposition of IgG occurs along the GBM, and the specific antibody can be eluted from the kidneys. However, in the rat, no proteinuria or deposition of C3 occurs, indicating little pathogenesis, even though both IgG1 and IgG2a are fixed (Stuffers-Heiman, Günther and Van Es, 1979) and both subclasses can fix and activate complement (Medygesi et al, 1978). This differs from the disease in sheep and rabbits, where anti-GBM antibodies are pathogenic (Steblay, 1962; Unanue and Dixon, 1967).

Mercuric chloride induces a spectrum of kidney diseases in experimental animals. In PVG and Wistar rats, the resulting disease has a single long term phase of immune complex membranous glomerulopathy, which in the PVG strain appears to be due to complexes of anti-nuclear antibody (Weening, Fleuren and Hoedemaeker, 1978). In rabbits, the disease is biphasic- in the initial phase antibodies to basement membrane and extracellular collagen matrix are found and result in linear staining along the GBM, while the second phase consists of antigen-antibody complexes in situ and in the circulation (Roman-Franco et al, 1978). A disease with some similarities occurs in BN rats injected with $HgCl_2$ (Druet et al, 1979).

1.2.7. Regulation of Autoimmune Responses

Control of autoantibody production may occur either spontaneously as autoregulation, or externally by various surgical, radiological, chemical or immune means. Control may be exerted positively, to reduce the autoimmune phenomenon, or negatively to increase it, and may be specific for the autoantibody, or non-specific. Study of experimental models of autoimmunity has greatly helped in an understanding of regulation of autoimmune processes.

Of external manipulations involving surgery, neonatal thymectomy in $HgCl_2$ induced (immune complex mediated) glomerulopathy in PVG rats (Weening, Hoedemaeker and Bakker, 1981) and spontaneous thyroiditis in Buffalo rats (reviewed in Rose et al, 1977), causes acceleration of autoantibody production and immunopathological features, while adult thymectomy has no significant effect. However, adult thymectomy, irradiation and bone marrow reconstitution in the rat RBC induced anti-erythrocyte response in mice, results in failure to produce autoantibodies after rat RBC injection (Naysmith, Ortega-Pierres and Elson, 1981). Irradiation of Lewis rats, under certain conditions, allows reinduction of allergic encephalomyelitis in convalescent animals normally resistant to reinduction (Willenborg, 1982).

Cytotoxic drugs may also affect autoantibody production, as has been shown for cyclophosphamide in rat RBC induced anti-erythrocyte autoimmunity (Naysmith, Ortega-Pierres and Elson, 1981), $HgCl_2$ induced anti-GBM disease (Pusey et al, 1983), and EAE (Paterson and Hanson, 1969). In the first model enhancement has been shown, in the second, dose dependent enhancement or inhibition, while for EAE inhibition occurs. Cyclosporin inhibits induction of $HgCl_2$ induced nephritis in BN rats (Baran et al, 1985; Prummel et al, 1985) and prevents/cures diabetes in the BB rat (Yale and

Marliss, 1984). However, each of these manipulations, thymectomy, irradiation and cytotoxic drugs, acts on the immune system in a non-specific manner, either removing T suppressor cells, removing T helper cells, or other cells aiding antibody production, or affecting humoral regulatory antibodies. In their non-specific actions, even if autoantibody synthesis is down regulated, other protective antibody production systems may also suffer.

Understanding of specific mechanisms for regulation of autoantibody synthesis, will have relevance to the design of therapeutic regimens in autoimmune diseases. Such specific mechanisms might be expected to occur in experimental animal models undergoing autoregulation as part of a self limiting disease, or in certain manipulations involving for example, heterologous anti-idiotypic antibodies. These mechanisms would be presumed to be similar to those described earlier for regulation of the immune response to foreign antigens, and might involve most notably T suppressor cells and anti-idiotypic antibodies.

Anti-idiotypic Antibodies

Heterologous anti-idiotypic antibody has been shown in a number of experimental models of autoimmunity to modify the autoantibody response. Thus, in rabbit TBM induced autoimmune tubulointerstitial nephritis in strain 13 guinea pigs, pretreatment with rabbit antisera raised to eluates from diseased kidneys reduces both disease activity and levels of circulating antibody to TBM compared to controls. This is especially marked with antisera raised to IgG2 as against IgG1 anti-TBM antibodies, and does not affect other antibody responses (Brown, Carey and Colvin, 1979).

In spontaneous experimental autoimmune thyroiditis in Buffalo rats, repeated injections of heterologous anti-idiotypic

antibodies into sublethally irradiated recipients exhibiting disease activity, results in a decrease in levels of circulating anti-thyroglobulin antibody (Zanetti and Bigazzi, 1981). However, heterologous anti-idiotype is also capable of inducing anti-thyroglobulin production when injected into young normal Buffalo rats (Zanetti, Rogers and Katz, 1984).

In murine lupus nephritis, where heterogeneity of the autoantibody is broader, treatment of diseased mice with a mouse monoclonal anti-idiotype to a major cross-reactive idiotype, results in (1) slight transient reduction in total anti-DNA antibody, (2) deletion of target idiotype, and (3) prolonged survival due to delay in onset of nephritis. However, due to a probable idiotype 'switch', suppression of the anti-DNA response is succeeded by the appearance of large amounts of anti-DNA antibodies of other idiotypes (Hahn and Ebling, 1984). In another murine SLE model, administration of heterologous anti-idiotype to the dominant idiotype results in augmentation of that idiotype and anti-DNA antibodies (Teitelbaum et al, 1984).

With heterologous anti-idiotypes being shown to have a mixed effect on autoantibody production, the role of auto-anti-idiotypes becomes more important. These may arise during an animals own autoregulation, or may be induced by other means and lead to control. In $HgCl_2$ nephritis in the BN rat, recent work has shown the presence of antigen-augmentable PFC (putatively representing the presence of anti-idiotype (Schrater et al, 1979)), occurring when circulating anti-GBM antibody levels peak and then fall during autoregulation. This is associated with humoral factors, which also behave as auto-anti-idiotypic antibodies (Chalopin and Lockwood, 1984).

In vitro suppression of anti-DNA antibody binding to DNA,

has been shown by anti-idiotypic antibody (localised in $F(ab')_2$ of IgG) present in the sera of patients with inactive SLE and normal donors in contact with lupus patients or sera, but not in patients with active SLE or non-contact normals (Abdou et al, 1981). This anti-idiotype has been found to inversely follow anti-DNA antibody (or idiotype) levels (Zouali and Eyquem, 1983).

In TBM induced interstitial nephritis in rats, no auto-anti-idiotype can be detected as part of auto regulation, although, anti-idiotypic antibodies can be induced by immunisation with a relevant monoclonal anti-TBM antibody (Neilson and Zakheim, 1983), or pretreatment with TBM antigen reactive (and idiotype expressing) T lymphoblasts, which also reduce disease activity (Neilson and Phillips, 1982a). Neilson has suggested that the failure to produce anti-idiotypes in the unmodulated model is a result of polyclonal suppression, subsequent to the disease, because other immunogens also fail to evoke an antibody response (Neilson and Phillips, 1982b).

Cells and suppression

In the rat RBC induced autoimmune haemolytic anaemia model in mice, a number of observations have been made with regard to the autoregulation of the anti-erythrocyte autoantibody. In this model suppression may be transferred from previously immunised mice to normal syngeneic recipients using lymphoid cells, whether lymph node, spleen or thymus (Cooke et al, 1978; Naysmith et al, 1980) and even blood leukocytes (Gare and Cox, 1980). The suppression is specific, with neither the anti-rat RBC (Naysmith et al, 1980) or primary anti-SRBC response (Cooke et al, 1978) being affected. The effect is dose dependent and active on the inductive phase of the disease (Naysmith et al, 1980). B lymphocyte or macrophage

depletion does not affect the transferrable suppression, while T cell depletion causes partial abrogation (Naysmith, Ortega-Pierres and Elson, 1981). Suppression is transferrable after 4 weeks and still occurs at 12 weeks, while in other studies detectable autoantibody production was not shown to be necessary for generation of suppressor cells (Naysmith et al, 1980).

In self regulating experimental EAE in Lewis rats, lymphoid cells (spleen, thymus, non-draining lymph node) taken from convalescent rats have been shown to transfer suppression to naive recipients, with resulting inhibition of EAE induction (Adda et al, 1977). The suppressive activity is short lived, existing between days 15 and 17, and disappearing on day 25 (Adda et al, 1977). A suppressor factor can be extracted from the non-draining lymph node cells and this is inhibitory in vivo. In addition, the non-draining lymph node cells, and 48 h culture supernatants from them, specifically inhibit antigen induced proliferation in vitro (Beraud et al, 1982).

In murine SLE, a lymphocyte derived non-specific suppressor factor (SIRS) has been shown to attenuate one of the suppressor cell defects, and markedly prolongs survival with respect to nephritis, and diminishes the autoimmune disease (Krakauer et al, 1977).

Suppressor cells have also been implicated in antigen specific control of induction of Heymann nephritis in rats, both with respect to the autoimmune response and proteinuria. Such suppressor cells could be induced in tolerised animals (Litwin et al, 1979). In mercuric chloride nephritis in the BN rat, a role for specific T suppressor cells during autoregulation has been shown (Bowman et al, 1984) and will be discussed in this thesis.

## 1.3. GOODPASTURE'S SYNDROME AND ANTI-GBM AUTOANTIBODY MEDIATED NEPHRITIS

Production of autoantibodies to the GBM in humans leads to a rapidly progressive glomerulonephritis which may also be associated with pulmonary haemorrhage (Goodpasture's syndrome). Diagnosis was initially made by detection of immunoglobulin deposits along the glomerular basement membrane in direct immunofluorescence of biopsy material, although it is often now made in conjunction with assays for circulating anti-GBM antibodies. Duration of antibody production in both forms of the disease has been found to average 11 to 14 months in untreated patients, but in all cases spontaneously ceases (Wilson and Dixon, 1981; Flores et al, 1986). Recurrence of anti-GBM antibodies is rare but not unknown (Hind et al, 1984).

### 1.3.1. Specificity

Anti-GBM antibody eluted from the glomeruli of kidneys from patients with Goodpasture's syndrome has been found to react with GBM, TBM and alveolar basement membrane (Koffler et al, 1969), and even to the choroid plexus (Sharon, Rhode and Lewis, 1978). This has been confirmed using the monoclonal antibody MCA-P1, to the Goodpasture antigen (Pressey et al, 1983), which binds to GBM, basement membrane from distal tubules, Bowman's capsule, alveolar basement membrane, choroid plexus, cochlea basement membrane, and retinal and lens capsule membrane (Pusey et al, 1986).

The Goodpasture antigen itself is found in collagenase digests of GBM (Wilson, 1980), and chaotropic (Marquardt, Wilson and Dixon, 1973) and guanidine hydrochloride extracts (Wieslander, Bygren and Heinegård, 1983). By SDS-PAGE it appears to be in some high molecular weight material, and two major peaks of approximately 25K and 50K (reviewed by Rand-Weaver and Price, 1983; Wieslander,

Bygren and Heinegård, 1984), although different preparative procedures have shown up to 6 bands on SDS-PAGE, with Western blotting, again centred around 25K and 50K. All sera tested from more than 30 patients with Goodpasture's syndrome, bound to each of the 6 bands, as did the monoclonal antibody, MCA-P1, showing high homology of reactivity and highly restricted specificity (Pressey et al, 1983; A. Dash-personal communication).

The antigen has the heteropolysaccharide nature of many partially purified glycoproteins (Wilson, Holdsworth and Neale, 1981), a non-collagenous nature (Wilson, Holdsworth and Neale, 1981; Wieslander, Bygren and Heinegård, 1983), and has been recently shown to be in the non-collagenous NC1 domain of type IV collagen (Wieslander et al, 1984). It has been suggested that antibodies from patients with Goodpasture's syndrome have a broader reactivity with other basement membranes than patients with anti-GBM nephritis alone (McPhaul and Dixon, 1970).

### 1.3.2. Genetics

The genetic restriction of anti-GBM disease has been shown by the high incidence of tissue type DRW2, with a relative risk of 15.9 (Rees et al, 1978). Severity of expression of the disease has also been linked to B7 (Rees et al, 1984). In addition, in a number of cases, anti-GBM disease in identical twins, siblings and first cousins has been reported (reviewed in Rees and Lockwood, 1986). Susceptibility to anti-GBM disease is also associated with the Gm allotype of Ig heavy chains, with a significant increase in the frequency of Gm 1,2,21, as compared to the general population (Rees, Demaine and Welsh, 1984).

1.3.3. Pathology

Light microscopy of renal biopsies shows proliferative crescentic glomerulonephritis, with some monocyte or polymorphonuclear leukocyte infiltration. Destruction of the GBM varies from extensive, to moderate with focal lesions, although the GBM may occasionally be normal. Tubulointerstitial changes also often occur. Electron microscopy of biopsies shows an increase in the mesangial matrix, detachment of endothelial cells from the basement membrane, and the presence of gaps or discontinuities along the GBM (reviewed by Wilson and Dixon, 1981; Glassock et al, 1981).

Immunofluorescence of the renal biopsy shows smooth linear deposition of immunglobulin, usually IgG, but occasionally with IgM and IgA (Wilson and Dixon, 1981). However, it should be noted that weak linear deposits may also occur in normal kidneys, and especially in diabetics (Gallo, 1970). Linear deposits, (either diffuse or focal) along the tubular basement membrane are also found in some patients with Goodpasture's syndrome (Lehman, Wilson and Dixon, 1975). C3 deposition occurs in 50-60% of patients (Williams and Peters, 1982). Furthermore, linear deposition of IgG may occur in the lung, even in the absence of pulmonary symptoms (Wilson, Holdsworth and Neale, 1981).

The pathogenicity of the anti-GBM antibodies has been demonstrated by Lerner, in experiments where antibodies which were eluted from Goodpasture's patients kidneys, with linear staining along the GBM, or from their serum, were capable of transfer of glomerulonephritis to other primates (Lerner, Glassock and Dixon, 1967). Additionally, nephrotoxicity of the antibody has been indicated in recurrence of anti-GBM disease in renal transplantation, especially where residual circulating anti-GBM antibody remained (Wilson, 1980).

1.3.4. Mechanisms

The mechanism by which tolerance is broken and anti-GBM autoantibody production is initiated in this disease is not known, but may occur by similar mechanisms to those postulated for other autoimmune diseases, as described earlier. Thus, perhaps damage to the kidney or lung, by environmental or immunologic factors, may result in the antigen being released into the circulation, to come into contact with immune cells. Chemicals or viruses might perhaps bind to the antigen and act as a foreign carrier, or release restricted stimulators of the immune system, or even act as cross reacting antigens. However, to whatever extent environmental factors, including viruses, either together or separately, induce the formation of anti-GBM antibodies, the role played by genetic factors cannot be ignored as seen by the genetic restriction of the disease.

Clinical associations which may be related to the aetiology of anti-GBM disease include the finding that approximately 40% of patients have a 'flu-like' illness shortly before the clinical manifestations of the disease (Wilson, Holdsworth and Neale, 1981), while some patients have coincidently manifested an influenza A2 infection (Wilson and Smith, 1972). Hydrocarbon exposure has been noted in others (reviewed in Rees and Lockwood, 1986). In some patients, anti-GBM antibodies evolve following membranous nephropathy, and Klassen has postulated that they are then due to immune complexes damaging the membrane, liberating antigenic fragments (Klassen et al, 1974). Anti-GBM antibodies have also been noted after renal transplantation, in patients with Alport's syndrome, who, it has been suggested, lack the Goodpasture antigen (McCoy et al, 1976; Wilson, Holdsworth and Neale, 1981).

1.3.5. Treatment

Treatment of anti-GBM disease has involved methods to reduce the levels of circulating antibody, which may be mediating the nephritis and lung haemorrhage as well as the treatment of the renal disease itself. Removal of antibody may be accomplished by plasma exchange, and down regulation of antibody production by immunosuppressive drugs (Savage et al, 1985). Since these forms of treatment carry various side effects, a more specific form of immunotherapy would be advantageous, but its development awaits further understanding of autoregulation of the disease.

The prognosis of anti-GBM induced Goodpasture's syndrome or rapidly progressive glomerulonephritis is still variable in terms of mortality and renal function (Wilson, Holdsworth and Neale, 1981; Savage et al, 1986). Patients treated with intensive plasma exchange and immunosuppression at an early stage (before their creatinine reaches 600μmol/l) have been shown to often regain kidney function, while those with more advanced renal failure tend to have irreversible kidney damage. In this latter group there is a relatively high mortality and 25% of these patients do not survive 8 weeks (Savage et al, 1986).

Identification of individuals at risk, coupled with development of a specific treatment to prevent induction of the disease, or to treat the disease at an early stage, would dramatically improve this situation.

## 1.4. MERCURIC CHLORIDE INDUCED NEPHRITIS IN THE BN RAT

### 1.4.1 Background

In the late 1970's, during testing of various rat strains for the effect of subcutaneous injections of $HgCl_2$ in inducing an immune complex type membranous nephropathy, Druet and Sapin

discovered that one strain, the Brown Norway rat, responded instead with an anti-basement membrane autoantibody mediated nephritis. This occurred within two weeks and was characterised by linear staining of IgG along the GBM (Sapin, Druet and Druet, 1977). Anti-basement membrane antibodies were also fixed in the majority of basement membranes (Bernaudin et al, 1977). Breeding studies then showed that the development of the anti-GBM antibodies was under genetic control (Druet et al, 1977). Anti-GBM antibodies could be detected in both serum Ig and renal eluates, using indirect immunofluorescence, and the disease could be transferred with the eluates (Sapin, Druet and Druet, 1977).

$HgCl_2$ in the BN rat was then shown to induce a biphasic glomerular disease, characterised firstly by the production of anti-basement membrane antibodies with linear staining along the GBM, followed by a second phase by day 60, of granular or immune complex staining, both in the kidney (Druet et al, 1978) and in extra renal localisations (Bernaudin et al, 1979). Temporary proteinuria of moderate proportions, occurred during the time of linear staining, and in some cases rats were found to die from acute renal failure, probably due to acute tubular necrosis (Druet et al, 1979). Evidence of complement activation was found (Druet et al, 1979).

By 1980, reconstitution experiments from (LEW x BN) $F_1$ hybrids into irradiated Lewis rats had shown that the susceptibility to develop anti-GBM antibodies could be transferred using bone marrow or spleen cells (Sapin, Druet and Mandet, 1980). In addition, transient clotting abnormalities and disseminated intravascular coagulation were found at the time of maximal proteinuria (Michaud et al, 1980).

In 1981, $HgCl_2$ was found to induce non-specific as well as specific effects in the BN rat. Marked increases in the total serum

IgE were observed, as were specific IgE anti-ovalbumin antibodies, when $HgCl_2$ and ovalbumin were injected. However, IgE anti-GBM antibodies could not be detected (Prouvost-Danon et al, 1981).

The kinetics of circulating IgG class anti-GBM antibodies were then investigated using an RIA, and circulating immune complexes detected (Bowman et al, 1981; Bellon et al, 1982). Initial studies on the self limiting nature of the disease showed that protection from subsequent rechallenge with $HgCl_2$ occurred (Bowman et al, 1981).

When specificity of anti-GBM antibodies was later evaluated, antibodies to several basement membrane constituents were also found (Bellon et al, 1982), and it was then discovered that $HgCl_2$ in the BN rat was acting as a polyclonal activator. Effects included enlargement of lymph nodes and spleen, transient increases in direct and indirect anti-SRBC and anti-TNP PFC and the appearance of DNA binding activity. Polyclonal activation was also shown to occur by the action of $HgCl_2$ on normal BN spleen cells in culture (Hirsch et al, 1982 ), and the concept of $HgCl_2$ induced immune dysregulation leading to autoantibody production in this model, was postulated (Druet et al, 1982). Proteinuria was shown to be complement independent (Capron et al, 1982). The effect of cyclophosphamide on anti-GBM antibody production and polyclonal stimulation (as indexed by rises in total IgG) was examined (Pusey et al, 1983) followed by further studies on the self limiting nature and autoregulation in this model (Bowman et al, 1984; Chalopin and Lockwood, 1984). Using hydridoma technology, monoclonal antibodies, produced by $HgCl_2$ stimulation, to autoantigens and 'foreign' specificities have been recently developed (Hirsch et al, 1984; Hirsch et al, 1985), and the effect of $HgCl_2$ on modifying T cells to induce proliferation has been examined (Pelletier et al, 1985).

1.4.2. The Model

Subcutaneous injection of $HgCl_2$ at 1mg/kg, thrice weekly into BN rats from several sources has been shown to result in production of anti-GBM antibodies, as detected by indirect IF (Sapin, Druet and Druet, 1977), RIA (Bowman et al, 1981; Bellon et al, 1982), ELISA (Michaelson, McCoy and Bigazzi, 1981) and magnetic EIA (Druet et al, 1982b). The disease is self limiting, and biphasic as characterised by immunofluorescence, consisting initially of linear deposits of IgG along the GBM (Sapin, Druet and Druet, 1977), followed by granular immune complex deposits (Druet et al, 1978). The disease is dose dependent on the $HgCl_2$, with fewer animals developing immunofluorescent changes, and IF changes being less marked together with a lower incidence of proteinuria, when lower doses are used (Druet et al, 1978).

1.4.3. Genetics

Using a standard regimen of 200µg $HgCl_2$/kg body weight, anti-GBM antibodies as detected by direct immunofluorescence have been searched for up to day 60 in a variety of inbred (Lewis, Wistar AG, August, PVG/c, BN) and outbred (Wistar, Sprague-Dawley) rats, and are only detectable in the BN rat, although there has been one report of an additional unnamed strain responding with mixed linear and granular fixation in the kidney, by day 15 (Druet et al, 1979). Breeding studies using F1, F2 and backcross hybrids with Lewis rats show that all F1 hybrids produce anti-GBM antibody on $HgCl_2$ injection, indicating that responsiveness is dominant, but F2 and backcross studies show the response to be determined by 2 or 3 dominant autosomal genes, one of which is RT1 linked (Druet et al, 1977; Sapin et al, 1982). In addition, the lower incidence of proteinuria in the hybrids suggests that another gene is involved in

tissue injury (Druet et al, 1977).

Since the disease is biphasic in the BN rat, with granular staining following the linear fixation, genetic control of susceptibility to the second stage of the disease, with immune complexes has also been examined and found to be an autosomal dominant trait, dependent on one major RT1-linked gene, with other non-RT1 linked gene(s) controlling the magnitude of the response (Sapin et al, 1981; Sapin et al, 1982).

Susceptibility to both phases of $HgCl_2$ induced nephritis, is expressed on immune cells as shown by transfer of bone marrow or spleen cells from (LEW x BN) F1 hybrids into the irradiated, but normally resistant Lewis rat (Sapin, Druet and Mandet, 1980). Analysis of the $HgCl_2$ induced polyclonal IgE increase in LEW x BN crosses shows that this feature is under genetic control by 4 loci, one of which is RT1-linked (Sapin et al, 1984).

### 1.4.4. Pathology and Immunopathology

Light Microscopy: During the first 15 days, there are no changes in the glomeruli, although when the dose of $HgCl_2$ is increased, tubular lesions are seen, similar to those found in other rat strains with mercury induced toxic tubular necrosis (Sapin, Druet and Druet 1977). In addition, large mononuclear cells as well as a few polymorphs are found in the glomeruli and interstitium (Hinglais et al, 1979).

During the second stage, at day 60, extramembranous deposits in kidney biopsies are apparent (Druet et al, 1979), but otherwise the kidney is normal. However the spleen suffers from hypertrophy of the white pulp due to immunoblasts and macrophages (Bernaudin et al, 1979).

Immunofluorescence: By day 9 a linear pattern of fixation

of IgG along the GBM can be seen, which is intense from day 12 (Hinglais et al, 1979). IgM and $C_3$ are also present in a linear though less intense or defined pattern (Druet et al, 1979). At this time intense IgG fixation also occurs in the splenic white pulp and occasionally on the reticulum and arterial wall (Bernaudın et al, 1979). No linear IgE is found in the kidneys (Prouvost-Danon et al, 1981). By days 30-60, the linear pattern becomes attenuated, and on this in most rats is superimposed a granular fixation of IgG in the glomeruli, and also renal artery walls (Druet et al, 1979). In addition extra renal immune deposits are found in the spleen (basement membrane of small arteries and capillaries), lung and liver (Bernaudin et al, 1979). Using immunofluorescence, it has been found that $HgCl_2$ injected BN rats lose their FX1A antigen (Druet et al, 1978).

Electron Microscopy: By day 9 ultrastructural examination by EM, reveals detachment of the glomerular epithelium, and infiltration of monocytes into both the glomeruli and interstitium. These are joined at day 15 by some polymorphs, at which time heterogeneous subendothelial material and subepithelial electron dense deposits are seen in the capillary walls. By 2 months the cellular infiltrate has disappeared and although the GBM is thickened, endothelial cells are normal (Hınglais et al, 1979). Focal and dense deposıts are also found in lungs and spleen (Bernaudin et al, 1979).

Proteinuria/Renal Failure: Transient proteinuria occurs in most rats in Druet's experiments, but with great variation, depending on the dose of $HgCl_2$, varying from 20-70mg protein/24 hr, up to greater than 1g/24 hr (Druet et al, 1979), yet in other studies is only 7-30mg (Druet et al, 1977). Proteinuria occurs during the fırst phase of the disease only. Lower doses of $HgCl_2$

sometimes cause prolonged proteinuria (Druet et al, 1978). Proteinuria does not occur in 100% of rats even in the presence of anti-GBM antibodies (Druet et al, 1977; Bowman and Lockwood, 1985a). Decomplementation does not reduce proteinuria (Capron et al, 1982). Acute renal failure, as shown by increases in serum creatinine and death does occur in some rats within 1 month but the creatinine on day 60 is normal (Druet et al, 1978).

Eluate Studies: Anti-GBM antibody can be eluted from kidneys with linear staining, and binds *in vitro* to kidneys from normal BN and other rat strains, along the GBM, some TBM and arteriolar basement membrane, as determined by indirect immunofluorescence (Sapin, Druet and Druet, 1977) and RIA (Bowman, Peters and Lockwood, 1983). In addition, i.v. injection of eluate causes linear fixation to GBM *in vivo* (Sapin, Druet and Druet, 1977). When kidneys with granular staining are eluted, the eluate fixes in a linear pattern to GBM, TBM and renal artery walls (Druet et al, 1978). Eluate from spleen with granular fixation does not fix to kidney or liver (Bernaudin et al, 1979).

### 1.4.5. Serum Proteins and Antibodies

Early studies on serum proteins did not show any gross changes during the disease, except that rats which were still proteinuric in the second phase of the disease, were hypoalbuminuric, with raised $\alpha 1$ and $\alpha 2$ globulin but decreased Ig fraction (Druet et al, 1978).

Later studies have indicated that the level of total serum IgG shows a transient 3-6 fold increase (Pusey et al, 1983; Bowman et al, manuscript in preparation) and that total serum IgE is also strikingly elevated, first apparent by day 5, peaking between days 16 and 22 and remaining elevated until day 60 (Prouvost-Danon et al,

1981). Peak total IgE does not correlate with the autoimmune process of IgG deposition in the spleen or kidney (Sapin et al, 1984).

Clotting abnormalities occur at the time of maximal proteinuria, with some clotting variations transient and related to the nephrotic syndrome (low antithrombine III, low factor XII, high factor V, high fibrinogen), and others suggestive of disseminated intravascular coagulation (fibrinogen degradation products, thrombopenia), (Michaud et al, 1980).

Complement changes occur during the first phase of the disease, with a transient drop in $CH_{50}$ to less than 25 units/ml, at the time of proteinuria, days 11-27 (Druet et al, 1979), which also coincides with a transient decrease in C4 haemolytic activity (Bellon et al, 1982). Circulating immune complexes (CIC) occur transiently, whether detected by conglutinin binding assay (Bowman et al, 1981) or the Raji cell RIA (Bellon et al, 1982) and peak around days 11-16. These CIC appear to be responsible for granular IgG deposits in the glomeruli, rather than the linear IgG converting to granular deposits during the disease (Houssin et al, 1983).

Circulating anti-GBM antibodies were first detected in the isolated serum Ig fraction by indirect immunofluorescence (Sapin, Druet and Druet, 1977). However the development of radioimmunoassays and enzyme immunoassays for the antibody (Michaelson, McCoy and Bigazzi, 1981; Bellon et al, 1982; Druet et al, 1982b; Bowman, Peters and Lockwood, 1983), has allowed the kinetics of production to be more closely examined. These vary from group to group. In Druet's studies, anti-GBM antibody is first detectable by day 11, peaks around day 20 and falls to become undetectable by day 60 (Bellon et al, 1982), while at Hammersmith, antibody is first detectable by day 8, peaks between days 11 and 14 and becomes

undetectable by day 36 (Bowman et al, 1981; Bowman et al, 1984). In all groups, anti-GBM antibody is detectable in 100% of $HgCl_2$ treated BN rats (Bowman et al, 1981; Michaelson, McCoy and Bigazzi, 1982; Bellon et al, 1982).

The kinetics of the PFC response to GBM in the spleens of $HgCl_2$ treated BN rats has also been examined. Peak PFC responses occur at day 9, and are usually only present from days 8-11, with both indirect and direct PFC being detectable (Chalopin and Lockwood, 1984). However, small secondary peaks of anti-GBM PFC activity do occur later in the disease (Chalopin and Lockwood, 1984; J. Savige - personal communication). In studies to show the specificity of the anti-GBM antibody, it has been reported that sera from strongly anti-GBM antibody positive animals would also react with laminin, procollagen IV and fibronectin (Bellon et al, 1982).

Other antibodies with both auto- and foreign-antigenic specificities have also been detected in the $HgCl_2$ treated BN rat. Both indirect and direct PFC specific for SRBC and 2,4,6 TNP occur transiently in the spleen on days 6 and 12, and anti- single stranded DNA antibody is also present (Hirsch et al, 1982). In recent studies examining the range of autoantibodies produced, antibodies to thyroglobulin, collagens types I and II, and single and double stranded DNA, have been detected (Bowman et al, manuscript in preparation). The kinetics of these antibodies show self-regulation (anti-GBM, anti-thyroglobulin), a prolonged response (anti-DNA) or intermediate partial regulation (anti-collagen).

Monoclonal antibodies produced from $HgCl_2$ stimulated BN rats are mainly IgE, while specificities range from anti-kidney autoantibodies, anti-DNA, to anti-TNP (Hirsch et al, 1985). A monoclonal IgG class anti-GBM antibody capable of inducing weak proteinuria has also been developed (Hirsch et al, 1984).

1.4.6. Mechanisms

The mechanism by which $HgCl_2$ results in the induction of anti-GBM nephritis in the BN rat, but not other rat strains has been shown to be dependent on immunocompetant cells from the spleen or bone marrow, as indicated by transfer of susceptibility into Lewis rats by cells from susceptible (LEW X BN) $F_1$ hybrids (Sapin et al, 1980). The antigen(s) against which the anti-GBM antibodies are directed had already been shown to be present in other rat strains (Druet et al, 1977), and results from transplantation experiments of kidneys from nonsusceptible Lewis rats into binephrectomized (LEW x BN) $F_1$ hybrids, or BN into LEW congenic rats, followed by $HgCl_2$ challenge show that induction of the disease is more dependent on the host immune system than on modification of the kidney determinants by $HgCl_2$ (Druet, Houssin and Druet, 1983).

Mercuric chloride has been shown to act as a polyclonal stimulator both *in vivo* and *in vitro* (Hirsch et al, 1982) and this action or the action of $HgCl_2$ on T suppressor cells, may be associated with Druet's sugggested mechanism of $HgCl_2$ as causing loss of immune homeostasis, resulting in immune dysregulation (Druet et al, 1982a). However, it is not known definitely whether such immune dysregulation, or differing immune response in the BN rat to $HgCl_2$ modified GBM determinants, or perhaps both are responsible for the susceptibility of BN rats to $HgCl_2$ induced anti-GBM nephritis (Druet, Houssin and Druet, 1983).

Recent work has shown that $HgCl_2$ acts *in vivo* or *in vitro*, by modifying BN T cells, especially T helper cells, such that these cells cause proliferation of T and B cells both *in vivo* and *in vitro*, resulting in polyclonal activation (Pelletier et al, 1985a,b).

1.4.7. Treatment

The effect of cyclophosphamide on anti-GBM antibody synthesis has been examined in this model, and shown to have 2 dose dependent effects. Low doses (2.5mg/kg) cause increased peak antibody levels, while high doses ($\geqslant$ 20mg/kg) prevent antibody synthesis. In addition, high dose cyclophosphamide reduces the response to rechallenge with $HgCl_2$ 3-4 months later (Pusey et al, 1983).

Cyclosporin has been shown to suppress $HgCl_2$ induced autoimmune phenomena, such as anti-GBM antibody production and CIC formation, and it prevents and cures the clinical manifestations of the disease - proteinuria, and weight loss, early death - (Baran et al, 1985; Prummel et al, 1985).

Plasma exchange causes attenuation of the disease and removal of circulating anti-GBM antibody but not reduction in linear Ig deposition (Vendeville and Druet, 1985; C. Pusey - personal communication).

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In the foregoing introduction, I have briefly examined the mechanisms which result in autoimmunity, as well as the factors which contribute to the pathogenic consequences of autoimmunity. In addition, a study has been made of regulation of the immune response, many aspects of which would apply to down regulation of the autoimmune response, a state to be sought after in therapeutic regimens for autoimmune disease. These three themes of mechanisms, pathogenicity and regulation were then applied to anti-GBM autoantibody mediated nephritis, a disease of longstanding interest to this department at Hammersmith Hospital, and then to an experimental model of autoantibody mediated nephritis - $HgCl_2$

induced disease in the BN rat. The following pages describe work carried out to initially examine the autoimmune phenomena and disease processes in this experimental model, and then examine regulation of anti-GBM antibody synthesis both *in vivo* and in anti-GBM antibody synthesising cell cultures.

# 2. MATERIALS AND METHODS

## 2.1. THE MODEL

### 2.1.1. Animal Protocol

Brown Norway rats were obtained from the Repgo Institute (Rijkswik , Holland) or Olac (Oxon. U.K.) and breeding colonies established. BN rats were also bought from Charles River Laboratories (U.S.A.), and used within 6 weeks of arrival. Animals were fed standard laboratory diet and water ad libitum. For induction of the disease rats (7-12 weeks old) were injected thrice weekly for 2 weeks with 1mg/kg mercuric chloride, given subcutaneously as a 0.1% solution in distilled water. Control animals were left untreated, or injected with equal volumes of PBS (phosphate buffered saline) or distilled water adjusted to the same pH as the $HgCl_2$ solution. Serial blood samples were taken by tail artery puncture, under ether anaesthesia. For cell studies, blood was collected into heparin (Monoparin, Weddel Pharmaceuticals Ltd., U.K.). Lewis, Wistar and Sprague-Dawley, and $F_1$ crosses were obtained from Charles River (U.K.) Ltd., or the Experimental Animal Breeding Unit, Royal Postgraduate Medical School.

### 2.1.2. Antisera and Proteins

Rat IgG was partially purified from normal rat serum (NRS), or serum from day 14 $HgCl_2$ treated BN rats by precipitation with sodium sulphate, as described by Kekwick (1940). Rabbit anti-rat IgG (Miles-Yeda Ltd., Rehovot, Israel) was affinity purified by adsorption to a column of normal rat IgG attached to Sepharose 4B (Pharmacia Ltd., Milton Keynes, U.K.) by CNBr (Fuchs and Sela, 1978). Bound antibody was eluted with 0.2M glycine/HCl, pH 2.2, immediately neutralised with 0.1M NaOH, then dialysed against PBS, pH 7.2. For some experiments rabbit anti-rat IgG was partially

purified by sodium sulphate precipitation and bound to Sepharose 4B-CNBr as a solid phase immunoadsorbent.

Rat IgM was enriched from NRS by euglobulin precipitation against distilled water (Heide and Schwick, 1973). Rabbit anti-rat IgM (Miles-Yeda) was affinity purified using a Sepharose 4B-'IgM rich fraction' column as described above for anti-rat IgG.

Rabbit Ig was partially purified from normal rabbit serum by precipitation with sodium sulphate. Goat anti-rabbit Ig (a kind gift of Miss D. Grennan) was absorbed with glutaraldehyde fixed NRS (Avrameas and Ternynck, 1969), and affinity purified using a Sepharose 4B-rabbit Ig column as described above.

Affinity purified rabbit anti-rat IgG, rabbit anti-rat IgM and goat anti-rabbit Ig were all radiolabelled with $^{125}I$ using a modification of the chloramine T method (McConahey and Dixon, 1967) to a specific activity of 4μCi/μg. Rat IgG was radiolabelled with $^{125}I$ or $^{131}I$ to 10 μCi/μg.

Rabbit anti-rat IgG2b, goat anti-rat IgG2a, goat anti-rat IgG2c and goat anti-rat IgA were purchased from Miles-Yeda. Rabbit anti-rat IgE and rabbit anti-rat IgG1 were obtained from Nordic Immunological Laboratories Ltd. (Berks., U.K.). Rabbit anti-goat IgG (H+L) was bought from Miles Laboratories Ltd. (Slough, U.K.), and the FITC anti-rat Ig for immunofluorescence studies from Wellcome Reagents Ltd., (London, U.K.). FITC goat anti-mouse and rabbit anti-mouse Ig were kind gifts from Miss D. Grennan. All polyclonal antisera were tested for reactivity against rat, rabbit or goat sera as appropriate by immunodiffusion (Ouchterlony and Nilsson, 1973).

Mouse monoclonal antibodies (mAb) to rat IgG1, IgG2b and IgG2c were bought from Serotec (Oxon, U.K.). The mouse monoclonal antibodies, MRC OX8, MRC W3/25, MRC OX19 and MRC OX12, which bind to

rat T suppressor, T helper (Brideau et al, 1980), T (Dallman, Thomas and Green, 1984) and B (Hunt and Fowler, 1981) cells respectively were initially donated by Dr. D.W. Mason and later purchased from Serotec. The mouse monoclonal antibody MCA-P1, to the GBM antigen(s) recognised by autoantibodies from patients with Goodpasture's syndrome (Pressey et al, 1983) was kindly supplied by Miss A. Pressey.

Type I collagenase (Sigma Chemical Co., Missouri, C-0130) was purified as described by Seifter and Gallop (1962).

Keyhole limpet haemocyanin (KLH) was kindly provided by Prof. J. Humphrey.

2.1.3. Rat and Human GBM Preparation

Rat GBM was prepared by a similar method to that described for human GBM by Spiro (1967). All stages were carried out at 4°C. Briefly - kidneys from normal BN, Lewis or outbred rats were decapsulated, the medulla partly removed and the cortex forced through a 106μm stainless steel sieve (Endecotts, London, U.K.), and washed with ice cold PBS/sodium azide (0.001%). The separated glomeruli were passed through a 250μm sieve to remove large aggregated fragments, and collected on a 63μm sieve. The isolated glomeruli were washed on the sieve with PBS/azide, then transferred to a centrifuge tube, centrifuged at 400g, 4°C for 5 min, and washed twice with PBS/azide. After examination by light microscopy, the isolated glomeruli were suspended in PBS/azide and sonicated at 18μm amplitude in 30 sec bursts, with ice cooling, then examined by light microscopy for disruption. Sonication was continued until all glomeruli were disrupted, usually 90 sec. The disrupted glomeruli were centrifuged at 121g, 4°C for 15 min, the supernatant discarded and the precipitate washed thrice with PBS/azide at the same speed.

The precipitate was then washed thrice in ice-cold distilled water at 400g for 5 min and lyophilised.

The freeze dried material was cut up finely, suspended in 0.1M Tris/0.005M calcium acetate buffer (pH 7.4) at 10mg/ml and 0.7% w/w collagenase (1mg/ml) solution added. The reaction mixture was incubated at 37°C with stirring for 60 min and the collagenase was then heat inactivated at 60°C for 5 min. The digest was centrifuged at 700g for 15 min. and the resulting supernatant of collagenase-solubilised rat GBM was stored at -70°C. Determination of optical density at 280nm ($OD_{280}$) showed that 20% GBM was solubilised. Where significant contamination with rat IgG occurred, the rat GBM digest was absorbed on a column of Sepharose 4B-anti-rat IgG.

Human GBM was prepared in a similar manner as described elsewhere (Bowman and Lockwood, 1985b).

### 2.1.4. Preparation of Renal Eluates

BN rats, injected with $HgCl_2$ were sacrificed at day 14, and the kidneys collected. Sonicated glomeruli were isolated from 20-30 kidneys as described above, and extensively washed in PBS/azide at 700g, until the $OD_{280}$ of the supernatant was less than 0.02. Antibody was eluted by incubating the glomeruli in 3ml of 0.1M glycine/HCl (pH 2.8) for 20 min, with gentle stirring at 4°C, centrifuging at 700g for 5 min and immediately neutralising the supernatant with 0.1M NaOH. Second and third elutions were also performed. The neutralised supernatants were dialysed against PBS/azide for 18 hr at 4°C. The first eluate typically had a protein concentration of 100-150µg/ml. Elution of normal BN kidneys was performed similarly.

2.1.5. Anti-GBM Antibody Radioimmunoassay (RIA)

IgG and IgM Class

Rat GBM (100µl/well of 5µg/ml GBM in Complement Fixation Diluent (CFD, Oxoid Ltd., Basingstoke, U.K., pH 7.2)) was coated to flexible polyvinyl chloride 'U' shaped microtitre plates (Dynatech Lab. Ltd., Sussex, U.K.) by 18 hr incubation at 4°C. Coated plates were washed thrice with CFD containing 0.05% Tween 20 (T20, Sigma) and used immediately or stored at 4°C. In all RIA systems, washing was performed thrice in CFDT20 and incubation volumes of 100µl were used. Rat serum (1/16 in CFDT20) was added to GBM coated wells and incubated at 37°C for 1hr. After washing, plates were drained and bound antibody detected by incubating wells with $^{125}I$ labelled anti-rat IgG ($3.5x10^5$cpm) in CFDT20 containing 0.1% bovine serum albumin (BSA) at 37°C for 1hr. Plates were then washed, drained and counted in an LKB 1260 Multigamma Counter. Each serum sample was assayed in triplicate. Each assay incorporated a negative control (from a pool of normal BN rat sera) and a positive control (from a pool of sera from day 14 $HgCl_2$ treated BN rats). Results were expressed as percentages of the binding obtained with a strong positive reference serum. Sera were also tested on human GBM coated wells for some experiments.

For detection of IgM class anti-GBM antibodies, in early experiments $^{125}I$ anti-rat IgM was used in a similar system to that described above and results expressed as cpm binding.

Other Classes and Subclasses

To detect anti-GBM antibodies of IgA, IgE, IgM classes and IgG1, IgG2a, IgG2b and IgG2c subclasses, rat sera (normal, day 14 or sequential samples) were incubated in rat GBM coated wells as described above. IgM, IgE, IgG1 and IgG2b antibodies were then

detected by incubating wells for 1hr at 37°C with 1/1000 dilution of the appropriate rabbit antisera in CFDT20/BSA, followed by washing, and incubation with $^{125}I$ goat anti-rabbit Ig in CFDT20/BSA/1%NRS at 37°C for 1hr. IgA, IgG2a and IgG2c antibodies were detected by incubating wells for 1hr at 37°C with 1/1000 dilution of the appropriate goat antisera in CFDT20/BSA, washing, then incubating wells with 1/1000 rabbit anti-goat IgG in CFDT20/BSA/1%NRS at 37°C for 1hr. This was followed by washing and incubation with $^{125}I$ anti-rabbit Ig as described above. All results were expressed as cpm $^{125}I$ anti-rabbit Ig binding. Controls without anti-class or anti-subclass reagents, were performed.

For some later experiments, IgG1, IgG2b and IgG2c anti-GBM antibodies were detected using mouse monoclonal reagents, and rabbit anti-mouse Ig in a similar system to that described above.

Specificity of Anti-GBM RIA

Experiments showing specificity of the RIA for IgG class anti-GBM antibodies have been described previously (Bowman, Peters and Lockwood, 1983). Specificity of the RIA for anti-GBM antibodies of other classes and subclasses were determined by inhibition studies. Dilutions of positive sera were chosen, which were on the linear section of the antibody dilutions curves. 350µl of the appropriate dilution of normal or day 14 BN rat serum were preincubated with 50µl CFDT20, rat or human GBM or KLH (2mg/ml) for 1hr at 37°C then 100µl volumes assayed in the RIA systems as above.

2.1.6. Anti-DNA RIA

Anti-DNA activity of IgG class was assayed as for the anti-GBM antibody described above, using plates coated with calf thymus DNA (Sigma) at 20µg/ml. Results were expressed as cpm $^{125}I$ anti-rat

IgG binding.

2.1.7. Rat IgG RIA

Concentration of rat IgG in eluates (or serum) was determined by incubating dilutions of test solutions in affinity purified anti-rat IgG coated plates (10µg/ml, in CFD, 18hr, 4°C) at 37°C for 1hr, washing, and detecting with $^{125}I$ anti-rat IgG (5 x $10^5$ cpm) at 37°C for 1hr. Each assay incorporated a standard curve of dilutions of a solution of rat IgG of known concentration, to which the test samples were compared.

2.1.8. Immune Complex RIA

Immune complexes were detected using a modification of the conglutinin binding assay (KgBA (Casali et al, 1977)). Polyvinyl microtitre plates were coated with conglutinin (5µg/ml CFD, a kind gift of Mr. A. Dash) for 18hr at 4°C. Plates were then washed and incubated with 1/10 test or normal sera in CFDT20 for 1hr at 37°C. After further washing, conglutinin bound immune complexes were detected by incubation for 1hr at 37°C with $^{125}I$ anti-rat IgG, as described above. Results were expressed a cpm $^{125}I$ anti-rat IgG bound. Immune complexes were deemed to be positive if cpm binding were greater than the mean for day 0 samples ± 1 standard deviation.

2.1.9. SDS-PAGE and Western Blotting

Immunoblotting of SDS-PAGE fractionated rat and human GBM was used to investigate the reactivity of the anti-GBM antibody in day 14 serum or renal eluates, as compared to the reactivity of sera from patients with Goodpasture's syndrome, or the MCA-P1 mouse monoclonal antibody reactive with the same antigen(s) (Pressey et al, 1983). Rat GBM digest in 0.1% T20 was concentrated 5-fold in

dialysis tubing on Aquacide II (Sigma). Concentrated rat GBM or unconcentrated human GBM (3mg/ml) were fractionated on a 5-20% gradient SDS-polyacrylamide gel (18hr, 40 volts), and either stained for protein with Coomassie blue or electrophoretically transferred to nitrocellulose sheets (Schleicher and Schüll, 0.45µm BA85 membrane filters) for 4hr at 30 volts using standard techniques (Towbin, Staehelin and Gordon, 1979). The sheets were quenched for 18hr at 4°C with 3% bovine haemoglobin (Hb) or a rat Hb rich fraction from lysed rat RBC, diluted in CFD/0.5% T20, washed and individual lanes overlaid with serum or sodium sulphate Ig fractions from normal or day 14 BN rats; BN kidney eluate; serum from patients with Goodpasture's syndrome or monoclonal anti-GBM antibody supernatant, diluted to 15ml with CFDT20, and incubated for 18hr with gentle mixing. After washing to remove unbound material, bound rat or mouse IgG antibody was detected with $^{125}I$ anti-rat IgG and human autoantibodies with $^{125}I$ anti-human IgG ($10^6$ cpm/lane). $^{125}I$ labelled marker proteins were kindly provided by Mr. A. Dash. Nitrocellulose lanes were then washed and autoradiographed using Ortho G X-ray film (Kodak Ltd., Herts., U.K.) and image intensifying screens.

2.1.10. Immunofluorescence - direct and indirect

Kidney biopsies were performed on rats 14 days after the first $HgCl_2$ injection. Direct immunofluorescence (IF) of kidneys, and indirect IF of sera were performed as described by Sapin, Druet and Druet (1977).

2.1.11. Proteinuria

To measure specific tissue injury, 24hr protein excretion was determined sequentially on $HgCl_2$ treated female BN rats by

trichloroacetic acid precipitation, followed by the Biuret reaction (Weichselbaum, 1946).

2.1.12. Total and Differential WBC

Total white blood cell counts were determined using a ZF model Coulter Counter, on heparinised blood samples lysed with Zaponin (Coulter Ltd., Luton, U.K.). Differential WBC counts were performed on ethanol fixed blood smears using Modified Giemsa stain (Raymond A. Lamb, London, U.K.) and standard techniques.

2.1.13. Flow Cytometry Cell Analysis and Lymphocyte Subsets

Lymphocytes were labelled with the monoclonal antibodies MRC OX19, MRC OX8, MRC W3/25 and MRC OX12 using a whole blood technique. From sequential samples of heparinised blood, 100µl aliquots were washed thrice in 10ml PBS and incubated with 20µl 1/100 dilution of mAb or medium for 30 min at room temperature (RT). After washing wth PBS, labelled cells were detected by incubation with microfuged FITC goat anti-mouse Ig in 20% rat serum for 20min at RT. Cells were washed with PBS and erythrocytes lysed by suspending the pellet in 1ml lysing buffer (0.15M $NH_4Cl$, 10mM $KHCO_3$,0.5mM EDTA, pH 7.4), incubating on ice for 5 min and washing with PBS. Cells were then fixed in 1% paraformaldehyde in PBS and analysed, preferably within 48hr. Analyses were performed on a Coulter EPICS C fluorescence activated cell sorter, which separated red cell debris and polymorphs and partially separated macrophages, from the lymphocytes. $10^5$ cells from the lymphocyte rich fraction of the mononuclear cells were analysed for % positive cells, and compared to the negative control (no first antibody), which was then subtracted. Initially negative controls were labelled with an irrelevant mouse monoclonal antibody to CMV (kindly provided by

Dr. B. Rogers) until this was found to give results not significantly different to controls incubated with medium.

Absolute numbers of positive lymphocytes/ml peripheral blood could be derived using %positive cells and total and differential WBC.

Percentage T cell subsets in spleens were determined in a similar manner using $10^6$ WBC/test, but replacing the lysis step with distilled water shock lysis.

### 2.1.14. Statistical Analysis

Statistical analysis was performed by Student's t-test, or in some cases by the Wilcoxon rank sum test.

## 2.2. REGULATION AND AUTOREGULATION OF ANTI-GBM ANTIBODY SYNTHESIS

### 2.2.1. Cell Transfer Studies

#### Spleen Cell Transfer Protocol

All spleen cell (SC) transfers were carried out in female BN donors and recipients. Spleens from normal rats or rats injected with $HgCl_2$, and at various stages of convalescence were taken into Dulbecco's A + B salt solution with 2% newborn or foetal calf serum (DAB/CS). A single cell suspension was prepared by sieving, filtration through cotton wool and washing twice at 4°C in DAB/CS. Erythrocytes and non-viable lymphocytes were removed by distilled water lysis. Spleen cells were adjusted to concentrations from $5x10^6$ to $2.5x10^8$ cells/ml DAB/CS and 1ml injected into the tail artery of recipients. Controls were injected with DAB/CS. All rats were then challenged with 4 injections of $HgCl_2$. In some experiments spleen cells were transferred into rats at day 7 in the $HgCl_2$ regimen.

Antigen Specific Suppression

To study specificity in SC-mediated suppression, the antibody response to BSA injected i.v. (1mg in 1ml DAB) at the same time as the first $HgCl_2$ injection was examined. Anti-BSA antibodies were detected by similar methodology to that for anti-GBM antibody detection, described above, using plates coated with BSA at 0.5mg/ml in 0.05M borate buffer, pH 8.6. Results were expressed as cpm $^{125}I$ anti-rat IgG binding.

Antibodies used in Depletion Experiments

The source of mAb to rat B, T and T cell subsets is described in section 2.1.2. MRC OX21 (a mAb to human C3b inactivator (Hsiung et al, 1982)) which was used as a control irrelevant antibody, was kindly donated by Dr. D.W. Mason. Rabbit $F(ab')_2$ anti-mouse Ig antibody (RaMIg), FITC-RaMIg and mouse immunoglobulin (MIg), were prepared by Steve Simmonds. Goat anti-mouse Ig antibody (GAMIg, raised by D. Grennan) was passed through a Sepharose 4B-rat Ig column and affinity purified on a Sepharose 4B-mouse Ig as described above for rabbit anti-rat IgG.

Lymphocyte Depletion

SC suspensions were prepared as above. Packed cells ($10^9$) were incubated with mAb MRC OX8, MRC OX12, MRC OX21 or MRC W3/25 (20µg antibody in 1ml RPMI with 10%CS) for 60 min at $4^oC$, then washed thrice with DAB/CS. Controls were incubated with DAB/CS.

Sheep red blood cells (SRBC) were coated with RaMIg, MIg, CS or GAMIg by the $CrCl_3$ method (Mason, 1981). Briefly, 0.625 ml washed, packed SRBC in 12.5ml normal saline, were incubated with 1.5mg protein and 1ml 0.1% $CrCl_3$ and then washed twice in DAB and once in DAB/CS.

MRC OX8 labelled SC ($10^8$/ml in DAB/CS containing 10% NRS) were rosetted with RaMIg-SRBC ($10^9$ SC/0.625ml packed, coated SRBC) by rotating at 4°C for 20 min. This was followed by corosetting with equivalent volumes of MIg-SRBC. MRC OX12 and MRC W3/25 labelled SC were rosetted with RaMIg-SRBC alone. In some later experiments, for MRC W3/25 cell depletions, GAMIg-SRBC was used. Rosettes were separated by centrifugation at 200g for 45 sec. The cell pellet, which contained some free leukocytes in addition to rosette forming cells, was gently resuspended and rerosetted to increase the yield of non-rosetting cells. Control depletions were performed using unlabelled cells and CS-SRBC or MRC OX21 labelled cells and RaMIg-SRBC.

The depleted cell suspension was washed once in DAB/CS and the packed cells incubated with 0.84% ammonium chloride for 10 min at room temperature to lyse any red blood cells. The cells were washed twice, adjusted to $6x10^7$ to $2x10^8$ cells/ml DAB/CS and transferred to recipients as described above. Efficiency of depletion was determined by flow cytometry using FITC-RaMIg or FITC-GAMIg. Depletion for MRC OX8 positive cells was routinely greater than 98%, and for MRC W3/25 positive cells greater than 80%.

### 2.2.2. Studies with Heterologous 'Anti-idiotypic' Antisera

#### Preparation and Characterisation of Anti-GBM Antibody containing Eluate (idiotype)

Renal eluates from day 14 $HgCl_2$ treated rats were prepared as described in section 2.1.4. Eluates were tested for anti-GBM activity by the RIA and indirect immunofluorescence as described previously. In addition day 14 eluate was radiolabelled with $^{125}I$ using chloramine T (McConahey and Dixon, 1966) to a specific activity of 15μCi/μg and tested for anti-GBM activity by a

modification of the solid phase RIA, and on lyophilised rat GBM fragments. For the RIA, $10^6$cpm $^{125}I$ eluate in CFDT20 was incubated in rat GBM coated microtitre wells for 1hr at 37°C and the wells washed and radioactivity counted on a gamma counter. Specificity of binding was tested by inhibition as described elsewhere (Bowman, Peters and Lockwood, 1983). Reactivity to crude rat GBM was tested by incubating $10^5$cpm of $^{125}I$ eluate and $10^4$cpm $^{125}I$ rat IgG with 2 or 4 mg lyophilised, undigested rat GBM at 37°C for 30 min, followed by centrifugation at 1500g for 10 min at 4°C, twice washing of the pellet with 1ml cold PBS, and counting radioactivity.

Preparation and Characterisation of Rabbit Anti-idiotypic Antibodies

Rabbit anti-idiotypic antibodies were raised by injecting rabbits with 250µg of day 14 kidney eluate in CFA, followed by 2 fortnightly booster injections of 100µg eluate in incomplete Freund's adjuvant (IFA). Sequential serum samples from test bleeding 7-21 days after each booster injection, which were reactive by Ouchterlony analysis against rat Ig, were pooled for each rabbit. Antisera to eluates from normal BN rats were prepared in a similar manner.

Rabbit antisera or normal rabbit serum were immunoabsorbed with glutaraldehyde insolubilised NRS, BN red blood cells and homogenised rat kidney. Antisera (5ml) were repeatedly absorbed with the equivalent of 2.5ml well washed insolubilised NRS for 30 min at room temperature until no reactivity against NRS remained by Ouchterlony analysis, and then absorbed again (usually 5 absorptions). Washed RBC from EDTA rat blood were then used as an immunoabsorbent at a ratio of 5ml antisera to 0.5ml packed cells in a similar manner. BN kidneys (n=2) were homogenised in PBS and

washed extensively in cold PBS until the $OD_{280}$ was less than 0.05, and used to absorb 5 ml antisera as above.

A polyethylene glycol (PEG) technique using $^{125}I$ eluate was used to determine reactivity of the antisera with the idiotypic anti-GBM antibodies present in the day 14 eluate. Absorbed antisera (4µl) were incubated with 2.5 x $10^5$cpm $^{125}I$ eluate and 1 x $10^4$cpm $^{131}I$ rat IgG in PBS/20%NRS for 1 hr at 37°C. An equal volume of ice cold 8% PEG 6000 in PBS was added and incubation continued at 4°C for 2 hr. Tubes were centrifuged at 1500g for 20 min at 4°C, the supernatant decanted and the precipitate washed twice in ice cold 4% PEG before counting on a gamma counter. In later experiments to more sensitively determine the presence of any remaining anti-rat IgG contamination, NRS was deleted from the incubation mixture.

In addition, solid phase RIAs were set up, whereby rat IgG and day 14 eluate were coated to polyvinyl plates at 5µg/ml and 10µg/ml respectively, as described for GBM coating, and dilutions of normal rabbit sera, rabbit anti-normal eluate and rabbit anti-day 14 eluate assayed on these and also rat GBM coated plates, using methodology as described for the anti-GBM assay. Bound activity was detected using $^{125}I$ anti-rabbit Ig. Remaining anti-rat IgG activity was compared to that in commercial rabbit anti-rat IgG.

### In Vitro Studies of Inhibition with Heterologous 'Anti-idiotypic' Antisera

In vitro inhibition of the anti-GBM antibody in BN day 14 eluate by heterologous anti-idiotypic antisera was shown by preincubating dilutions of unlabelled day 14 eluate in 1/40 NRS/CFDT20 with rabbit antisera to day 14 or normal eluate, or buffer in glass for 1 hr at 37°C, before assaying for remaining anti-GBM activity as previously described. As a control of

inhibition due to residual anti-rat IgG activity, eluate was also preincubated with a dilution of rabbit anti-rat IgG having the same activity as that remaining in the anti-day 14 eluate.

In addition, 4 x $10^5$cpm $^{125}I$ eluate was preincubated with 50µl rabbit antisera, before assaying in rat GBM coated plates.

In vitro inhibition of anti-GBM antibody in day 14 sera by heterologous anti-idiotypic antisera was shown by preincubating dilutions of day 14 sera in CFDT20, with rabbit antisera in glass for 1 hr at 37°C before assaying in rat GBM coated plates as above.

In Vivo Studies of Inhibition with Heterologous 'Anti-idiotypic' Antisera

To test for in vivo inhibition of anti-GBM antibody synthesis, rats were injected i.p. with 1.5ml normal rabbit sera, rabbit anti-day 14 eluate or rabbit anti-normal eluate, and then challenged with $HgCl_2$ as in the standard protocol of disease induction. As a specificity control, rats were injected with 500µg BSA in IFA i.p. on day 4. Anti-GBM and anti-BSA activity were assayed in sequential serum samples. In some later experiments, the volume of rabbit serum injected was increased to 2.3ml.

2.2.3. Studies with Putative Autologous 'Anti-idiotype'

In Vitro Studies using Sequential Serum Samples

The presence of putative auto-anti-idiotypic antibodies was tested by incubating $^{125}I$ eluate (4 x $10^5$cpm) with 100µl of 1/8 sequential serum samples in CFDT20 for 1 hr at 37°C in glass, before assaying the eluate in rat GBM coated wells. Inhibition of eluate binding by the sequential sera was compared to the level of anti-GBM antibody, which might cause competitive inhibition.

In Vivo Studies of Convalescent Serum/plasma Transfer

To determine whether soluble factors such as anti-idiotypic antibodies, in convalescent serum or plasma might be suppressive, a pilot experiment was initiated, whereby 1ml of pooled plasma from convalescent $HgCl_2$ treated BN rats at days 20 to 25, or normal BN rats, was injected i.p. into naive syngeneic recipients, which were then challenged with $HgCl_2$. On day 3, the animals were challenged with 1mg BSA in IFA as a specificity control. Sequential serum samples were tested for anti-GBM and anti-BSA antibody activity.

## 2.3. IN VITRO CELL CULTURE STUDIES

### 2.3.1. Culture Conditions

Rat SC suspensions were prepared aseptically from female normal BN or Lewis rats, or $HgCl_2$ treated BN rats, by dispersion through a stainless steel sieve, and washed three times with RPMI 1640 medium. In some experiments non-viable cells were removed by a 60 sec distilled water lysis step followed by filtration through a small wad of cotton wool and washing in medium. SC were resuspended in RPMI 1640 supplemented with 12mM $NaHCO_3$, 10% FCS, 2mM L-glutamine, 100 units/ml penicillin and 10µg/ml streptomycin, at $10^7$ cells/ml. Viability was determined by Trypan Blue exclusion.

Cultures consisted of 1ml SC suspension ($10^7$ cells) in 24 well Linbro tissue culture plates or 200µl (2 x $10^6$ cells) in 96 well flat bottomed Falcon tissue culture plates. All cultures consisted of 3-6 replicate wells. Bulk cultures were also prepared, by culturing 10ml aliquots of SC suspensions in 25ml Linbro tissue culture flasks. Cultures were maintained for 4 or 7 days in a 5% $CO_2$ saturated, humid atmosphere. On termination of culture, plates were centrifuged at 400g for 10 min and the supernatants harvested.

2.3.2. IgG Class Anti-GBM Antibody Synthesis

Supernatants from SC cultured as above, were assayed for IgG class anti-GBM antibody at a 2/3 dilution in CFDT20, in the RIA described in section 2.1.5. using 'rat GBM-IgG' coated plates, and the amount of antibody expressed as cpm $^{125}I$ anti-rat IgG bound.

Specificity of the anti-GBM antibody was tested by preincubating supernatants with dilutions of rat GBM or Hb, prior to assaying in GBM coated wells.

SC from Day 9 or 10 $HgCl_2$ treated BN rats at day 0 of culture were sonicated until lysed, as determined by microscopy and the supernatant assayed for anti-GBM antibody to determine the release of preformed antibody as compared to newly synthesised antibody.

To examine the requirement for adherent cells/macrophages in the culture, in some experiments SC suspensions were passed through a sterile Sephadex G-10 column, as described by Ly and Mishell (1974).

To examine the effect of washing cells and replacing the medium, part way through the culture, a procedure which would be required when studying the effect of soluble regulators on in vitro antibody synthesis, SC from day 10 $HgCl_2$ treated BN rats were cultured for 6 or 24 hr, then washed twice with medium and cultured for a further 5 days. As a control, cells were manipulated in a similar manner, but without removing the medium.

2.3.3. Regulation of IgG Class Anti-GBM Antibody Synthesis

Studies with Heterologous 'Anti-idiotypic' Antisera

Rabbit antisera to day 14 eluate, normal eluate, or normal rabbit sera, prepared and absorbed as described above, as well as commercial rabbit anti-rat IgG, were diluted 1/4 in medium and

sterilised by passage through a 0.22μm filter (Millipore, Bedford, MA). Spleen cells from day 9 or 10 $HgCl_2$ treated BN rats were cultured with dilutions of antisera for 5-7 days and supernatants harvested and assayed for IgG class anti-GBM antibody. For some experiments the antisera were heat inactivated at 56°C before use.

The effect of the same concentrations of antibody on preformed anti-GBM antibody in supernatants was determined by incubating bulk culture supernatants having various levels of anti-GBM activity, with dilutions of antisera, in glass at 37°C for 1 hr, before assaying in the anti-GBM RIA.

Studies with Convalescent serum

Serum from day 21 $HgCl_2$ treated convalescent BN rats, or normal BN rat serum was extensively absorbed on a rat GBM-Sepharose 4B column, prepared by CNBr coupling, to remove any anti-GBM antibody activity, until activity was no longer detectable in the RIA. Sera were heat inactivated at 56°C for 30 min, diluted in RPMI 1640 and sterilised by passage through a 0.22μm Millipore filter. SC from day 9 or 10 $HgCl_2$ treated BN rats were cultured with dilutions of normal or convalescent sera for 7 days as described above, and supernatants harvested and assayed for IgG class anti-GBM antibody.

The effect of the same concentrations of sera on preformed anti-GBM antibody binding in the RIA, was determined by preincubating day 5 supernatants with dilutions of convalescent or normal sera for 1hr at 37°C in glass before assaying in the anti-GBM RIA.

Studies with Cell Cocultures

In cell coculture experiments, SC from day 9 or 10 $HgCl_2$

treated BN rats were cocultured with 5 to 80% SC from normal BN rats, or $HgCl_2$ treated BN rats convalescent at days 19 to 23, as described above.

For some experiments macrophages/adherent cells were removed from the convalescent SC suspensions by passage through Sephadex G-10. In other experiments, the SC suspensions were enriched for T cells by passage through nylon wool columns (Julius, Simpson and Herzenberg, 1973). Efficiency of enrichment was indicated by labelling cells with MRC W3/25 mAb, and analysis by flow cytometry. Routinely, MRC W3/25 labelled T helper cells were increased from 35% to 75% by a single passage through nylon wool.

2.3.4. IgM Class 'Non-specific Anti-GBM' Activity from Normal Cells

The in vitro IgM anti-GBM antibody response in normal BN rats was determined by culturing SC from normal BN rats, or BN rats injected i.m. 4 days previously with 1mg human GBM or BSA in CFA, as described above. SC from normal Wistar or Lewis rats were used as controls. Supernatants were harvested and assayed for IgM anti-GBM activity, as described in section 2.1.5.

Specificity studies of this IgM class activity involved preincubating supernatants from cultured normal SC, or dilutions of day 14 serum with a similar level of IgM anti-GBM activity, with soluble rat GBM before assaying in rat GBM wells previously blocked with 5%FCS for 2 hr at RT. In other studies, supernatants from cultured normal SC were preincubated with lyophilised rat GBM, human GBM or collagen; or rabbit anti-day 14 or normal eluate; or passed through rat GBM-Sepharose, DNA-Sepharose or control-Sepharose columns before assaying for activity.

Coculture with antigen was performed by culturing SC from normal or GBM immunised BN rats at $10^7$ cells/ml with dilutions of

collagenase solubilised human GBM (3mg/ml in RPM/1640/FCS) previously sterilised by passage through a 0.22μm Millipore filter. Day 7 supernatants were then assayed for IgM anti-GBM activity.

# 3. RESULTS

## 3.1. THE MODEL

### 3.1.1. Anti-GBM Antibody Production

#### Time Course and Sex Incidence

Male and female BN rats were injected thrice weekly for 6 weeks and anti-GBM antibody levels followed sequentially. The time course is shown in Figure 1. All animals produced circulating anti-GBM antibody, which was detectable by day 7 and peaked between days 11 and 14. Levels then fell rapidly and became undetectable by day 36. In some rats smaller secondary peaks of antibody could be detected after day 20. Anti-GBM antibodies were found in both male and female rats.

#### Dose Response

Female BN rats were injected 1, 3 or 5 times with $HgCl_2$ or thrice weekly up to 5 weeks. It was found that the levels of circulating anti-GBM antibody were dependent on the number of $HgCl_2$ injections up to 5 (Fig. 2). Maximum levels were produced by 5 injections of $HgCl_2$; 3 injections induced significantly less antibody ($p<.01$), and with 1 injection, low levels of antibody were detectable in only 2/5 animals. No difference was seen in the timing of the peak response or its termination, in rats given fewer than 5 injections (Fig. 3).

Female BN rats were injected thrice weekly with $HgCl_2$ at the standard dose of 1mg/kg, or 1/10th of the dose, 0.1mg/kg, or distilled water adjusted to the same pH as the $HgCl_2$ solution, for a total of 5 injections, and anti-GBM antibody levels followed sequentially for 4 weeks. Using 1/10th of the standard dose, only 1/4 of the rats produced detectable anti-GBM antibody, and only in very low levels (Fig. 4).

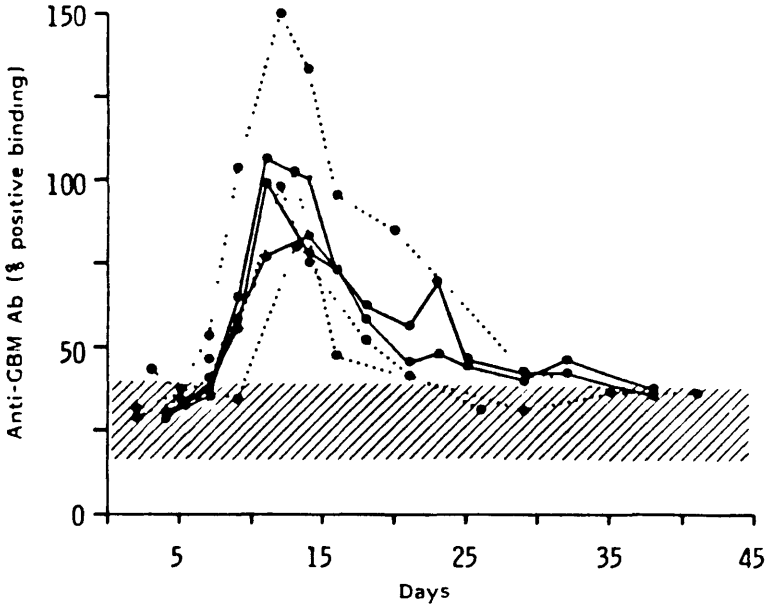



Figure 1. Time course of anti-GBM production in male (●·····●) and female (●——●) BN rats. Animals were injected with $HgCl_2$ thrice weekly for 40 days. Results are expressed as % binding of a strongly positive reference serum (% positive binding). The hatched area represents the normal range (mean of 30 normal sera ± SD).

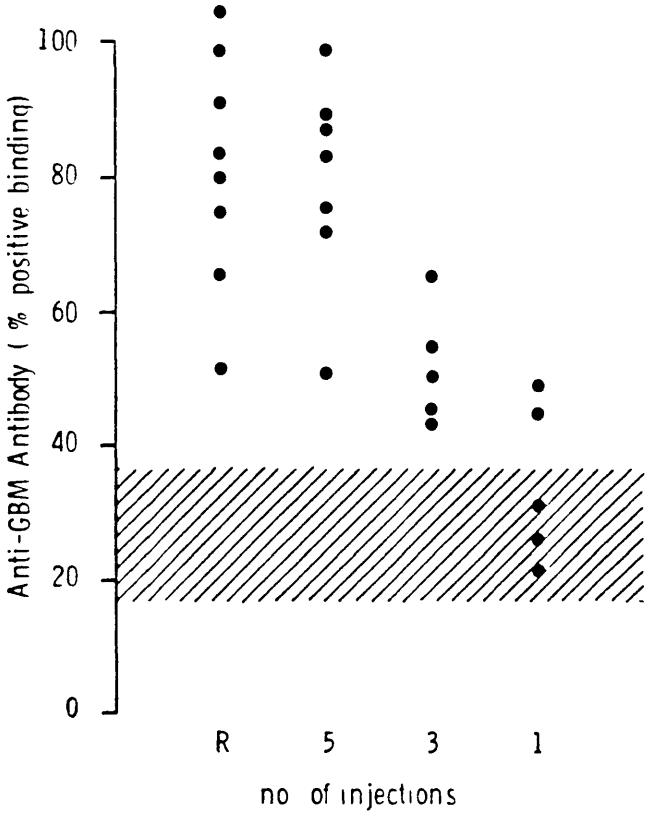



Figure 2. Effect of number of $HgCl_2$ injections on peak anti-GBM antibody levels. Female rats were injected throughout the experiment (R) or given a total of 1, 3 or 5 injections. Results of peak antibody levels are expressed as % positive binding. The normal range is hatched.

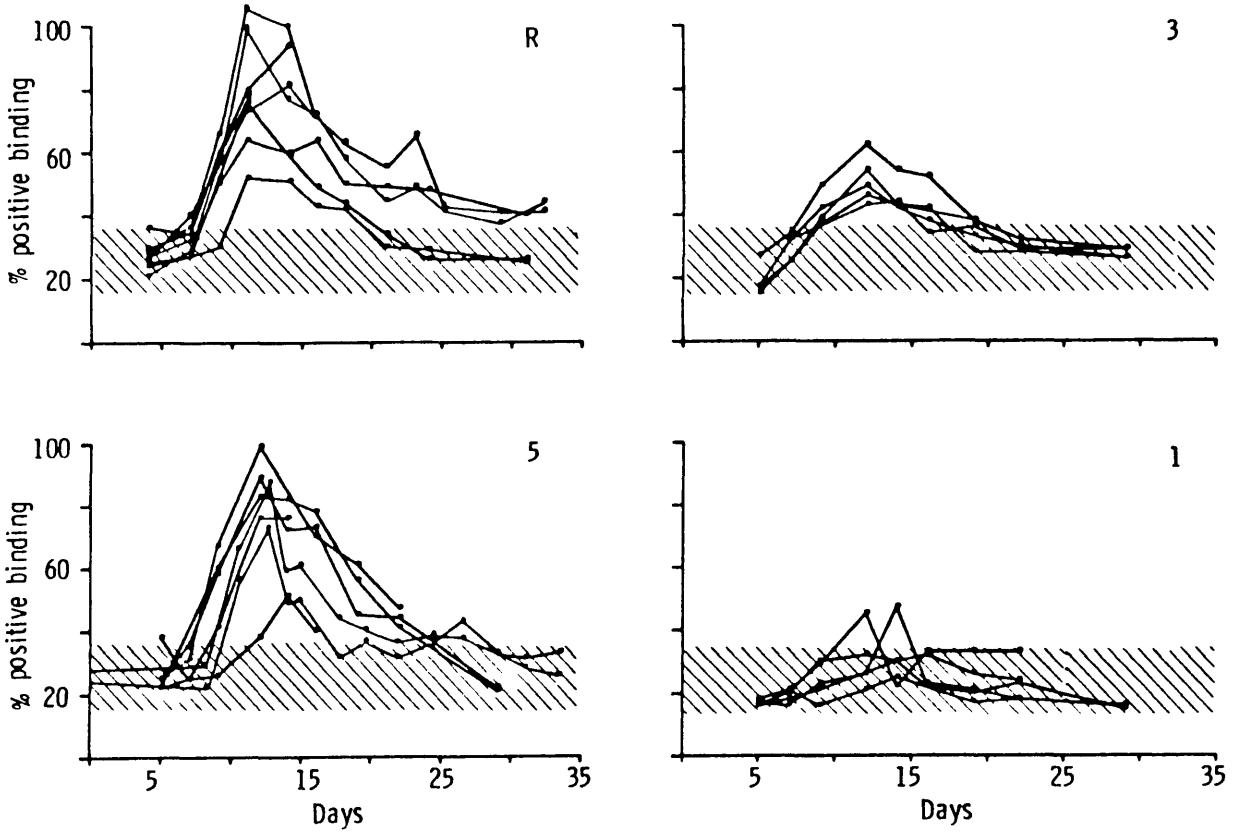



Figure 3. Effect of number of $HgCl_2$ injections on time course of anti-GBM antibody production. Female rats were injected throughout the experiment (R), or given a total of 1, 3 or 5 injections. Sequential anti-GBM antibody results of individual rats are shown, expressed as % positive binding. The normal range is hatched. Similar kinetics of synthesis and down regulation were observed in all rats producing anti-GBM autoantibodies.

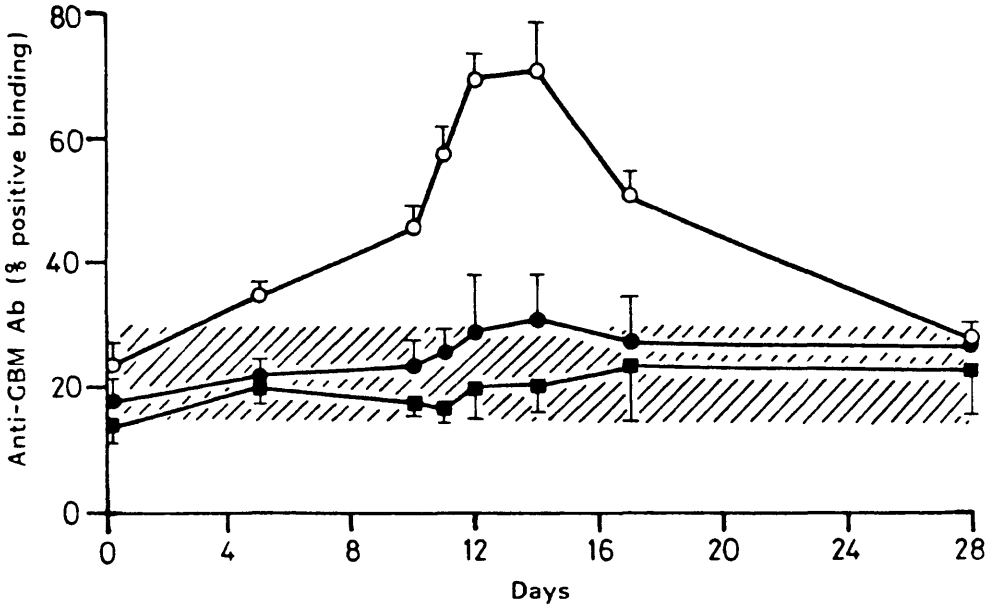



Figure 4. Dose response of $HgCl_2$ on time course of anti-GBM antibody production. Groups of female BN rats (n=4) were injected with 5 injections of distilled $H_2O$, adjusted to pH 5 (■——■), 0.1mg/kg $HgCl_2$ (●——●), or 1mg/kg $HgCl_2$ (○——○). Results are expressed as % positive binding of the mean ± SD of each group. Normal range is hatched. Only 1/4 of the rats injected with 1/10th of the standard $HgCl_2$ dose produced detectable anti-GBM antibody.

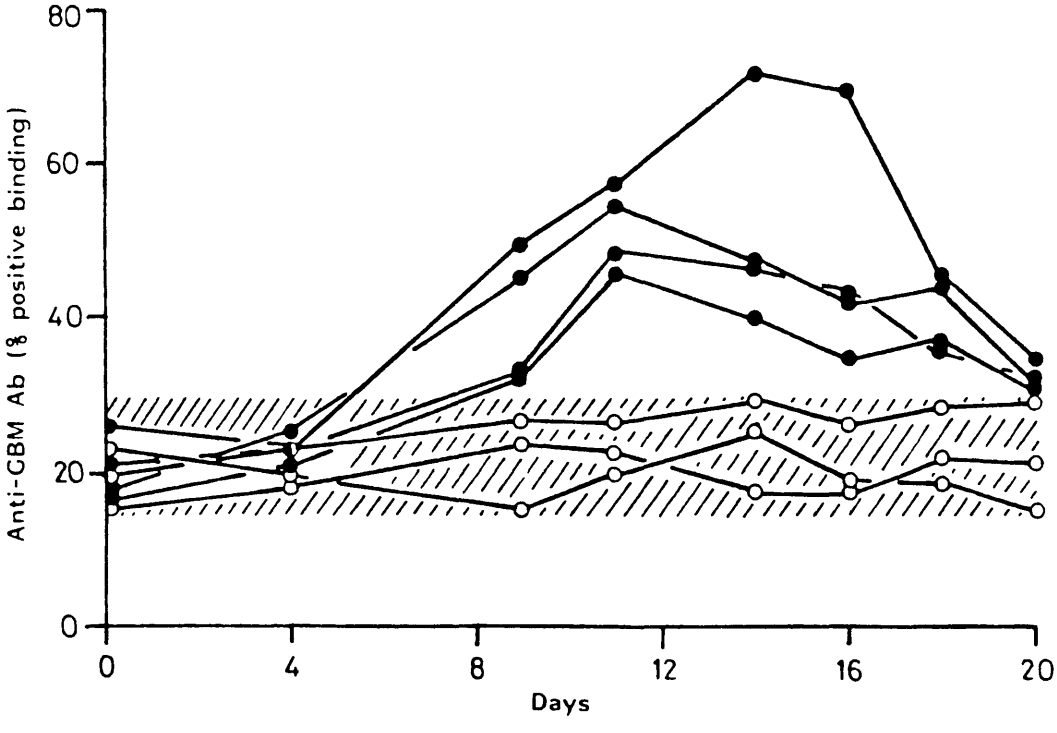



Figure 5. $HgCl_2$ induced anti-GBM antibody production in BN and Lewis rats. Female BN (●——●) or age and sex matched Lewis (○——○) rats were subjected to the standard $HgCl_2$ injection protocol. Results are expressed as % positive binding. Normal range is hatched. Lewis rats did not respond.

Strain Dependence

Female BN or age and sex matched Lewis rats were subjected to the standard $HgCl_2$ injection protocol and followed sequentially for 20 days. While all BN rats synthesised anti-GBM antibody, no change from baseline binding could be detected in the Lewis rats (Fig. 5). Wistar rats also failed to respond to $HgCl_2$ (data not shown).

Class of Anti-GBM Antibody

Normal BN serum and pooled serum from day 13 $HgCl_2$ treated BN rats were assayed for IgG, IgM, IgA and IgE classes of anti-GBM antibody. All classes were detectable (Fig. 6) and specifically inhibitable in inhibition studies, although IgE anti-GBM antibody was present only in very low levels.

Class of anti-GBM antibody was studied sequentially in $HgCl_2$ injected animals. IgG, IgM, IgA and IgE classes showed similar kinetics - in each case peaking between days 11 and 14 (Fig. 7,8).

Specific IgG, IgA and to a lesser extent IgM anti-GBM antibodies were also detected in day 14 kidney eluates (data not shown).

IgG Subclass of Anti-GBM Antibody

Sera from normal or day 13 $HgCl_2$ treated BN rats were assayed for IgG subclass of the anti-GBM antibodies, using polyclonal anti-subclass reagents. IgG1, IgG2a and IgG2c were present (Fig. 9) and specifically inhibitable. IgG1 and IgG2a antibodies were most readily detectable. IgG2b anti-GBM antibodies were only occasionally detectable and required inhibition tests to discriminate their presence from non-specific IgG2b binding in the assay.

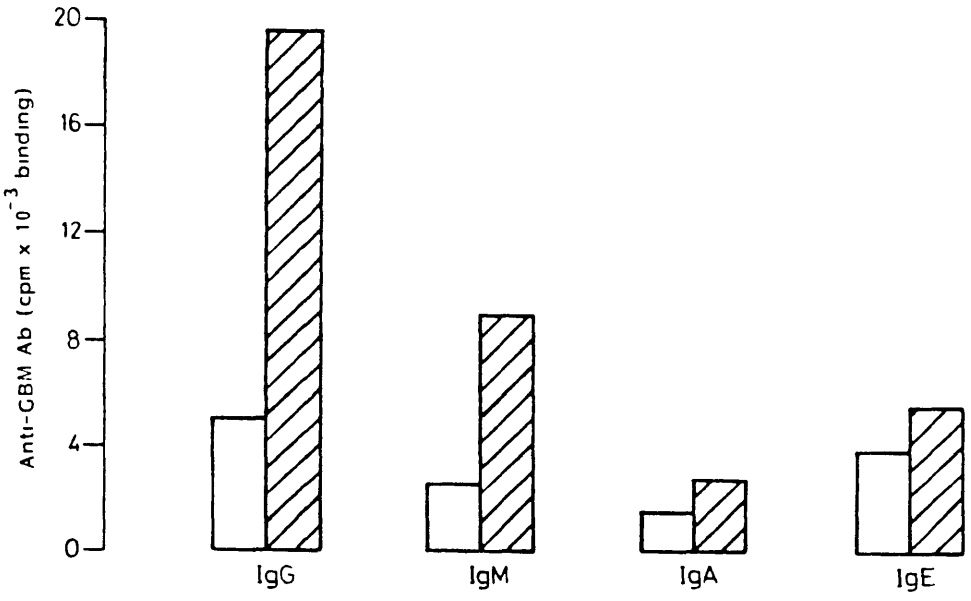



Figure 6. Class of anti-GBM antibody in $HgCl_2$ treated BN rats. Normal BN serum (unhatched bars) and pooled serum from $HgCl_2$ treated BN rats at day 13, the time of peak IgG response (hatched bars), were assayed for IgG, IgM, IgA and IgE class of anti-GBM antibodies. Results are expressed as cpm binding of the final layer of detecting $^{125}I$ anti-Ig. All classes were detectable and specifically inhibitable.

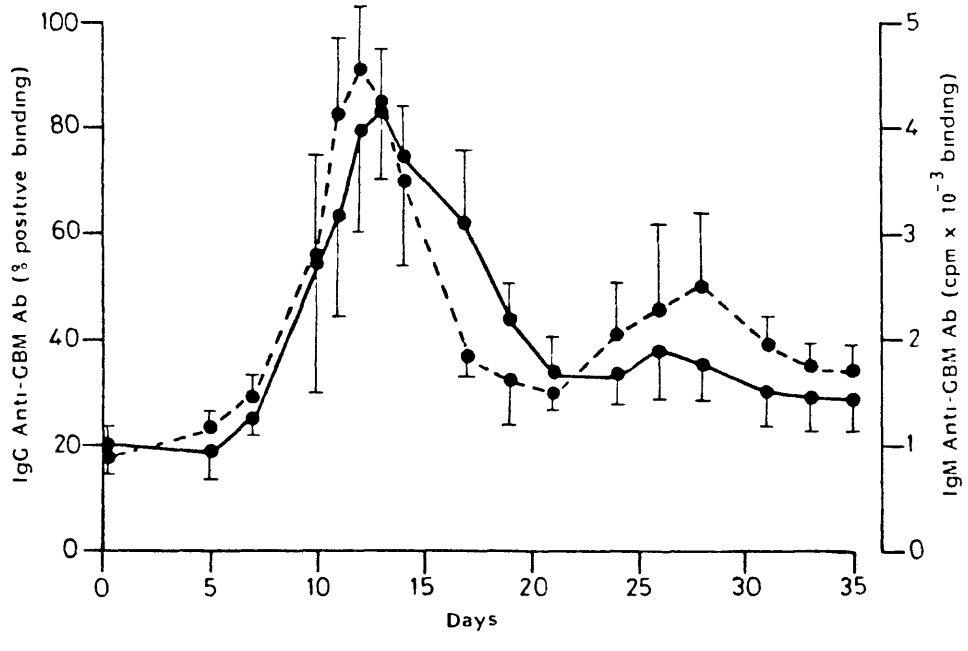



Figure 7. Time course of IgG and IgM class anti-GBM antibody production. Female BN rats (n=10) were subjected to the standard $HgCl_2$ injection protocol. Results show the mean ± SD for IgG anti-GBM antibody (●——●), expressed as % positive binding, and IgM anti-GBM antibody (●– –●), expressed as cpm binding of $^{125}I$ anti-IgM.

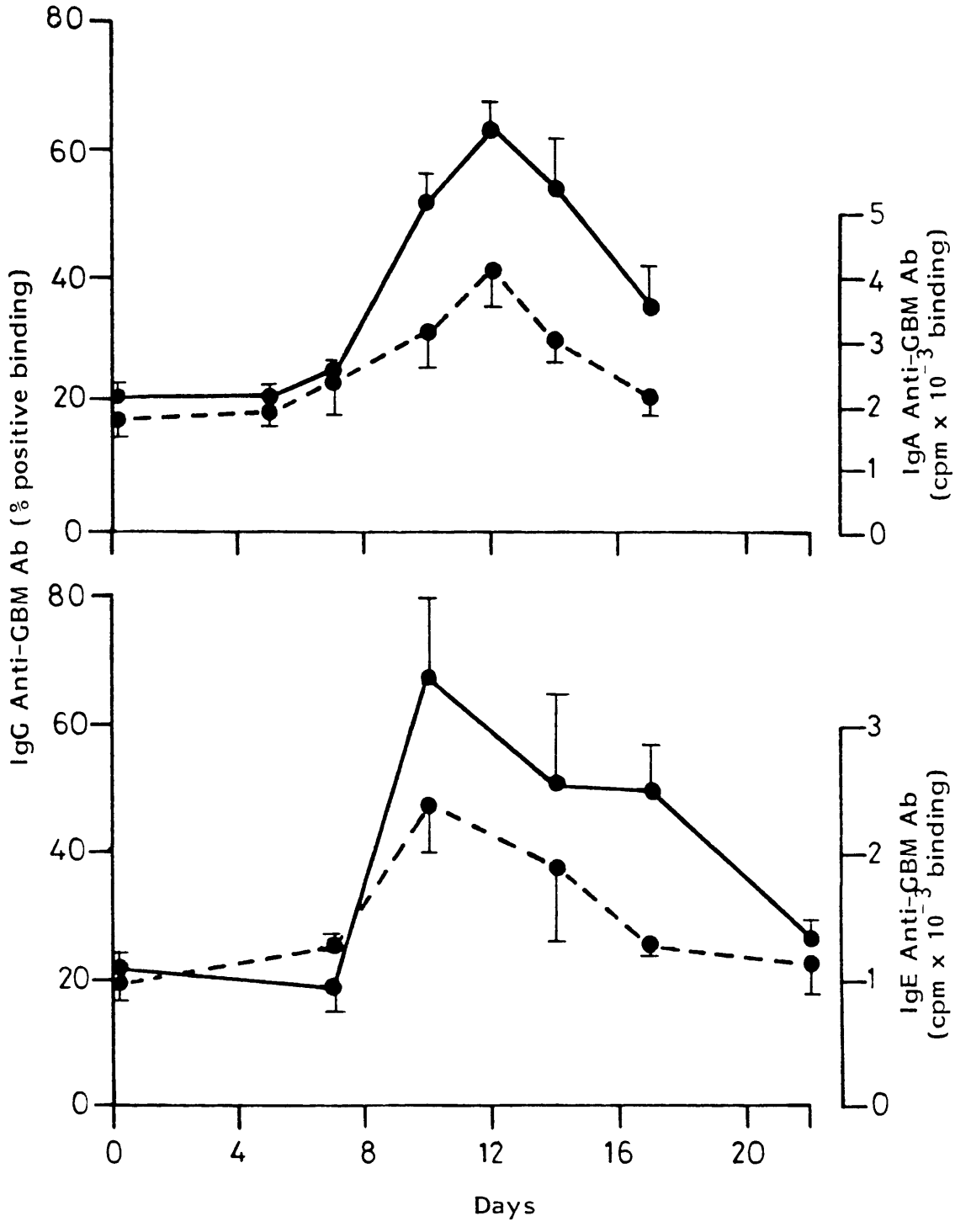



Figure 8. Time course of IgG, IgA and IgE class anti-GBM antibody production. Female BN rats (n=4) were subjected to the standard $HgCl_2$ injection protocol. The mean ± SD of anti-GBM antibody levels is shown, with IgG class expressed as % positive binding, and IgA or IgE class as cpm binding of the detecting $^{125}I$ anti-rabbit Ig. In one experiment, shown by the upper graph, IgG (●——●) and IgA (●– –●) class of antibody were compared, and in another experiment, shown by the lower graph, IgG (●——●) and IgE (●– –●) class of antibody were compared.

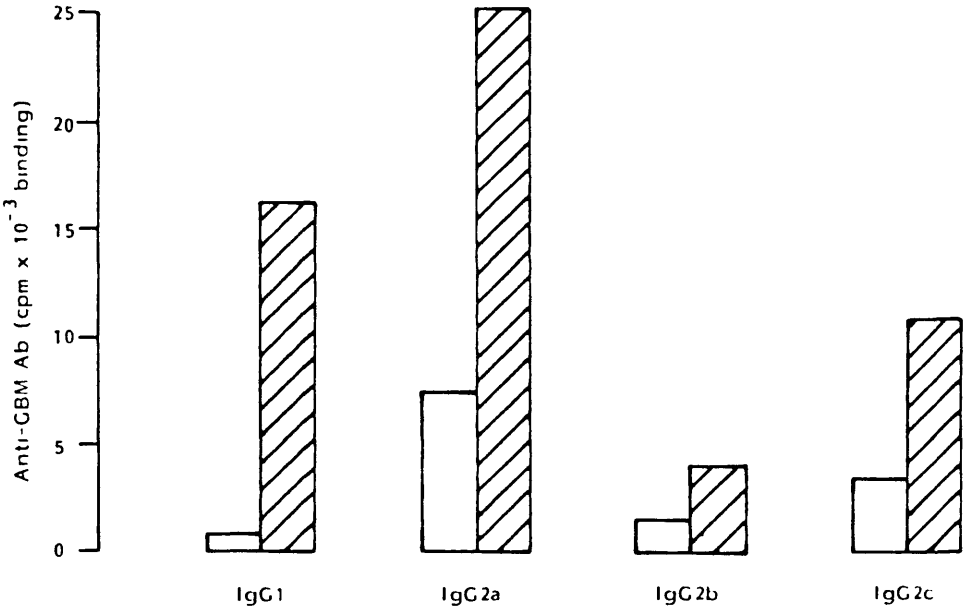



Figure 9. IgG subclass of anti-GBM antibody in $HgCl_2$ treated BN rats. Normal BN serum (unhatched bars) and pooled serum from $HgCl_2$ treated BN rats at day 13, the time of peak response (hatched bars) were assayed for IgG1, IgG2a, IgG2b and IgG2c anti-GBM antibodies using polyclonal anti-subclass reagents. Results are expressed as cpm binding of the final layer of detecting $^{125}$anti-Ig. Anti-GBM antibodies of subclasses IgG1, 2a and 2c were all specifically inhibitable, and of IgG2b only occasionally so.

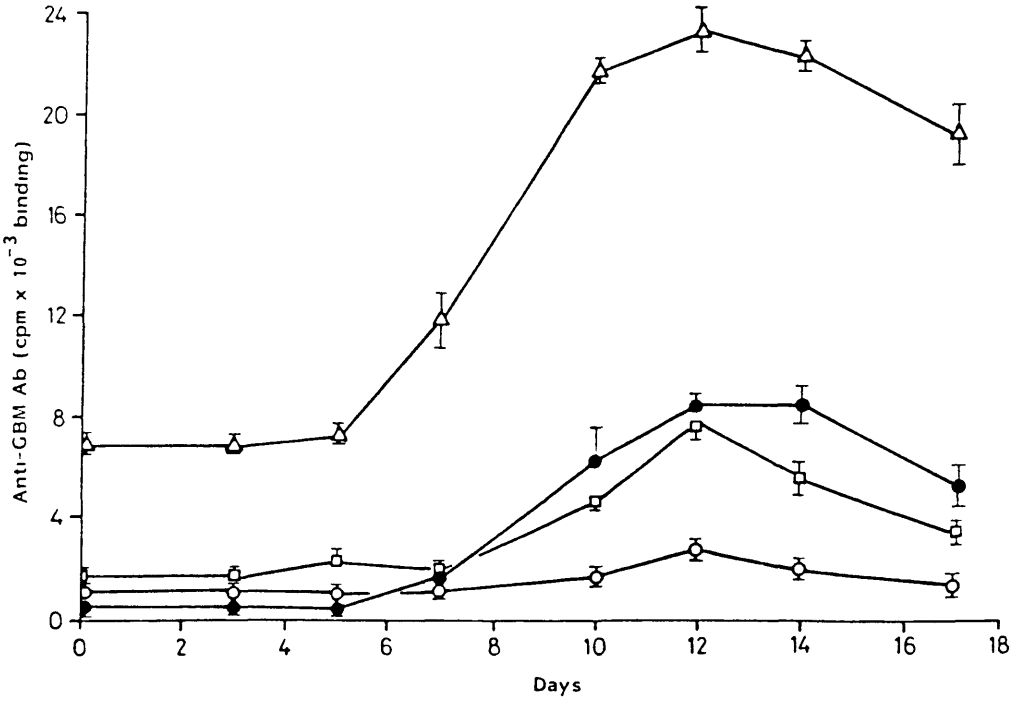



Figure 10. Time course of IgG subclass of anti-GBM antibody production. Female BN rats (n=6) were subjected to the standard $HgCl_2$ injection protocol. Results show the mean ± SD of the IgG1 (●—●), IgG2a (Δ—Δ), IgG2b (○—○) and IgG2c (□—□) anti-GBM antibodies expressed as cpm binding of the detecting $^{125}$I anti-rabbit Ig, using polyclonal anti-subclass reagents.

IgG subclass of anti-GBM antibody was studied sequentially in $HgCl_2$ treated rats, using polyclonal anti-subclass reagents. IgG1, IgG2a and IgG2c showed similar kinetics to the IgG class response, peaking between days 11 and 14 (Fig. 10).

Anti-GBM antibodies of all IgG subclasses were easily detectable in day 14 kidneys eluates, which showed substantial purification of specific activity of antibody as compared to serum antibody, when expressed with respect to IgG concentration (Fig. 11). When monoclonal anti-subclass reagents to IgG1, IgG2b and IgG2c were used in the detection system, these three subclasses of anti-GBM antibody were readily detectable in eluate, although in serum both IgG2b and IgG2c were now barely detectable (Fig. 12).

### 3.1.2. Anti-DNA Activity

Anti-DNA activity was detected in $HgCl_2$ treated BN rats between days 9 and 14, with a further peak of apparently similar intensity at days 24-26 (Fig. 13). However, there was great variation in timing of the response within these 'peaks' between rats.

### 3.1.3. Circulating Immune Complexes

Circulating immune complexes were searched for using the C1qBA and solid phase KgBA. Day 0 values in the C1qBA were very high and variable, so the KgBA was used for sequential studies. Immune complexes were detectable after $HgCl_2$ treatment in BN rats but not Lewis rats (Fig. 14). Relationship of timing of anti-GBM antibody and immune complexes was performed by sequential analysis. Immune complexes were detectable in all rats, occurring after day 11, when anti-GBM antibody was present (Fig. 15). Analysis of individual rats showed that maximal levels of circulating immune

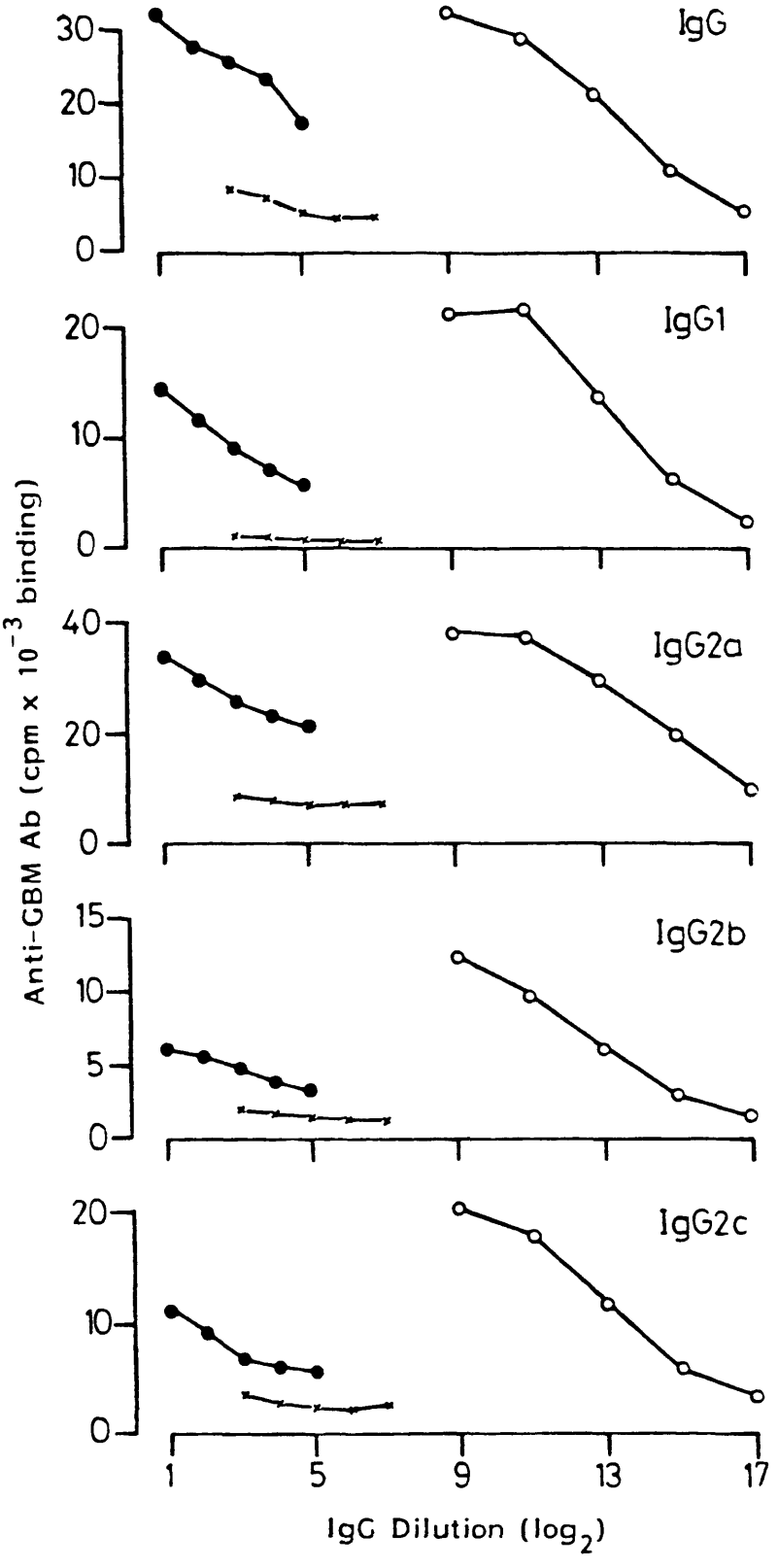



Figure 11. Dilution curves of IgG subclass of anti-GBM antibody in serum and eluate- polyclonal anti-subclass reagents. Pooled sera from normal BN rats (x——x), and day 13 $HgCl_2$ treated BN rats (●——●), and day 14 kidney eluate (o——o) were assayed for total IgG concentration, and then dilutions assayed for IgG and IgG subclass of the anti-GBM antibody, using polyclonal antisera. Graphs show the dilution curves of antibody, as cpm binding of detecting $^{125}I$ anti-Ig, when expressed as a function of IgG concentration, starting at 2.5mg/ml. All subclasses appeared to be detectable in both serum and eluate.

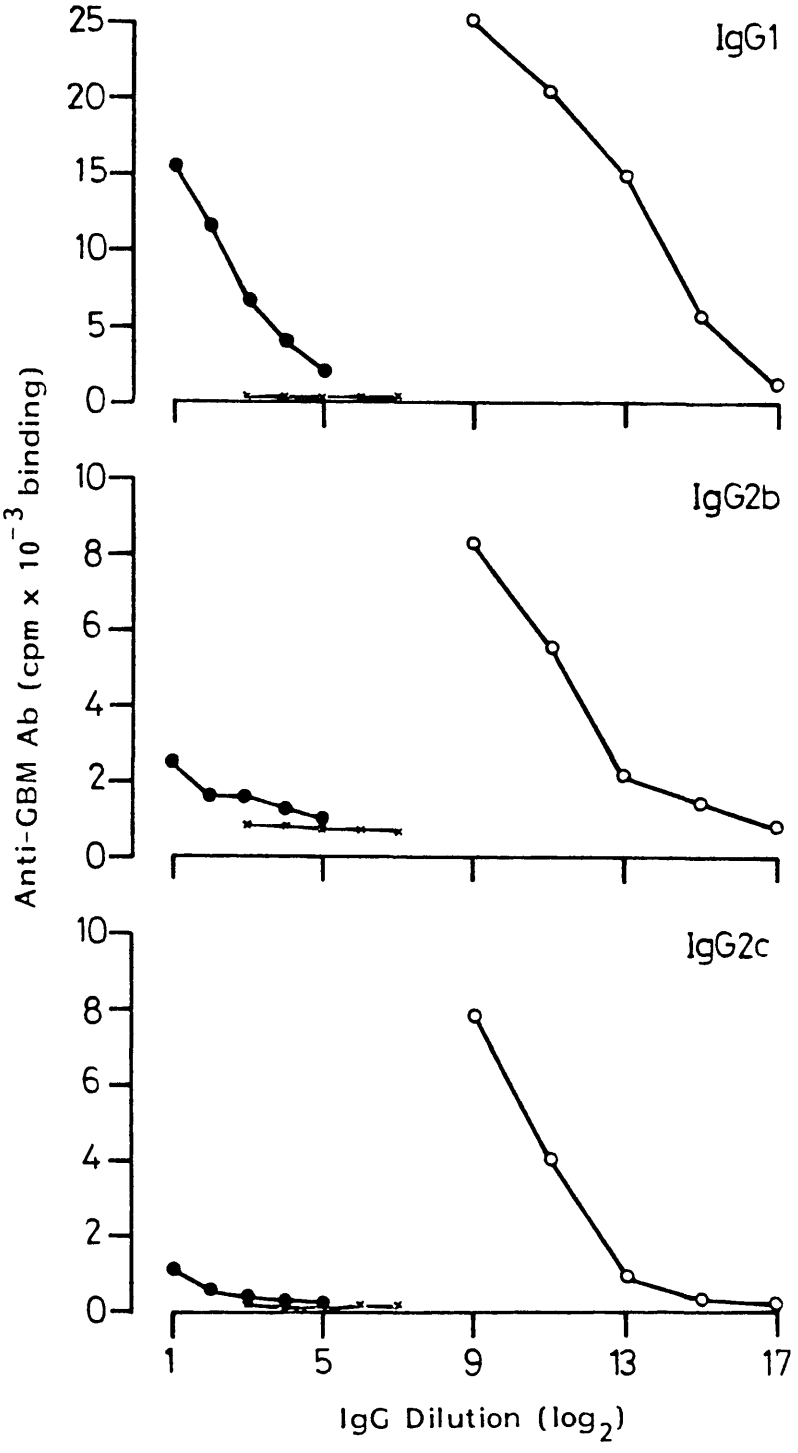



Figure 12. Dilution curves of IgG subclass of anti-GBM antibody in serum and eluate- monoclonal anti-subclass reagents. Pooled sera from normal BN rats (x——x), and day 13 $HgCl_2$ treated BN rats (●——●), and day 14 kidney eluate (o——o) were assayed for total IgG concentration, and then dilutions assayed for IgG1, IgG2b and IgG2c subclasses of anti-GBM antibody using monoclonal antibodies. Graphs show dilution curves of antibody, as cpm binding of detecting $^{125}I$ anti-Ig, when expressed as a function of IgG concentration, starting at 2.5mg/ml. Both IgG2b and 2c antibodies were barely detectable in serum but readily detectable in eluate.

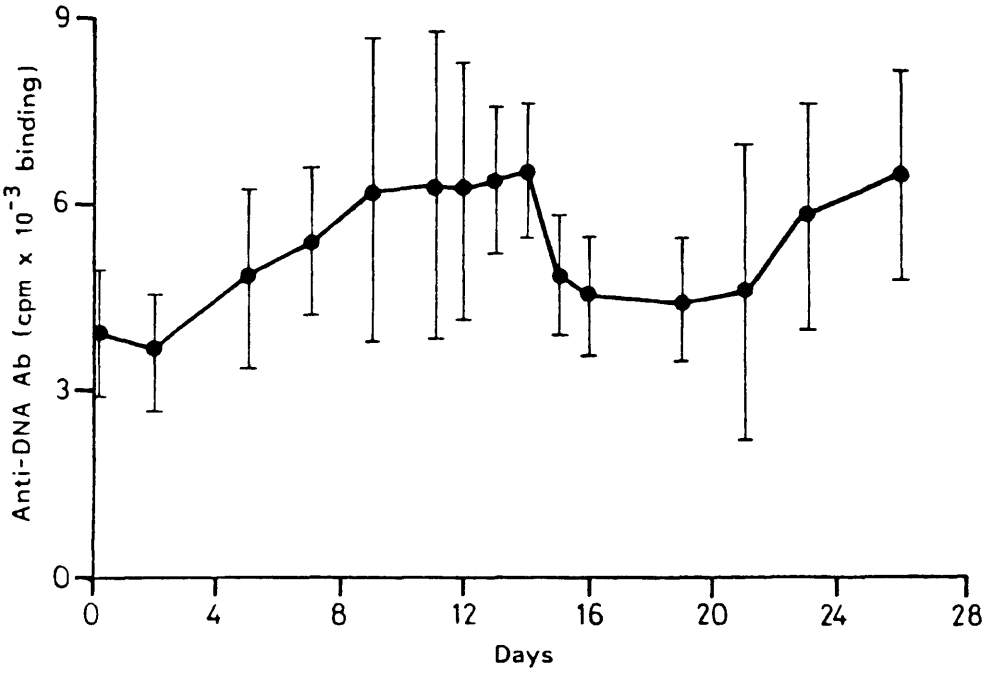



Figure 13. Time course of anti-DNA activity in $HgCl_2$ treated BN rats. Female BN rats (n=5) were subjected to the standard $HgCl_2$ injection protocol. The mean ± SD is shown, expressed as cpm binding of $^{125}I$ anti-Ig.

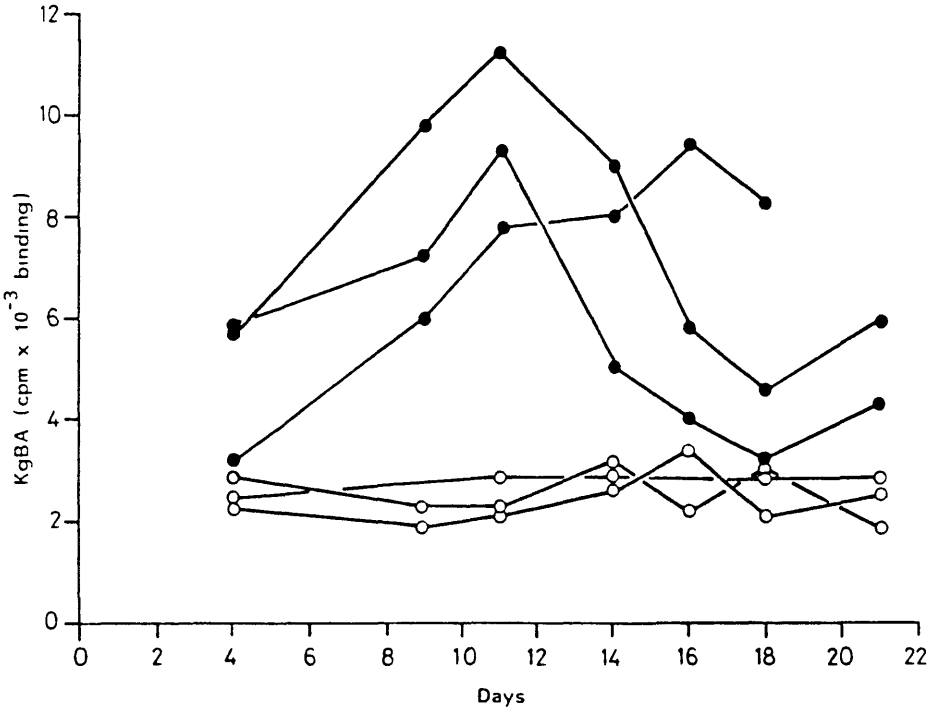



Figure 14. Time course of circulating immune complexes (CIC) in $HgCl_2$ treated BN or Lewis rats. Female BN (●——●) or age and sex matched Lewis (○——○) rats were subjected to the standard $HgCl_2$ injection protocol. Results are expressed as cpm binding of $^{125}I$ anti-IgG in the KgBA. CIC were detectable in BN but not Lewis rats.

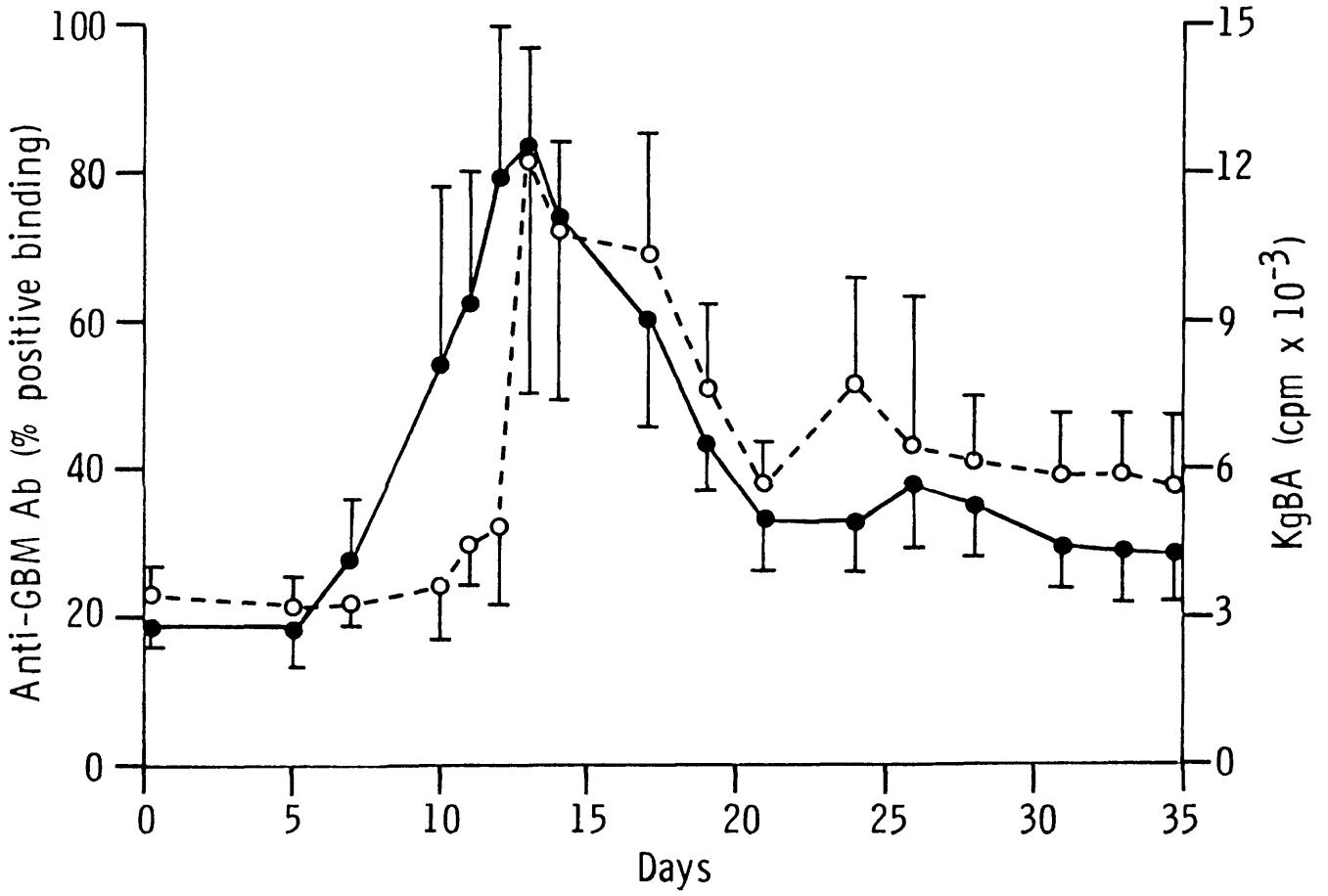



Figure 15. Relationship of immune complexes and anti-GBM antibody in $HgCl_2$ treated BN rats. Female BN rats (n=5) were subjected to the standard $HgCl_2$ injection protocol. The mean ± SD of sequential estimations of anti-GBM antibody (●——●), expressed as % positive binding, and circulating immune complexes (o– –o), expressed as cpm binding of $^{125}I$ anti-IgG in the KgBA, is shown. Immune complexes were detectable at the time of, and following, peak anti-GBM antibody levels.

complexes usually occurred after the peak of anti-GBM antibody and subsequently they too fell towards day 0 values, although later, smaller peaks were commonly found (Fig. 16).

### 3.1.4. Specificity of Anti-GBM Antibody

Specificity of the anti-GBM antibody in serum and kidney eluate was determined by inhibition studies and immunoblotting after SDS-PAGE (Western blotting). Rat GBM but not an irrelevant protein such as KLH caused inhibition of binding of antibody in serum or eluate to GBM in the RIA. Examination of cross species reactivity showed that human GBM caused inhibition although less than similar concentrations of rat GBM (Table 1).

When BN rat serum was incubated with nitrocellulose sheets immunoblotted with rat GBM separated on 5-20% gradient SDS-polyacrylamide gels, the background was found to be quite high. Use of sodium sulphate IgG fractions of normal or day 14 serum, or use of different quenching reagents on the nitrocellulose sheets, such as rat Hb instead of bovine Hb, failed to alleviate this high background. In addition, normal BN serum showed a propensity to bind to any protein bands non-specifically. Figure 17 shows a typical Western blot, using rat and human GBM and rat serum. Both control and day 14 serum bind to several protein bands in rat and human GBM, including the 6 bands detected by serum from patients with Goodpasture's syndrome, or a monoclonal antibody, MCA-P1, and also to a protein band in rat GBM of MW approx. 80K, which was also immunoreactive with the Goodpasture sera. However, day 14 serum gave more intense staining of the bands than normal BN serum.

Eluate was also blotted onto SDS-PAGE separated GBM, in order to reduce the problems associated with non-specific binding of serum IgG. With rat GBM, immunoreactive bands were seen at approx.

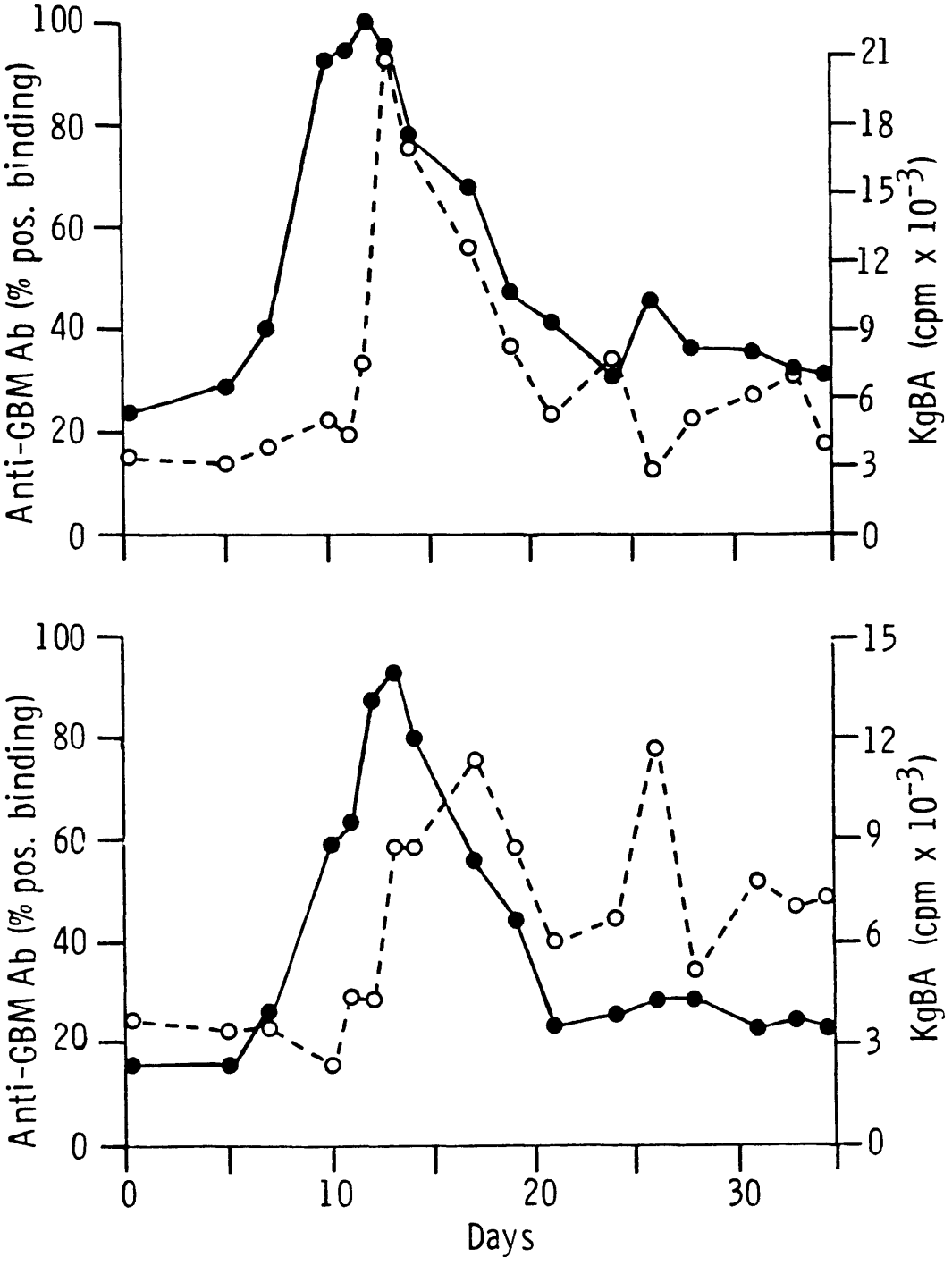



Figure 16. Relationship of immune complexes and anti-GBM antibody in individual $HgCl_2$ treated BN rats. Female BN rats were subjected to the standard $HgCl_2$ injection protocol. Graphs show anti-GBM antibody (●——●) expressed as % positive binding and circulating immune complexes (o- - -o), expressed as cpm binding of $^{125}I$ anti-IgG in the KgBA for 2 individual rats. Immune complexes were detectable slightly following the peak of anti-GBM antibody.

Table 1: Inhibition of Anti-GBM Activity

| Sera/Eluate | CFD | RGBM | HGBM | KLH |
|---|---|---|---|---|
| Normal Sera (1/64) | 1911 ± 188 | 1876 ± 130 | 1822 ± 316 | 1993 ± 196 |
| Day 14 Sera (1/64) | 5434 ± 249 | 3488 ± 323 | 4080 ± 252 | 5582 ± 702 |
| Day 14 Eluate (1/16) | 6078 ± 269 | 2122 ± 221 | 4876 ± 416 | 5546 ± 810 |

Day 14 or normal sera, or day 14 kidney eluate were preincubated with rat or human GBM or KLH (as described in section 2.1.5) before assaying in the anti-GBM RIA, in triplicate. Results show the mean ± SD of cpm binding of $^{125}I$ anti-IgG. Anti-GBM antibodies in serum and eluate were specifically inhibitable by rat GBM and to a lesser extent, by human GBM.

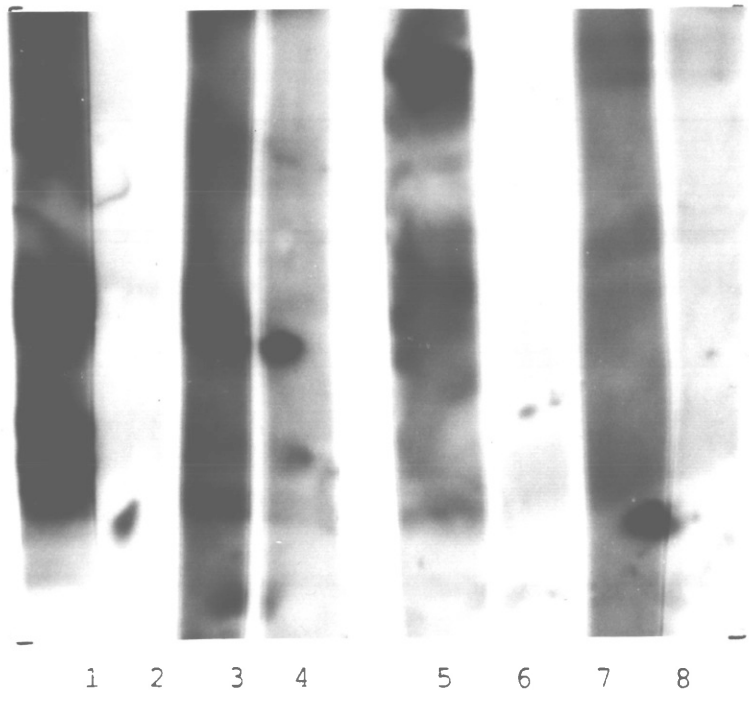



Figure 17. Immunoblot of BN sera and serum from patients with Goodpasture's syndrome on rat and human GBM separated by SDS-PAGE. Human GBM (lanes 1-4, 250 µg/lane) or rat GBM (lanes 5-8, 250µg/lane) were separated on a 5-20% SDS- polyacrylamide gel with spacer gel, and blotted onto nitrocellulose, which was quenched and overlaid as follows:

Lanes 1,5: 1ml serum from patient with Goodpasture's synd.
Lanes 2,6: 1ml serum from patient with non G-P RPGN.
Lanes 3,7: 2ml serum (pooled) from day 14 $HgCl_2$ treated rats.
Lanes 4,8: 2ml normal BN serum.

Detection was by $^{125}I$ anti-human IgG or $^{125}I$ anti-rat IgG. Photograph shows resulting autoradiograph. Note the binding of day 14 serum (lane 3) and to a lesser extent normal BN rat serum (lane 4) to bands in human GBM recognised by autoantibodies in a patient with G-P disease (lane 1). Note also similar and additional bands in rat GBM recognised by day 14 serum (lane 7) and sera from a patient with G-P disease (lane 5).

10K and 40K. In addition a weak band at 50K was detected, similar to that found with the mAb to GBM (Fig. 18). With human GBM, only a very weak band at 50K could be detected.

Separation of both human and rat GBM on 5-20% gradient SDS-PAGE produced a number of protein bands, many of which were similar (Fig. 19).

### 3.1.5. Immunofluorescence

Direct immunofluorescence of FITC anti-rat IgG stained kidney sections from $HgCl_2$ treated BN rats, showed characteristic linear IgG along the GBM by day 11, bright linear immunofluorescence at day 17, attenuating by day 26 to be replaced by granular deposits still present at day 36 (Fig. 20).

### 3.1.6. Proteinuria

Proteinuria (greater than 10mg protein excreted/24hr) occurred in 4/7 female rats examined, with peak levels ranging from 28-143 mg. The peak of urinary protein excretion took place at the same time as or shortly following, the peak of anti-GBM antibody (Table 2). Maximal levels of urinary protein excretion did not correlate with peak levels of anti-GBM antibody. Analysis of the timing and levels of circulating immune complexes also failed to show any relationship with proteinuria (Fig. 21).

### 3.1.7. Changes in Lymphoid Cells/Organs

#### Spleen Size

$HgCl_2$ treatment of BN rats caused an increase in spleen size, up to 2 fold, as determined by extractable yield of spleen cells, at days 10 and 21. The mean number of spleen cells approached day 0 values by day 36 (Table 3).

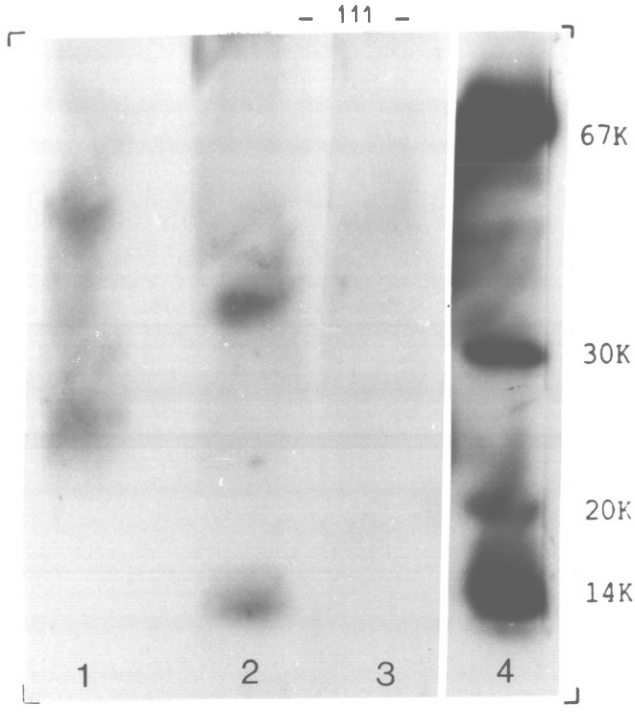



Figure 18. Immunoblot of BN eluate and MCA-P1 on rat GBM separated by SDS-PAGE. Rat GBM (250µg/lane, lane 2), human GBM (250µg/, lanes 1,3) and $^{125}I$ marker proteins (lane 4) were separated on a 5-20% SDS polyacrylamide gel, with spacer gel, and blotted onto nitrocellulose, which was quenched and overlaid with:

Lane 1: 10ml supernatant MCA-P1 (mAb to G-P antigen)
Lane 2,3: 1ml day 14 kidney eluate

Detection was by $^{125}I$ anti-rat IgG. Photograph shows resulting autoradiograph. Note the bands recognised by eluate but not MCA-P1.

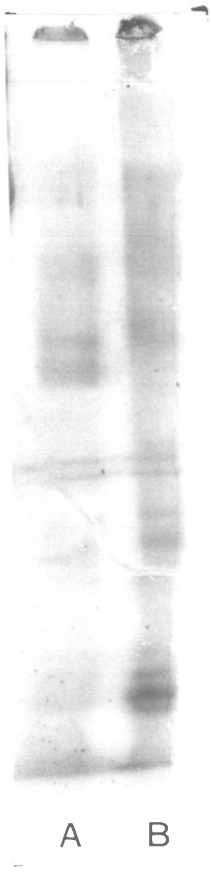



Figure 19. SDS-PAGE of rat or human GBM digests. Rat GBM (500µg, A) and human GBM (250µg, B) were separated on a 5-20% SDS-polyacrylamide gel, with spacer gel, and protein bands stained with Coomassie Blue. Many identical protein bands were found in both rat and human GBM.

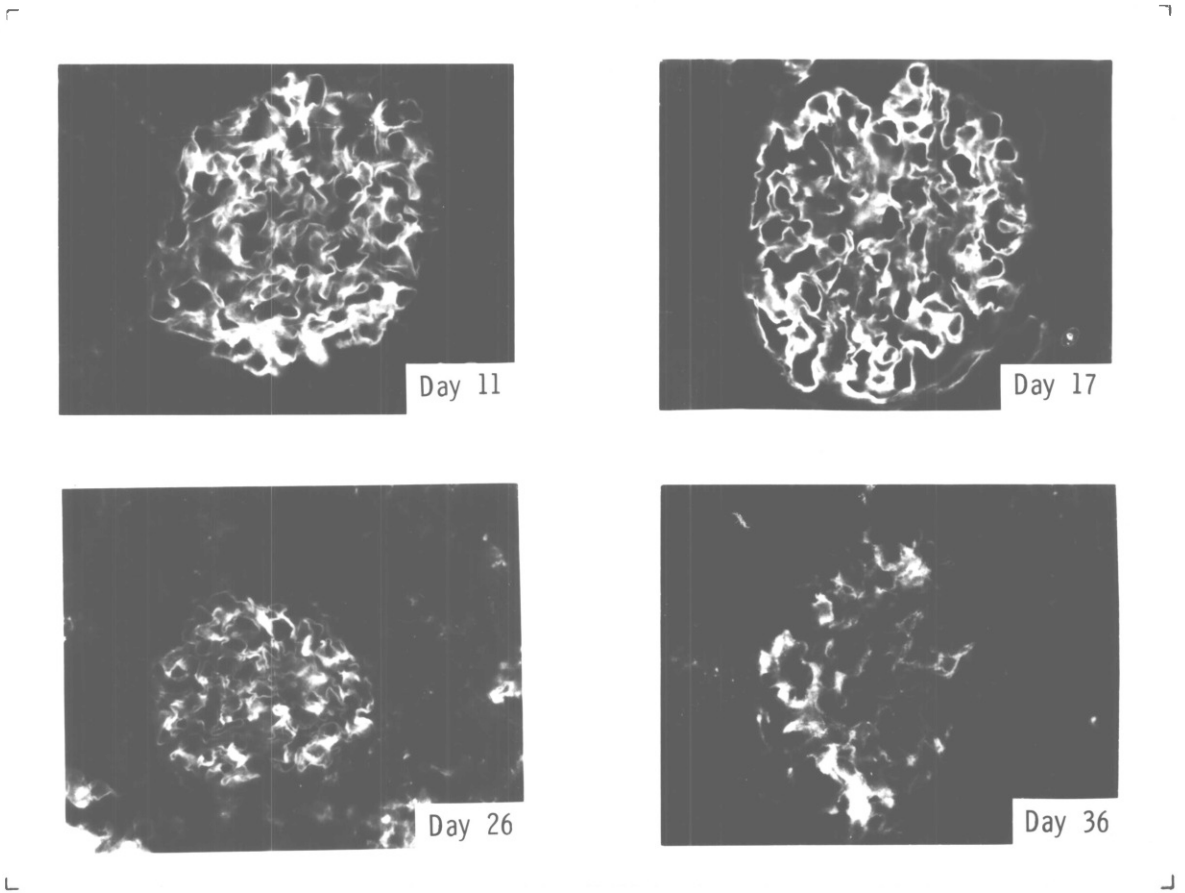



Figure 20. Direct immunofluorescence of kidney sections from day 11,17,26 and 36 $HgCl_2$ treated BN rats. Female BN rats were subjected to the standard $HgCl_2$ injection protocol. Rats were sacrificed at various times, and kidneys sectioned and stained with FITC anti-rat IgG. Note the linear pattern of IgG fixation at day 11 and the granular pattern at day 36.

Table 2. Proteinuria and Anti-GBM Antibody in $HgCl_2$ Treated BN Rats

| Rat | Peak Proteinuria (mg/24 hr) | Time (day) | Peak anti-GBM Ab (% positive binding) |
|---|---|---|---|
| 1 | 143 | 15 | 73 |
| 2 | 61 | 17 | 55 |
| 3 | 44 | 12 | 93 |
| 4 | 28 | 13 | 64 |
| 5 | 8 | - | 64 |
| 6 | 8 | - | 54 |
| 7 | 9 | - | 42 |

Note the variable susceptibility to proteinuria even with similar levels of anti-GBM antibody.

Table 3. Time Course of Number of Spleen Cells in $HgCl_2$ Treated BN Rats

| Day | 0 | 10 | 21 | 36 |
|---|---|---|---|---|
| Number of Animals | 8 | 11 | 6 | 3 |
| Mean yield | $2.6 \times 10^8$ ± 0.4 | $5.0 \times 10^8$ ± 1.14 | $4.1 \times 10^8$ ± 0.43 | $3.1 \times 10^8$ ± 0.81 |

Results show extractable yield of spleen cells from $HgCl_2$ treated BN rats, expressed as mean SC ± SD. Note the transient increase in spleen size.

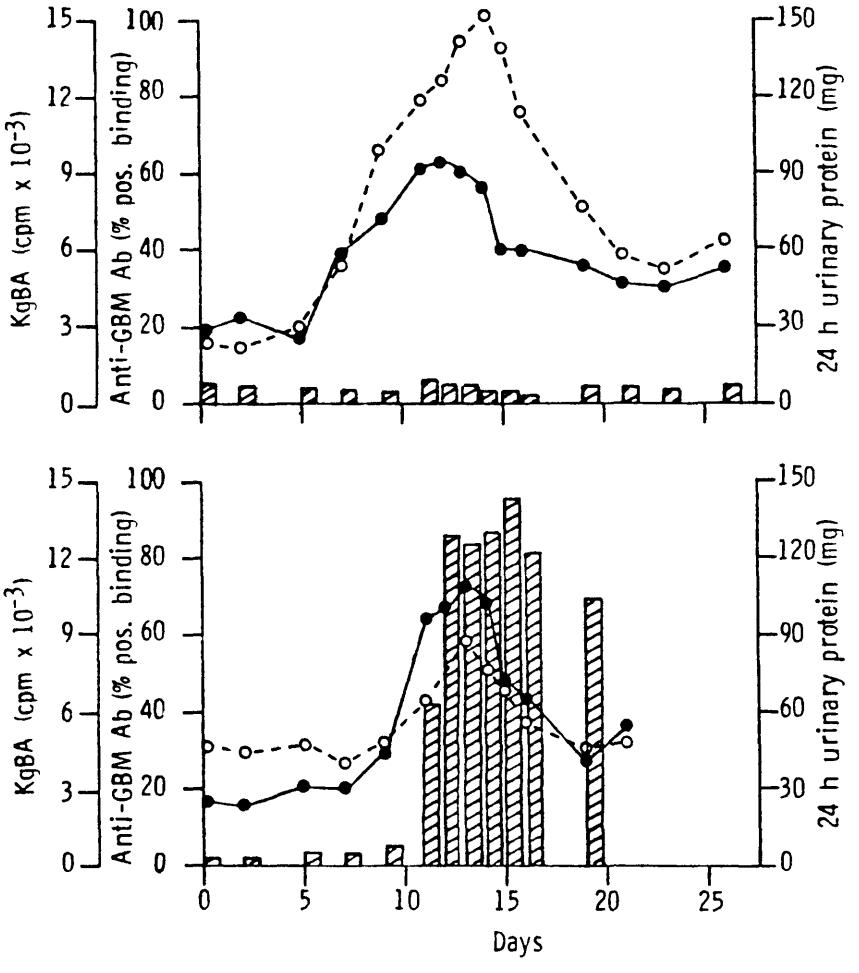



Figure 21. Relationship between anti-GBM antibodies, immune complexes and proteinuria in individual rats. Female BN rats were subjected to the standard $HgCl_2$ injection protocol. Data shown is for two rats, with sequential measurements of anti-GBM antibody (●——●), expressed as % positive binding, immune complexes (o- - -o) assayed by the KgBA and expressed as cpm binding, and proteinuria (hatched bars) as mg urinary protein excreted /24 hr. Proteinuria, when present, occurred slightly following peak anti-GBM antibody and immune complexes.

Total and Differential WBC

Peripheral white blood cell counts increased after day 12, but with great variability between rats, and fluctuated despite discontinuance of $HgCl_2$ injections. In some experiments they remained slightly elevated at day 36 (Fig. 22). Wistar rats, which are not susceptible to $HgCl_2$ induced nephritis, did not show this cellular proliferation (Fig. 23).

The increase in total circulating WBC coincided with an increase in neutrophils, peaking at day 17. Number of circulating neutrophils returned to day 0 values by day 26. Both % and total neutrophils increased during this period. No significant change occurred in lymphocyte numbers, although great variability was present (Fig. 23). Less variation was seen in analysis of % lymphocytes.

Lymphocyte Subsets

The effect of $HgCl_2$ treatment in BN rats, on lymphocyte subsets in peripheral blood was examined. T cells (MRC OX19 positive) generally decreased while B cells (MRC OX12 positive) increased in a reciprocal manner (Fig. 24). This reciprocity was especially marked early in the disease. In more detail, the percentage of T cells showed a decrease after day 10 from day 0 values of 37% to 22% by day 19 (Fig. 25). In some experiments an early transient decrease at day 7 was noted (Fig. 24,26). Levels rose to normal by day 40. The decrease(s) in % T lymphocytes could be accounted for by the decrease in MRC W3/25 positive (T helper) cells, which followed similar kinetics to the T cells (Fig. 25,26). Non-susceptible Wistar rats did not have significant changes in MRC OX19 or W3/25 positive cells (Fig. 26).

However, MRC OX8 positive (T suppressor) cells were often

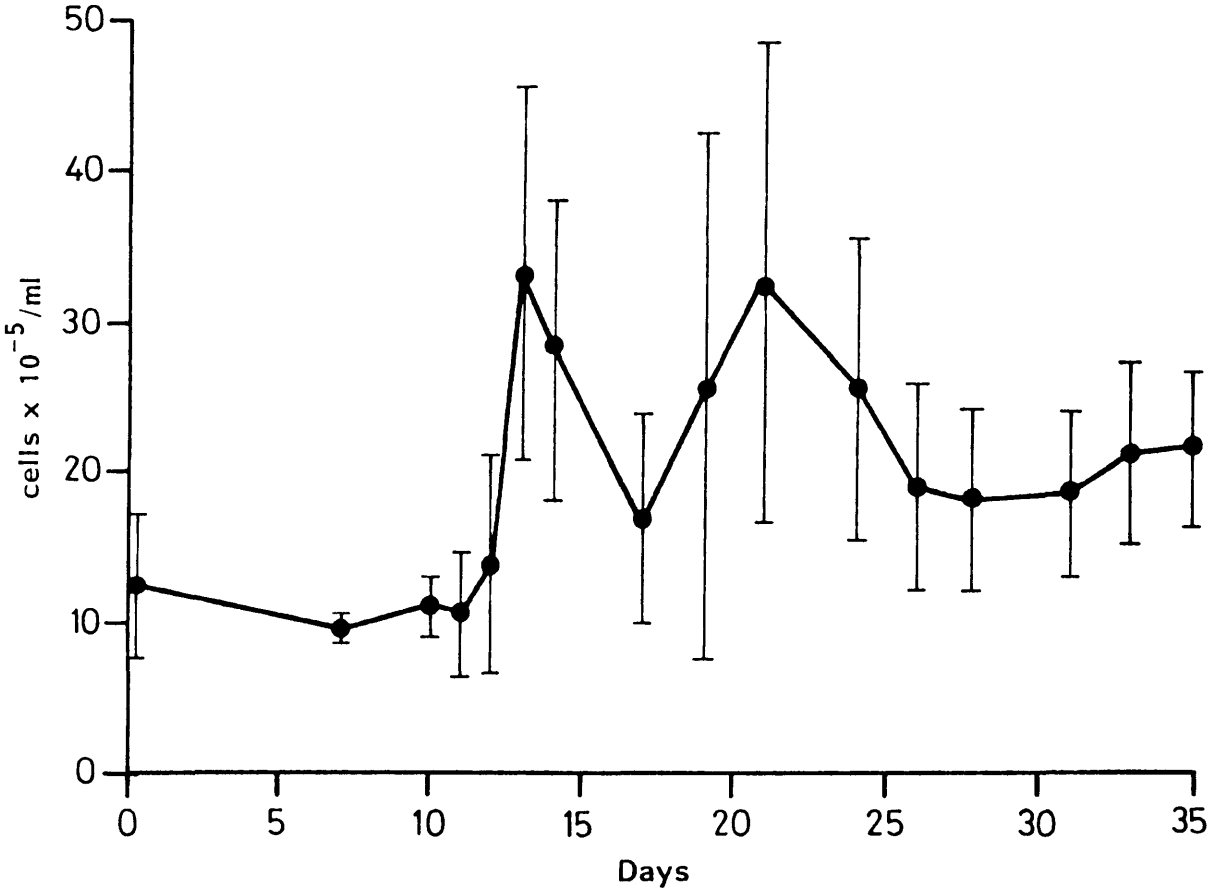



Figure 22. Time course of circulating WBC in $HgCl_2$ treated BN rats. Female BN rats (n=10) were subjected to the standard $HgCl_2$ injection protocol. Results show the mean ± SD for total WBC as determined on a Coulter counter.

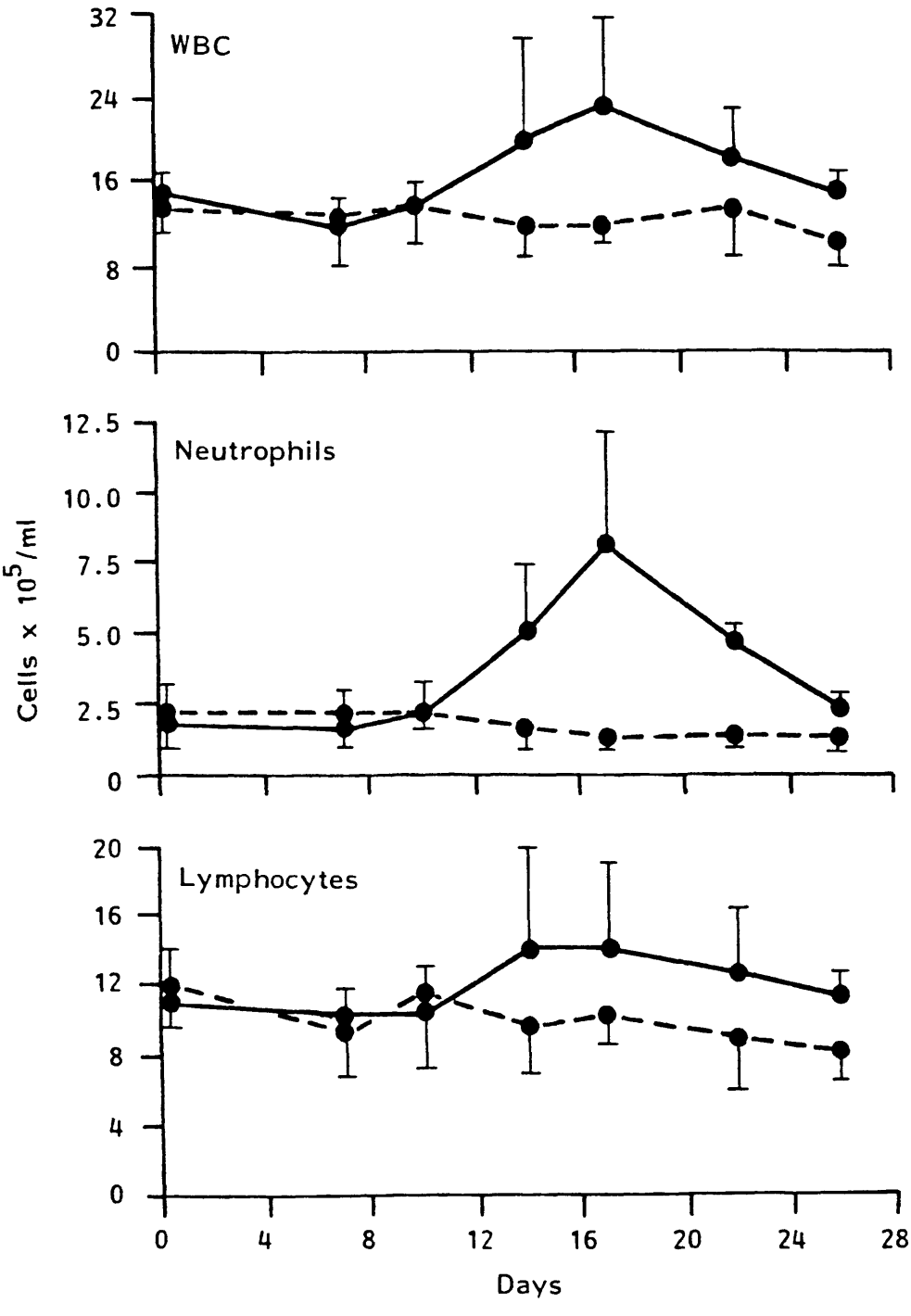



Figure 23. Time course of total WBC, neutrophils and lymphocytes in $HgCl_2$ treated BN and Wistar rats. Groups of female BN (●——●) or Wistar (●- -●) rats (n=3) were subjected to the standard $HgCl_2$ injection protocol and sequential total and differential WBC performed, from which were also derived the number of neutrophils and lymphocytes/ml. Results shown represent the mean ± SD of each group expressed as cells/ml.

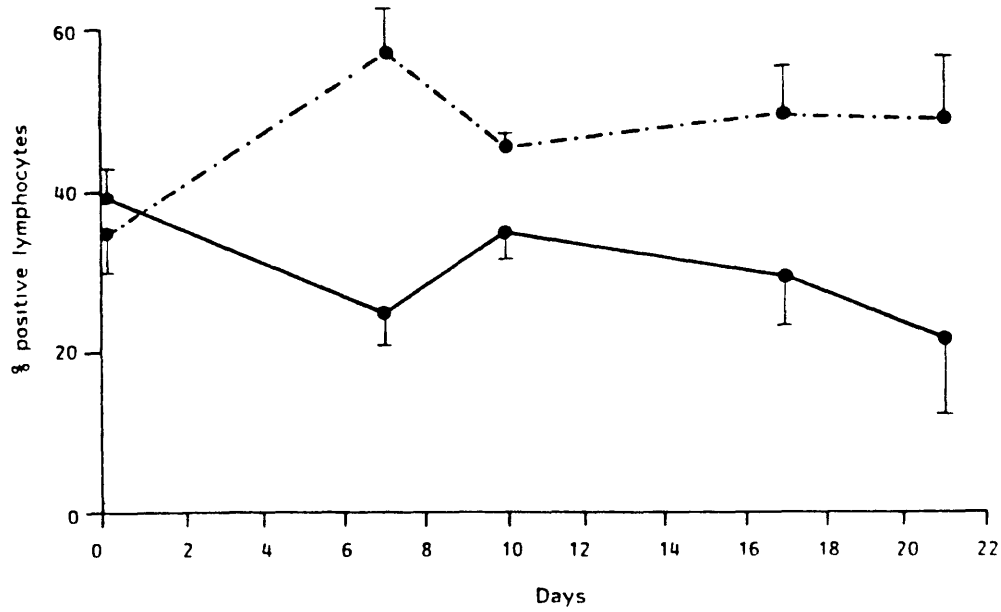



Figure 24. Reciprocal changes in % peripheral B and T lymphocytes in $HgCl_2$ treated BN rats. A group (n=6) of female BN rats were subjected to the standard $HgCl_2$ injection protocol and sequential B (MRC $OX12^+$, ●—·—●) and T (MRC $OX19^+$, ●——●) cells determined on a Coulter EPICS C cell analyser. A time course of the mean ± SD of the % positive lymphocytes is shown.

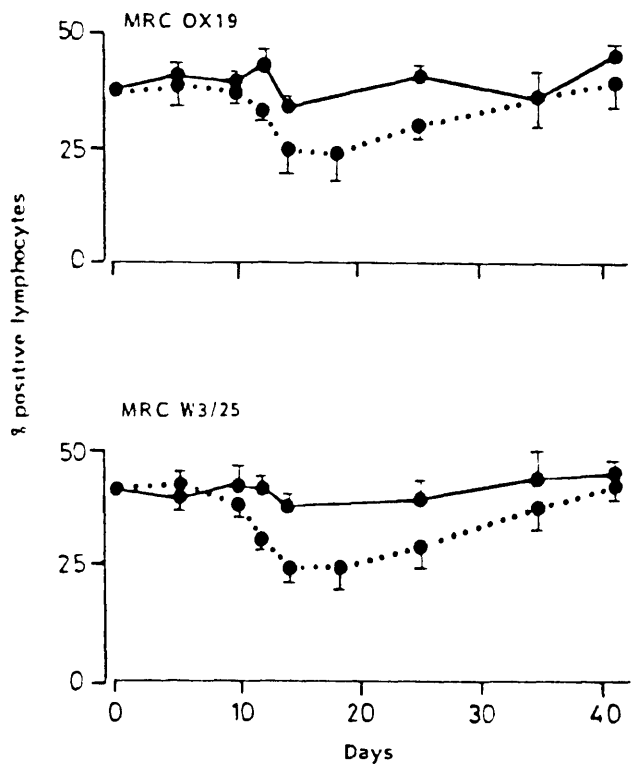



Figure 25. Sequential changes in % peripheral T and $T_h$ cells in $HgCl_2$ treated and control BN rats. Groups of BN rats (n=4) were subjected to the standard $HgCl_2$ injection protocol (●····●), or injected with PBS (●——●) and sequential MRC $OX19^+$ (T cells, upper graph) or MRC $W3/25^+$ ($T_h$ cells, lower graph) cells analysed by flow cytometry. Results show the mean ± SD, expressed as % positive lymphocytes. Similar transient decreases occurred in % of MRC $OX19^+$ and MRC $W3/25^+$ cells in $HgCl_2$ treated but not control BN rats.

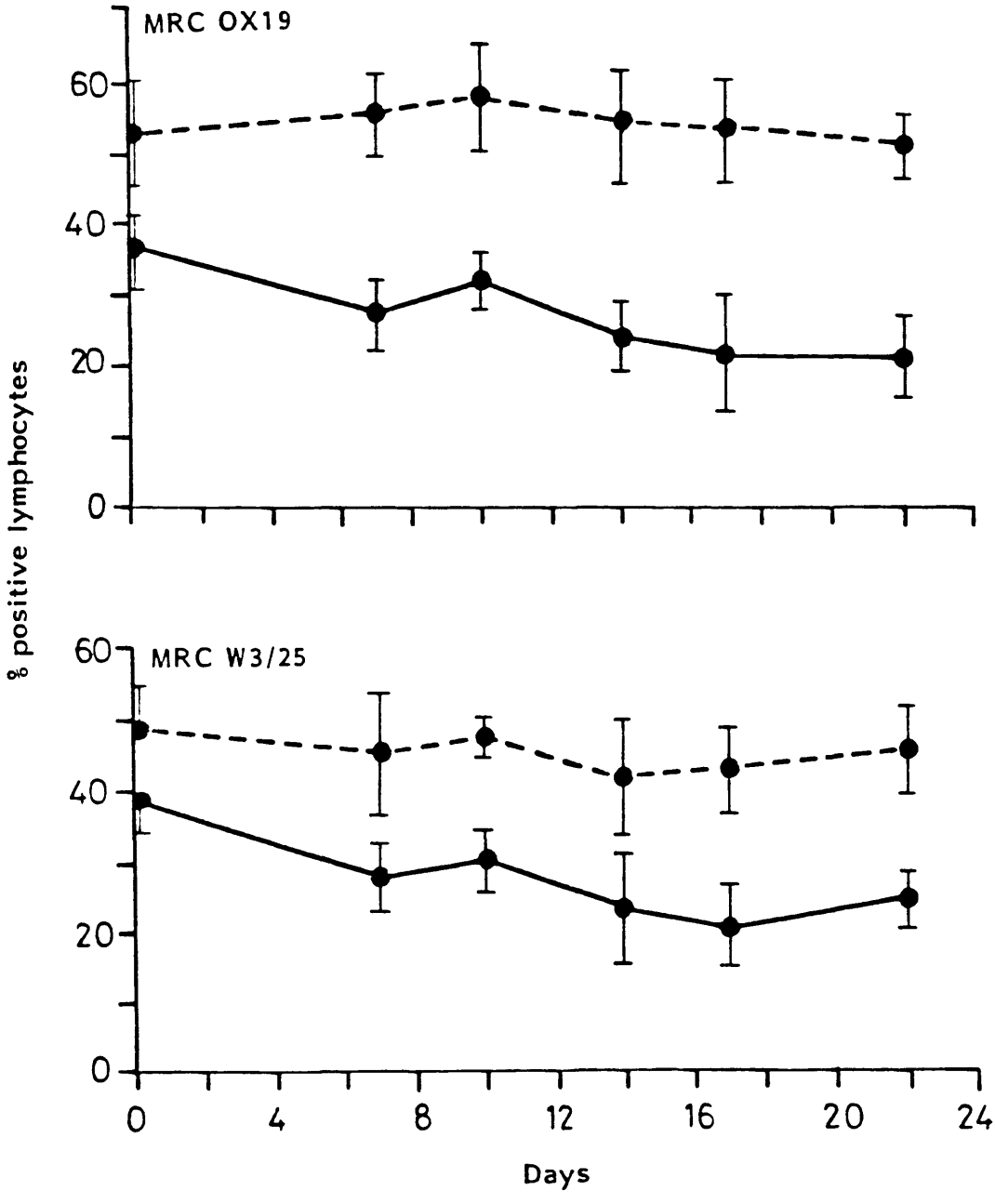



Figure 26. Sequential changes in % peripheral T and $T_h$ cells in $HgCl_2$ treated BN and Wistar rats. Groups (n=6) of BN (●——●) or Wistar (●- - -●) rats were subjected to the standard $HgCl_2$ injection protocol and sequential MRC $OX19^+$ (T cells, upper graph) or MRC $W3/25^+$ ($T_h$ cells, lower graph) cells analysed by flow cytometry. Results show the mean ± SD, expressed as % positive lymphocytes. Decreases in MRC $OX19^+$ and MRC $W3/25^+$ cells occurred in $HgCl_2$ treated BN but not Wistar rats.

found to have an early decrease at days 5-10 (Fig. 27). This occurred in 12/15 rats examined, and additionally, some rats (7/15) showed a later rise above the normal range at days 12-22 (Fig. 28). $HgCl_2$ treated Wistar rats, with more than twice the day 0 suppressor cells of BN rats, failed to show this early decrease and late increase, but sometimes exhibited a decrease in percentage $T_s$ after day 12.

When the number of positive cells/ml was calculated instead of percentages, similar trends in MRC OX19, W3/25 and OX8 positive cells emerged, although greater variation was seen between rats due to the large variation in number of lymphocytes/ml in the rats.

In a small number of spleens examined, percentages of MRC OX19 and W3/25 positive lymphocytes also decreased during the disease, while % MRC OX12 positive cells showed a transient increase (data not shown).

## 3.2. REGULATION AND AUTOREGULATION OF THE RESPONSE

### 3.2.1. Rechallenge Experiments

#### Time Course

To determine whether rats, in which antibody levels had fallen spontaneously, were still resistant to the induction of further anti-GBM antibody synthesis, groups of animals were allowed to convalesce for between 3 and 10 weeks after their peak antibody response and were then rechallenged with $HgCl_2$. All rechallenged rats had markedly reduced responses (Fig. 29). This resistance to rechallenge was time dependent and no protection remained after 9 months (Table 4).

#### Low Dose $HgCl_2$

In other experiments using 5 injections of 0.1mg/kg $HgCl_2$

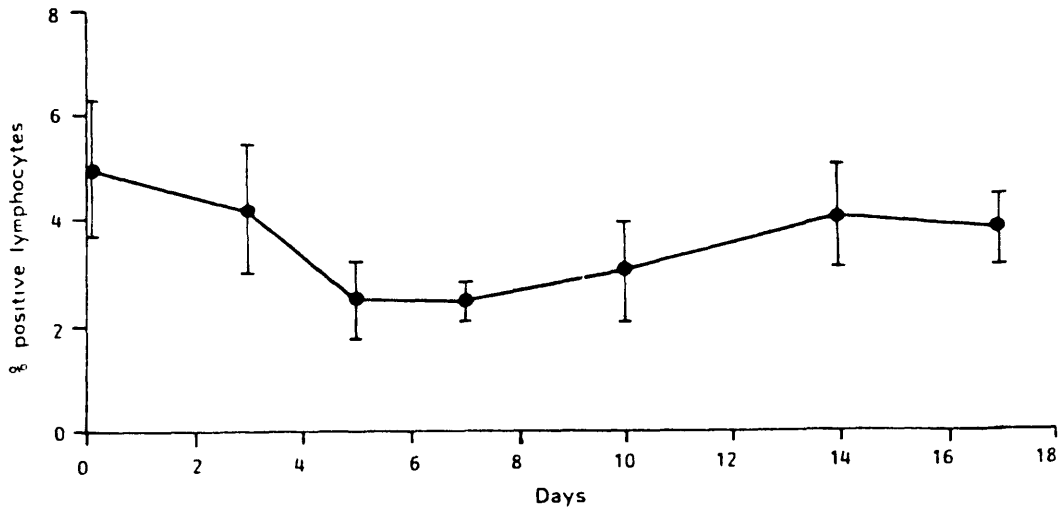



Figure 27. Sequential changes in % peripheral MRC OX8$^+$ ($T_S$) cells in $HgCl_2$ treated BN rats. Female BN rats (n=6) were subjected to the standard $HgCl_2$ injection protocol and MRC OX8$^+$ cells analysed by flow cytometry. Results show the mean ± SD, expressed as % positive lymphocytes.

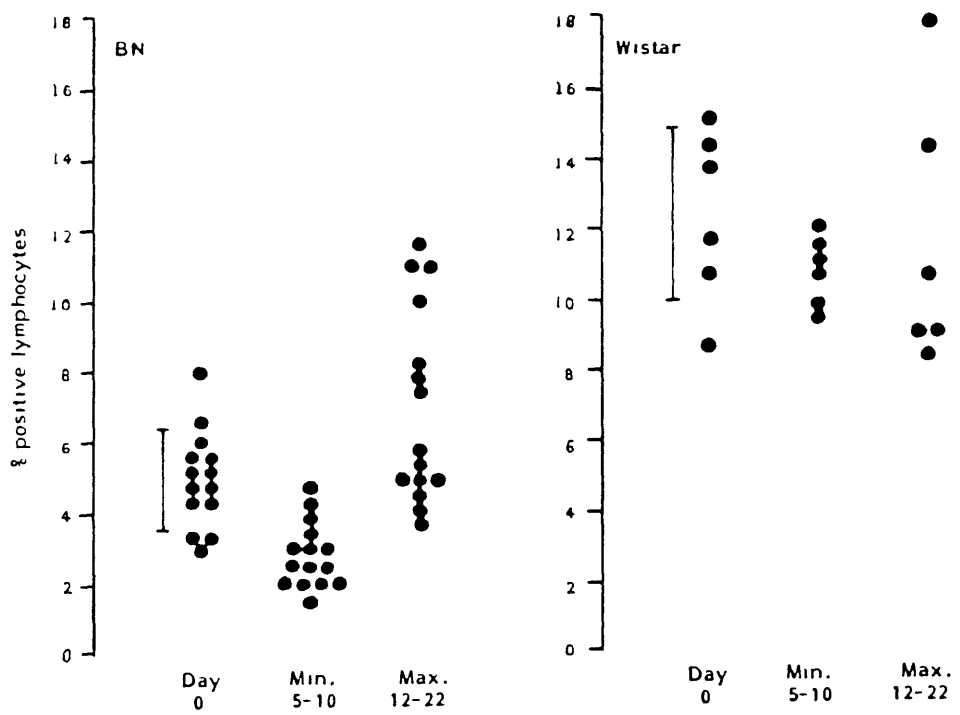



Figure 28. MRC OX8 positive cells in peripheral blood of individual $HgCl_2$ treated BN or Wistar rats. BN or Wistar rats were subjected to the standard $HgCl_2$ injection protocol and MRC OX8$^+$ cells analysed sequentially by flow cytometry. Data shown represent the day 0 values, the minimum value at an early stage (days 5-10) and the maximum value at a later stage (days 12-22), expressed as % positive lymphocytes. The normal range (mean ± SD) of day 0 values is shown by the bar for each strain. Note that an early decrease and late rebound in $T_S$ cells occurred in $HgCl_2$ treated BN but not Wistar rats.

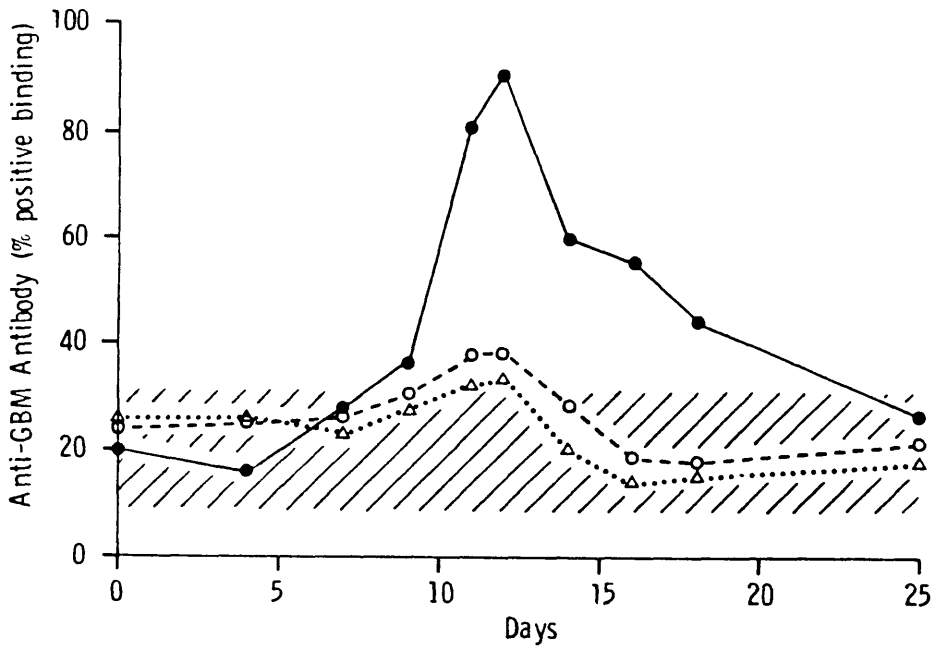



Figure 29. Time course of anti-GBM antibody synthesis in BN rats rechallenged with $HgCl_2$. Groups of rats at 3-5 weeks (n=5, Δ• • • • •Δ) or 7-10 weeks (n=5, o– – –o) after the peak anti-GBM antibody response induced by 5 injections of $HgCl_2$, were then rechallenged. Controls (n=4, ●——●) were age-matched, naive rats. Results are expressed as % positive binding of the mean of each group. Normal range is hatched. Rechallenged rats were resistant to further induction of anti-GBM antibodies by $HgCl_2$.

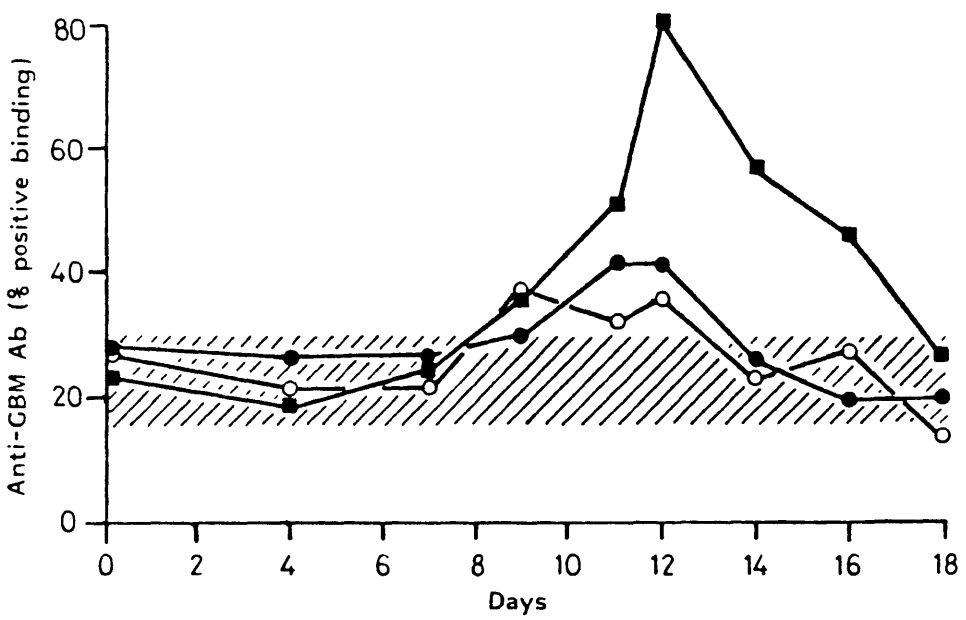



Figure 30. Effect of primary low dose $HgCl_2$ on anti-GBM antibody synthesis in rats rechallenged with $HgCl_2$. Groups of rats (n=4) were injected with 5 injections of PBS (■——■), 0.1mg/kg $HgCl_2$ (o——o) or 1mg/kg $HgCl_2$ (●——●) and after 9-11 weeks all groups were injected with 1mg/kg $HgCl_2$. Results after rechallenge are expressed as % positive binding of the mean of each group. Normal range is hatched. Note that low dose $HgCl_2$ pretreatment still conferred resistance to rechallenge.

Table 4. Duration of Resistance to Rechallenge with $HgCl_2$

| Time | 3-5 weeks | 7-10 weeks | 4 1/2 months | 9 months |
|---|---|---|---|---|
| n | 5 | 5 | 2 | 2 |
| % suppression | 90% | 81% | 74% | -5% |

BN rats were allowed to convalesce for various times after peak anti-GBM antibody response and then rechallenged with $HgCl_2$. Age-matched control rats were subjected to a primary course of $HgCl_2$ injections for each experiment. Data shown represent the % suppression calculated by:-

$$\frac{100}{1} \times \frac{\text{mean peak anti-GBM Ab control group} - \text{mean peak anti-GBM Ab rechallenge group}}{\text{mean peak anti-GBM Ab control group}}$$

Note that resistance to rechallenge decreased with time.

(1/10th of the standard dose), followed by convalescence for 9-11 weeks and challenge with 1mg/kg $HgCl_2$, it was found that low doses of $HgCl_2$ which induce no or a minimal antibody response, still conferred protection against later rechallenge (Fig. 30).

Rechallenge after Renal Transplantation

To determine whether lack of available antigen after a primary response was responsible for the resistance of convalescent rats to rechallenge with $HgCl_2$, rats, 2 months after a primary response, were subjected to nephrectomy and syngeneic kidney transplantation, or sham operated, and 1 week later, rechallenged with $HgCl_2$. An analysis of peak anti-GBM antibody response at day 13, showed that transplanted, rechallenged rats had a markedly reduced response compared to naive controls when challenged with $HgCl_2$, although they had apparently higher levels than unoperated rechallenged rats (Fig. 31). However, the sham operated, rechallenged rat also had a higher level of binding than unmanipulated rechallenged rats, and all animals subjected to surgical procedures had day 0 values above the normal range, due to non-specific binding.

Rechallenge with GBM

To determine whether resistance to rechallenge with $HgCl_2$ was associated with resistance to challenge with GBM antigen, a pilot experiment was set up injecting homologous rat GBM into rats 3 or 4 1/2 months convalescent after $HgCl_2$ treatment, or age matched controls. $HgCl_2$ pretreated rats appeared to make a markedly reduced response (Fig. 32).

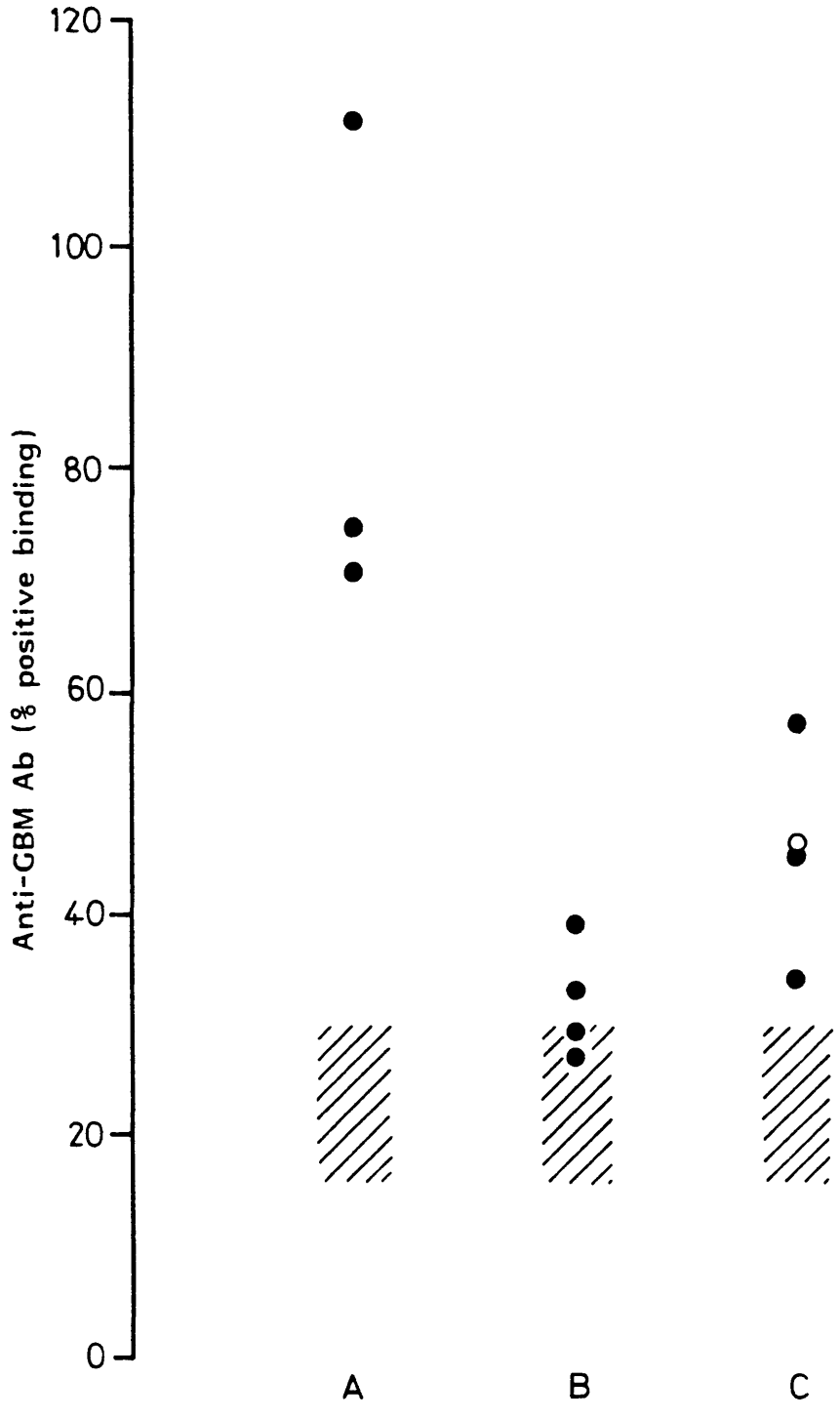



Figure 31. Effect of kidney transplantation on resistance to rechallenge with $HgCl_2$, as indexed by peak anti-GBM antibody response. Naive control BN rats (A), rats 3 months convalescent after primary $HgCl_2$ challenge (B) or 3 months convalescent after $HgCl_2$ and then subjected to nephrectomy and syngeneic kidney transplantation (C,●) or sham operated (C,o) were, after a further 7 days, challenged with $HgCl_2$. Peak anti-GBM antibody levels are shown, expressed as % positive binding, for each rat. The normal range is hatched. Transplant recipients were still resistant to rechallenge.

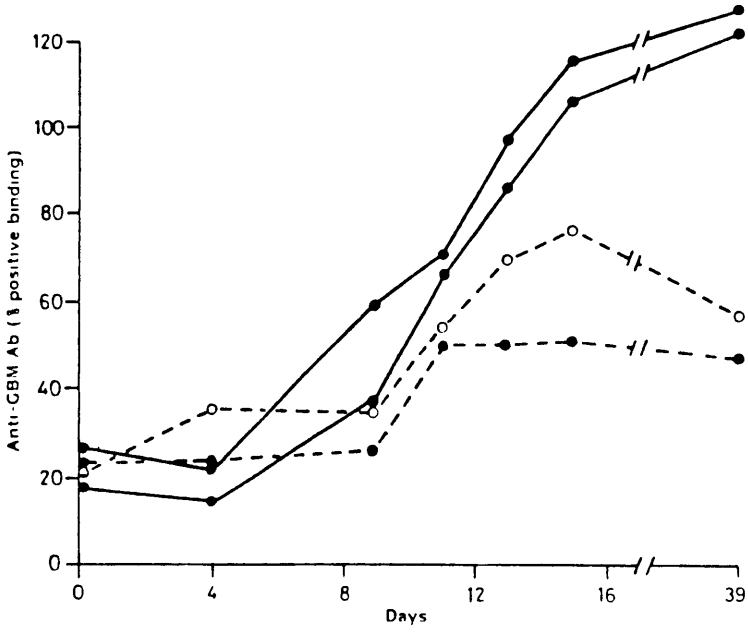



Figure 32. Effect of challenge with GBM in rats convalescent after a course of primary $HgCl_2$ injections. BN rats 3 months (●– –●) or 4 1/2 months (o– –o) convalescent after 5 injections of $HgCl_2$, or age matched controls (●——●) were challenged with 200µg rat GBM i.m.. Results of anti-GBM antibody are expressed as % positive binding. Note that rats convalescent from $HgCl_2$ treatment synthesised less antibody than controls, after GBM challenge.

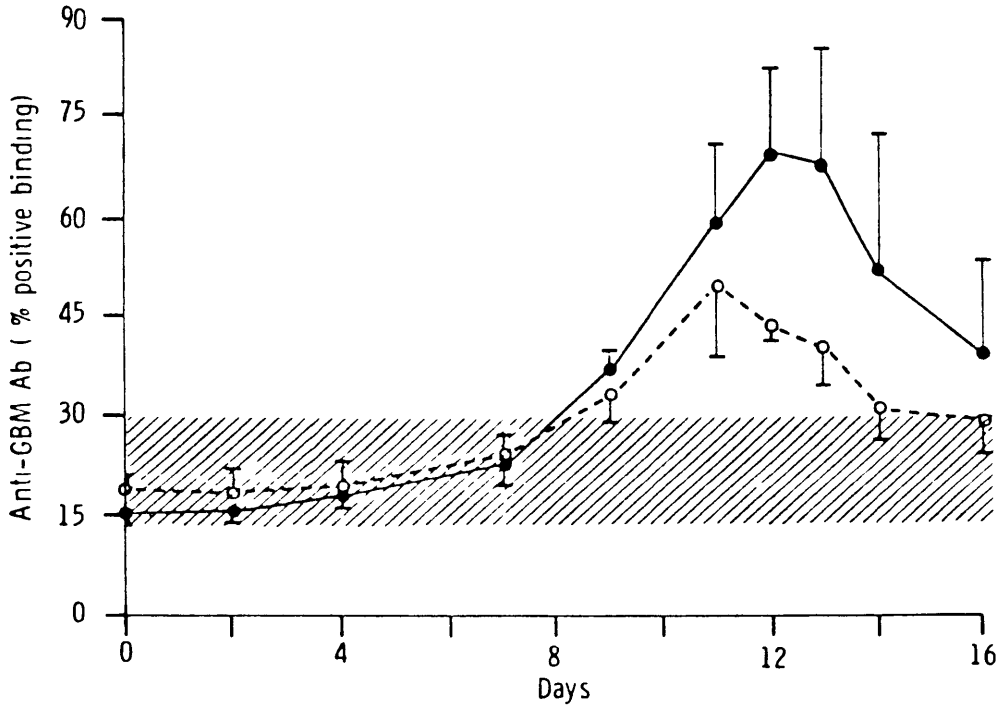



Figure 33. Effect of convalescent SC transfer on $HgCl_2$ induced anti-GBM antibody synthesis. Groups of rats received 6 x $10^7$ SC from day 21 convalescent rats, in DAB/CS (n=4, o– –o), or DAB/CS alone (n=6, ●——●), and were then given 3 injections of $HgCl_2$. Results (% positive binding) are expressed as the mean ± SD for each group. Normal range is hatched. Significant suppression of anti-GBM antibody synthesis occurred in recipients of convalescent cells at day 12 ($p<0.01$) and day 13 ($p<0.05$).

3.2.2. Spleen Cell Transfer

The resistance to $HgCl_2$ rechallenge in convalescent animals was investigated further by cell transfer experiments. Spleen cells were pooled from day 21 convalescent rats, and were transferred to naive recipients, which were then injected with $HgCl_2$ (Fig. 33). The recipients of convalescent SC made significantly less anti-GBM antibody than controls receiving medium.

Dose Effect

To determine the minimum dose of SC required to produce suppression, doses from 5 x $10^6$ to $10^8$ cells were transferred to groups of recipients, which were then injected with $HgCl_2$ (Fig. 34). Doses of 5 x $10^6$ to 5 x $10^7$ cells resulted in early augmentation of the antibody response, with no subsequent suppression. Doses of greater than 5 x $10^7$ convalescent cells were required to transfer suppression. In other experiments the number of cells required to produce significant suppression of the anti-GBM antibody response was found to range between 6 x $10^7$ and 2 x $10^8$ cells (Fig. 34,35).

Effect of Spleen Cells from Normal Animals

To determine the effect of normal BN spleen cells on $HgCl_2$ induced generation of anti-GBM antibody, groups of animals received from 6 x $10^7$ to 2 x $10^8$ normal or convalescent cells or medium. Peak antibody levels were compard between recipients of normal or convalescent cells and medium (Fig. 35). While recipients of convalescent cells produced significantly less antibody ($p<.001$) than those receiving medium, those receiving normal cells were not significantly different from recipients of medium. Only occasionally (3/22) did recipients of normal cells appear to be suppressed.

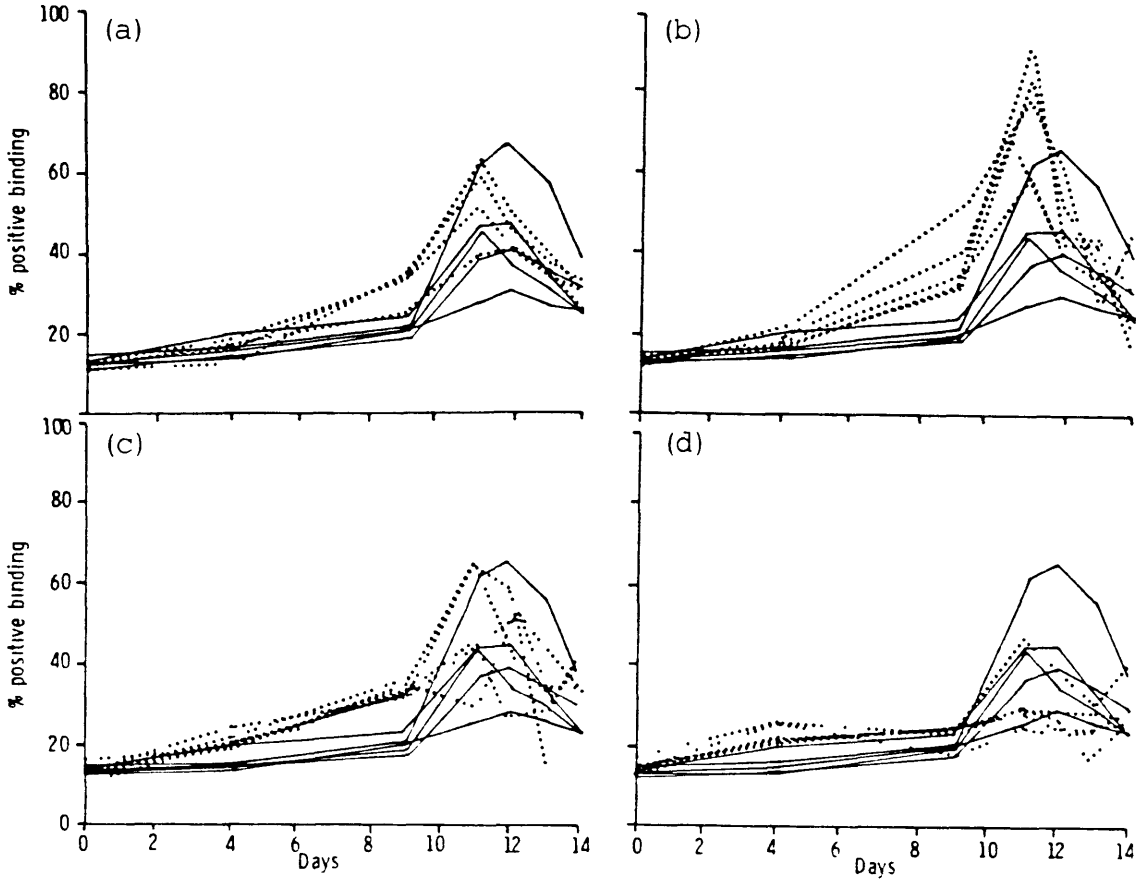



Figure 34. Dose response effect of convalescent SC on anti-GBM antibody production. Groups of naive rats (n=5) received doses of (a) 5 x $10^6$, (b) $10^7$, (c) $5x10^7$ or (d) $10^8$ SC in DAB/CS from day 21 convalescent rats (·····), or DAB/CS alone (_____), and were then given 4 injections of $HgCl_2$. Results are expressed as % positive binding for each rat during the time course. Significant augmentation of anti-GBM antibody response, as compared to recipients of medium (DAB/CS) occurred at day 9 with doses of 5 x $10^6$ cells ($p<0.002$), $10^7$ cells ($p<0.002$), 5 x $10^7$ cells ($p<0.001$) and at day 11 for $10^7$ cells ($p<0.005$). Transfer of $10^8$ convalescent SC caused suppression in 4/5 rats at the time of peak antibody response and one control rat failed to respond. The probability that this is a chance distribution is 25/252 i.e. $p=0.1$.

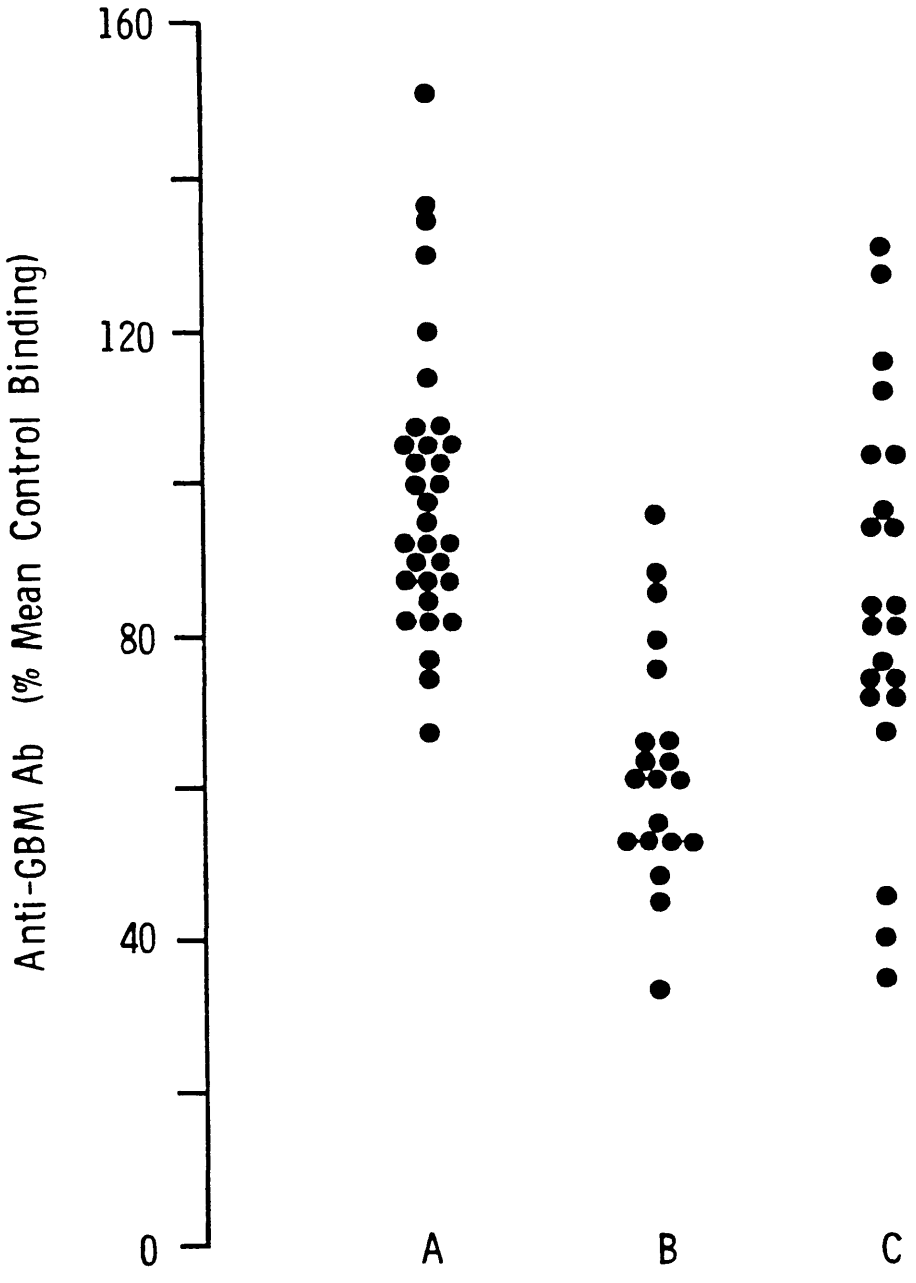



Figure 35. Effect of convalescent and normal SC transfer on peak anti-GBM antibody levels. Composite data from 7 SC transfer experiments. Rats were injected with day 21-28 convalescent SC (B, n=20) or normal SC (C, n=22) in doses from 6 x $10^7$ to 2 x $10^8$ and then given $HgCl_2$. Controls in each experiment received medium and $HgCl_2$ (A, n=32). Peak anti-GBM antibody levels are shown, expressed as % mean control group binding for each experiment. Significant suppression occurred in recipients of convalescent cells as compared to controls ($p<0.001$), and compared to recipients of normal cells ($p<0.01$). Although there was a significant difference between recipients of normal cells and the composite control group ($p<0.05$), there was no difference in experiments (5) undertaken specifically to compare antibody levels in recipients of medium and normal cells.

Duration of Transferrable Suppression by Spleen Cells

In separate experiments, we found this suppression to be transferrable from animals as early as day 14 during the disease, and as late as day 35 (Table 5). However, not all intervening time points yielded SC capable of transferring suppression.

Timing of Recipient

To determine whether suppression could be transferred to animals already committed to anti-GBM antibody production, convalescent spleen cells from day 21 rats were transferred to BN rats at day 7 in the $HgCl_2$ treatment regimen. Analysis of peak anti-GBM antibody showed that convalescent cells were capable of transferring suppression to the day 7 recipients (Table 6).

Specificity of Suppression

To determine the specificity of suppression, rats were given an i.v. injection of BSA (with or without $10^8$ day 21 SC) at the same time as their first $HgCl_2$ injection. No suppression of the anti-BSA antibody response was observed (Table 7), but rather an increased response.

Depletion Experiments

To determine which population of SC was responsible for the transfer of suppression, the mAb MRC OX8 was used to deplete convalescent SC suspensions of T suppressor cells prior to administration to naive recipients. The peak anti-GBM antibody levels, as a composite of three experiments are shown in Fig. 36. The group receiving MRC OX8 depleted cells had significantly higher levels of anti-GBM antibody than the group receiving undepleted or control depleted convalescent cells, but still significantly lower

Table 5. Suppression Produced by Spleen Cells Transferred from Animals at Different Stages of Convalescence (a)

| Donors (Day) | No of Recipients | Peak Anti-GBM Ab. (% mean control binding) | Significance (cf medium recipients) |
|---|---|---|---|
| 8 | 4 | 87.3 ± 33.8(b) | N.S. |
| 14 | 10 | 71.5 ± 15.2 | p <.001 |
| 19 | 5 | 83.0 ± 13.7 | N.S. |
| 21 | 23 | 70.6 ± 12.1 | p <.001 |
| 23 | 7 | 71.5 ± 23.6 | p <.05 |
| 28 | 10 | 69.8 ± 26.5 | p <.005 |
| 35 | 5 | 79.6 ± 11.7 | p <.05 |

(a) In other experiments with male donors and recipients, suppression could not be transferred from 3 month convalescent rats (Pusey, unpublished observations).

(b) Results show mean ± SD peak anti-GBM antibody, in each group.

N.S. = not significant

Note that suppression was transferrable at numerous time points during convalescence.

Table 6. Suppression Produced by Convalescent Cells Transferred into Day 7 Recipients

| Recipients | No. | Peak anti-GBM Ab (% mean control binding) |
|---|---|---|
| Medium | 10 | 100.0 ± 14.0 |
| Convalescent Day 21 cells | 10 | 73.7 ± 16.1[a] |

(a) Data represent the mean ± SD of peak anti-GBM antibody levels in 2 experiments, using SC from day 21 convalescent rats. The antibody in the group receiving convalescent cells was significantly lower than in the group receiving medium ($p < .01$).

Table 7. Specificity of Suppression in Convalescent Spleen Cell Recipients

| Recipients | No. | Peak Anti-GBM Ab.[a] (% positive binding) | Peak Anti-BSA Ab[a] (cpm) |
|---|---|---|---|
| Medium | 5 | 86.8 ± 19.3 | 7640 ± 2831 |
| Convalescent cells | 4 | 64.0 ± 12.5[b] | 25425 ± 7654[c] |

(a) Day 12

(b) $p < .05$ compared with medium recipients

(c) $p < .001$ compared with medium recipients

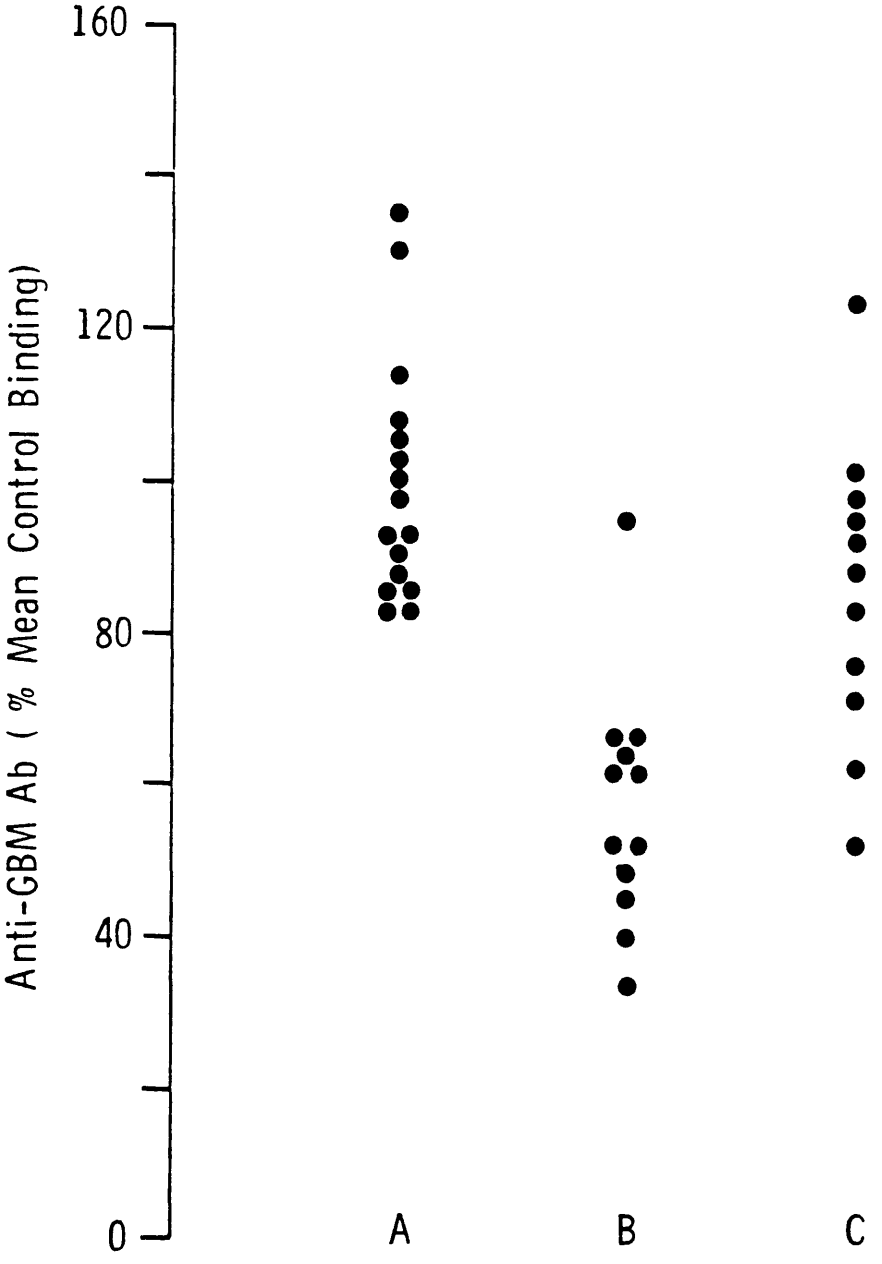



Figure 36. Effect of depletions of MRC OX8$^+$ lymphocytes from convalescent SC on suppression of $HgCl_2$ induced anti-GBM antibody response. Composite data from 3 SC transfer experiments. Rats were injected with 6 x $10^7$ to 2 x $10^8$ convalescent (day 21-28) SC which had been left undepleted or subjected to a control depletion with NCS-SRBC or MRC OX21 (B, n=12), or depleted with MRC OX8 (C, n=11). Controls in each experiment received medium (A, n=16). All animals were then challenged with $HgCl_2$. Peak antibody levels are shown, expressed as % mean control group binding for each experiment. The group receiving MRC OX8 depleted cells had antibody levels significantly higher than the undepleted/control depletion group ($p<0.002$), but also significantly lower than the group receiving medium ($p<0.05$).

than the control group receiving medium.

When MRC OX12, which binds to Ig kappa chains on rat B cells (Hunt and Fowler, 1981) was used in similar depletion experiments, there was no abrogation of suppression (Table 8). Depletion with MRC W3/25, which binds to T helper cells and macrophages (Brideau et al, 1980; Barclay, 1981), was also performed. It is of interest that antibody levels in recipients of MRC W3/25 depleted cells polarised into 2 groups- with or without suppression (Fig. 37). Because of this polarisation the more appropriate statistical analysis of Wilcoxin ranking was used, and showed that the group receiving MRC W3/25 depleted cells had anti-GBM antibody levels not significantly different to the undepleted/control depleted group.

### 3.2.3. Studies with 'Anti-idiotypic' Antibody

#### Characterisation of Anti-GBM Idiotype Containing Eluate

The binding of radiolabelled kidney eluate to rat GBM coated microtitre plates was low, often less than 1% of the added cpm. However, this binding was specific, and gave a linear inhibition curve with rat GBM (Fig. 38). In specificity studies, greater than 95% of the cpm binding could be inhibited with rat GBM, but only 15% with Hb. Separate studies, using purified, lyophilised, solid GBM to absorb the eluate showed that 52% of the radiolabelled eluate was immunoreactive. Only 15% of the control $^{131}I$ rat IgG bound to the GBM. A fall in immunoreactivity when antigen is coated to a solid surface has been noted by others (Randolph, Kibler and Fritz, 1977).

#### Characterisation of Heterologous 'Anti-idiotypic' Antisera

Using the PEG precipitation technique, 27% of $^{125}I$eluate (6% of $^{131}I$ rat IgG) was precipitated by absorbed rabbit anti-day 14 eluate, 6% of eluate (4% of rat IgG) by rabbit anti-normal eluate,

Table 8. MRC OX12 Depletion of Convalescent Spleen Cells[a]

| Recipients | No | Peak Anti-GBM Ab. (% mean control binding) |
|---|---|---|
| Medium | 10 | 100.0 ± 18.6 |
| Convalescent Control (MRC OX 21) Depleted cells | 8 | 74.5 ± 19.0 |
| Convalescent MRC OX 12 Depleted Cells | 8 | 80.9 ± 19.7 |

(a) Data represent the mean ± SD of peak anti-GBM antibody levels in 2 experiments. The antibody in the group receiving control depleted (MRC OX 21) convalescent cells was significantly lower than in the group receiving medium ($p < .02$), but not significantly different than in the group receiving B lymphocyte (MRC OX 12) depleted cells.

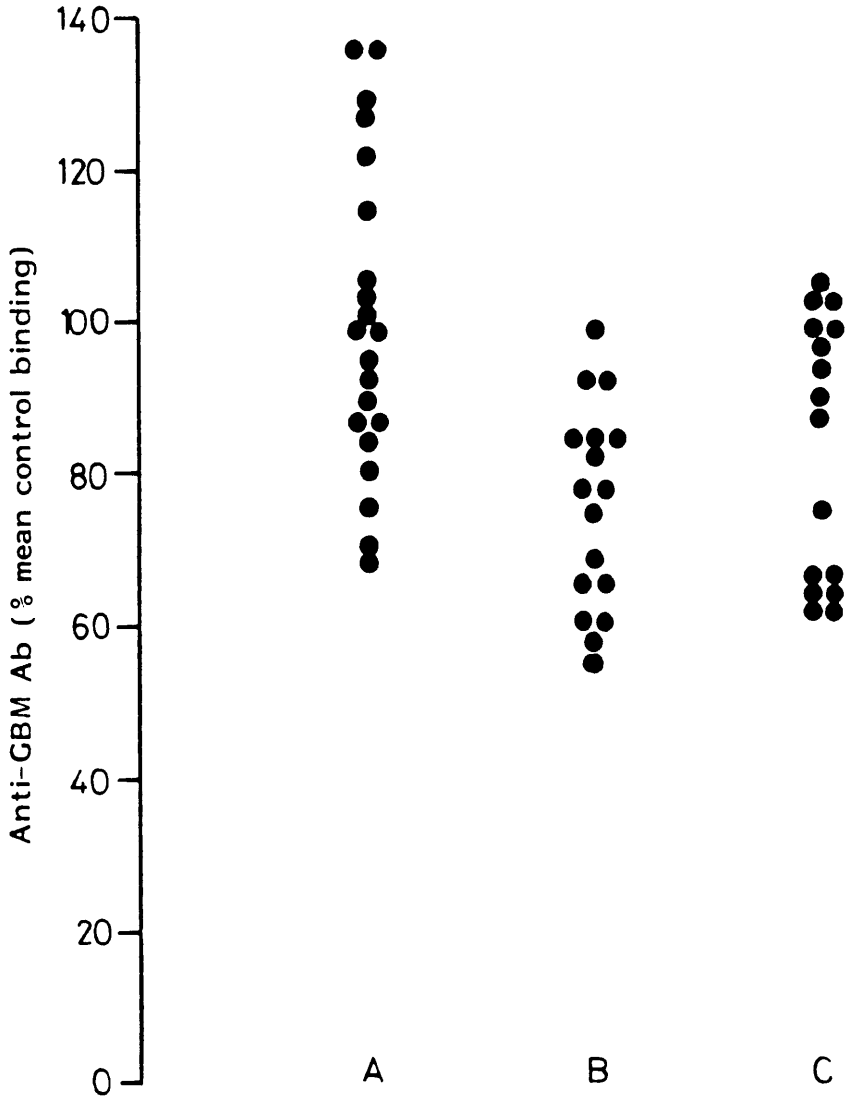



Figure 37. Effect of depletions of MRC W3/25$^+$ lymphocytes from convalescent SC on suppression of $HgCl_2$ induced anti-GBM antibody response. Composite data from 4 SC transfer experiments. Rats were injected with 6 x $10^7$ to 2 x $10^8$ convalescent (day 21-28) SC which had been left undepleted or subjected to a control depletion with NCS-SRBC or MRC OX21 (B, n=12), or depleted with MRC W3/25 (C, n=16). Controls in each experiment received medium (A, n=21). All animals were then challenged with $HgCl_2$. Peak antibody levels are shown, expressed as % mean control group binding for each experiment. Using the Wilcoxin rank sum method, the group receiving MRC W3/25 depleted cells had antibody levels not significantly different to the undepleted/control depleted group, although antibody of the latter was significantly suppressed compared to recipients of medium ($p<0.02$).

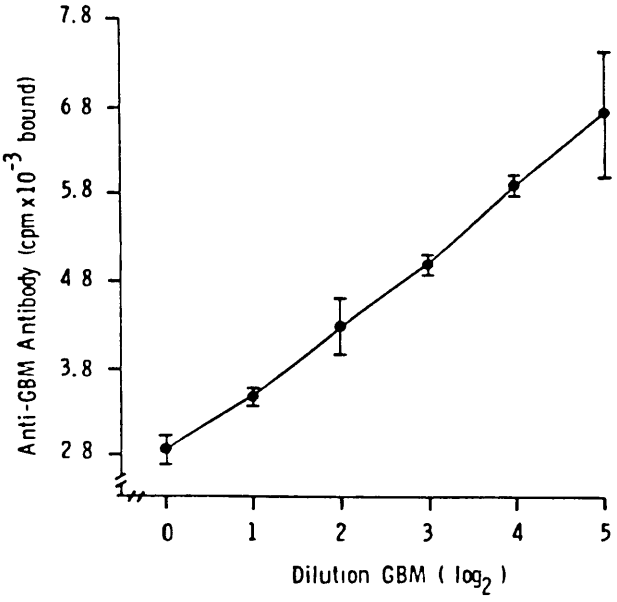



Figure 38. Inhibition of $^{125}I$ eluate binding to solid phase rat GBM by added GBM. $^{125}I$ eluate from day 14 kidneys was pre-incubated with rat GBM (initially 2mg/ml) in doubling dilutions before incubating in GBM coated wells. Results show the mean ± SD for cpm binding of triplicate wells.

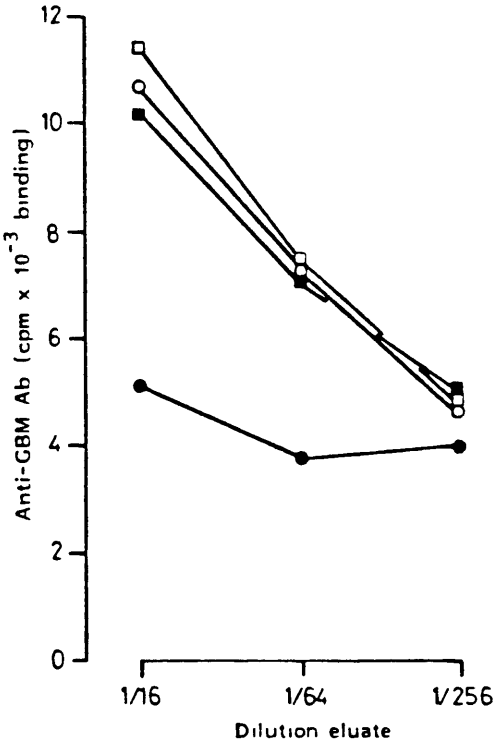



Figure 39. In vitro inhibition of eluate binding to rat GBM, by heterologous 'anti-idiotypic' antiserum. Dilutions of unlabelled day 14 kidney eluate (300µl) in 1/40 NRS/CFDT20 were pre-incubated with 30µl of rabbit antisera to day 14 eluate (●), or normal eluate (□), or 1/50 rabbit anti-rat IgG (Δ), or CFDT20 (o), before assaying for remaining anti-GBM antibody as previously described. Results show the mean cpm binding of duplicate estimations, when activity was detected with $^{125}I$ anti-rat IgG. Note that only anti-day 14 eluate caused inhibition.

and 4% of eluate (6% of rat IgG) by normal rabbit serum. When NRS was deleted from the reaction mixture, anti-day 14 eluate precipitated 35% of $^{125}I$ eluate and 11% $^{131}I$ rat IgG. The anti-day 14 eluate showed no precipitation with NRS by Ouchterlony analysis.

When more sensitive solid phase radioimmunoassays were employed, the anti-eluate was discovered to have activity remaining against rat IgG and rat GBM, despite extensive absorptions (Table 9). However, residual anti-GBM activity for the antisera to day 14 eluate was less than that for anti-normal eluate, so the anti-normal eluate was used as a control in inhibition experiments. Residual anti-rat IgG activity in the antisera to day 14 eluate was found to be equivalent to 1/50 of a commercial rabbit anti-rat IgG preparation, which was also then used as a control.

In Vitro Inhibition of Anti-GBM Binding, with Heterologous 'Anti-idiotypic' Antisera

Inhibition of the dilution curve of unlabelled eluate binding to solid phase rat GBM, by antisera to day 14 or normal kidney eluate, normal rabbit serum or 1/50 rabbit anti-rat IgG was examined (Fig. 39). Inhibition occurred only with the anti-day 14 eluate, not with anti-normal eluate or anti-rat IgG. In addition the anti-day 14 eluate was capable of inhibiting 35% of the cpm of $^{125}I$ eluate binding to GBM in the RIA compared to the binding in the presence of anti-normal eluate.

Rabbit antisera to day 14 eluate was also capable of inhibiting the binding of anti-GBM antibodies in day 14 serum, when compared to binding in the presence of anti-normal eluate (Fig. 40).

Table 9. Antibody Specificities in Heterologous Anti-eluate Antibody

| Antisera | Dilution | RGBM | Rat IgG | Day 14 Eluate |
|---|---|---|---|---|
| CFD | - | 172 | 336 | 169 |
| Anti-day 14 | 1/10 | 18580 | 11649 | 32704 |
| Eluate | 1/50 | 20072 | 9862 | 32512 |
| | 1/250 | 9179 | 3925 | 28325 |
| Anti-normal | 1/10 | 25670 | 3105 | 28018 |
| Eluate | 1/50 | 24197 | 1723 | 22490 |
| | 1/250 | 12624 | 1129 | 12867 |
| Normal Rabbit | 1/10 | 736 | 2030 | 684 |
| Serum | 1/50 | 630 | 1371 | 760 |
| | 1/250 | 434 | 659 | 681 |

Dilutions of absorbed rabbit antisera raised against day 14 and normal kidney eluates, or absorbed normal rabbit serum were assayed by solid phase RIA on plates coated with rat GBM, rat IgG and day 14 eluate. Results show the mean cpm binding of duplicate estimations when detected with $^{125}I$ anti-rabbit Ig. Note that antisera to day 14 eluate also reacted with rat GBM but to a lesser extent than did anti-normal eluate, which could be used as a control.

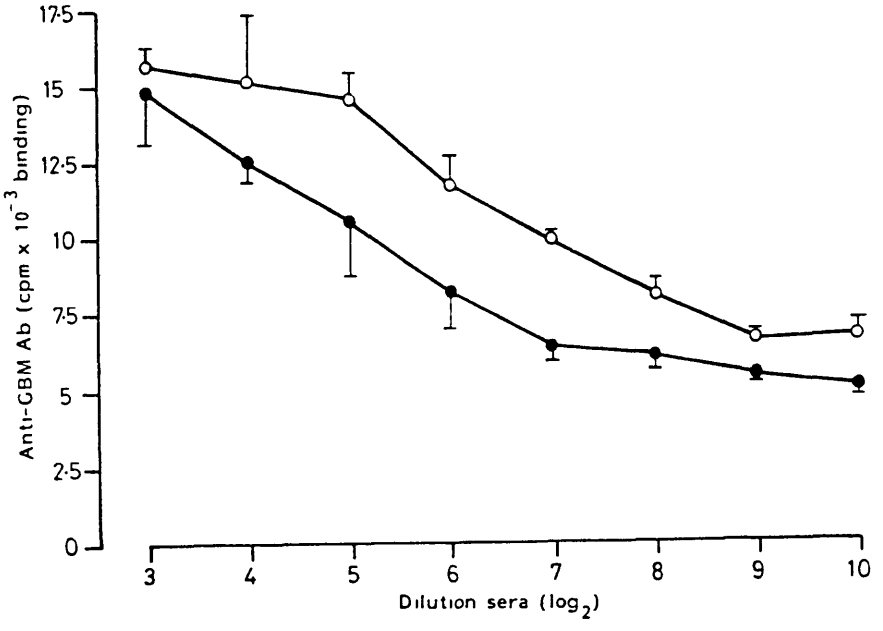



Figure 40. In vitro inhibition of the binding of anti-GBM antibodies in day 14 serum to rat GBM, by heterologous 'anti-idiotypic' antiserum. Dilutions of pooled sera from day 14 $HgCl_2$ treated BN rats (300µl) were preincubated with 20µl rabbit antisera to normal eluate (o——o) or day 14 eluate (●——●) before assaying in triplicate in rat GBM coated plates, for remaining anti-GBM activity. Results show the mean ± SD of the anti-GBM antibody, expressed as cpm $^{125}I$ anti-rat IgG binding.

Inhibition of $^{125}I$ Eluate Binding, with Sequential Sera from $HgCl_2$ Treated Rats

To determine whether factors, putatively 'anti-idiotypic' antibodies, were present in sera during the phase of anti-GBM antibody regulation, pilot experiments were set up to examine the inhibition of $^{125}I$ eluate binding, by sequential serum samples. Figure 41 shows two of such experiments where inhibition of binding is compared to anti-GBM antibody levels. In addition to time points when competitive inhibition might be expected due to the presence of anti-GBM antibodies in serum, there are other peaks of inhibition which coincide with falling antibody levels, as well as 'troughs' of inhibition coinciding with a rise in antibody levels.

In Vivo Studies with Heterologous 'Anti-idiotypic' Antisera

In a pilot experiment, examining the *in vivo* effect of rabbit anti-day 14 eluate on induction of anti-GBM antibody by $HgCl_2$, it was found that the 2 recipients of anti-eluate made less antibody than the 2 recipients of normal rabbit serum, which synthesised less antibody than a control rat after $HgCl_2$ treatment (Fig. 42). Anti-BSA antibody did not appear to be suppressed in these recipients, over the time course studied. In another experiment, 2/5 recipients of anti-day 14 eluate appeared to be partially suppressed compared to recipients of normal rabbit serum, or controls (Fig. 43). However, in scaled up experiments, variation of anti-GBM antibody levels of recipients of any rabbit sera, was too great to determine its effect.

In Vivo Studies with Convalescent Plasma

In a pilot experiment to determine the effect of transferred convalescent plasma on *in vivo* anti-GBM antibody synthesis, the two

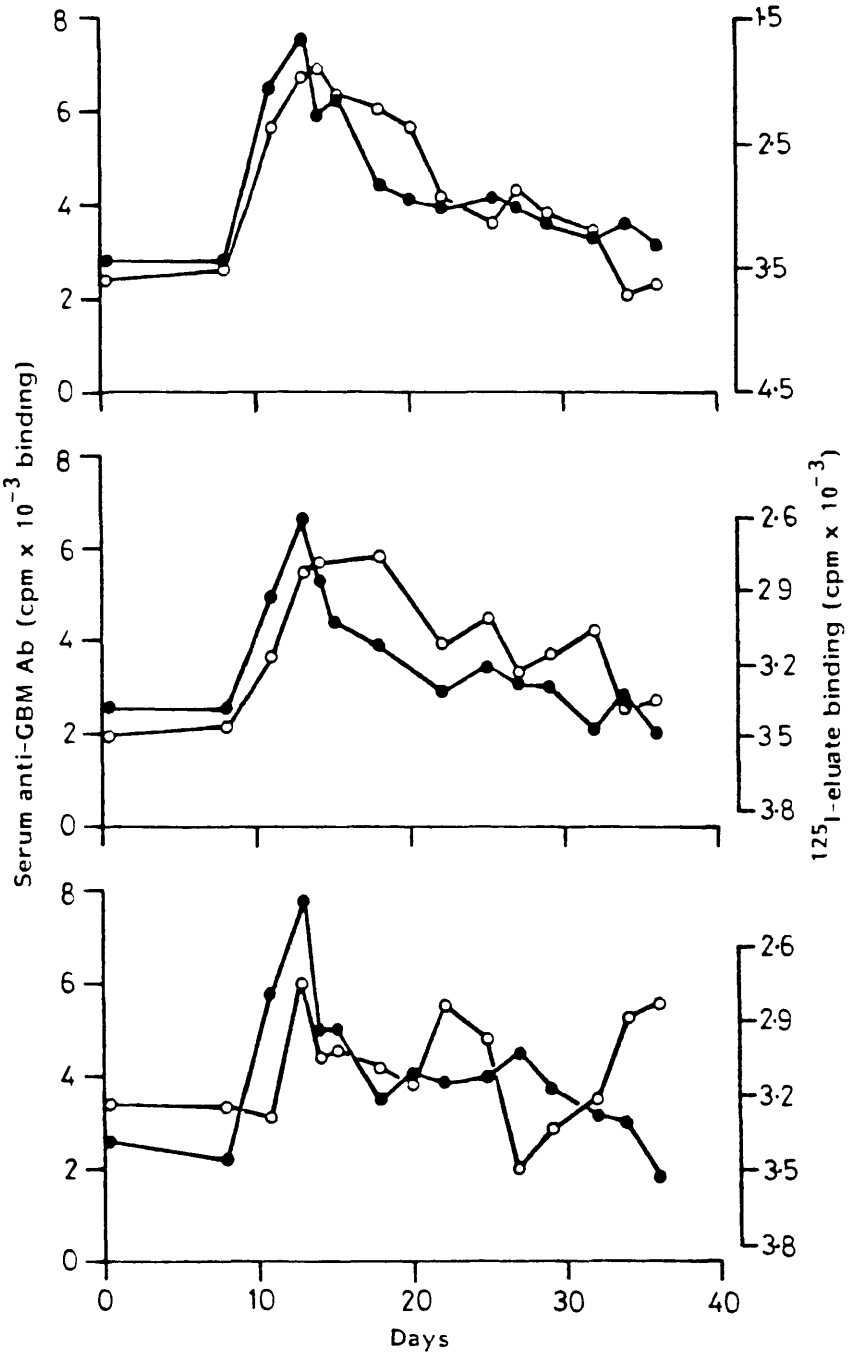



Figure 41. Inhibition of $^{125}I$ eluate binding, with sequential serum samples from $HgCl_2$ treated rats. $^{125}I$ eluate (4 x $10^5$ cpm) was incubated with 100µl of sera before assaying in GBM coated wells. Results show the residual binding of $^{125}I$ eluate (o——o) (note reversed scale to indicate inhibition), after incubation with sequential serum samples from 3 $HgCl_2$ treated rats. Anti-GBM antibody levels of the serum samples, expressed as cpm binding, are also shown (●——●). Inhibition occurred both in the presence of unlabelled antibody and also during down regulation.

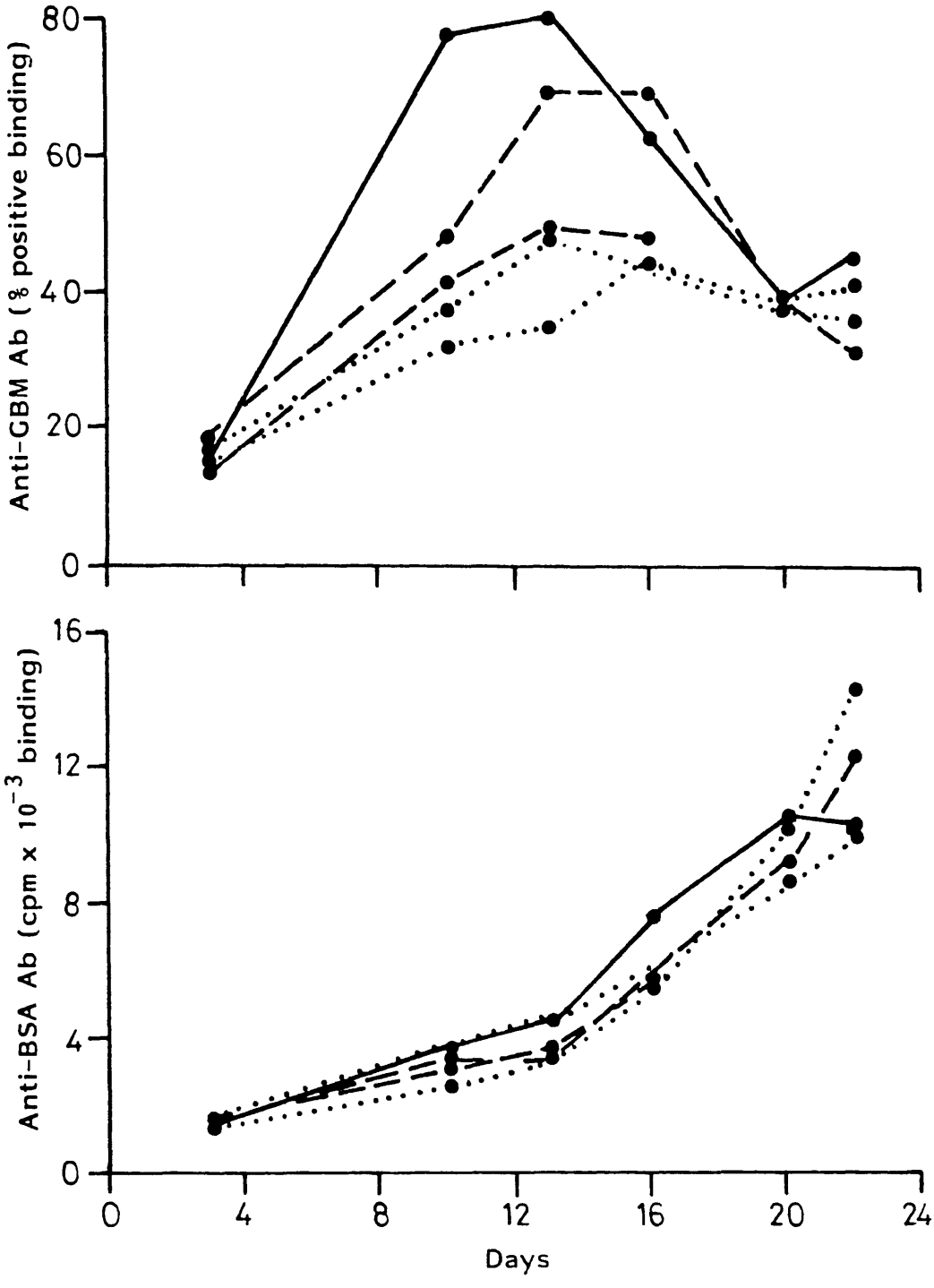



Figure 42. In vivo effect of heterologous 'anti-idiotypic' antisera on anti-GBM and anti-BSA antibody levels. BN rats were injected i.p. with 1.5ml normal rabbit serum (●– – –●), or anti-day 14 eluate (●·····●), or left as controls (●——●), and then challenged with $HgCl_2$. On day 4 all animals were injected with 500µg BSA in IFA. Results show sequential levels of anti-GBM antibody (upper graph), expressed as % positive binding, and anti-BSA antibody (lower graph), expressed as cpm binding for individual rats. Suppression of anti-GBM but not anti-BSA antibody synthesis occurred in recipients of anti-day 14 eluate.

Figure 43.

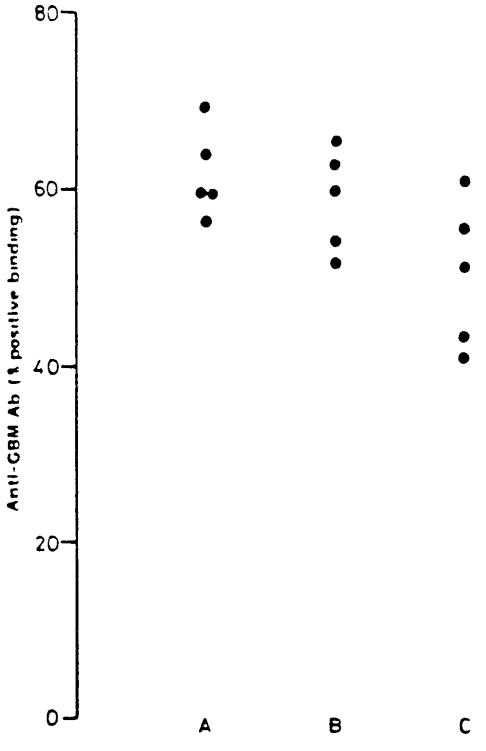



Figure 44.

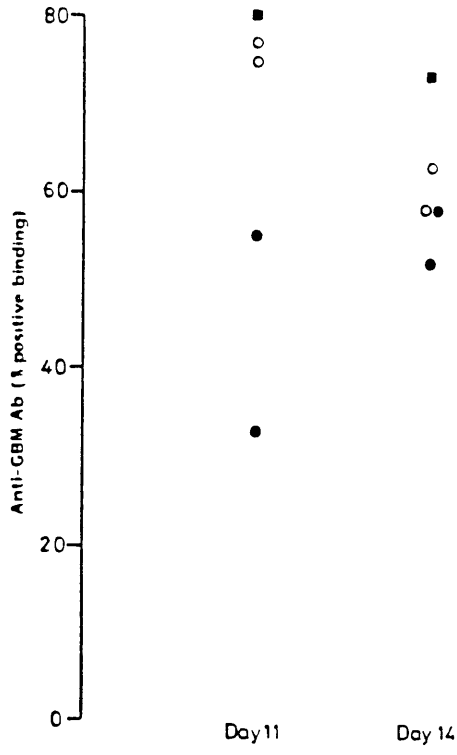



Figure 43. In vivo effect of heterologous 'anti-idiotypic' antisera on peak levels of anti-GBM antibody. Groups of BN rats (n=5) were injected i.p. with 1.5ml normal rabbit serum (B), or anti-day 14 eluate (C), or left as controls (A), and then challenged with $HgCl_2$. Results show the peak levels of anti-GBM antibody for individual rats, expressed as % positive binding. 2/5 recipients of anti-day 14 eluate appeared to be suppressed compared to controls.

Figure 44. In vivo effect of transfer of convalescent plasma on induction of anti-GBM antibody by $HgCl_2$. BN rats were injected i.p. with 1ml plasma from day 20-25 convalescent BN rats (●), or normal BN rats (o) or left as controls (■), and then all were challenged with $HgCl_2$. Results show the anti-GBM antibody levels on days 11 and 14, expressed as % positive binding for individual rats. On day 11 the 2 recipients of convalescent plasma appeared to be suppressed compared to controls.

recipients of convalescent plasma, when compared to recipients of normal BN plasma, had markedly lower levels of anti-GBM antibody on day 11, but only slightly lower levels on day 14 (Fig. 44). The presence of anti-BSA antibodies in some rats on day 0, prevented the use of anti-BSA antibody analysis as a specificity control.

## 3.3. IN VITRO CELL CULTURE STUDIES

In order to circumvent some of the problems encountered in studying regulation of the antibody response *in vivo*, especially with regard to scale and the multitude of 'factors influencing' in the whole animal, an *in vitro* anti-GBM antibody synthesising cell culture system was developed, to screen for potential regulators prior to their ultimate reintroduction *in vivo*.

### 3.3.1. IgG Class Anti-GBM Antibody Specificity, Time Course , Cell Dose and Culture Requirements

Seven day bulk culture supernatants derived from cultured SC from day 10 $HgCl_2$ treated BN rats were tested for IgG class anti-GBM activity and an inhibition curve constructed to test for specificity in the assay. Binding to rat GBM was found, which was specific resulting in a linear inhibition curve (Fig. 45). Lysed, uncultured day 10 SC released only minimal activity.

The time course of anti-GBM antibody production from SC from day 10 $HgCl_2$ treated BN rats is shown in Fig. 46. Antibody was already detectable after 8 hours and only minimal antibody production occurred after 3 days. Most cultures were carried out for 5-7 days.

When SC from day 10 animals were cultured at $2.5 \times 10^6$ or $10^7$ cells/ml for 5 days, anti-GBM antibody was not detected in the

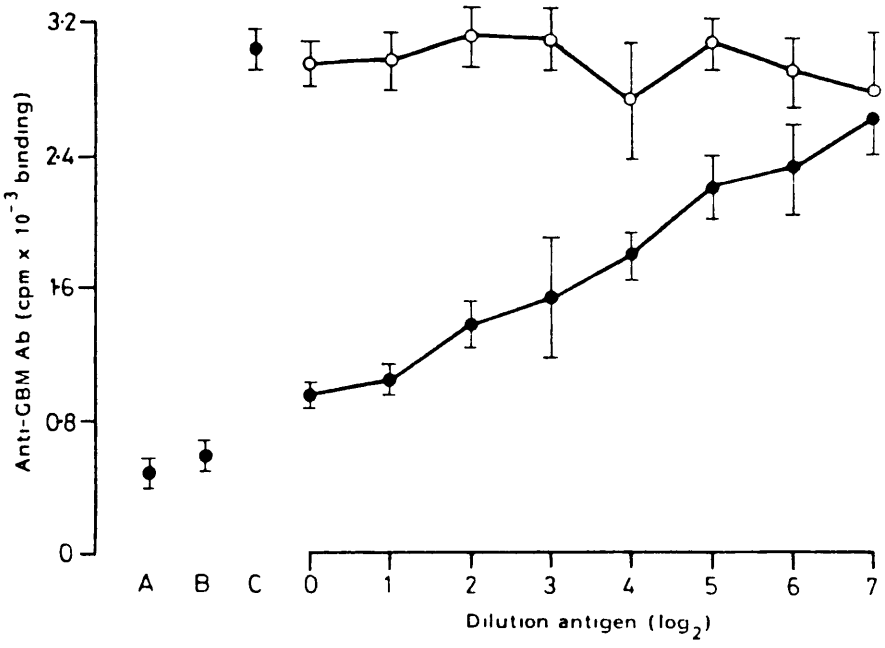



Figure 45. In vitro inhibition of the binding of anti-GBM antibody synthesised in culture, to rat GBM, by added GBM or haemoglobin. Seven day supernatants from SC from day 10 $HgCl_2$ treated BN rats, were preincubated with CFDT20 (C), or rat GBM (●——●) or haemoglobin (o——o), initially 2mg/ml, in doubling dilutions, before assaying for IgG class anti-GBM antibody in the RIA. Controls consisted of medium (A) and supernatants from lysed, uncultured day 10 SC (B). Results, expressed as cpm binding, show the mean ± SD of triplicate anti-GBM assay wells. Antibody was inhibited specifically by rat GBM.

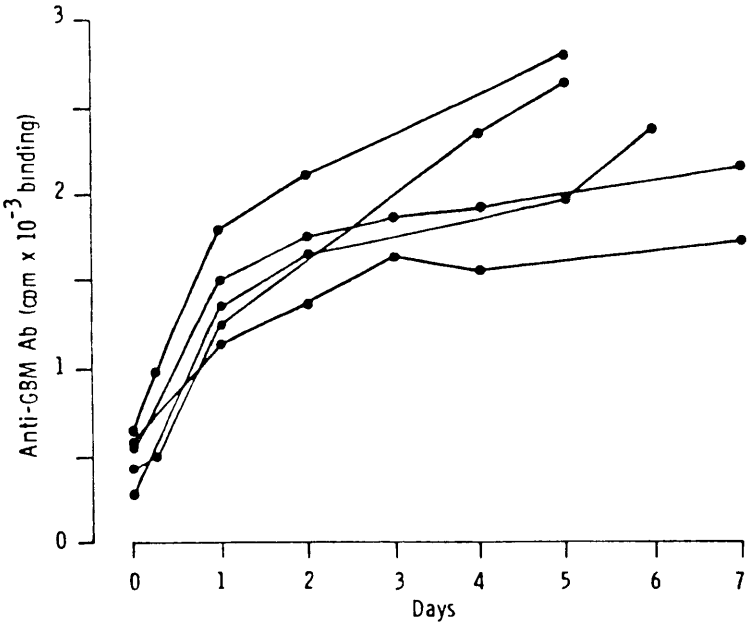



Figure 46. Time course of in vitro IgG class anti-GBM antibody synthesis by SC from day 10 $HgCl_2$ treated BN rats. Spleen cells (2 x $10^6$) from $HgCl_2$ treated BN rats (n=5) were cultured in 96 well plates for 5 or 7 days. Six wells from each rat culture were harvested after various time periods. Results show the mean anti-GBM antibody, expressed as cpm binding, for each rat.

supernatants of low cell doses, but readily detectable from $10^7$ cells/ml. Higher cell doses were not used, to prevent overcrowding (Fig. 47).

Spleen cells from rats treated with $HgCl_2$ for varying periods were cultured under standard conditions. Normal BN SC made only minimal antibody, as did those from day 6 $HgCl_2$ treated animals. Spleen cells from both day 8 and day 10 rats synthesised anti-GBM antibody in culture, with day 10 SC donors being the most effective (Fig. 48). In separate experiments SC from day 7,11,16 and 21 $HgCl_2$ treated BN rats were compared to SC from day 10 animals, in terms of *in vitro* anti-GBM antibody production. Minimal antibody was produced from day 7 derived SC while day 11 and 16 derived SC produced similar amounts of antibody to those from day 10 rats, and by day 21 the ability to synthesise antibody in culture had begun to wane. SC from day 9 or 10 $HgCl_2$ treated BN rats were routinely used in subsequent cultures, as these days probably preceded autoregulatory phenomena.

To test for the requirement for macrophages in *in vitro* anti-GBM antibody synthesis, SC from day 10 $HgCl_2$ treated rats were passed through a Sephadex G-10 column. Removal of G-10 adherent cells caused a marked reduction in subsequent antibody synthesis, although some antibody was still detectable, presumably due to incomplete depletion (Fig. 49).

Daily feeding of SC cultures with fresh medium had no effect on anti-GBM antibody production.

### Effect of 'Washing Cells' on In Vitro Anti-GBM Antibody Synthesis

To determine whether the cell washing procedure, which might be required during cultures for assaying soluble regulatory factors,

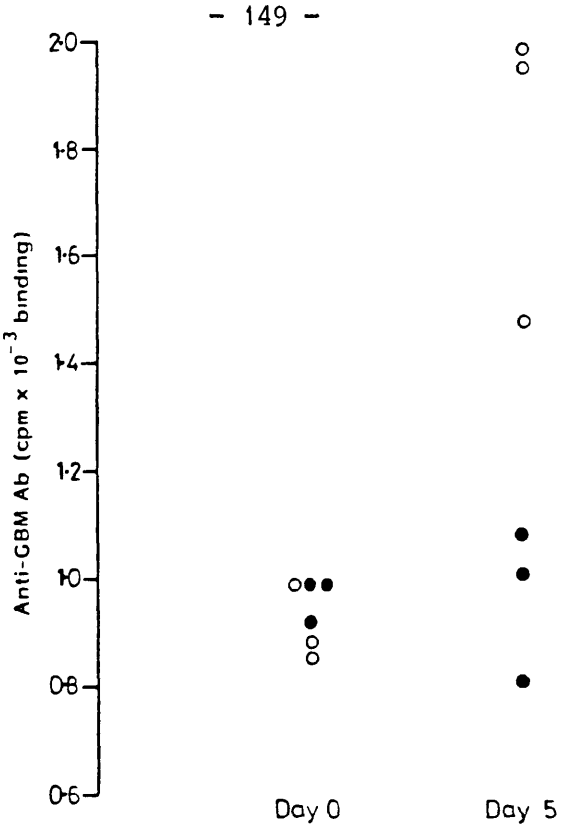



Figure 47. Cell numbers and in vitro anti-GBM antibody synthesis. SC from day 10 $HgCl_2$ treated BN rats were cultured at 2 x $10^6$ cells/ml (●) or $10^7$ cells/ml (o), in 96 well plates for 5 days. Results show IgG class anti-GBM antibody in individual wells (n=3), expressed as cpm binding. $10^7$ cells/ml was the optimal concentration.

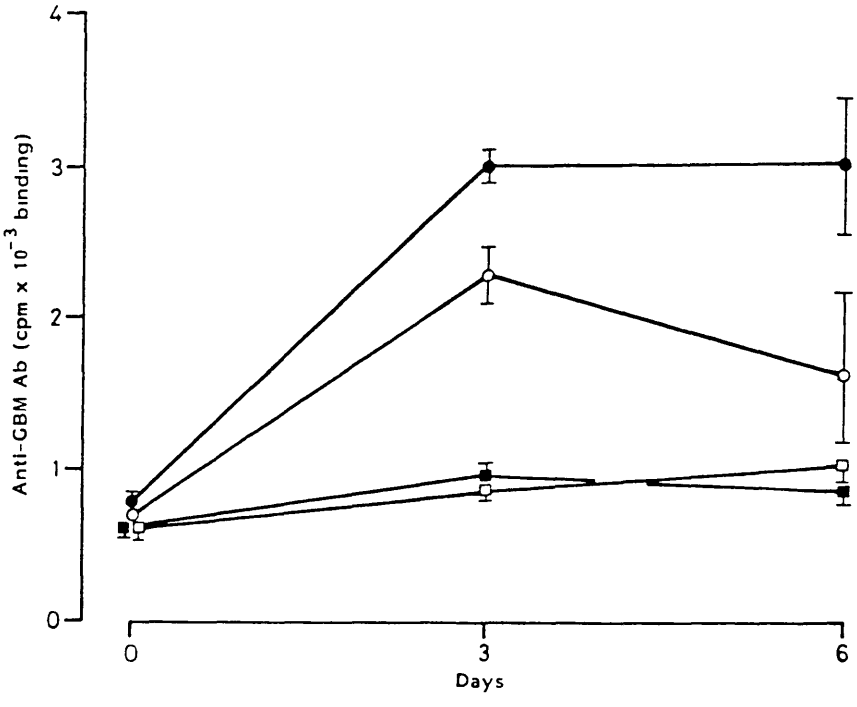



Figure 48. In vitro anti-GBM antibody synthesis by SC from BN rats between 0 and 10 days after commencement of $HgCl_2$ injections. SC from normal (■) and day 6 (□), 8 (o) or 10 (●) $HgCl_2$ treated BN rats, were cultured at $10^7$/ml for 5 days. Results show the mean ± SD of IgG anti-GBM antibody in 6 replicate wells, expressed as cpm binding. Antibody was synthesised most adequately by SC from day 8-10 rats.

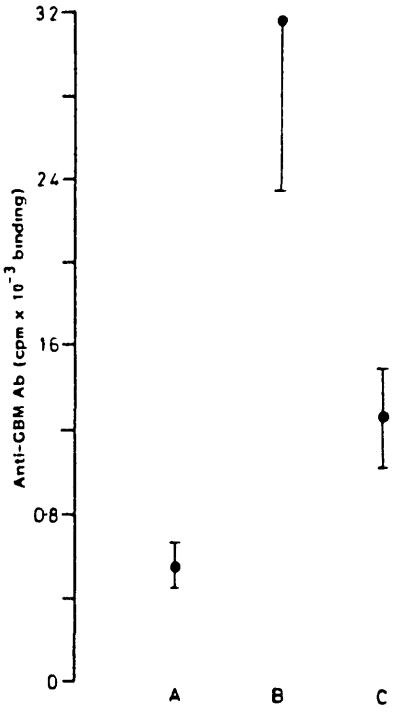



Figure 49. Effect of removal of Sephadex G-10 adherent cells on in vitro anti-GBM antibody synthesis. SC from a day 10 $HgCl_2$ treated BN rat, unfractionated (B), or depleted of Sephadex G-10 adherent cells (C) were cultured at $10^7$ cells/ml for 5 days. Results show the ± SD of anti-GBM antibody in 6 replicate wells, expressed as cpm binding. Background binding of medium is also shown (A). Sephadex G-10 adherent cells were necessary for in vitro antibody synthesis.

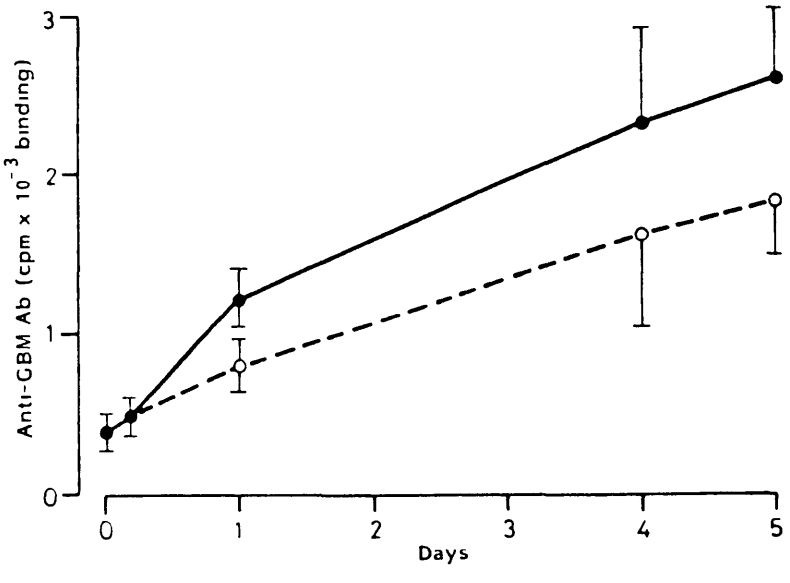



Figure 50. Effect of 'washing cells' on in vitro anti-GBM antibody synthesis. SC from a day 10 $HgCl_2$ treated BN rat were cultured at $10^7$ cells/ml. After 6 hr, certain wells (o- - o) were subjected to centrifugation and washing twice before the culture was continued for a further 4 days. Controls (●——●) were centrifuged and the cells sucked up and down with a pipette but the medium was not replaced. Supernatants from 6 replicate wells were harvested at days 1, 4 or 5. Results show the mean ± SD of IgG anti-GBM antibody, expressed as cpm binding. Note that cells subjected to 'washing' synthesised less antibody than controls.

would affect the ability of SC to synthesise antibody in vitro, day 10 derived SC were cultured for 6 hr, washed twice with medium, and cultured for a further 4 days. SC subjected to this treatment synthesised far less anti-GBM antibody than SC subjected to a similar procedure, but without replacing the media (Fig. 50). The extent to which this reduction in antibody was greater than the amount actually synthesised during the first 6 hours, varied from culture to culture. In all cases, anti-GBM antibody was still readily detectable. However, when cells were washed after 24 hr in culture, they subsequently synthesised only minimal amounts of anti-GBM antibody.

Regulation by Heterologous 'Anti-idiotypic' Antisera

Heterologous anti-day 14 eluate was found to inhibit preformed anti-GBM antibody in supernatants of day 10 derived SC, from binding to GBM coated microtitre wells, with the degree of inhibition dependent on the amount of anti-GBM antibody present (Fig. 51,52), while anti-normal eluate only caused inhibition at high concentrations, in low level antibody supernatants. However, when heterologous anti-day 14 eluate or normal eluate were added at day 0 of culture, to test for suppression of in vitro anti-GBM antibody synthesis, results proved to be highly variable. While in some experiments heterologous anti-eluate suppressed antibody production when compared to anti-normal eluate, when used at dilutions which had no effect on preformed antibody (Fig. 52: e.g. 1/16, 1/64 of high antibody responder; 1/64 of moderate antibody responder), in other experiments any apparent suppression was explicable by the effect of the antisera on preformed antibody. In case the presence of complement caused lysis of cells with antibody bound, decomplementation of the antisera by heat treatment was

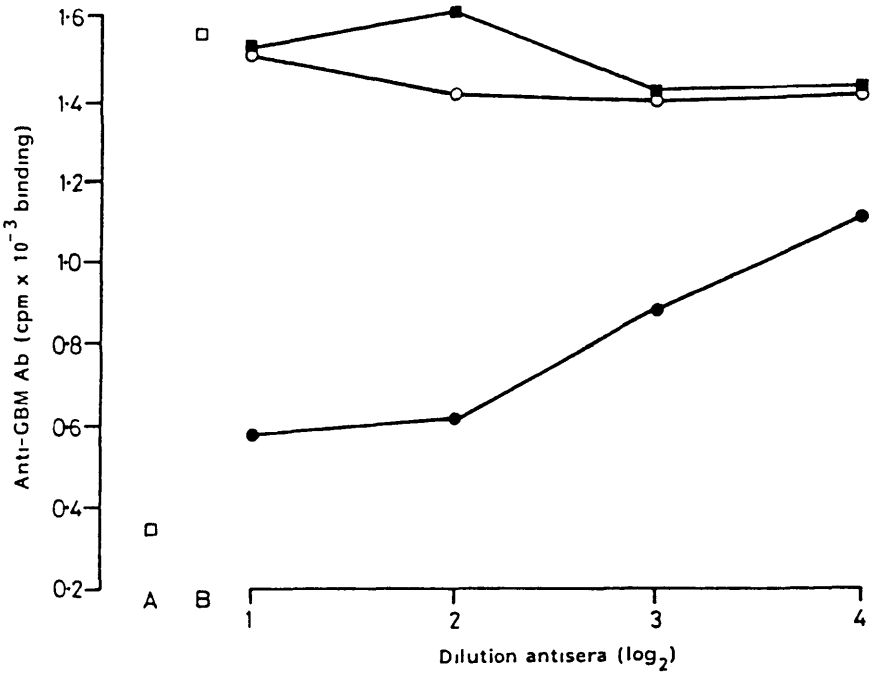



Figure 51. In vitro inhibition of binding of preformed anti-GBM antibody from supernatants, to rat GBM, by heterologous 'anti-idiotypic' antiserum. Seven day supernatants from SC from day 10 $HgCl_2$ treated BN rats (300µl of 2/3 diln.) were preincubated with 30µl of dilutions of normal rabbit serum (■——■), rabbit anti-normal eluate (o——o) or rabbit anti-day 14 eluate (●——●) before assaying for remaining IgG class anti-GBM antibody. Controls consisted of medium (A), or supernatant incubated with PBST20 instead of rabbit serum (B). Results, expressed as cpm binding are for the mean of triplicate RIA wells. Anti-day 14 eluate, but not normal rabbit serum or anti-normal eluate, could inhibit binding of preformed anti-GBM antibody in supernatants, to rat GBM.

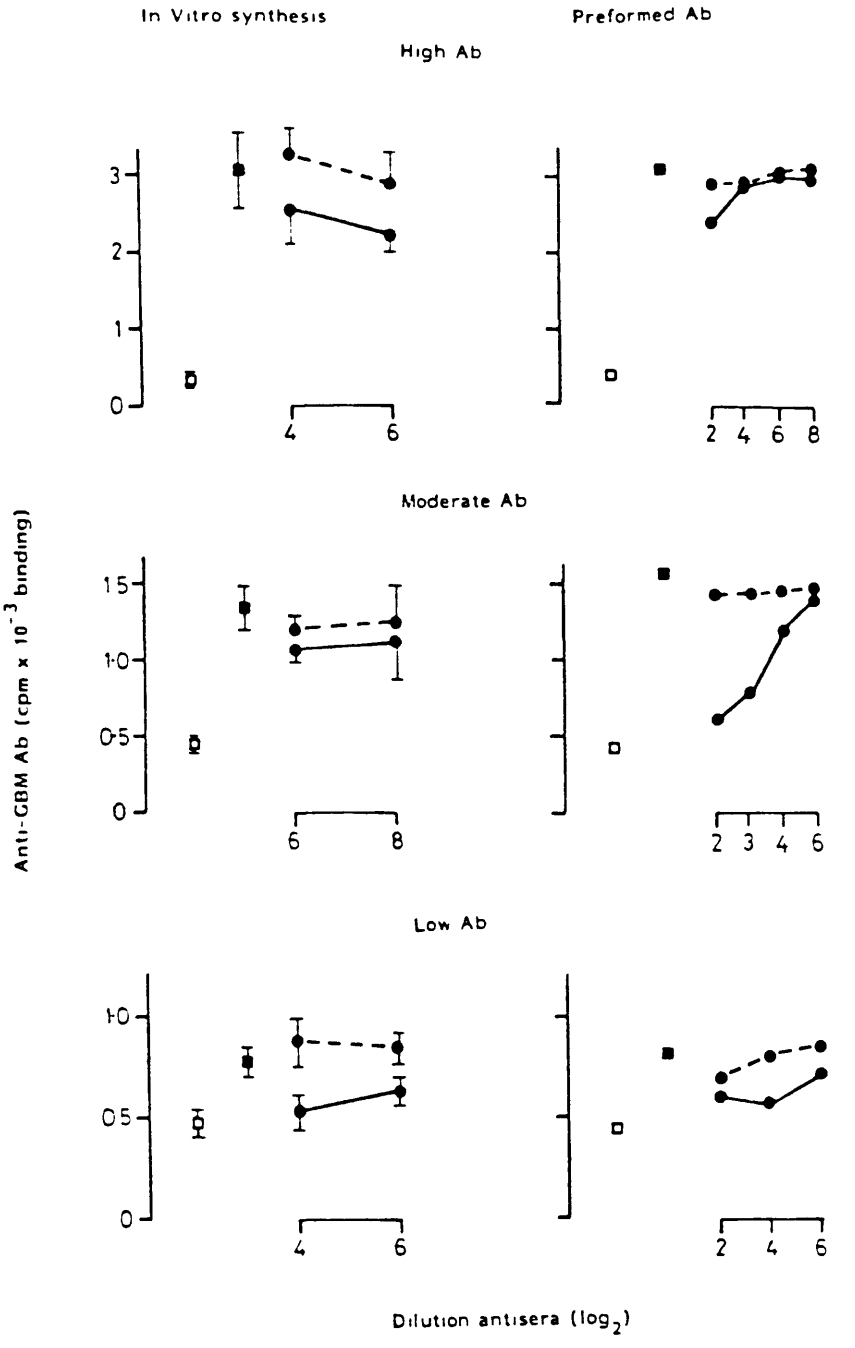



Figure 52. Effect of heterologous 'anti-idiotypic' antiserum on binding of preformed anti-GBM antibody to GBM and on *in vitro* synthesis of anti-GBM antibody. SC from day 10 $HgCl_2$ treated BN rats (2 x $10^6$ cells/200µl) were cultured with 20µl of dilutions of rabbit anti-normal eluate (●- - -●) or rabbit anti-day 14 eluate (●——●) for 5 days. Results, expressed as cpm binding in the RIA, on the left, show the mean ± SD of 6 replicate wells, for 3 experiments in which high, moderate or low levels of anti-GBM antibody were produced in control wells. Graphs on the right represent inhibition of preformed anti-GBM antibody in pooled 7 day cultures from SC from day 10 rats, where supernatants with high, moderate or low antibody activity were chosen. Protocol was as for Fig. 51. Controls for both preformed antibody, and *in vitro* synthesis were medium (□), or supernatant with PBS (■).

performed, but this had no effect on the presence, absence of magnitude of the suppression (Fig. 53).

To minimise the effect of the test antisera on anti-GBM antibody after synthesis (equivalent to preformed antibody), as compared to suppression of antibody synthesis, experiments were attempted to wash out the antisera after 6 or 24 hr, and then continue culturing. Removal of antisera after 24 hr caused too great a reduction in subsequent antibody synthesis, even in controls. In cultures with antisera removed after 6 hr, results were as variable as in cultures not subjected to this manipulation.

Regulation by Anti-rat IgG

As expected, rabbit anti-rat IgG had a dose dependent inhibitory effect on the binding of preformed IgG class anti-GBM antibody from supernatants, to rat GBM (Fig. 54). However, as with the heterologous anti-eluate, use of the rabbit anti-rat IgG to suppress *in vitro* anti-GBM antibody synthesis proved highly variable, and often dilutions of anti-rat IgG which would inhibit preformed antibody, had no effect on *in vitro* anti-GBM antibody synthesis (Fig. 54).

Regulation by Convalescent Serum

Convalescent (day 21) serum and normal BN rat serum were absorbed of anti-rat GBM reactivity and tested for inhibition of antigen binding of anti-GBM antibody in supernatant, and suppression of *in vitro* anti-GBM antibody synthesis. At low dilutions, the presence of either convalescent or normal serum in the RIA, increased cpm binding, with convalescent serum having the greatest effect (Fig. 55), and this interfered with subsequent assays for anti-GBM antibody synthesised after 5 days in culture. However,

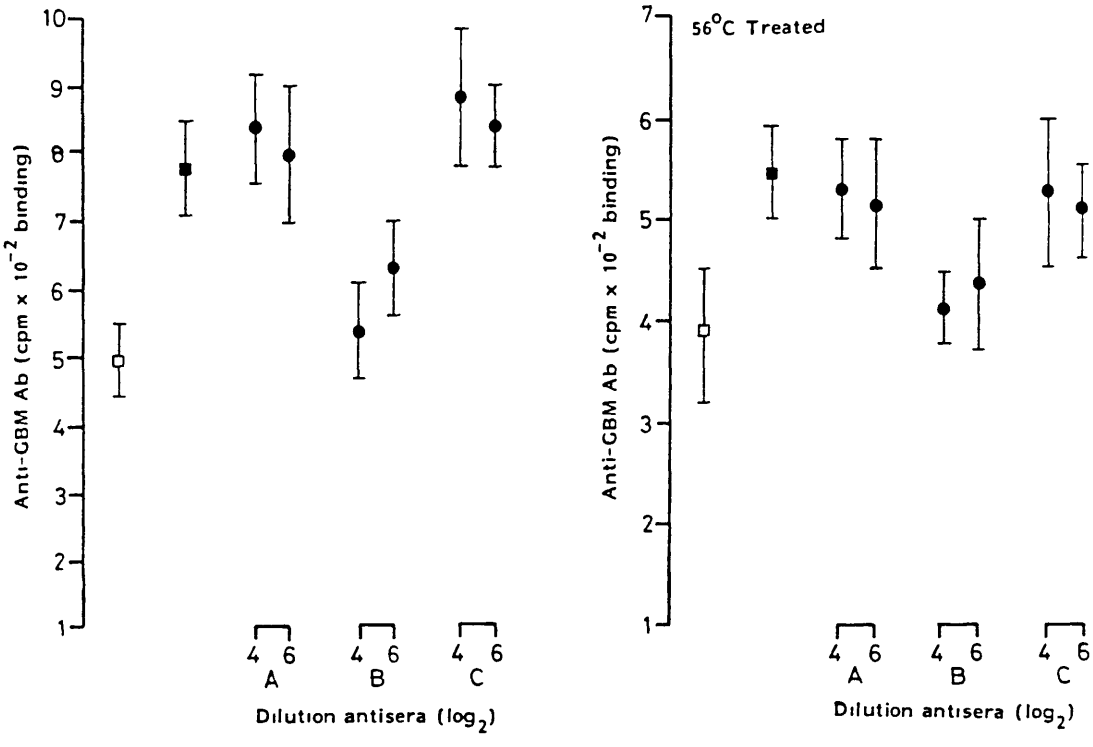



Figure 53. Effect of 56°C treatment on heterologous 'anti-idiotypic' suppression of *in vitro* anti-GBM synthesis. SC (2 x $10^6$ cells/200µl) from day 10 $HgCl_2$ treated rats were cultured for 5 days, with 20µl of dilutions of normal rabbit serum (A), rabbit anti-day 14 eluate (B) or rabbit anti-normal eluate (C). Results, expressed as cpm binding, show the mean ± SD for 6 replicate wells, incubated with untreated antisera (left) or 56°C treated antisera (right). Controls for each were medium (□) or control supernatant with PBS (■). Both 56°C treated and untreated anti-day 14 eluate caused suppression of *in vitro* anti-GBM antibody synthesis.

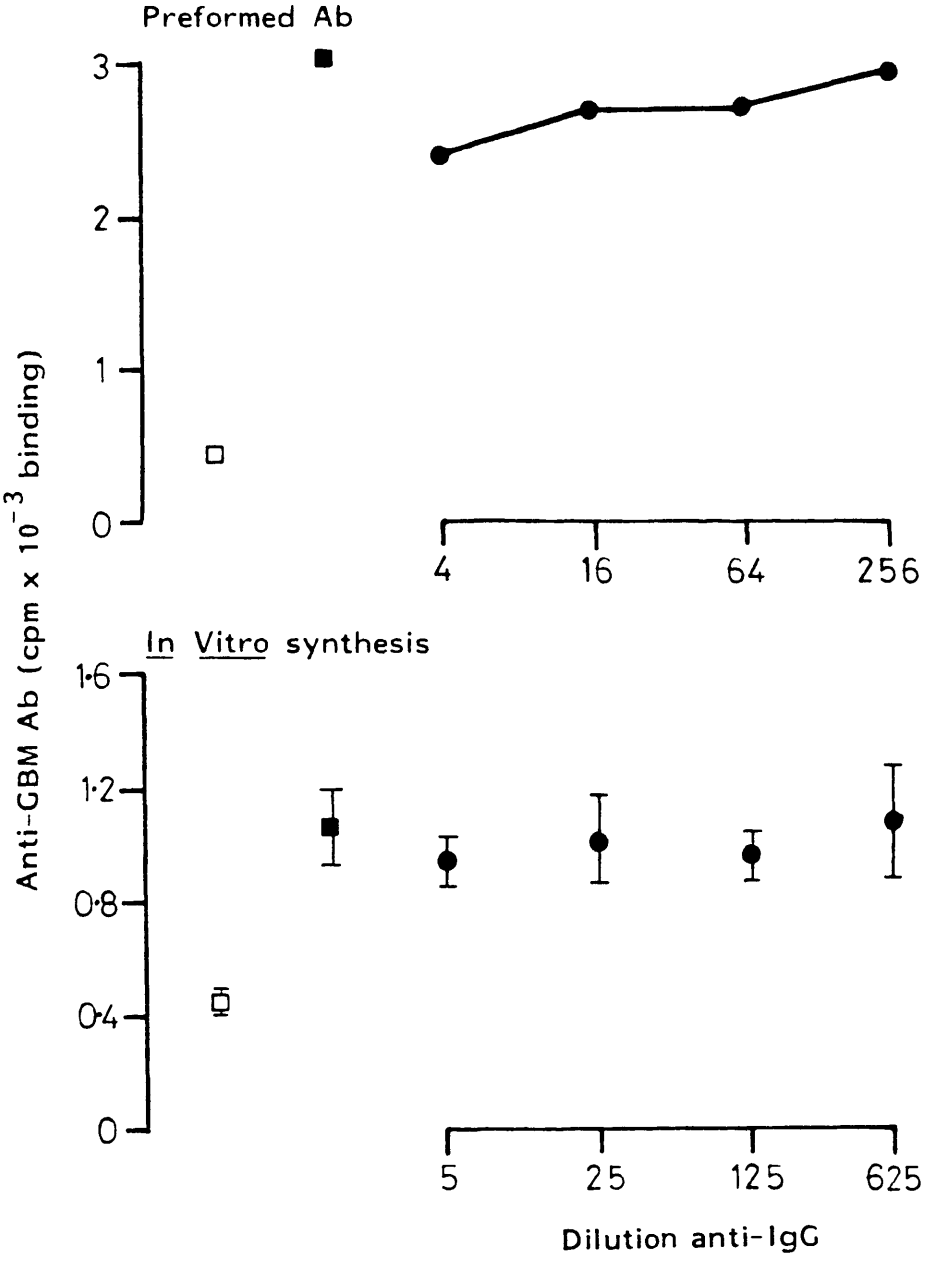



Figure 54. Effect of rabbit anti-rat IgG on binding of preformed anti-GBM antibody to GBM, and on in vitro synthesis of anti-GBM antibody. SC (2 x $10^6$ cells/200µl) from day 10 $HgCl_2$ treated BN rats were cultured for 5 days with 20µl of dilutions of rabbit anti-rat IgG (lower graph). Results, expressed as cpm binding in the RIA show the mean ± SD of 6 replicate wells. The upper graph shows the inhibition of the binding of preformed anti-GBM antibody in 7 day bulk cultures (no anti-Ig during culture), preincubated with dilutions of rabbit anti-rat IgG before assaying in the anti-GBM RIA. Results, expressed as cpm binding, show the mean of the triplicate assay wells. In each case controls were medium (□) or supernatant without rabbit anti-rat IgG (■). Anti-rat IgG had no effect on in vitro anti-GBM antibody synthesis.

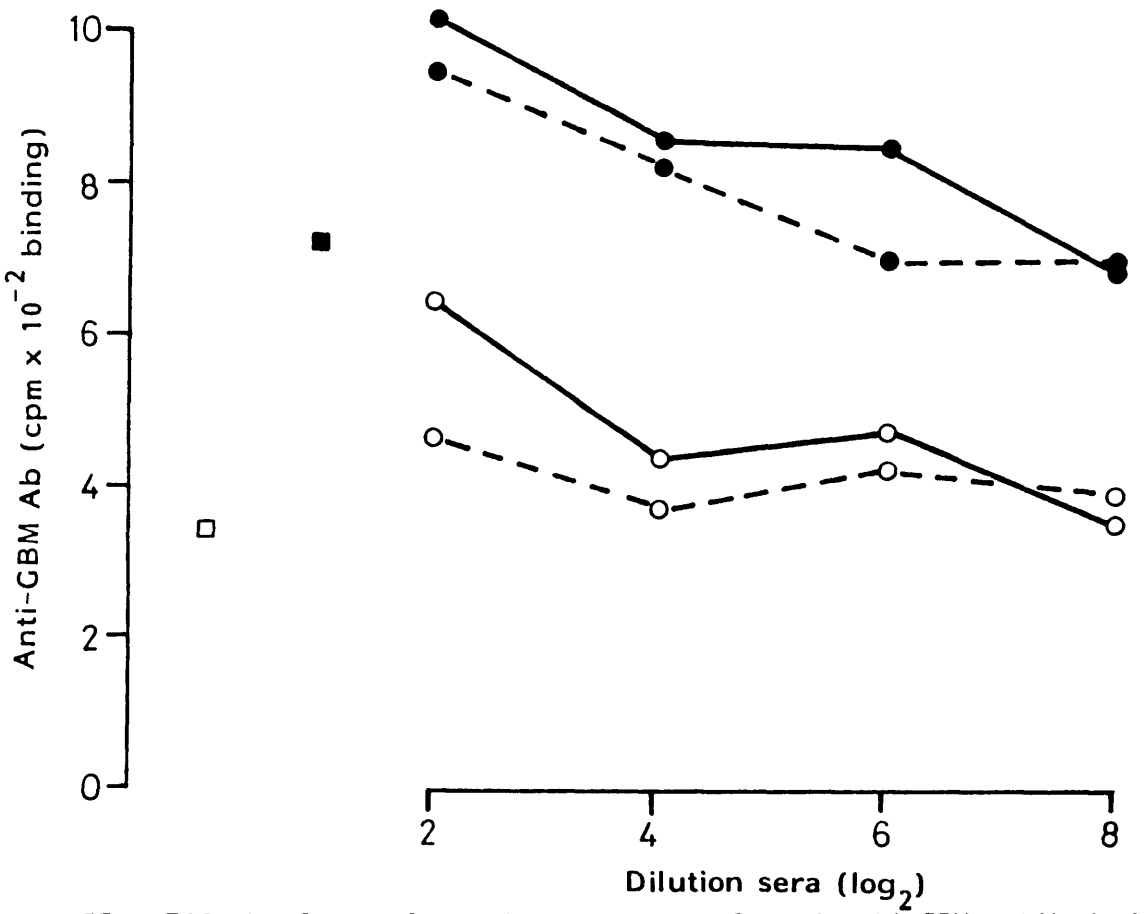



Figure 55. Effect of convalescent serum on preformed anti-GBM antibody binding in the RIA. 300µl of 2/3 day 5 bulk culture supernatant from SC from day 10 $HgCl_2$ treated rats (o) or medium (●) were preincubated with 30µl of dilutions of convalescent (——) or normal (– – –) BN sera then assayed in the anti-GBM RIA. Results, expressed as cpm binding, show the mean of triplicate assay wells. Controls were medium (□) or day 5 supernatant without added sera (■). Note that high concentrations of convalescent, or to a lesser extent normal serum, caused increased binding of both positive supernatant and medium in the RIA.

Fig. 56 shows the results of several experiments, in which the cpm binding from convalescent serum treated culture wells was always lower than that in NRS treated culture wells, indicating possible suppression of anti-GBM antibody synthesis. At high dilutions of serum, suppression in convalescent serum treated wells compared to untreated control wells as well as compared to normal serum treated wells could occasionally be seen.

When cells were washed after incubation with sera for 24 hr, and the cultures continued, antibody levels were very low even in untreated wells. However, anti-GBM antibody production in convalescent serum treated wells, did appear to be suppressed compared to that in both NRS treated and untreated (but washed) wells (Fig. 57). In cultures where cells were washed after 6 hr, results were similar to those where sera were present throughout the culture.

Regulation by Cells

When day 21 convalescent cells were cocultured with SC from day 9 $HgCl_2$ treated BN rats, partial suppression of anti-GBM antibody synthesis was observed with greater than 20% extra convalescent cells, in a dose dependent manner (Fig. 58). In one experiment where day 10 derived SC were cultured with normal BN SC or SC from $HgCl_2$ treated rats before the peak antibody response (day 8), or convalescent SC, suppression was only observed with the convalescent SC (Fig. 59). However, inhibition from normal cells was sometimes observed, especially if high concentrations of cells were added (Fig. 60). In addition, variability was noted in the capacity to suppress and be suppressed, both on the part of the convalescent donors, and the antibody producing recipients.

To try and increase the potency of the convalescent cell

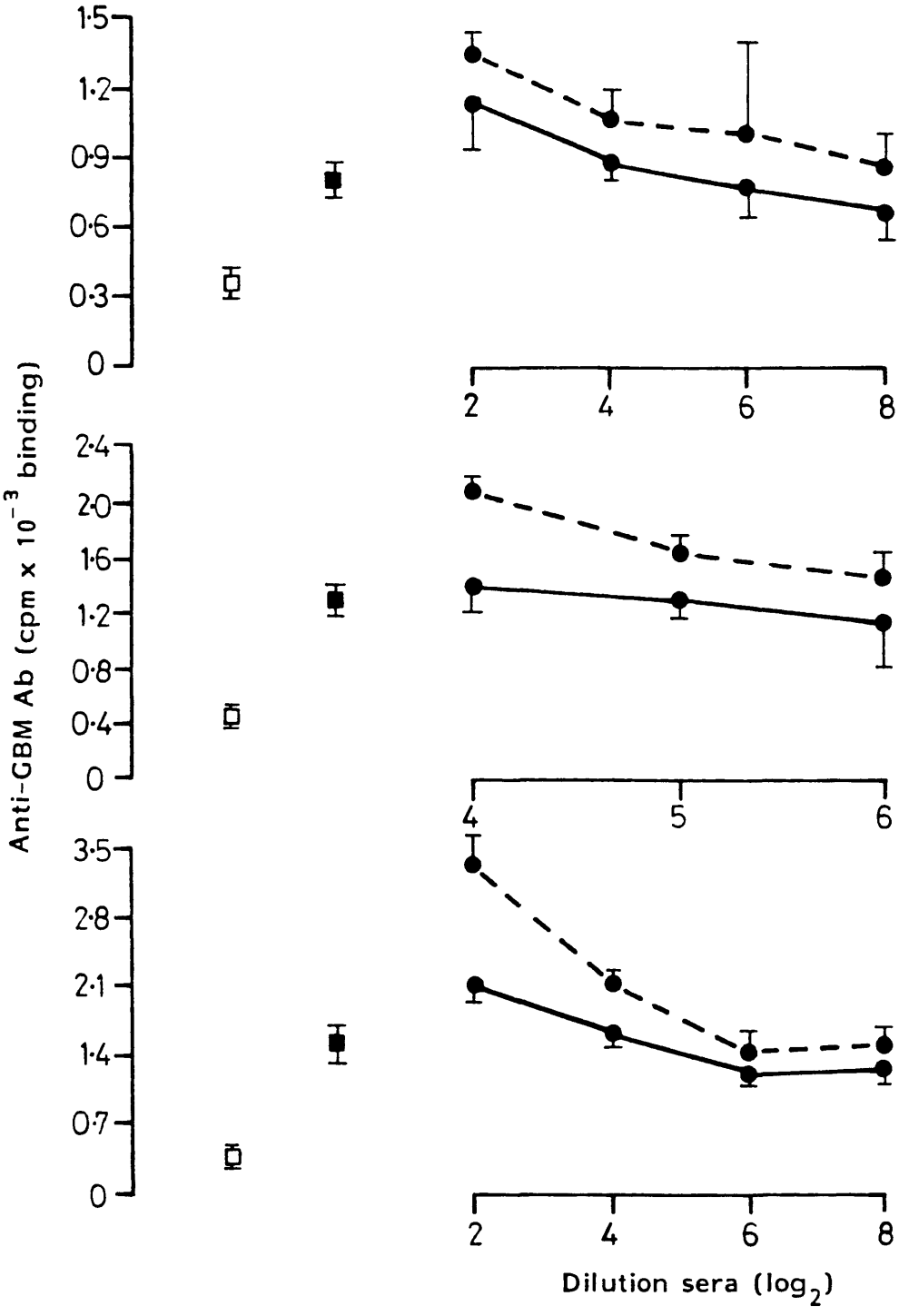



Figure 56. Effect of convalescent serum on in vitro anti-GBM antibody synthesis. SC (2 x $10^6$/200µl) from day 10 $HgCl_2$ treated rats, were cultured for 5 days with 20µl of dilutions of day 21 convalescent (●——●) or normal (●- - -●) BN sera. Results, expressed as cpm binding for the mean ± SD of 6 replicate wells, are from 3 separate experiments. Controls in each case were medium (□) or day 5 control supernatants without added sera (■). In each case SC cultured with convalescent serum synthesised less antibody than SC cultured with normal serum.

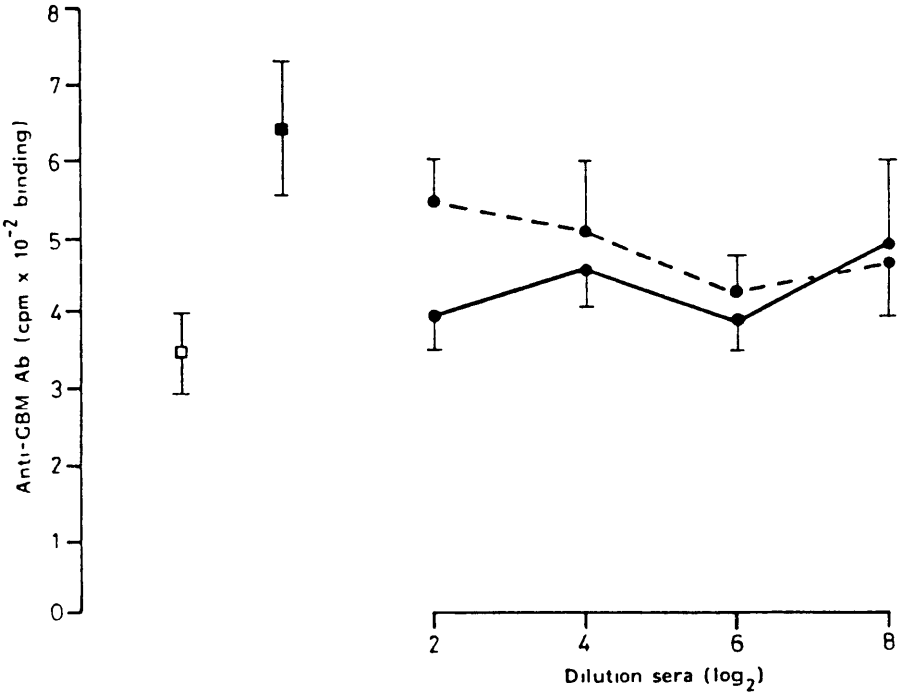



Figure 57. Effect of 24 hr incubation of SC with convalescent serum on subsequent in vitro anti-GBM antibody synthesis. SC (2 x $10^6$/200µl) from day 10 $HgCl_2$ treated BN rats were cultured with 20µl of dilutions of day 21 convalescent (●——●) or normal (●- - -●) BN sera, washed twice with medium and the culture continued for a further 5 days. Results, expressed as cpm binding, are of the mean ± SD of 6 replicate wells. Controls were medium (□) or supernatant without added sera ( ■ ). Pretreatment with convalescent serum suppressed subsequent anti-GBM antibody synthesis.

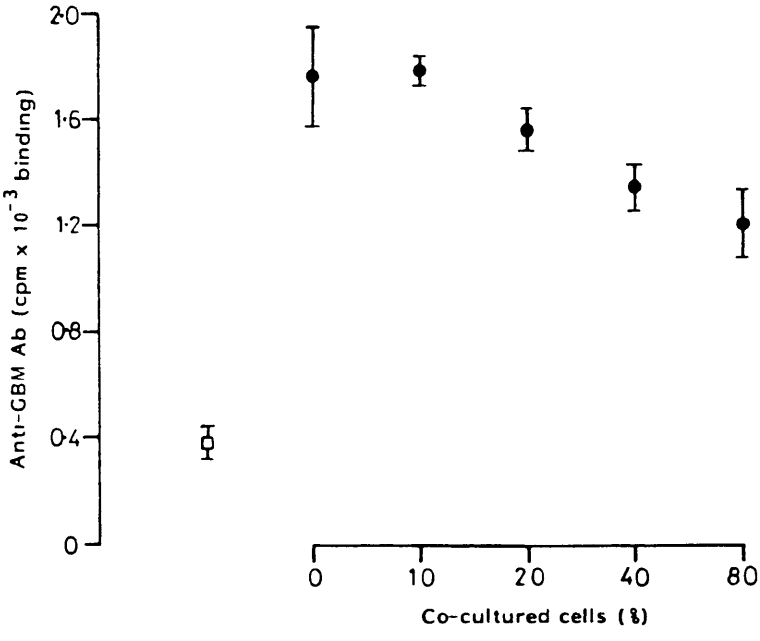



Figure 58. Effect of coculture with day 21 convalescent SC on in vitro anti-GBM antibody synthesis by day 10 SC. Spleen cells (2 x $10^6$/200µl) from day 10 $HgCl_2$ treated BN rats, were cultured for 5 days with 0 to 80% additional SC from day 21 convalescent BN rats. Results, expressed as cpm binding, are of the mean ± SD of 6 replicate wells. Background binding of medium is shown (□). Day 21 convalescent SC caused dose dependent suppression of in vitro anti-GBM antibody synthesis.

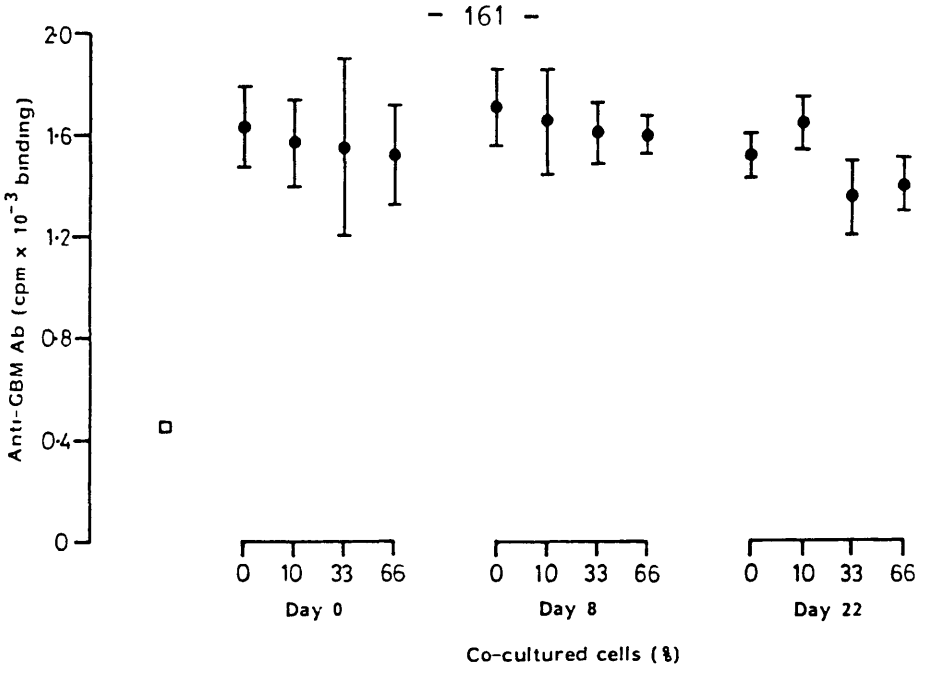



Figure 59. Effect of coculture with normal, day 8 or day 22 SC on in vitro anti-GBM antibody synthesis by day 10 SC. SC (2 x $10^6$/200µl) from day 10 $HgCl_2$ treated BN rats were cultured for 5 days with additional SC from normal or day 8 or 22 treated BN rats. Results, expressed as cpm binding are of the mean ± SD of 6 replicate wells. Background binding of medium is shown (□). Slight suppression of anti-GBM antibody synthesis was caused by day 22 but not day 8 or day 0 SC.

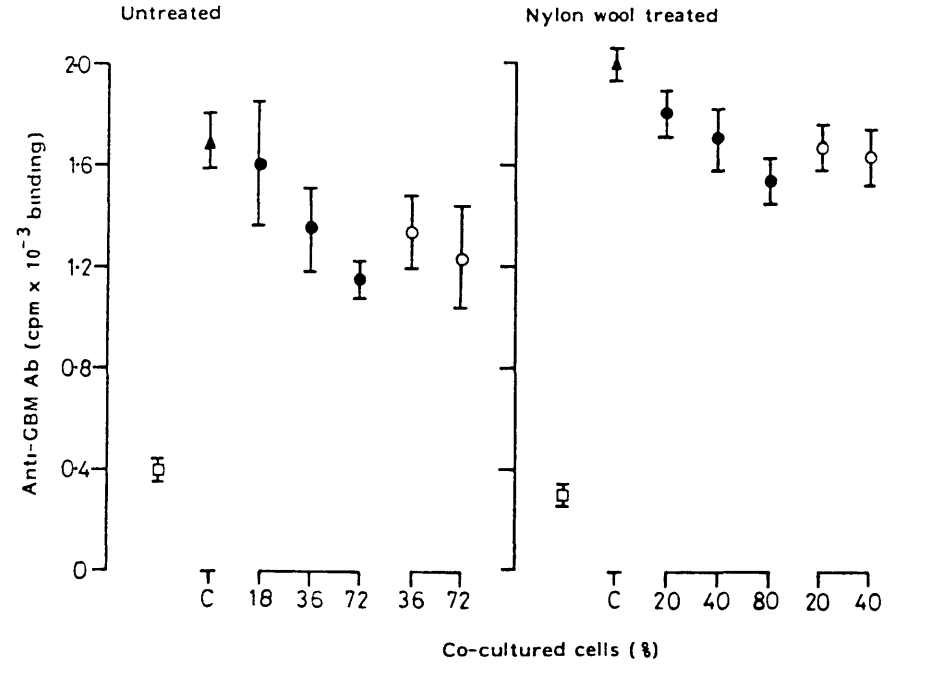



Figure 60. Effect of nylon wool treated or untreated normal or day 21 convalescent SC on in vitro anti-GBM antibody synthesis by day 10 SC. SC (2 x $10^6$/200µl) from day 10 $HgCl_2$ treated BN rats were cultured for 5 days with additional untreated or nylon wool passed SC from normal (o) or day 21 convalescent (●) rats. Results, expressed as cpm binding, are of the mean ± SD of 6 replicate wells. Binding with control supernatants (no added cells, ▲ ) or medium (□) is shown. Note that both normal and convalescent SC, whether untreated or nylon wool depleted, suppressed anti-GBM antibody synthesis.

suspension in suppression, and differentiate this from a non-specific suppression which may have been mediated by macrophages in normal BN SC, SC suspensions were passed through Sephadex G-10 columns prior to coculturing with day 10 derived SC. However, results were still variable. In one experiment such treatment removed the capacity to inhibit, while in another, it resulted in normal SC having greater suppression than convalescent cells.

Donor SC were also passed through nylon wool columns to enrich for T cells. Fig. 61 shows the resulting effect on anti-GBM antibody synthesis, when T enriched donor SC were cultured with recipient SC from 2 $HgCl_2$ treated rats. Inhibition was found with the convalescent SC, but was absent or barely present with normal SC. However, results were variable in other experiments, and T enriched normal SC also sometimes caused inhibition (Fig. 60).

Attempts to coculture with MRC W3/25 depleted (T suppressor enriched) convalescent spleen cell suspensions proved unsuccessful due to failure of the untreated or control depleted cells to cause inhibition.

### 3.3.2. IgM Class Activity and Normal Cells

When SC from normal BN rats were cultured for 8 days, they produced a low level IgM class anti-GBM activity. Similar low levels of activity were produced from day 10 $HgCl_2$ treated, or GBM or BSA injected BN rats. However, the activity was not found in culture supernatants from Wistar or Lewis rats (Fig. 62).

Results of inhibition studies using fluid phase GBM were highly variable and repeatable specific inhibition could not be seen, even at concentrations sufficient to inhibit IgM anti-GBM antibodies in day 14 serum, diluted to similar levels of activity. Inhibition tests using solid phase GBM (lyophilised or Sepharose

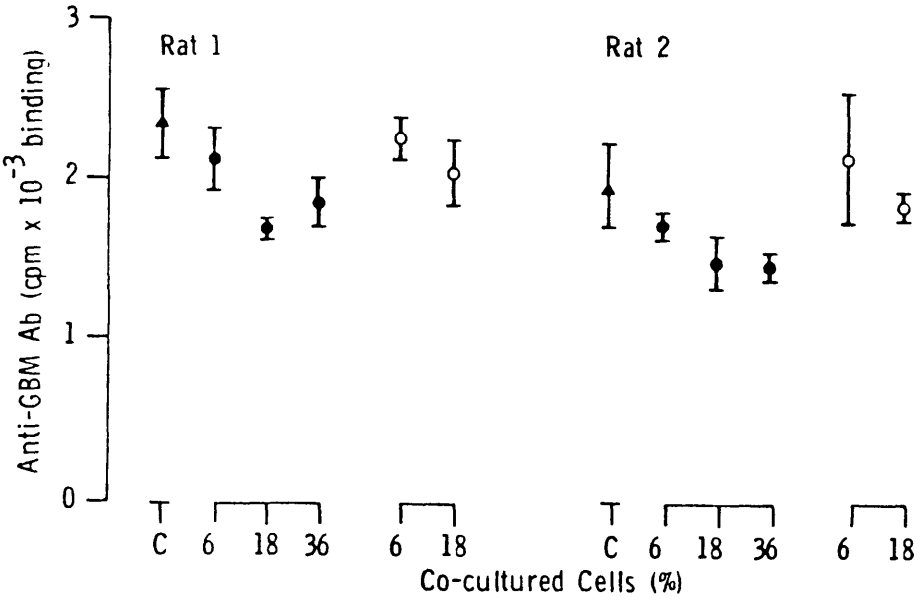



Figure 61. Effect of nylon wool treated normal or day 21 convalescent SC on in vitro anti-GBM antibody synthesis by day 10 SC. SC (2 x $10^6$/200µl) from 2 day 10 $HgCl_2$ treated rats were cultured for 5 days with additional nylon wool passed SC from normal (o) or day 21 convalescent (•) BN rats. Results, expressed as cpm binding, are of the mean ± SD of 6 replicate wells. Binding with control supernatants (no added cells) is shown (▲). Note that suppression by normal or convalescent SC varied between recipient rats.

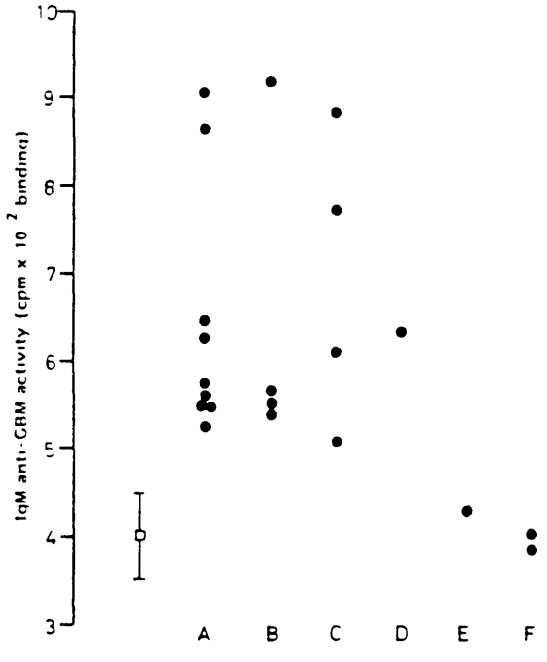



Figure 62. In vitro synthesised IgM anti-GBM activity from SC of BN and other rats. SC (2 x $10^6$/200µl) from normal BN (A), day 10 $HgCl_2$ treated BN (B), GBM immunised BN (C), BSA immunised BN (D), or normal Lewis (E) or Wistar (F) rats were cultured for 7 days without further stimulation. Results, expressed as cpm binding, show the mean of 6 replicate wells for each spleen cell culture. Background binding of medium is shown (□). Activity was only detectable in cultures with BN SC.

bound) also failed to show specific inhibition. However, rabbit anti-day 14 eluate but not anti-normal eluate or normal rabbit serum, caused a dose dependent inhibition (Fig. 63).

The time course of this IgM class anti-GBM activity produced from normal BN SC is shown in Fig. 64. Activity was rarely detectable before 24 hr and peaked between days 4 and 8.

When SC from normal or GBM immunised BN rats, were cultured with or without human GBM, it was found that the presence of GBM in culture inhibited synthesis of IgM anti-GBM antibody in cultures from GBM immunised rats, but not that from normal BN rats, even when similar levels of activity were present in both (Fig. 65). This effect was found to be dose dependent and at high levels of GBM an inhibitory effect was noted on SC from normal as well as GBM immunised rats.

The effect of washing the cells and replacing the medium after 24 hr, then culturing for a further 7 days was examined, and it was found that this procedure prevented further production of IgM anti-GBM activity, even though in controls large amounts of activity were subsequently produced (Fig. 66).

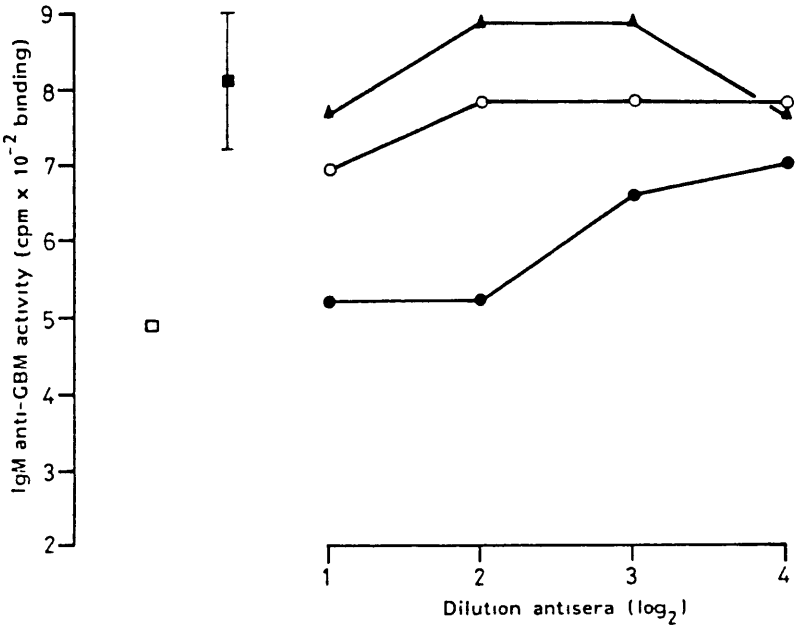



Figure 63. In vitro inhibition of binding of IgM anti-GBM activity from cultured normal BN SC, to GBM, with heterologous 'anti-idiotypic' antiserum. Day 7 supernatants (300µl of 2/3 diln.) from normal BN SC were preincubated with 20µl of dilutions of normal rabbit serum (▲——▲), rabbit anti-normal eluate (o——o) or rabbit anti-day 14 eluate (●——●) before assaying in the IgM class anti-GBM RIA. Results, expressed as cpm binding, show the mean of triplicate assay wells. Controls consisted of medium (□) or supernatant preincubated with PBS (■). IgM class activity was specifically inhibited by anti-day 14 eluate in the RIA.

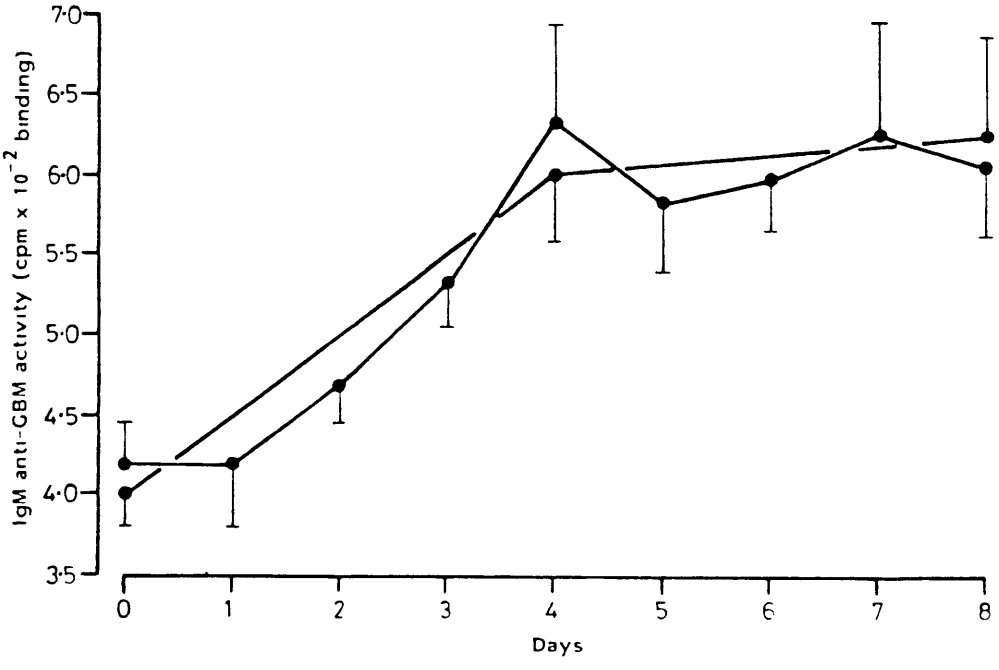



Figure 64. Time course of in vitro production of IgM anti-GBM activity from normal BN SC. Normal BN spleen cells ($10^7$/ml) were cultured for up to 8 days. Results, expressed as cpm binding, show the mean ± SD of triplicate culture wells for two separate experiments.

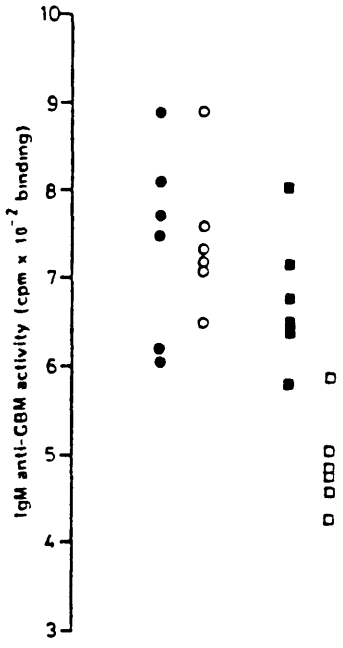



Figure 65. Effect of GBM on in vitro production of IgM anti-GBM activity by SC from normal or GBM immunised BN rats. SC (2 x $10^6$/200µl) from normal (●,o) or a day 4 GBM immunised BN rat (■,□) were cultured for 8 days in the presence (open symbols) or absence (closed symbols) of 20µl of 3mg/ml human GBM. Results, expressed as cpm binding, show the IgM anti-GBM activity in 6 wells of each combination. Coculture with GBM caused suppression of antibody synthesis in cultures of SC from GBM immunised but not normal BN rats.

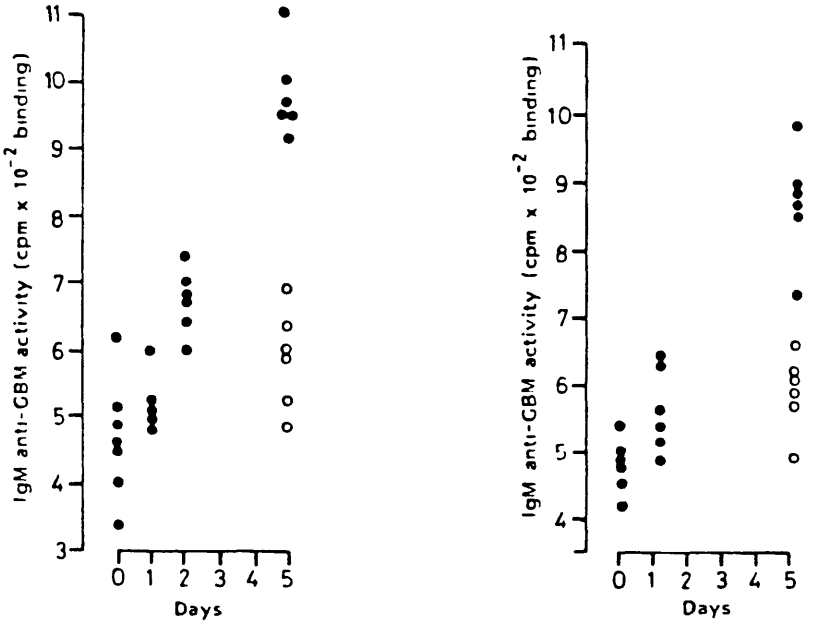



Figure 66. Effect of 'washing cells' on production of in vitro IgM anti-GBM activity by normal BN SC. SC (2 x $10^6$/200µl) from normal BN rats were cultured for 24 hr and then washed twice (o), or manipulated similarly but without replacing the medium (●), and cultured for a further 4 days. Results, expressed as cpm binding, show activity at various time points, in 6 replicate wells, in two separate experiments. 'Washed' SC synthesised less antibody than control SC.

# 4. DISCUSSION

## 4.1 THE MODEL

### 4.1.1 Anti-GBM Antibody Response

The work described in this thesis shows that $HgCl_2$ induces anti-GBM autoantibodies in Brown Norway rats, and that these can be detected in the circulation as well as bound or eluted from the kidney. The antibody response is dose dependent on the $HgCl_2$, both with respect to number of injections and concentration. The generation of anti-GBM antibodies is not sex linked as has been found in some other experimental autoimmune diseases (Smith and Steinberg, 1983), but appears to be genetically restricted, since Lewis and Wistar rats do not respond. This confirms Druet's finding, using immunofluorescence studies (Druet et al, 1977). In such restriction the disease is similar to experimental anti-TBM disease (Hyman, Colvin and Steinberg, 1976), as well as autoantibody mediated glomerulonephritis in humans, in which there is an association with the tissue type DRW2 (Rees et al, 1978).

Levels of anti-GBM antibody were found to fall spontaneously after 14 days, in the presence or absence of continuing $HgCl_2$ stimulation, suggesting rapid autoregulation of autoantibody synthesis. The overall time course of the $HgCl_2$ induced anti-GBM response was truncated compared to other autoimmune models, involving CFA or heterologous antigens, such as in anti-erythrocyte autoimmunity in mice (Naysmith, Ortega-Pierres and Elson, 1981) and EAT (Lillehoj and Rose, 1982); for example, antibody was undetectable 5 weeks after initiation. In anti-GBM nephritis in humans, antibody production continues for an average of 11 to 14 months in untreated patients but then ceases spontaneously (Wilson and Dixon, 1981; Flores et al, 1986).

The detection of anti-GBM antibody in the circulation

preceded that detectable as bound to the kidney, although this may also have been related to the far greater sensitivity of the RIA, as compared to immunofluorescence. However, linear IgG staining in the kidney was still present at day 26, when circulating anti-GBM antibody levels were falling towards background, perhaps representing active adsorption of autoantibody from the circulation to the target organ. The peak of circulating anti-GBM antibodies at days 11 to 14, however, occurred after the splenic GBM PFC response, which occurred at day 9 (Chalopin and Lockwood, 1984).

Troughs, following smaller peaks of anti-GBM antibody, could occasionally be detected after day 14, indicating a possible antibody/homeostatic control response. This phenomenon, with a similar interpretation, has previously been noted in phosphoryl-choline injected mice (Rose and Lambert, 1980), and in SLE (Zouali and Eyquem, 1983). These smaller anti-GBM antibody peaks are also preceded by peaks of GBM PFC in the spleen (J. Savige - personal communication). Splenic PFC responses occurring prior to a detectable circulating antibody response in other systems, have been well characterised (Wigzell, 1966).

As well as IgG, other classes of anti-GBM antibody, such as IgM, IgA and IgE were also detectable in the serum, with similar kinetics of synthesis and down regulation to the IgG class, although this is in contrast to the usual IgM to IgG switch in the immune response to foreign antigens. IgM class autoantibodies have been found in a number of autoimmune diseases and models of autoimmunity (Naysmith, Ortega-Pierres and Elson, 1981; Stuart et al, 1979; Hughes, 1982), including occasionally Goodpasture's syndrome, whether detected in the kidney (Wilson and Dixon, 1981) or in the circulation (Bowman, Ambrus and Lockwood, unpublished data), but are less common in others (Torrigiani, Roitt and Doniach, 1968). The presence of IgA autoantibodies in this model contrasts with their

absence in anti-erythrocyte autoimmunity in mice (Naysmith, Ortega-Pierres and Elson, 1981), although IgA anti-DNA autoantibodies are present during the polyclonal activation found in SLE (Hughes, 1982), and occasionally in the more specific autoantibody response in thyroiditis (Torrigiani, Roitt and Doniach, 1968). Anti-GBM antibodies of IgA class are also found in a minority of patients with Goodpasture's syndrome, both in the kidney (Wilson and Dixon, 1981) and in the circulation (Bowman, Ambrus and Lockwood, unpublished data). IgE autoantibodies, although present in very low titre, were particularly interesting, due to the IgE association primarily with the allergic response to foreign antigens, or the normal antibody response to helminth infections (Jarrett and Bazin, 1974), rather than an autoantibody response. When monoclonal antibodies were raised from $HgCl_2$ stimulated BN rats, almost all were of the IgE class (Hirsch et al, 1985), although previously specific IgE class anti-GBM antibodies were not detectable by the same group (Prouvost-Danon et al, 1981).

In kidney eluates, class of anti-GBM antibody was mainly IgG, although small amounts of IgA and IgM specific antibodies were detectable. This contrasts with heterologous GBM induced glomerulonephritis in BN rats, where IgA and IgM anti-GBM antibodies were not found (Stuffers-Heiman, Günther and Van Es, 1979).

In the circulation, IgG1, IgG2a and IgG2c anti-GBM antibodies were readily detectable, and IgG2b only occasionally so, using polyclonal anti-subclass reagents, while with the more specific monoclonal reagents, both IgG2b and IgG2c antibodies were barely detectable. However, no comment can be made about quantitative differences in the subclasses, due to inadequate knowledge of the relative potency of the detection antisera, even though dilutions were optimised. Subclass of autoantibody responses

has been found to be restricted only in some autoimmune diseases such as idiopathic thrombocytopenic purpura (Karpatkin et al, 1973), and primary biliary cirrhosis (Riggione, Stokes and Thompson, 1983), and not in others, such as anti-erythrocyte autoimmunity in mice (Naysmith, Ortega-Pierres and Elson, 1981). However, in another model of autoimmunity in rats, spontaneous autoimmune thyroiditis in Buffalo rats, only circulating autoantibodies of subclasses IgG1 and IgG2a are detectable (Zanetti and Bigazzi, 1981). In human autoimmune glomerulonephritis, studies examining subclass in kidney eluates are conflicting, with restriction or no restriction being reported (Lewis, Busch and Schur, 1970; McPhaul and Dixon, 1971; Bannister et al, 1983), while in studies on subclass of anti-GBM antibody in the circulation, restriction to IgG1 and IgG4 has been found in almost all patients (Bowman, Ambrus and Lockwood, manuscript in preparation). Subclass of autoantibody in kidney disease is important with respect to tissue injury and the possible masking of the GBM by non-complement fixing antibodies such as IgG4.

In SLE disease expression appears to be linked to subclass of the anti-nuclear antibody, with complement fixing antibodies occurring when nephritis is present (Tojo, Friou and Spiegelberg, 1970; Puritz et al, 1973). It is not known whether the levels of complement fixing anti-GBM antibodies in the BN rat model, correlated with disease expression as indexed by proteinuria.

Interestingly, in eluate, anti-GBM antibodies of all 4 subclasses of IgG were readily detectable with both the polyclonal and monoclonal reagents. It is not known whether this is due to the relative avidity of the different subclasses, with IgG2b and perhaps IgG2c binding preferentially to the GBM and thus being selectively removed from the serum, or whether the target antigens for all subclasses are the same. Changes in avidity with different

subclasses in the rat have been previously noted (Medgyesi et al, 1981), and there is evidence that antibodies depositing in the kidney differ from those in the circulation (Winfield, Faiferman and Koffler, 1977). Alternatively, kidney bound anti-GBM antibodies could bind anti-idiotypic antibodies which may be of different subclass to the idiotype. However, studies using foreign antigens have suggested that anti-idiotypic antibodies in the rat are IgG1 (Rousseaux-Prévost et al, 1983) rather than the IgG2b and IgG2c found additionally here.

The lack of restriction in eluates contrasts with the heterologous GBM induced model of nephritis in BN rats, where only IgG1 and IgG2a are present in the kidney (Stuffers-Heiman, Günther and Van Es, 1979). It is interesting that in autoimmune glomerulopathy in the mouse, analysis of subclass of IgG by immunofluorescence studies in the kidney, show that IgG1 is the predominant subclass and remains linear, whereas the other subclasses are less marked and change from linear to granular, perhaps because they are directed against abnormal GBM (Bolton, Benton and Sturgill, 1978).

In all subclass studies, whether described herein or published by other workers, it should be noted that detection of antibodies with polyclonal anti-subclass antisera may not always reveal restriction because although such reagents may be monospecific by standard immunoprecipitation techniques such as Ouchterlony analysis, they may still exhibit some cross reaction when used in more sensitive radioimmunoassay techniques. Studies using monoclonal anti-subclass reagents will help to clarify this, and may reveal restriction not previously apparent.

### 4.1.2 Specificity of Anti-GBM Antibodies

Binding of anti-GBM antibody in the RIA was shown to be

specific, and could be inhibited with human GBM as well as rat GBM indicating common antigens in the two species. This is in agreement with the binding of sera from patients with anti-GBM disease or Goodpasture's syndrome to rat GBM as well as human GBM. However, the mAb MCA-P1, reactive with the Goodpasture antigen (Pressey et al, 1983) binds to human GBM but not rat GBM in the RIA (unpublished data), and does not bind to rat kidney sections by immunofluorescence (S. Cashman - personal communcation).

Use of immunoblotting to detect the relevant $HgCl_2$ induced anti-GBM antibody binding antigen(s) in rat and human GBM preparations proved difficult due to the high level of non-specific binding of normal and especially day 14 BN sera to any protein bands on the immunoblot, as well as the nitrocellulose sheets themselves, whether blocked with bovine Hb in CFDT20 or rat Hb. Use of Ig preparations also failed to prevent this binding. By examining intensity of staining for normal and day 14 sera, it appeared possible that antibody in day 14 sera, might bind to the same bands (centred around 25K and 50K and in rat GBM, also 80K) that are detectable with sera from patients with anti-GBM disease, and that these bands were present in both rat and human GBM.

Immunoblotting with eluate from day 14 kidneys also proved difficult to interpret, due to variability between blots, although background binding was far less than with sera. While weak immunoreactive bands could be found in the 50K region, similar to those detected with patients' sera, in both rat and human GBM, additional bands at 10K and 40K were found with rat GBM, which had no counterpart to those detected using human sera.

### 4.1.3 Immune Complex Phenomenon

$HgCl_2$ induced nephritis in the BN rat is a biphasic disease

in that by day 30, immunofluorescence studies of kidney biopsies show attenuation of the linear IgG and the appearance of granular deposits (Druet et al, 1979). The studies described herein confirm this finding and extent it to the appearance of immune complexes in the circulation. Although not all immune complex assays proved suitable, especially the fluid phase C1q Binding Assay, due to high day 0 values, it was found that by using the solid phase KgBA, all BN but not Lewis rats, developed significantly raised levels of immune complexes, compared to day 0 values. The detection of circulating immune complexes preceded their detection in kidney biopsies. However, immune complexes (granular deposits) may have been present at an earlier stage in the kidney, but been masked by the intensity of linear staining. A peak of circulating immune complexes occurred at the time of, or slightly following the peak of anti-GBM antibody, and in addition, other peaks of varying levels occurred later in the time course. The close timing of circulating immune complexes and anti-GBM antibodies was suggestive of a functional association. The possibility of immune complexes consisting of anti-GBM antibodies and a regulatory factor such as anti-idiotype, was considered, as has been found in the mouse phosphorylcholine system, where immune complexes occurring at this time were demonstrated to consist of idiotype and anti-idiotype (Rose and Lambert, 1980). However, pilot experiments using a similar detection protocol, with $^{125}I$ eluate as the idiotype, were unsuccessful in demonstrating the presence of anti-idiotype in conglutinin bound immune complexes. Additionally, the presence of other products of the simultaneous polyclonal activation in this model, was felt to be a complicating factor in analysis of the immune complexes. In anti-GBM nephritis in humans, immune complexes are not a common feature of the disease, although there have been

reports of Goodpasture's syndrome, with linear staining in biopsies being preceded, followed or coinciding with granular deposits in the kidney (Klassen et al, 1974; Agodoa et al, 1976; Pasternack, Törnroth and Linder, 1978).

4.1.4. The Polyclonal Response

Hirsch et al (1982) have found that $HgCl_2$ in the BN rat acts as a polyclonal activator, both *in vivo* and *in vitro*, with reports of increases in the PFC response to various antigens, and the appearance of anti-DNA antibodies in the circulation. The presence of anti-DNA antibodies was confirmed in this study, although with an uncharacterised assay. However, similar results have been found in an ELISA system for antibodies to double and single stranded DNA, although the down regulatory mechanism does not appear to be as definitive as for anti-GBM antibodies (Bowman et al, manuscript in preparation). Anti-collagen I and II antibodies have also been found in this model, as have anti-thyroglobulin antibodies, with the latter having similar kinetics of synthesis and down regulation to anti-GBM antibodies (Bowman et al, manuscript in preparation).

Serum IgG concentrations have been found to rise by up to 4 fold, as might be expected during a polyclonal response, and these increases in serum IgG were found to be cyclical in nature (C. Pusey, J. Savige - personal communication), perhaps reflecting changes in efficacy of control mechanisms.

Polyclonal activation was also indicated by changes in lymphoid cells and organs. Spleen size increased dramatically but transiently, falling to day 0 values by day 36. This transient increase in spleen size has been noted by others (Hirsch et al, 1982) and found to be strain dependent. Total circulating WBC also showed a temporary increase. However, although $HgCl_2$ is a

polyclonal B cell activator (Hirsch et al, 1982), it was not the total circulating lymphocytes which increased, but instead the circulating neutrophils. It is not known to what extent the neutrophil increases were a consequence of any $HgCl_2$ induced inflammatory responses. However, neutrophilia after $HgCl_2$ treatment was not found in another non anti-GBM antibody susceptible strain. If neutrophils are under T cell control, as has been found for eosinophils (Walls et al, 1971) then perturbation of T cell control, which results in autoantibody production, might also be accompanied by neutrophilia.

4.1.5. Changes in Lymphocytes

Changes in T cell function and T cell subsets have been found in a number of autoimmune diseases (reviewed by Miller and Schwartz, 1982; Shoenfeld and Schwartz, 1984) and may involve decreases in T suppressor levels, increases in T helper cells or decreased T cells. Certain human kidney diseases with a suspected autoimmune aetiology also show changes in the normal ratio of T cell subsets (Chatenoud and Bach, 1981). In addition, in an experimental model of EAE, sequential studies after induction have shown a reduction in T helper cells in the circulation and their appearance in the central nervous system (Hauser et al, 1984).

In $HgCl_2$ treated BN rats, a transient reduction in T cells was found, which did not occur in another non-suspectible strain - the Wistar rat. It occurred both slightly preceding (day 7) and also at the time of (day 12 onwards) the anti-GBM antibody response, and the B cells showed a corresponding increase. Analysis of % cells, mirrored that of actual numbers in the circulation, with the result that although the polyclonal response and increase in total WBC was not due to large increases in total lymhocytes, the actual B

cell numbers did rise, although not dramatically.

The decrease in T cells was found to be due to decreases in T helper cells as in EAE (Hauser et al, 1984), and the diabetes prone BB rat (Yale and Marliss, 1984). However, in $HgCl_2$ treated rats there is only a little mononuclear cell infiltrate into the kidney (Hinglais et al, 1979) or thyroid (A. Weetman - personal communcation) to account for the loss of T helper cells from the circulation. The fate of these $T_h$ was not known, although other organs have not been studied.

Of interest was the early decrease in T suppressor cells at days 5 or 10, preceding the autoantibody response. This timing might indicate an additional cause of the resulting autoantibody response. If, for example, $T_s$ cells from BN rats were more susceptible to the toxic effects of $HgCl_2$, their subsequent low numbers might cause an imbalance and result in temporary loss in autoregulation and unhindered B cell derived autoantibody production. It is interesting that mercury treatment of PVG rats, which results in a glomerulopathy, also causes a reduction in general T cell reactivity to PHA and Con A induced suppressor function (Weening, Hoedmemaeker and Bakker, 1981). Additionally, onset of disease in spontaneously occurring SLE in NZB/NZW mice is associated with loss in suppressor cell function (Kraukauer, Waldmann and Strober, 1976), while a similar mechanism has been suggested for autoimmune thyroiditis, where a specific T suppressor dysfunction has been postulated (Kidd et al, 1980). In multiple sclerosis, loss in proportion of T suppressor cells occurs in active disease but not in remission (Craig et al, 1985).

Since in many rats, during the time of down regulation of autoantibody production, T suppressor cells recover and increase beyond day 0 values, this could represent the regaining of auto-

regulation. However, no relationship was noted between peak levels of anti-GBM antibody and the minimum level of $T_S$ during the early phase or the maximum $T_S$ levels during the down regulatory phase. One important feature of these changes in T suppressor cells may be that BN rats have a particularly low percentage of $T_S$ compared to other rats such as the Wistar. A low level of $T_S$ and high % of B cells in BN compared to Lewis spleens, has been noted by others (Rosenberg and Feldman, 1982).

The T suppressor cell decrease and recovery also precedes the rise and fall in number of neutrophils, suggesting a possible cause/effect relationship.

In all these studies on flow cytometry analysis of T cell subsets, recognition must be given to the fact that differences in intensity of staining may occur during the disease, giving rise to weakly positive cells, such that the cells labelled with the mAb, may not be representative of the normal distribution of cells of that particular phenotype. This has been found in the MRL-lpr/lpr mouse, where cells stained with Ly-1 have an abnormally low intensity compared to other mouse strains (Lewis, Giorgi and Warner, 1981). In addition, care must be taken in the studies involving the whole blood technique of labelling, that an efficient lysing technique is used, as the presence of more than minimal amounts of red cells during flow cytometry analysis, causes a falsely low percentage of positive cells, as the unlabelled, contaminating red cells move into the area sampled by the cell analyser, as lymphocytes.

### 4.1.6. Tissue Injury

Proteinuria occurred in only a proportion of $HgCl_2$ treated BN rats in this study. This contrasts with the complete lack of

proteinuria found in BN rats immunised with heterologous or autologous GBM, even when anti-GBM autoantibodies are present (Stuffers-Heiman, Günther and Van Es, 1979; Pusey, Sinico and Lockwood, 1985), but the presence of proteinuria in 100% of $HgCl_2$ treated BN rats of a separately derived colony which still had similar levels of anti-GBM antibody to the rats in this study (J. Savige - personal communication).

No relationship was found between circulating levels of anti-GBM antibody or immune complexes and degree of tissue injury as reflected by proteinuria. Both this lack of relationship and the variation in proteinuria between individual rats, colonies and in different models all involving anti-GBM autoantibodies, could be explained by variation in the complement fixing ability, specificity and affinity of the antibodies. Low or non-complement fixing antibodies - IgG1 and IgG2c for example - may be non-destructive, while complement fixing antibodies, such as IgG2a by promoting polymorph infiltration and release of phlogistic enzymes, may cause substantial membrane injury and proteinuria. However, it should be noted that in Capron's study of proteinuria in $HgCl_2$ treated BN rats, decomplementation had no effect (Capron et al, 1982). Because the RIA uses a crude collagenase extract of GBM, individual fine specificities of antibody to different components in the GBM, such as structural as against filtration components, which might vary between animals and models, and vary in their pathogenicity, would not be distinguished.

Similar considerations need to be taken into account when examining the varying abilities of immune complexes to produce tissue injury. Class, subclass and affinity of the antibody component of immune complexes have all been shown to be important (Steward, 1976).

Cellular mechanisms may also be important in mediation of tissue injury, yet vary between individual rats and models of disease. Hinglais has noted an influx of monocytes in the glomeruli, which slightly precedes the onset of proteinuria in $HgCl_2$ induced nephritis (Hinglais et al, 1979). Experiments using another polyclonal activator, complete Freund's adjuvant, show that in nephrotoxic nephritis, lymph node cells from CFA stimulated animals may potentiate tissue injury: the amounts of kidney bound or circulating anti-GBM antibody are not affected by the CFA (Moorthy and Abreo, 1983).

The presence of other autoantibodies in these $HgCl_2$ treated BN rats also did not necessarily correlate with tissue injury. All rats produced anti-thyroglobulin antibody, yet thyroids were normal (Bowman et al, manuscript in preparation).

### 4.1.7. Mechanisms of Development of the Anti-GBM Antibody

The mechanism by which $HgCl_2$ induces anti-GBM autoantibody production is unknown, although various effects of $HgCl_2$ may result in the disease: (1) $HgCl_2$ acts as a polyclonal activator both *in vitro* and *in vivo* as shown herein and in previous studies (Hirsch et al, 1982). Other drugs, such as penicillamine, have also been shown to act as polyclonal activators of both B and T lymphocytes (Goodman and Weigle, 1981b), and in BN rats, penicillamine induces a similar disease to $HgCl_2$, with production of various autoantibodies (Donker et al, 1984). (2) $HgCl_2$ may be preferentially cytotoxic to T suppressor cells, as the above results might suggest. Various chemicals have been shown to inhibit T suppressor function or alter lymphocytes, and be associated with the production of autoantibodies (Bluestein et al, 1979; Kirtland, Mohler and Horwitz, 1980), while $HgCl_2$ is associated with loss in T suppressor function in immune

complex glomerulopathy in the PVG rat (Weening, Hoedemaeker and Bakker, 1981). (3) $HgCl_2$ binds to autoantigens such as the GBM, modifying the tissue structure, such that it becomes antigenic. In rabbits $HgCl_2$ has been shown to bind to renal tubular basement membrane, although not to the GBM, and the $HgCl_2$ treated rabbits develop anti-basement membrane antibodies (Roman-Franco et al, 1978). In addition, other drugs which result in autoantibody production, notably penicillamine, react with the targeted antigens (Munthe, Jellum and Aaseth, 1979).

That $HgCl_2$ causes anti-GBM nephritis in the BN rat, by inducing a loss of autoregulation, or immune dysregulation, has been previously suggested (Druet et al, 1982a), and may involve a combination of its first two effects described above. However, a cumulative effect of all three of the above, resulting in autoantibody production, cannot be overlooked. In addition to this, there may be a mechanism involving the recently discovered effect of $HgCl_2$ in modifying T helper cells, which then cause proliferation of both B and T cells (Pelletier et al, 1985a,b).

## 4.2. REGULATION OF THE IMMUNE RESPONSE

### 4.2.1. Rechallenge Experiments

In some experimental autoimmune models, including induced EAE in Lewis rats, rat erythrocyte induced autoimmunity in mice and EAT in rats, as well as $HgCl_2$ nephritis in BN rats, the disease is spontaneously self limiting (Willenborg, 1979; Naysmith, Ortega-Pierres and Elson, 1981; Lillehoj and Rose, 1982), with spontaneous reductions in autoantibodies and/or clinical symptoms. The mechanism of recovery may involve active regulation, and therefore in rechallenge experiments, convalescent animals might be expected to be resistant to reinduction of the disease. BN rats,

convalescent from $HgCl_2$ induced nephritis were almost totally protected from reinduction of the disease, in terms of anti-GBM antibody production. However, they differed from other self-limiting, reinduction resistant models in 2 respects: (1) This resistance to reinduction decreased with time and was totally lost by 9 months after initial challenge. IN EAE, resensitisation after a short convalescence leads to a brief, mild, clinical episode, but resistance to reinduction increases with time (Willenborg, 1979) as it does in anti-erythrocyte autoimmunity in mice (Naysmith, Ortega-Pierres and Elson, 1981); (2) The kinetics of anti-GBM antibody production in $HgCl_2$ injected convalescent rats were the same as in $HgCl_2$ treated naive rats, while in the anti-erythrocyte model, rechallenge resulted in an early but short lived burst of autoantibody activity (Naysmith, Ortega-Pierres and Elson, 1981).

The extent to which these differences reflect variation in the induction of the diseases or the active control mechanisms, is not known. In EAE in particular, although convalescent rats are resistant to active induction of the disease, they are still susceptible to passive induction, using sensitised lymphocytes (Willenborg, 1979).

Of associated interest, is the effect on rechallenge after certain immunosuppressive procedures. In studies by other workers in this group, it has been shown that a single dose of cyclophosphamide, sufficient to prevent $HgCl_2$ induced synthesis of autoantibody, also confers complete resistance to rechallenge at 3 months (Pusey et al, 1983), and even at 12 months (C. Pusey - personal communication). However, it is not known whether this may be due to clonal deletion or induction of a very powerful suppressor cell. In EAE in Lewis rats, resistance to rechallenge could be overcome by total body irradiation possibly by preventing an early, low level,

secondary antibody response which Willenborg believes is associated with resistance to reinduction of the disease (1982). This was apparently different to the mechanism responsible for recovery from the disease. Separate regulatory mechanisms may explain why rats suffering from thyroglobulin induced EAT, which is a self limiting disease, nevertheless are not resistant to reinduction (A. Weetman - personal communcation). It is of interest that anti-GBM nephritis in humans only rarely recurs (Hind et al, 1984), although the events in man which would lead to the equivalent of rechallenge in experimental animals, are not understood.

It was also found that low doses of $HgCl_2$, insufficient to provoke a detectable anti-GBM antibody response, could still induce resistance to subsequent challenge with standard doses of $HgCl_2$. This suggested that autoregulatory mechanisms could be engaged without the concommitant induction of significant levels of autoantibody. This could occur as part of classical 'low dose tolerance', and has been found in another drug induced model of autoimmunity in BN rats - the penicillamine induced model - where low doses of the drug induced tolerance to subsequent potentially fatal doses (Donker et al, 1984).

One explanation for the resistance to rechallenge in the $HgCl_2$ treated rat, at least with respect to anti-GBM antibodies, is that after the first cycle of the disease, all readily accessible antigen has been destroyed, and that antigen was required in conjunction with $HgCl_2$ to further stimulate autoantibody production. However, convalescent animals which received renal transplants also failed to subsequently develop anti-GBM antibodies on $HgCl_2$ rechallenge. Interestingly, day 0 values of antibody in the transplanted animals were raised above the normal range. However, since a similar effect was noted in the sham operated animal, it was

thought this was a non specific effect due to acute phase reactants in post operative animals, rather than an effect due perhaps to immunisation with kidney fragments during the transplantation procedure. Therefore resistance to rechallenge in this model, was believed to be due to active suppression rather than exhaustion of antigen.

Of interest also, was the partial resistance of $HgCl_2$ treated convalescent animals to anti-GBM antibody production induced by challenge with rat GBM. This would suggest that the specificities, and perhaps even idiotypes of the antibodies in the two models, have some features in common, even though the induction mechanisms differ. This might perhaps indicate a basic defect in BN rats with respect to anti-GBM antibody production and control, and that regulatory mechanisms may be shared, although since antibody production in the GBM induced model continues for 9 months, it is difficult to induce regulation. The effect of $HgCl_2$ pretreatment on the GBM model and its effect on established anti-GBM antibody production have been studied in more detail by others (Pusey, Sinico and Lockwood, 1985). They found that $HgCl_2$ treatment temporarily increased the anti-GBM antibody levels when given during a time of constant antibody production, but did not induce down regulation. Thus control was limited to induction of the autoantibody production, and even then was incomplete, suggesting separate as well as shared specificities and idiotypes.

### 4.2.2. Suppressor Cells

Investigation of the mechanisms responsible for both the active down regulation of anti-GBM autoantibody production and active resistance to reinduction led to an examination of the possible role of suppressor cells in conferrment of resistance. In

common with two other models of autoimmunity, EAE in Lewis rats (Adda, Beraud and Depieds, 1977) and rat erythrocyte induced autoimmunity in mice (Naysmith et al, 1980), lymphoid cells from convalescent animals were able to transfer suppression to naive recipients. In Heymann nephritis, transfer of spleen cells from tolerised animals to naive recipients has been shown to not only affect the autoimmune response, but also, as a consequence of this, the clinical expression of autoimmunity- proteinuria (Litwin et al, 1979). Similarly, in EAE, transfer of spleen cells from convalescent animals affects the disease on clinical as well as histological grounds, in the recipients (Adda, Beraud and Depieds, 1977). Although lymphoid cells from spleen, thymus, lymph node or blood have been shown to transfer suppression in EAE and erythrocyte autoimmunity, the best suppression in the latter, which more closely resembles $HgCl_2$ induced disease in that it is autoantibody mediated, is spleen cells (Naysmith et al, 1980), so spleen cells were used in these studies. In $HgCl_2$ treated rats, convalescent spleen cells suppress anti-GBM autoantibody synthesis on transfer to naive recipients. However, the great variability in disease expression in this model, as indexed by proteinuria, precluded any study relating suppressor cells to suppression of the clinical disease. Irradiation of recipients, which is required when demonstrating transferrable suppression by cells, to foreign antigens in rats (Brideau et al, 1980) was not necessary in this model of autoimmunity in the BN rat.

Suppression of the anti-GBM antibody response was not complete, even with transfer of high numbers of spleen cells. This is in contrast to the complete suppression reported by Gare and Cox (1980) using blood leukocytes in the rat erythrocyte induced model of autoimmunity, and the almost complete suppression using spleen

cells in that same model, by Naysmith's group (Naysmith et al, 1980). However, it is similar to the partial suppression conferred by convalescent lymphoid cells in EAE (Adda, Beraud and Depieds, 1977). This failure to obtain complete suppression may be because the cellular suppression is idiotype specific, and a proportion of the antibodies produced have private idiotypes, rather than cross-reactive idiotypes present in all animals. Alternatively, insufficient suppressor cells may have been transferred. Increasing the number of cells transferred did not increase suppression, but this may have been due to the transfer of increased numbers of accompanying antibody producing cells, offsetting the suppressive effect. Interestingly, at lower cell doses, increased peak levels of anti-GBM antibody were observed. One possible explanation for these results is that the cells conferring suppression were numerically fewer, but more potent, than cells conferring help, and were subject to a dose threshold effect. Similar dose dependant help and suppression has been described in a human in vitro system (Dosch, Lam and Gelfand, 1982). The cell numbers required to transfer suppression were similar to that required in EAE in rats (Adda, Beraud and Depieds, 1977) and three times those required in the erythrocyte model of autoimmunity in mice (Naysmith et al, 1980).

For reasons not yet understood, occasionally recipients of normal cells produced low levels of anti-GBM antibody in response to $HgCl_2$. One explanation for this is that these animals showed non-specific suppression, mediated by lysed, normal cells, as described by Goodman and Weigle (1981a).

The ability to confer suppression varied with time. Suppression was transferrable from donors at the peak of anti-GBM antibody production, day 14, as well as during antibody down

regulation, from days 21 to 35, but not from day 8 animals, in which total circulating T suppressor cells are reduced, or from day 19 animals. In other experiments with male donors and recipients, suppression could still be transferred from 3 month convalescent rats (C. Pusey - personal communication). One possible explanation for the lack of transferrable suppression at day 19 could be that this time may slightly precede one of the smaller secondary peaks of anti-GBM antibody, and so may represent a time of inadequate suppression even in the donor. Such oscillation of antibody production and regulatory T cells has been noted in PFC responses to TNP (Zöller et al, 1985). The timing of the donor in this model is similar to in the EAE model, in that spleen cells from the time of peak response could transfer suppression. However, while in EAE, donor timing was critical, with spleen cells from convalescent rats rapidly losing potency (and lymph node cells taking over (Adda, Beraud and Depieds, 1977)), in this model, suppression could be transferred from donor spleen cells during convalescence, over a wider time range.

When timing of recipients was examined, to determine what phase of the anti-GBM antibody response was susceptible to the activity of the suppressor cells, it was found that cells could suppress when transferred at day 0, at the induction of the disease, as well as at day 7, when the animal is already committed to antibody production. In erythrocyte autoimmunity in mice, only if mice are injected with spleen cells at day -1, are all phases of the subsequent autoantibody response suppressed (Naysmith et al, 1980). In Heymann nephritis, lymphoid cells from rats tolerised with antigen in IFA, rather than from convalescent rats, can transfer suppression, but only if transferred to animals prior to induction of the disease. They have no effect when transferred into

recipients with active early Heymann nephritis (Litwin et al, 1979). Although it was determined that in $HgCl_2$ induced nephritis convalescent spleen cells can act both on the induction, and to some extent the effector phases of the autoantibody response, it is not known whether they act directly on the B cells, the appropriate $T_h$, or though macrophages.

Suppressor cells may be induced in the absence of overt autoimmunity, as seen by their induction in tolerised animals in Heymann nephritis (Litwin et al, 1979), and also in lightly irradiated mice in the absence of detectable anti-erythrocyte autoantibody in the anti-erythrocyte model of autoimmunity (Naysmith et al, 1980). In $HgCl_2$ induced disease, detectable anti-GBM antibodies were only rarely produced when low doses of $HgCl_2$ were used, and yet these animals were still resistant to rechallenge with standard doses of $HgCl_2$. Although not performed, it might be expected that lymphoid cells from these low dose tolerised donors might also be capable of transferring suppression to naive recipients.

The specificity of the suppression mediated by the convalescent cells was tested by i.v. administration of BSA at the same time as cell transfer. Antibody responses under these circumstances are critically dependent upon normal spleen cell function (Lockwood, 1983). Rather than conferring non-specific suppression, an enhanced anti-BSA response was seen in those animals receiving convalescent cells. It is not known to what extent this enhancement may have been due to macrophages present in the SC suspensions, acting as antigen presenting cells. However, from these results it could be inferred that at least in terms of exogenous antigens, the convalescent cell mediated suppression was antigen specific. In the rat erythocyte induced model of auto-

immunity in mice, transferrable suppression acts only on the autoantibody response, and not on the associated anti-rat erythrocyte response (Naysmith, Ortega-Pierres and Elson, 1981), while in EAE transferrable suppression is also antigen specific (Adda, Beraud and Depieds, 1977). Antigen specificity of suppressor cells, if they are to be used therapeutically in an autoimmune disease, would be a prerequisite, since the antibody response of the recipient to heterologous antigens, such as microorganisms, must not be affected.

The cell type responsible for transfer of suppression was examined using depletion experiments. When the mAb MRC OX8 was used to deplete convalescent cell suspensions, prior to transfer, the suppression was abrogated. Depletions using an irrelevant monoclonal antibody had no effect. Therefore it could be inferred that the MRC $OX8^+$ cell, presumably acting as a conventional $T_S$ cell, plays an important role in regulating anti-GBM autoantibody responses. In addition, a role for Natural Killer cells, which are also labelled by MRC OX8 (Cantrell et al, 1982), in regulation cannot be excluded, as they have been implicated in control of the immune response to foreign antigens (Abruzzo and Rowley, 1983). Other mechanisms may be involved as well, since the abrogation by MRC OX8 depletion is not complete. Indeed, if control exerted by $T_S$ cells was the only mechanism by which anti-GBM antibody synthesis was regulated, then it would be expected that recipients of SC depleted of MRC $OX8^+$ cells would make a higher antibody response than controls receiving medium alone, since the helper effect, dominant when suppressor cells were diluted out at low cell doses, would appear. In erythocyte autoimmunity in mice, depletion of T cells also resulted in only partial abrogation of the transferrable suppression, and Naysmith has suggested that this was evidence for T

and B cell mediated suppression, or at least T and non T cell suppression, since B cell depletions had no effect on the transferrable suppression (Naysmith et al, 1980). In $HgCl_2$ induced disease, it does not appear that B cells, either as autoantibody producing cells, or as B suppressor cells (Gilbert and Hoffman, 1983), are involved in this transferrable suppression, since depletions using MRC OX12, which binds to rat B lymphocytes, had no effect.

When MRC W3/25, which binds to T helper/inducer cells and macrophages, (Barclay, 1981), was used in depletion experiments, results were not significantly different to when control depletions were performed, as determined by Wilcoxin ranking. However, the recipients of MRC W3/25 depleted SC did appear to be polarised into two groups - those which were suppressed, and those not suppressed. Whether this polarisation was an artefact of insufficient numbers, or whether it represents a division in the ability of the recipients to be suppressed by MRC $W3/25^-$ cells, is not known. One explanation for the latter could be if suppression by $OX8^+$ cells acted through $T_h$ cells (or macrophages), which are present in the SC suspensions as well as the rat recipients. Any rat/rat variation in the accessible $T_h$ cells (or macrophages) in the recipients, could make the recipient rat dependent on transfer of MRC $W3/25^+$ cells with the MRC $OX8^+$ cells in the SC suspensions. In heterologous systems, suppression may act as the level of the $T_h$ cell (Sherr et al, 1979), or cells with a T helper/inducer phenotype may cause suppression (Dorf and Benacerraf, 1984). In addition, the action of some T suppressor cells is macrophage dependent (Yoshinaga, Yoshinaga and Waksman, 1982), while macrophages themselves may cause suppression (Allison, 1978).

Although in other systems transfer of antigen reactive T

lymphoblasts induces idiotype specific suppressor and cytotoxic cells (Binz and Wigzell, 1978; Flood et al, 1980) as well as anti-idiotypic antibodies (Nielson and Phillips, 1982a), their presence in convalescent spleens is unlikely to be a major explanation for the suppressive effect in this model because when antigen reactive blasts would be present, early in the disease, suppression could not be transferred, and such blasts would not be depleted by MRC OX8 antibody.

### 4.2.3. Anti-idiotypic Antibodies

Heterologous anti-idiotypic antibodies have been shown to suppress an autoimmune response in a number of systems, including experimental autoimmune tubulointerstitial nephritis (Brown, Carey and Colvin, 1979), and spontaneous autoimmune thyroiditis (Zanetti and Bigazzi, 1981). In addition, a mouse monoclonal antibody to an idiotype present in murine lupus has been shown to suppress both idiotype, the total anti-DNA antibodies, and retard the incidence of nephritis, although the effects on total anti-DNA antibodies were transient, possibly due to idiotype switch (Hahn and Ebling, 1984). For studies in $HgCl_2$ induced nephritis in the BN rat, a heterologous anti-idiotypic antisera was raised in rabbits to the anti-GBM antibodies present in kidney eluates from day 14 $HgCl_2$ treated rats, and was found to be capable of inhibiting *in vitro* binding of anti-GBM antibodies in both unlabelled and radiolabelled eluate, as well as serum, to solid phase GBM. Inhibition was not due to residual anti-IgG activity, as shown by the lack of inhibition by rabbit anti-rat IgG diluted to a similar anti-IgG activity as that remaining in the antisera to the eluate. Neither was inhibition due to any anti-GBM activity in the heterologous antisera binding to contaminating GBM fragments in the eluate, as anti-normal eluate,

with a greater anti-GBM activity, caused no inhibition. In addition the heterologous anti-idiotypic preparation was found by other workers to be capable of inhibiting GBM specific PFC formation (Chalopin and Lockwood, 1984). Recent work by Dr. J. Savige, using a $F(ab')_2$ preparation of the day 14 eluate, has confirmed the *in vitro* inhibition of anti-GBM antibodies by rabbit anti-idiotypic antisera (personal communication).

This *in vitro* inhibition was similar to that described for heterologous anti-idiotypic antibodies in spontaneous thyroiditis in rats (Zanetti and Bigazzi, 1981). However, unlike the latter, or autoimmune tubulointerstitial nephritis in guinea pigs, repeatable *in vivo* suppression could not be demonstrated, even though similar protocols were used. Only in some experiments were some of the recipients found to be suppressed with respect to anti-GBM antibody. It is not known whether rats which were not suppressed made anti-GBM antibodies of different idiotypes than those to which the anti-idiotypic antisera was directed. Not all anti-idiotypic antibodies are suppressive (Eichmann and Rajewsky, 1975; Teitelbaum et al, 1984), and this may also have been a contributory factor in some experiments, as could interference by the oligoclonal response to the foreign proteins in recipients of both test and control rabbit sera. An *in vitro* test system of anti-GBM antibody synthesis was required to screen in more detail any such regulatory substances.

Auto-anti-idiotypic antibodies have also been found in some autoimmune diseases during convalescent or inactive phases (Abdou et al, 1980; Zouali and Eyquem, 1983) as well as during the immune response to foreign antigens (Geha, 1982). In addition they have been induced by injection of antigen reactive T lymphoblasts, which can transfer suppression in anti-TBM disease (Neilson and Phillips, 1982a). However, no auto-anti-idiotypic antibodies have been found

in untreated rats with anti-TBM disease, or in the anti-erythocyte model of autoimmunity in mice (Neilson and Phillips, 1982b; Naysmith et al, 1980). In $HgCl_2$ induced anti-GBM disease in BN rats, antigen augmentable plaques, which Goidl has suggested are a result of auto-anti-idiotypic antibodies (Goidl et al, 1979), were found during down regulation of the anti-GBM antibody response, and earlier in recipients of convalescent SC (Chalopin and Lockwood, 1984). This has been confirmed in recent work by Dr. J. Savige, and the antigen augmentable PFC response also shown to occur following the smaller secondary peaks of anti-GBM antibody (J. Savige - personal communication).

Difficulties were encountered in assaying for anti-idiotypic antibodies in the studies of this model described herein. However, the results of experiments where sequential sera from $HgCl_2$ treated BN rats were used to inhibit the binding of the $^{125}I$-eluate to antigen coated plates, would support the hypothesis that auto-anti-idiotypic antibodies occur in this model during convalescence. Such inhibition occurred not only when anti-GBM antibodies were present, due to competitive inhibition, but also coincided with falling antibody levels, while troughs of inhibition later in the disease coincided with rises in antibody levels.

If auto-anti-idiotypic antibodies occur during convalescence and are partly responsible, in addition to $T_s$ cells, for either the down regulation or resistance to rechallenge, then transfer of convalescent serum to normal animals prior to $HgCl_2$ treatment, should be protective. Pilot experiments to examine for this suppression did suggest a possible effect. However, the difficulty in absorbing out all anti-GBM antibody in large quantities of convalescent serum, together with examining whether the effect was due to anti-idiotypic antibodies, soluble T suppressor factors, as

in EAE (Beraud et al, 1982), or cytotoxic mediators, led to a search for a small scale *in vitro* anti-GBM antibody production system, to screen for substances capable of autoregulation.

In human autoimmune diseases, demonstration of the presence of auto-anti-idiotypic antibodies has also proved difficult. While they have been shown to be present during inactive stages of a patient with SLE (Zouali and Eyquem, 1983), a definitive demonstration of their presence during convalescence in Goodpasture's syndrome has been the subject of much recent work, but has proved elusive (J. Savige, C. Savage - personal communication).

## 4.3. IN VITRO ANTI-GBM ANTIBODY SYNTHESIS

### 4.3.1. IgG Anti-GBM Antibody Synthesis in Culture

In order to provide a sensitive test system, under defined conditions, for characterising both cellular and soluble inhibitors of antibody production, prior to their use *in vivo*, a spleen cell culture system of anti-GBM autoantibody synthesis was set up. Specific antibodies to 'foreign' antigens can be detected in cultures of lymphoid cells, both human and animal, whether they are polyclonally stimulated (Fauci and Pratt, 1976), or stimulated with antigen (Dosch and Gelfand, 1976; Delfraissey et al, 1977), or even apparently spontaneously (Mishell and Dutton, 1967). The detection systems may involve plaque forming cell assays for the antibody producing cells (Mishell and Dutton, 1967), or immunoassays such as the RIA, for secreted antibody (Morimoto, Rheinherz and Schlossman, 1981). More recently, autoantibodies have been synthesised in culture (Sawada and Talal, 1979; McGregor et al, 1979; Willcox, Newson-Davis and Calder, 1983).

*In vitro* antibody formation in the rat has often proved to be difficult, due to the presence of an inhibitor cell present in

normal spleens (Corvalan and Howard, 1978; Morisaki et al, 1983), and due to a requirement for IL2 (Rosenberg, Gilman and Feldman, 1982). However, data from Hirsch's experiments suggests that spleen cells from normal Lewis rats can be cultured to spontaneously produce an anti-TNP PFC response, both in the absence of additional IL2 or any procedure to remove the inhibitor cell (Hirsch et al, 1982).

In this study, IgG class anti-GBM antibody was shown to be spontaneously synthesised in cultures of spleen cells from $HgCl_2$ treated BN rats. This antibody was actively synthesised during the culture rather than being released from dying cells. The time course was short, with most antibody synthesised within the first three days, and there was no lag phase, which suggests that B cells secreting antibody were already fully differentiated and activated. The ease with which BN spleen cells treated *in vivo* with $HgCl_2$, could be induced to synthesise autoantibody in culture contrasts with Rosenberg's study of anti-immunoglobulin induced activation of spleen cells in culture, where BN SC were poor responders, giving only one quarter of the response of Lewis SC (Rosenberg and Feldman, 1982), and also Hirsch's study, in which spontaneous anti-TNP PFC from cultured BN SC were far fewer than from Lewis SC, both for direct and indirect plaques. However, in the latter study, $HgCl_2$ *in vitro*, increased the BN PFC response but not the Lewis response (Hirsch et al, 1982).

The appearance of spleen cells capable of synthesising anti-GBM antibody in culture coincided with the timing of the splenic PFC response. However, while the PFC response had begun to wane after day 10, spleen cells from day 16 $HgCl_2$ treated rats could still synthesise antibody in culture. It has been suggested that down regulation of the splenic anti-GBM PFC response after day 10, is due

to masking by anti-idiotypic antibody, since added antigen can reveal plaques even at day 14 or later (Chalopin and Lockwood, 1984). This effect does not appear to prevent the spleen cells synthesising antibody in culture.

The effect of washing cells after 6 or 24 hours in culture was examined because (1) antigens/inhibitors present throughout the culture period could cause interference in the RIA subsequently employed, and (2) such washing would simulate stresses on lymphocytes during plasma exchange therapy in humans - such therapy has been suggested to contribute to down regulation of the anti-GBM antibody response as well as its physical removal (Rees and Lockwood, 1986). Cells treated in this manner subsequently synthesised less antibody than unwashed cells. This was partly due to removal of antibody synthesised before washing, but additional factors, such as removal of growth factors released early in the culture, or an inhibitory effect analogous to that suggested for plasma exchange, may be important.

The removal of inhibitory adherent cells required in other reports of rat spleen cell cultures (Corvalan and Howard, 1978; Morisaki et al, 1983), was found to be unnecessary in this system. In fact removal of Sephadex G-10 adherent cells caused a reduction in subsequent antibody synthesis. This may be because the macrophages which were removed were required as secretors of growth factors. Rosenberg has previously shown that anti-Ig induced, non-specific proliferation of rat spleen cells is macrophage and T dependent, but that replacement of macrophages and T cells with IL2, restores the proliferative response of the B cells (Rosenberg and Feldman, 1982). In addition, spontaneous anti-DNA antibody production in mouse spleen cell cultures has been found to be both macrophage and T cell dependent (Sawada and Talal, 1979).

4.3.2. Regulation of Anti-GBM Antibody Synthesis In Vitro

Systems of *in vitro* specific antibody synthesis have previously been used to dissect immunoregulation of B cell responses. While T suppressor cells may suppress PFC responses in rat cell culture in a non-specific manner (Rosenberg, Gilman and Feldman, 1982), anti-idiotypic antibodies have been shown to specifically stimulate antigen specific antibody responses *in vitro* (Weissberger, Shenk and Dickler, 1983), or suppress idiotype synthesis *in vitro* (Takeuchi et al, 1985). In the former report, evidence is given for stimulation of T helper cell function, and in another report, incubation of anti-idiotypes with SC *in vitro* induces T suppressor cells which suppress a specific secondary IgE response *in vivo* (Malley and Dresser, 1983).

However, in the studies described herein, the effect of heterologous anti-idiotypic antisera on *in vitro* anti-GBM synthesis was highly variable, as had occurred in *in vivo* studies of its effects. It is not known whether SC in cultures in which suppression did not occur, produced an idiotype of different specificity to that recognised by the heterologous anti-idiotypic antisera. Since the presence of heterologous serum remaining in the culture when supernatants were assayed, caused interference in the RIA, by either inhibiting preformed antibody, or increasing non-specific binding, in some experiments the heterologous serum was washed out after 6 hours, and culture continued. However, variability in the suppressive capacity of the heterologous anti-idiotype remained. Use of purified anti-idiotype may have circumvented some of these problems, if heterologous serum alone caused polyclonal stimulation.

In rat spleen cell cultures, addition of antibodies to rat $\lambda$ light chains, IgG, IgM or IgD has been reported to stimulate B

lymphocytes and augment $^{3}$H-TdR incorporation (Rosenberg, Gilman and Feldman, 1982; Rosenberg and Feldman, 1982). F(ab')$_2$ preparations of anti-Ig cause a similar stimulation. However, these effects are non-specific. Interestingly, BN spleen cells are far less susceptible to stimulation by anti-Ig reagents than Lewis spleen cells (Rosenberg and Feldman, 1982). It is therefore not surprising that in the studies described herein, addition of anti-rat IgG to cultures failed to provoke stimulation of anti-GBM antibody production. In fact, the effect of anti-rat IgG was highly variable, with neither overall inhibition or stimulation being noted.

To investigate the possibility that soluble factors in convalescent serum, whether auto-anti-idiotypes or T suppressor factors, could suppress in vitro anti-GBM antibody synthesis, and so confirm the preliminary finding in the in vivo model, but in a more defined system, convalescent serum was added at day 0, to the spleen cell cultures from $HgCl_2$ treated BN rats. Convalescent serum was repeatedly absorbed with solid phase GBM to remove any anti-GBM idiotypes. However, the presence of both convalescent and normal rat serum in supernatants during assaying caused high background binding. Despite this, convalescent serum always suppressed the antibody synthesis compared to NRS.

When sera were present only for the first 6 or 24 hours of culture, again convalescent serum suppressed subsequent anti-GBM antibody production compared to NRS, although antibody production was low, due to the effect of washing cells during culture, as described above. It should be acknowledged that the effect of convalescent serum may have been due to other more non-specific factors than anti-idiotypes or antigen specific T suppressor factors, such as cytotoxic compounds generated from the $HgCl_2$

treatment, or the presence of acute phase reactants. Difficulties in developing a system where serum still had an effect, while sufficient antibody was produced, to examine suppression, prevented this phenomenon from being examined in more detail.

T suppressor cells have been shown to suppress an *in vitro* PFC response to SRBC (Rosenberg, Gilman and Feldman, 1982), and in this model were shown to be capable of transferring antigen specific suppression from convalescent rats to normal recipients. Attempts to dissect this phenomenon, using *in vitro* anti-GBM antibody synthesis, did not prove to be beneficial. While unfractionated convalescent spleen cells were often found to cause partial suppression of the *in vitro* antibody response, normal cells were also occasionally observed to cause suppression, especially in high concentrations. It is possible that this effect of normal cells was similar to the non-specific suppression mediated by lysed normal cells, as reported by Goodman and Weigle (1981a), and may parallel the finding that some recipients of normal spleen cells *in vivo* were also occasionally suppressed.

This cell mediated suppression *in vitro* may have been due to either antigen specific T suppressor cells as in the *in vivo* transfer experiments, or non-specifically mediated by macrophages. Removal of macrophages on a Sephadex G-10 adherent column failed to differentiate these two possibilities. Similarly T cell enrichment of the convalescent cells gave only variable results.

Therefore, although a system was devised for synthesising anti-GBM antibody *in vitro*, it failed to be susceptible to immunoregulation by anti-idiotypic antibodies, or convalescent cells, and so failed to allow a more detailed examination of the immunoregulation found *in vivo*, but under controlled conditions. Other cell culture systems, perhaps involving *de novo* induction of anti-GBM

antibody synthesis in vitro may prove more useful.

### 4.3.3. IgM Class Activity and Normal Spleen Cells

Spontaneous synthesis of specific antibodies has been previously reported both from apparently normal animals, although perhaps stimulated by the FCS in culture medium (Mishell and Dutton, 1967) and in the case of autoantibodies, by patients with autoimmune disease (McGregor et al, 1979; Willcox, Newson-Davis and Calder, 1983). Spontaneous anti-DNA antibody production has been found in cultures of both autoimmune and normal mice (Sawada and Talal, 1979).

The culture of SC from normal or $HgCl_2$ or antigen treated BN rats, but not normal Wistar or Lewis rats, was found to result in the spontaneous production of an IgM activity, which was reactive with GBM. However, unlike the spontaneous anti-double stranded DNA antibody in mice (Sawada and Talal, 1979), extensive inhibition tests with both solid phase and fluid phase GBM, failed to confirm the specificity of the antibody. However, a heterologous anti-idiotypic antisera, raised in rabbits to day 14 kidney eluate, did inhibit the binding of the IgM 'antibody' in the RIA, while anti-normal eluate or normal rabbit serum did not. It is not known whether the difficulty in obtaining specific inhibition of the 'antibody' with antigen was due to low affinity.

The IgM reactivity from normal SC or GBM immunised BN SC could be differentiated on the basis of their response to coculture with antigen, since SC from GBM immunised rats were far more sensitive to the inhibitory effects of GBM in culture. Washing of cells after 24 hours also prevented further production of anti-GBM activity, even though the IgM activity was rarely detectable until after 24 hr in unmanipulated cultures. This effect may have been

due to removal of secreted growth factors, as described in the IgG class anti-GBM antibody synthesis system.

This IgM reactivity to GBM is particularly interesting even though its specificity lacks complete definition. In spontaneous *in vitro* anti-DNA antibody production in mice, it is the non-responder strains or young animals from responder strains (which would not yet have SLE) which produce primarily IgM, while the older animals of responder strains (presumably with active disease) produce IgG class antibodies (Sawada and Talal, 1979). The spontaneous IgM autoantibody production from normal BN SC but not from non-responder strains may indicate the level of precursor cells for the autoantibody. In addition, it may also represent the ease with which regulatory mechanisms are abrogated when the cells are placed in tissue culture and therefore in contact with plastic or FCS; or *in vivo* under $HgCl_2$ stimulation.

## 4.4. Regulation of Anti-GBM Antibody in $HgCl_2$ Induced Disease in BN Rats, and Treatment of Human Autoimmune Disease

Studies of regulation of anti-GBM autoantibody synthesis in the $HgCl_2$ treated BN rat have implicated a role for T suppressor cells and possibly anti-idiotypic antibodies. The development of specific forms of treatment for anti-GBM nephritis in humans, or in fact any human autoimmune disease, may one day be based on similar studies. Such a treatment could, if the pathogenic antibodies exhibit cross reactive idiotypes, involve purification of T suppressor cells or anti-idiotypic antibodies from convalescent patients undergoing spontaneous down regulation of the autoimmune response. Use of anti-idiotypic antibodies, whether heterologous, homologous or autologous, would require stringent testing, in order to segregate stimulatory from suppressive anti-idiotypes. Using

appropriate conditions *in vitro*, it may even be possible to specifically stimulate the patients own cells, and select out antigen specific T suppressor cells for clonal expansion and therapeutic manipulation. Such cells could suppress even private idiotypes and if fused to a continuous cell line and placed within membranes, might be capable of continuous secretion of antigen specific regulatory factors. However, such therapeutic developments await a greater understanding of the autoimmune response as well as the pathogenicity of various types of antibody, both *in vivo* and *in vitro* in both experimental animal models, and humans.

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In summary, the studies on autoantibody regulation described in this thesis show that $HgCl_2$ treatment in the BN rat results in a rapidly self limiting anti-GBM antibody response as well as the transient appearance of circulating immune complexes. Decreases in peripheral blood T suppressor cells precede the appearance of anti-GBM antibody, while autoregulation of antibody is associated with above normal numbers of $T_S$ cells. Convalescent rats are resistant to rechallenge with $HgCl_2$, a phenomenon which may be due to active suppression by T suppressor cells, since spleen cell transfers from convalescent rats into naive recipients manifest an antigen specific suppression. In addition, a role for anti-idiotypic antibodies in regulation is suggested. Anti-GBM antibody synthesis also occurs *in vitro*, but is not as susceptible to such regulation.

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