ANALYSIS OF B LYMPHOCYTE FUNCTION IN PROSPECTIVE RENAL TRANSPLANT PATIENTS

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SUMMARY

It is well established that uraemia impairs the in vivo cell-mediated immune responses while the in vitro lymphocyte responses of patients on maintenance dialysis have been reported to be impaired or normal. The work in this thesis deals predominantly with production of immunoglobulin in vitro by peripheral blood mononuclear cells obtained from normal and uraemic subjects.

The protein A plaque forming cell assay was utilised to enumerate immunoglobulin secreting cells and several parameters affecting its performance were examined. Thus, it was found that plaque forming cell numbers were dependent on the concentrations of sheep red blood cells, anti-serum, DEAE-dextran and complement while PEG 6000 was shown to improve the quality of the plaques. The optimal conditions for storing the cells before plating and incubating them after plating were also established and in addition, it was shown that cryopreservation does not affect plaque forming cell activity.

The optimal conditions for both proliferation and immunoglobulin production in response to mitogenic stimulation were also examined and once again the suitability of cryopreserved peripheral blood mononuclears for use in mitogen-stimulated cultures was confirmed. A normal range for both spontaneous and mitogen induced plaque forming cells was established and it was shown that pokeweed mitogen(PWM) was the most effective polyclonal B cell activator in inducing plaque forming cells while

Epstein-Barr-Virus(EBV) induced similar IgM-PFC as PWM. Co-stimulation of control peripheral blood mononuclear cells with Concavalin A(ConA) showed that both PWM and Staphylococcus Aureus Cowan I(SAC) cultures were suppressed although the former proved to be much more sensitive. Steroids are known to inhibit suppressor T cells and when methyl-prednisolone was added in PWM and SAC cultures of control peripheral blood mononuclear cells, PWM induced production of immunoglobulin was more readily enhanced. I propose that these polyclonal B cell activators differ in their susceptibility to regulation by suppressor T cells. When indomethacin was added in the cultures to inhibit prostaglandin synthesis, it was observed that SAC responses were slighty but non-significantly enhanced.

Uraemia was not found to have a significant effect on the number or proportion of lymphocyte populations but it impaired the proliferative responses to PHA and PWM. However, the relative in vitro immunosuppressive effect of steroids was not found to be stronger in these cultures. EBV and PWM induced plaque formation was normal in uraemic patients while both spontaneous and SAC induced immunoglobulin production were reduced. In addition, the combination of PWM and SAC did not produce the synergistic effect found in control cultures. Pre-incubation of control cells with uraemic serum affected the quality and suppressed slightly the numbers of PWM-induced plaque forming cells. Addition of methyl-prednisolone to SAC cultures did not produce the enhancing effect found in controls indicating that the reduced SAC responses in uraemia were not caused by

increased suppressor T cell activity. However, the SAC responses of uraemic peripheral blood mononuclear cells seemed to benefit more by the addition of indomethacin than the SAC responses of control cells. My results indicate strongly that the reduced production of immunoglobulin in uraemia is not due to reduced numbers of lymphocytes but reflects a true functional abnormality and the observed reduced proliferative responses of uraemic peripheral blood mononuclears to T cell mitogens indicate that T lymphocyte function is impaired. The studies using polyclonal B cell activators suggested that B cell function and T-B cell co-operation remained intact in uraemic patients and indicated that the defective immunoglobulin responses in both the spontaneous and SAC system were due to a functional abnormality of the helper T cell.

Third party blood transfusions were shown to reduce PWM-driven B cell differentiation and to enhance(in some patients) the spontaneous PFC responses. SAC induced PFC responses were not affected and since SAC responses are less affected than PWM responses by suppressor T cell activity, it was concluded that the differential inhibitory effects of blood transfusions on B cell differentiation into immunoglobulin secreting cells was due to an increase in suppressor T cell activity.

In studies of spontaneous immunoglobulin production in transplanted patients, IgG-PFC were found to increase during rejection episodes. Furthermore, a greater number of azathioprine treated patients had increased plaque forming cell activity compared with cyclosporine A treated recipients and this correlated very well with the number of rejection episodes in the two groups.

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DECLARATION

The experimental work presented in this thesis was completed by the author with the exception of the surface marker assays and the preparation of SAC which were carried out with the assistance of Mr Eric Galloway.

PUBLICATIONS

Parts of this thesis have been used in the following publications:

1.Degiannis, D., Tsakiris, D., Briggs, J.D., Mowat, A.McI., Junor, B.J.R. & Parrott, D.M.V. (1985)
Comparison of Protein-A Plaque Assay in Cyclosporine- and Azathioprine-Treated Renal Allograft Recipients.
Transplantation Proceedings, 17(6):2593-2596.

2.Degiannis, D., Tsakiris, D., Briggs, J.D., Mowat, A.McI., Bradley, J.A. & Junor,B.J.R.
Monitoring the Effect of Pre-Transplant Transfusion on IgG Secreting Cells Using a Protein A Plaque Assay.
Transplantation Proceedings(In Press, 1986).

3.Tsakiris, D., Watson, M.A., Degiannis, D., Briggs, J.D. & Junor, B.J.R.
Association Between Blood Transfusion, HLA-DRW6, and Response to Dinitrochlorobenzene(DNCB) Skin Test.
Transplantation Proceedings,(In Press, 1987).

ABBREVIATIONS

B Lymphocyte	Bursa or Bone Marrow Derived cell
BCDF	B Cell Differentiation Factor
BCGF	B Cell growth Factor
BT	Blood Transfusion
CAPD	Continuous Ambulatory Peritoneal Dialysis
cmm	Cubic milli-meter
CNB	Core Needle Biopsy
CO2	Carbon Dioxide
ConA	Concavalin A
CPM	Counts per Minute
CTL	Cytotoxic T Lymphocytes
DEAE-Dextran	Diethylaminoethyl-Dextran
dL	Deci-liter
DMSO	Dimethyl-sulfoxide
DTH	Delayed Type Hypersensitivity
EBNA	Epstein-Barr-Virus Nuclear Antigen
EBV	Epstein-Barr-Virus
Fab2	Pepsin Cleaved Fragment of Immunoglobulin
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
FNA	Fine Needle Aspiration
hr(s)	Hour(s)
Ig	Immunoglobulin
IL-2	Interleukin-2
INDO	Indomethacin
ISC	Immunoglobulin Secreting Cell
Kg	Kilo-gram

KLH	Keyhole Limpet Haemocyanin
LPS	Lipopolysaccharide
M	Molar
µg	µicro-gram
mg	Milli-gram
MHC	Major Histocompatibility Complex
µl	µicro-liter
ml	Milli-liter
MLR	Mixed Lymphocyte Reaction
mm	Milli-meter
µmol	µicromolar
mmol	Millimolar
MP	Methyl-Prednisolone
6-MP	6-Mercaptopurine
MRBC	Mouse Red Blood Cell
N	Normal
n.s.	Non-Significant
OKT3	Monoclonal Antibody Recognising Human T Cell
OKT4	Monoclonal Antibody Recognising Human Helper T cell
OKT8	Monoclonal Antibody Recognising Human Suppressor/Cytotoxic T cell
PBA	Polyclonal B Cell Activator
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PEG-6000	Polyethylene Glycol 6000
PFC	Plaque Forming Cell
PGE2	Prostaglandin E2

PHA	Phytohaemagglutinin
PPD	Purified Protein Derivative of Tuberculin
PWM	Pokeweed Mitogen
RHPA	Reverse Haemolytic Plaque Assay
SAC	Staphylococcus Aureus Cowan I
SD	Standard Deviation
SEM	Standard Error of Mean
SIg	Surface Immunoglobulin
SKSD	Streptokinase/Streptodornase
SRBC	Sheep Red Blood Cell
T Lymphocyte	Thymus Derived Cell
TT	Tetanus Toxoid
Tx	Transplantation
VLDL	Very Low Density Lipoprotein

CHAPIER I

INTRODUCTION

URAEMIA AND ITS TREATMENT

The kidneys are the organs responsible for the regulation of fluid and electrolyte balance, the retention and production of substances vital to body economy and the elimination of metabolic waste products, including many endogenous toxins and drugs. The most important of these wastes are the breakdown products of protein metabolism, such as urea and creatinine, which become toxic if they accumulate in large amounts due to renal failure. Renal failure may arise when the renal parenchyma is damaged either directly by disease or drugs or, indirectly, when extrarenal disorders affect adequate renal blood flow, or when obstruction at any point of the urinary tract prevents the outflow of urine. The impairment of renal function may be of variable severity and it may be acute or chronic. If untreated, the syndrome which results presents a serious clinical problem and may be life-threatening. Although the term "uraemia" is used to describe this syndrome, the symptoms of renal failure are not only attributable to the retention of urea but also to disturbances of water and electrolyte balance, to the accumulation of other toxic products, and to arterial hypertension. In this thesis, "uraemia" is used as a general term to describe the clinical state associated with chronic renal failure.

The principal methods of treatment of chronic renal failure are haemodialysis, continuous ambulatory peritoneal dialysis(CAPD) and transplantation. Both haemodialysis and

CAPD are used to preserve the lives of uraemic patients by allowing uraemic toxins to diffuse across a semipermeable membrane from high concentrations in the patient's blood to low concentrations in dialysis fluid. In haemodialysis this semipermeable membrane is a synthetic membrane while in CAPD the patient's peritoneum is employed as the dialysis membrane. Although both these methods of treatment are effective means of rehabilitation, they do not offer a permanent solution to the problem. If the patient is suitable for transplantation they are also used as a maintenance therapy until a suitable graft is found.

The first kidney transplantations were performed in dogs and goats by Ullmann(Hamilton, 1984) and the first recorded kidney transplantations in humans were performed when Jaboulay implanted pig or goat xenografts in the arm and thigh of uraemic patients(Jaboulay, 1906). Many unsuccessful attempts were made in the following fifty years and the breakthrough came in the early 1950s when cadaver kidneys were transplanted into patients with end-stage chronic renal failure(Hume et al, 1955). Although, some kidneys never functioned, others functioned well for a time and most of the symptoms and signs of uraemia were reversed. However, after three to five months most of the transplanted kidneys were rejected due to an immunological reaction against foreign antigens of the graft. Cortisone and ACTH were administered to suppress these rejection episodes but it was believed that the co-existing uraemia also produced

immunosuppression and that this was mainly responsible for the eventual favourable outcome. Although most of these early conclusions were later confirmed, rejection reactions limited the use of organ transplantation and until 1959 very few operations were performed. Around that time total body irradiation and 6-mercaptopurine(6-MP) were successfully introduced as means of suppressing the rejection episodes(Calne, 1960 & Zukoski et al, 1960) and, shortly afterwards azathioprine(Calne et al, 1962, Murray et al, 1963) and prednisolone in combination with 6-MP(Kuss et al, 1962) were first used for this purpose. Since then, the results have improved progressively and transplantation has become an accepted method of treatment of uraemic patients.

IMMUNE ABNORMALITIES IN URAEMIA

Uraemic patients have abnormalities of virtually every organ system and in the last three decades many immunological studies have shown that the immune response is also impaired. Clinically, this is evidenced by a high incidence of malignancies(Matas et al, 1975) and infections(Merrill, 1968) and the latter is a very common cause of morbidity and death. Many workers have examined cellular and humoral immunity of uraemic subjects in vivo and, more recently, attention has been given to the study of lymphocyte function in vitro and to the study of possible immunosuppressive factors in the uraemic serum. Although many different abnormalities have been described, it is

generally agreed that there is a decrease in the intensity of cellular and humoral responses both in vivo and in vitro. In addition, several immunosuppressive factors have been detected and some of these have been isolated.

1.IN VIVO STUDIES OF IMMUNITY IN URAEMIA

The first indications that uraemic patients had suppressed immune responsiveness in vivo came from studies by Hume et al(1955) who reported prolonged kidney allograft survival in patients with end-stage chronic renal failure and, as noted above it was suggested that immunosuppression due to the uraemia was responsible for this finding. In 1957, Dammin et al observed prolonged skin allograft survival in six uraemic patients and similar observations were subsequently made in dogs(Mannick et al, 1960) and in rabbits(Smiddy et al, 1961). Following this, Kirkpatrick et al(1964) found that both delayed and immediate cutaneous hypersensitivity were depressed in patients with renal failure. In 1969 Huber et al, studying delayed dermal hypersensitivity, confirmed that 50% of uraemic patients were anergic and Selroos et al(1973) then showed that the degree of skin test response decreased with increasing severity of uraemia. Boulton-Jones et al(1973) then showed that both delayed type hypersensitivity skin reactions and antibody responses were depressed and, although, one report (Kauffman et al, 1975) suggested that 78% of the uraemic patients had intact delayed dermal hypersensitivity, most

recent studies have confirmed the depressed cutaneous hypersensitivity in uraemia(Rolley, 1978, Cascianni et al, 1978, Ala-Houhala et al, 1979).

When antibody responses were first examined in uraemia, early reports by Balch et al(1955) and Stollof et al(1958) suggested that uraemic patients had normal antibody responses to foreign antigens such as tetanus toxoid and diphtheria toxoid. However, Wilson et al(1965) reported that the production of humoral antibodies to typhoid vaccine was depressed in patients with renal failure while Weeke et al(1971) found that one third of haemodialysis patients had low levels of immunoglobulins in the serum. Subsequently, it was shown that IgA and IgM levels in uraemic patients were within the normal range but IgG levels were variable and IgE levels were low(Cascianni et al, 1978). The finding of low IgE levels has since been confirmed, but interestingly, does not appear to be a correlation between IgE levels and length of dialysis treatment(Hallgren et al, 1982).

Studies of antibody responses in experimental animals with uraemia have produced conflicting results and Gowland & Smiddy(1962) found that the primary but not the secondary response to bovine serum albumin(BSA) was decreased in uraemic rabbits. Souhami(1973) showed that both primary and secondary antibody responses to sheep red blood cells(SRBC) and BSA were decreased in mice. In contrast, Nelson et al(1980) showed that the humoral response of uraemic animals was comparable to that of sham operated or control animals

by challenging uraemic rats with bacterial, viral, T-cell dependent and T-cell independent antigens. A possible explanation of these discrepancies may come from the study of Mezzano et al(1982) who found that while primary antibody responses to BSA were reduced in uraemic rats irrespective of the route of immunisation or the adjuvant used, the secondary antibody response was depressed only if antigen was given with a weak adjuvant. Interestingly, these authors also showed that it was easier to suppress the primary antibody response of uraemic rats by intravenous administration of soluble BSA and that only uraemic animals had suppression of their secondary antibody responses under these conditions.

In summary, while it is clear that delayed type hypersensitivity(DTH) is impaired in uraemic subjects there is still controversy over the humoral immune status of these subjects. Although several reports do indicate that antibody responses are impaired, it seems that this may affect the primary and secondary responses differently and may vary with the type of antigen and the route by which it is encountered. Furthermore, although studies discussed above have contributed to defining the immunosuppression of uraemia, they have not provided information about individual cells or the mechanisms responsible for the altered immune responsiveness reported in uraemia. Therefore, more recent studies have investigated lymphocyte number and function in vitro in an attempt to analyse and explain in more detail the immune defects associated with uraemia.

2.STUDIES OF LYMPHOCYTE POPULATIONS

A. BACKGROUND

Lymphocytes,macrophages,and granulocytes are the major cellular components of the immune response and abnormally low numbers of white blood cells may be associated with reduced immunological responsiveness. For many years, total white blood cell and lymphocyte counts provided the only information about immune status and these tests are still used routinely as a rapid means of screening the immune status of patients with disorders like chronic renal failure. Nevertheless, in recent years, the ability to differentiate between lymphocyte populations has led to more detailed investigations of the mechanisms involved.

Lymphocytes population can be divided into two major classes of immunocompetent cells: The T(thymus-derived) and B(bursa or bone marrow-derived) lymphocyte. T lymphocytes differentiate under the influence of thymus in foetal life and, after sensitisation act either directly as cytotoxic lymphocytes or indirectly through the cellular products called lymphokines. In addition, several classes of regulatory T cells are essential for the normal maturation and control of the immune response, the most important being the helper T cells and the suppressor T cells. These populations differ in phenotype and as they can be enumerated separately, this has greatly helped the diagnosis and investigation of various immunodeficiency states.

In mammals, B lymphocytes probably differentiate in the bone-marrow and their role in the immune response is

mediated entirely by their secreted cellular products, the antibodies. In addition, cell surface antibody also acts as an antigen-specific receptor which determines triggering and activation of the B cell. As noted above, the production of antibody is not only dependent on B cells, but also requires functional helper T cells for efficient proliferation and differentiation of antibody secreting cells. In addition, suppressor T cells may have a negative effect on antibody production.

T and B lymphocytes are morphologically identical but can be differentiated by the presence of specific surface or cytoplasmic markers. B lymphocytes are identified by the presence of surface immunoglobulin while, T lymphocytes can be identified by their ability to form rosettes with sheep red blood cells(SRBC) or by the OKT3 monoclonal antibody. Within the T cell population, helper T cells and suppressor T cells are differentiated by using the OKT4 and OKT8 monoclonal antibodies respectively. During the last ten years these techniques have been used extensively to investigate the immune status of uraemic patients.

B. LYMPHOCYTE COUNTS IN URAEMIA

Lymphopenia was first recognised as a complication of acute renal failure by Jensson et al(1958) and this was later confirmed for patients with chronic renal failure by Riis et al(1959). Subsequently Wilson et al(1965) found that advanced uraemia reduces the number of circulating

lymphocytes, despite a normal number of neutrophils. Subsequently, Boulton-Jones et al(1973) showed that patients with severe renal failure were lymphopenic and this was not improved by dialysis. Since then, the association of lymphopenia has been suggested by many groups, although its exact incidence may vary from 14% to virtually all patients(Kauffman et al, 1975, Cascianni et al, 1978, Lortan et al, 1982, Raska et al, 1983).

In recent years studies of lymphocyte numbers in uraemia have focussed on which lymphocyte population accounts for the reduced immune response. In the first study of this type, it was shown that only two of the twenty eight uraemic patients had diminished numbers of proportions of T cells, as assessed by their ability to form rosettes with SRBC(Kauffman et al, 1975). Using the same technique, Quadracci et al(1976) found that the total number of both T and B lymphocytes was significantly reduced in the peripheral blood of uraemic patients and the proportion of B cells was also reduced. Although, Kunori et al(1980) confirmed that the proportion of T cells was normal, they found also normal proportions of B cells. The first study using monoclonal antibodies to enumerate T lymphocytes also reported an absolute T and B cell lymphopenia in most uraemic patients and found a significant reduction in the number of T4+ cells(helper). The number of T8+(suppressor) cells was not different from that found in normal controls although their percentage was slightly higher than

controls(Lortan et al, 1982). In a more extensive study, Raska et al(1983) used monoclonal antibodies to study T cell subpopulations in uraemic patients in relation to age and the duration of haemodialysis. In patients under thirty years of age and over sixty, the absolute number of helper T cells was markedly reduced, whereas the number of suppressor/cytotoxic T cells was not changed. In contrast, both subpopulations were reduced in patients between thirty and sixty years old. The absolute number of T cells and the absolute count of helper T cells were lower in patients on dialysis for more than one year.

In summary, these studies indicated that uraemic patients have lymphopenia which affects particularly the numbers of B lymphocytes and helper/inducer T cells and it is still unclear whether or not suppressor/cytotoxic T cells are also depleted.

3.STUDIES OF LYMPHOCYTE FUNCTION IN VITRO

I. T CELL FUCTION

Response to Mitogens

While counts of lymphocyte numbers can provide some information on immune status, this can be achieved more readily by assays of lymphocyte function. Many different techniques are currently being used to assess the function of both T and B cells the majority of which measure the

ability of lymphocytes to proliferate in response to an antigenic or mitogenic stimulus. Phytohaemagglutinin A(PHA) has been used most widely to study T lymphocyte responsiveness in uraemic patients and many workers have shown that PHA responses of uraemic lymphocytes are depressed(Huber et al, 1969, Nakhla & Goggin, 1973, Kauffman et al, 1975, Quadracci et al, 1976, Holdsworth et al, 1978, Kunori et al, 1980, Alevy et al, 1981, Alevy et al, 1983, Langhoff & Ladefoged, 1984, Raskova et al, 1984). Although others have found that PHA responses may remain unaffected(Kasakura & Lowenstein, 1967, Daniels et al, 1971, Boulton-Jones et al, 1973, Sengar et al, 1975) the response of uraemic peripheral blood lymphocytes to Concavalin A(ConA) has consistently been shown to be depressed(Quadracci et al, 1976, Stewart & Miller, 1980,Alevy et al, 1981,Alevy et al, 1983). Therefore, these studies of T lymphocyte activation indicate that T lymphocyte function in vitro may be impaired in uraemic patients and it is likely that the discrepancies between different reports are because chronic renal failure has many aetiologies while lymphocyte function may be affected by the different treatment regimes, such as chemotherapy, haemodialysis, CAPD, blood transfusions, surgery and renal transplantation. This is supported by a report from Holdsworth et al(1978) who found that the poor responsiveness of uraemic lymphocytes was corrected for around 20 hours after each session of haemodialysis. Thus, it is clear that the time at which the sample was taken could markedly influence the results of in vitro tests. In

addition, Byron et al(1976) found that the blastogenic response to PHA in uraemic patients appeared to be delayed relative to the controls and suggested that differences in the pattern of response may account for inconsistencies in reports.

Alloantigen Responses

Although only a minute proportion of T cells will respond to the majority of specific antigens, up to 10% of T cells will respond to alloantigens coded by the major histocompatibility complex(MHC). This unusually high number means that alloreactive T cell responses provide another convenient means of assessing T cell reactivity in vitro. The one way mixed lymphocyte reaction(MLR) system has been used to investigate the reactivity of lymphocytes from patients with uraemia. Kasakura & Lowenstein(1967), Kunori et al(1980),and Alevy et al(1984) have shown that uraemic lymphocytes respond poorly to alloantigens and these findings correlate well with the depressed mitogen responses discussed above.

Antigen Responses

Whereas mitogens stimulate a significant proportion of the lymphocyte population, antigens stimulate only those lymphocytes which are sensitised to the antigen employed. In the majority of cases, the antigens employed to study lymphocyte activation in vitro have been those used for skin

testing in vivo, including tetanus toxoid(TT), candida albicans, streptokinase/streptodornase(SKSD), keyhole limpet hemocyamin(KLH) and purified protein derivative of tuberculin(PPD). In 1973, Boulton-Jones et al, Selroos et al, and Nakhla & Goggin, reported that the lymphocytes from uraemic patients responded poorly to antigens like PPD, SKSD or KLH and Selroos et al also found that there was no correlation between in vivo skin test reactivity and in vitro blast transformation to PPD.

All these studies provided information about T cell function in vitro and it is generally agreed that the proliferative responses of uraemic T lymphocytes in response to antigens, mitogens, and alloantigens coded by the MHC, are impaired.

II. B CELL FUNCTION

A.BACKGROUND

POLYCLONAL B CELL ACTIVATION

The provision that studies of lymphocyte number and phenotype provide only limited information on their behaviour in vivo is especially relevant when considering B cell function because the phenotype of functional B cell subpopulations is poorly defined. Therefore, considerable efforts have been made to develop sensitive assays which can dissect B cell function in vitro. These assays all rely heavily on the use of polyclonal B cell activators which

stimulate B cells in different ways to produce immunoglobulin. Many authors use the term polyclonal B cell activator instead of the term mitogen since mitogenesis is not regarded by some as a prerequisite for B cell activation(Coutinho & Moller, 1975, Janossy & Greaves, 1975). As with T cell mitogens, polyclonal B cell activators are substances which stimulate a large proportion of B cells and so their use allows a large proportion of the total B cell population to be studied. Although there is a wide range of polyclonal B cell activators, including lipopolysaccharide(LPS), anti-IgM etc, only a small group is particularly useful in dissecting mechanisms of B cell activation and differentiation under clinical conditions.

Pokeweed Mitogen

The first polyclonal B cell activator to be used in human studies was the mitogen obtained from pokeweed(PWM). In 1966, Chessin et al and, in 1967, Douglas et al found that PWM induces plasma blast development and it was shown subsequently that PWM induced both immunoglobulin synthesis and secretion as well as B cell proliferation(Greaves & Janossy, 1972, Wu et al, 1973, Waldmann et al, 1974). Early studies also indicated that T cells contributed to the response to the PWM and it is known that there is an absolute requirement for T cell help in the proliferation and differentiation of B cells in response to PWM(Janossy & Greaves, 1975, Fauci et al, 1976). Thus, PWM is a useful means of examining T cell dependent B cell function.

Staphylococcus Aureus Cowan I

Another polyclonal B cell activator which has been used increasingly in recent years is Staphylococcal protein A following work which showed that an insoluble preparation of protein A when presented on formalinised Staphylococcus aureus Cowan Type I bacteria(SAC) stimulated purified human B cells(Forsgren et al, 1976). Ringden & Rynnel-Dagoo(1978) presented evidence that the soluble form of protein A was mitogenic for both T and B cells and it has also been shown that T cell depleted peripheral blood mononuclear cells proliferate in response to SAC. Nevertheless, there is no differentiation into immunoglobulin secreting cells unless autologous T cells are added to the T cell-depleted population(Falkoff et al, 1982). Thus, the proliferative response of B cells to SAC is entirely T cell independent but differentiation into immunoglobulin secreting cells is T dependent. For these reasons, Cowan I bacteria are used in human immunological studies to analyse the regulation of both B cell proliferation and differentiation.

Epstein-Barr virus

The final polyclonal B cell activator in the group of major clinical importance is Epstein-Barr virus(EBV). All peripheral blood B cells bind EBV via a receptor whose structure is similar but not identical to the C3b receptor(Greaves et al, 1975), and blast transformation and DNA synthesis occur shortly after expression of Epstein-Barr virus nuclear antigen(EBNA, Einhorn & Ernberg, 1978). As

activation of B cells by EBV is not dependent on T cells and is not regulated by suppressor T cells(Bird & Britton, 1979a), EBV has been widely used as an entirely T cell independent polyclonal B cell activator.

REGULATION OF IN VITRO B CELL FUNCTION BY CORTICOSTEROIDS AND PROSTAGLANDINS

Most B cell studies have been performed within the framework of the polyclonal induction of B cell responses and these systems have been also widely used to examine the in vitro effect of potent drugs on human B lymphocyte functions. Many commonly used drugs, such as corticosteroids and indomethacin, have a wide range of effects on virtually every phase and component of the human immune and inflammatory responses.

Corticosteroids

In addition to their in vivo effects, pharmacological doses of corticosteroids suppress most immune functions in vitro including proliferative responses to antigen-, PHA-, ConA-, and PWM(Heilman, 1972, Webel & Ritts, 1977, Gordon & Nouri, 1981, Heilman et al, 1973) as well as the allogeneic and autologous mixed lymphocyte reactivity(Webel & Ritts, 1977, Ilfield et al, 1977) and NK cell activity(Parrillo & Fauci, 1978b). Nevertheless one possible exception to this is suggested by reports that immunoglobulin production in vitro may increase in the presence of corticosteroids, despite the suppressive effects of steroids on antibody production in vivo(Butler & Rossen, 1973, Posey et al,

1978). Thus, it has been shown that immunoglobulin production as detected in the supernatant of cultures with fetal calf serum(FCS) is significantly increased by in vitro corticosteroids(Smith et al, 1972), and also that there is an increase in the number of immunoglobulin secreting cells in the same cultures(Grayson et al, 1981). An increase in supernatant immunoglobulin production was also shown when cells were cultured with FCS and PWM(Cooper et al, 1979) and this finding was confirmed by other workers who found an increase in the number of plaque forming cells in PWM cultures with human serum(Fauci et al, 1977). Therefore it has been suggested that corticosteroids increase immunoglobulin production in the PWM system by abrogating suppressor cell activity(Haynes & Fauci, 1979).

Prostaglandins

Mononuclear phagocytes have been shown to be an essential cellular component in the induction of human lymphocyte responses and have also been shown to suppress human B lymphocyte differentiation in vitro(Knapp & Baumgartner, 1978). One possible mechanism by which macrophages exert their effect is through cyclooxygenase pathway products of arachidonic acid metabolism, such as prostaglandin E2(Goodwin & Webb, 1980). Prostaglandin E2(PGE2) has been reported to inhibit PHA- and ConA-induced blastogenesis but it had a minimal effect on PWM-induced blastogenesis(Goodwin et al, 1977). The authors suggested that PGE2 suppressed T cell but not B cell blastogenesis since PWM is more

effective in stimulating B cells. In contrast to this study, PGE2 has been found to inhibit human B lymphocyte colony responses(Whisler et al, 1982) while animal studies have shown that the in vivo antibody response of mice can be markedly increased by the administration of a cyclooxygenase inhibitor such as indomethacin(Webb & Osheroff, 1976).

B. B CELL FUNCTION IN URAEMIA

As discussed above, studies of humoral immunity in vivo have produced conflicting results(Gowland & Smiddy, 1962, Wilson et al, 1965, Boulton-Jones et al, 1973, Souhami, 1973, Byron et al, 1976, Mezzano et al, 1982, Balch et al, 1955, Stoloff et al, 1958, Nelson et al, 1980). There has been very little work on B cell function in vitro in uraemic patients or animals, despite the conflicting results obtained from studies of humoral immunity in vivo.

Although the first such study reported normal proliferative responses to PWM(Sengar et al, 1975) subsequent studies have found depressed responses to the B cell mitogen rabbit anti-human b2 microglobulin in man(Quadracci et al, 1976) and to LPS in rats(Alevy et al, 1981). The status of immunoglobulin secreting cells in uraemia is also controversial. Thus, Kunori et al(1980) showed that the number of SAC stimulated immunoglobulin secreting cells was decreased in uraemic patients, while a recent study showed that the number of circulating immunoglobulin secreting cells was normal in uraemic

patients, although uraemics had a wider scatter of values(Horsburgh et al, 1983). Very little work has been done on human B cell responses in vitro in uraemia and nothing is known about how uraemic B lymphocytes respond to different polyclonal B cell activators which differ on their dependence on T cells. It is also not known whether inhibition of immunoglobulin production by uraemic B cells is due to reduced numbers of B cells, reduced function, or both. One further aspect of B cell function which may be unusual in uraemia is the possibility that uraemic B cell differentiation may be particularly sensitive to the regulatory effects of corticosteroids. Nelson et al(1980) showed that the immune responsiveness of the uraemic host is normal and speculated that uraemia potentiates the pharmacological activity of some drugs which depress immune mechanisms. Very recently, the intra-individual consistency of uraemic lymphocyte responses to the in vitro suppressive effect of corticosteroids has been studied by measuring DNA, RNA, and protein synthesis by PHA-stimulated lymphocytes and it was found to be relatively stronger in uraemic cultures(Langhoff & Ladefoged, 1984). As corticosteroids may selectively inhibit the effect of suppressor T cells on immunoglobulin production in vitro, it would be interesting to examine the relative steroid sensitivity of mitogen stimulated immunoglobulin production of uraemic and control cells.

Finally, it has been shown that the activity of spleen cells from uraemic rats to PHA, ConA, and LPS is

significantly suppressed compared with cells from control animals and that this suppression is mediated by a glass-adherent suppressor cell(Alevy et al, 1981). Further studies by the same authors showed that the suppression is mediated by an adherent cell that differs from adherent cells present in control animals by being Ia-negative and indomethacin-insensitive(Alevy & Slavin, 1981). They speculated that the suppression in uraemic rats is either not mediated by prostaglandins or may be mediated by preformed prostaglandin synthetase products. The effects of PGE2 on uraemic B cell function have not been studied. Monocytes may suppress B cell function by release of PGE2 and indomethacin may increase B cell responses in vivo and in vitro. As noted above, uraemia may induce a population of adherent suppressor cells which inhibit T cell function in vitro and it would be interesting to determine whether the inhibition of immunoglobulin production in uraemia is PGE2 mediated.

III. THE ROLE OF IMMUNOSUPPRESSIVE SERUM FACTORS IN URAEMIA

The data discussed above show clearly that lymphocyte function may be markedly reduced in patients with uraemia, but it is difficult to distinguish whether this impairment in lymphocyte function is due to a primary defect at the level of cellular differentiation or whether uraemic serum can inhibit the expression of effector lymphocyte functions

in vitro. Many workers have attempted to determine whether the metabolites which accumulate in chronic renal failure are toxic to lymphocytes in vitro and have begun to identify these factors. Although several studies of this nature have been performed in humans, the results have been conflicting presumably due to the variety of causes leading to the uraemic state and to different therapeutic regimens. Thus it has been reported that uraemic serum depresses PHA responses of control peripheral blood lymphocytes(Silk, 1967, Newberry & Sanford, 1971, Sengar et al, 1975, Holdsworth et al, 1978, Raskova & Raska, 1981, Langhoff & Ladefoged, 1984) whereas others found that PHA responses were not affected by uraemic serum(Kasakura & Lowenstein, 1967, Webel et al, 1974, Kauffman et al, 1975). In contrast, all workers agree that the MLR is greatly depressed if performed in the presence of uraemic serum(Kasakura & Lowenstein, 1967, Sengar et al, 1975, Raskova & Morrison, 1978, Raska et al, 1980, Raskova & Raska, 1981) and it has also been shown that ConA responses of control rat spleen cells are suppressed by uraemic serum from rats(Stewart & Miller, 1980, Mezzano et al, 1984). Mezzano et al also showed that uraemic serum suppressed antibody formation to bovine serum albumin(BSA) in vitro but interestingly, the response to sheep red blood cells was not affected. This indicates that uraemic serum may suppress one mechanism of antibody production while leaving others unaffected.

In view of these findings, many attempts have been made to identify the factors in uraemic serum which are responsible for the immunosuppressive activity but not definitive conclusion has been obtained. In addition, very little is known about the possible effect of uraemic serum on in vitro immunoglobulin secretion by normal B cells in response to polyclonal B cell activators.

IMMUNOLOGICAL ASPECTS OF BLOOD TRANSFUSIONS IN RENAL TRANSPLANTATION

The medical treatment of uraemia without resort to renal transplantation does not alter the progress of the underlying disease but can improve the overall condition of the patient. As noted earlier, haemodialysis or peritoneal dialysis form the mainstay of these regimes but special care must also be given to treating co-existing complications such as anaemia. Until the demonstration of the harmful effects of lymphocytotoxic antibodies in renal transplantation(Terasaki et al, 1971), uraemic patients were routinely transfused when their haematocrit dropped below normal levels. When it was realised that blood transfusions were the main cause of generation of lymphocytotoxic antibodies in these patients, it was decided to restrict the number of blood transfusions in pretransplant patients. However, over the next few years it became apparent that limiting pretransplant blood transfusions was actually accompanied by a decrease in graft survival and patients who received blood transfusions before kidney transplantation had an improved renal allograft survival(Opelz et al, 1973a). Despite initial scepticism, this finding was confirmed by many studies in the following years(Opelz & Terasaki, 1974, Murray et al, 1974, Opelz & Terasaki, 1976, Persijn et al, 1977, Walter et al, 1977, Solheim et al, 1977, Briggs et al, 1978, Blamey et al, 1978), and there is

now general agreement that blood transfusions in uraemic patients improve the chances of survival of a subsequent kidney transplant. However, there is confusion about the best protocol to use, with considerable disagreement over the use of third party or donor specific transfusions, whole blood or packed cells as well as over the number of transfusions and the optimal time interval between each transfusion. There are two principal approaches using third party blood transfusions. Opelz and co-workers recommend that at least five units of third party blood transfusions are required every 2-3 months while Persijn et al have shown that one third party blood transfusion is sufficient and that its effect is long lasting(Van Rood, 1983). Other regimes which have been described include the use of blood transfusion which are HLA-,A,B,C matched with the recipient(Nube et al, 1981), or fully HLA-matched with the kidney donor(Cochrum et al, 1979). In addition, transfusions of pure platelets from random donors have also been shown to improve graft survival in rhesus monkeys(Van Rood, 1983). However, third party blood transfusion protocols are used most commonly and usually five units of packed red blood cells are given every 2-4 weeks, followed by one unit every 6 months until transplantation. This large number of different transfusion protocols and the fact that graft survival depends on many other additional factors, have made it difficult to determine the exact mechanism of the blood transfusion effect.

It has been suggested that one possible mechanism of the apparent improved progress of transfused patients is that in many centres the patients responding to transfusion with the production of lymphocytotoxic antibodies are frequently excluded from transplantation. This possibility has been supported by a study in which one third of uraemic patients given five units of third party blood developed lymphocytotoxic antibodies(mostly against B cells) but the overall in vitro cellular immune reactivity remained unchanged(Fehrman et al, 1983). The authors suggested that since fewer patients with antibodies received grafts, blood transfusion seemed to have led to a selection effect. Although, this selection theory has gained considerable support in protocols using donor specific blood transfusions it has been discarded by most of the workers as a possible mechanism in patients receiving cadaver transplants unrelated to the transfusion(Opelz & van Rood, 1983). Most other proposed mechanisms attribute the benefits of blood transfusion on the host immune response and early studies showed a depression of skin test reactivity which correlated with the administration of blood transfusions(Watson et al, 1979). Blood transfusion induced anti-idiotypic antibodies reacting with T cell antigen receptors have been also suggested as a possible protective mechanism(Singal et al, 1983) while non-cytotoxic Fc-receptor blocking antibodies developed after blood transfusion have been shown to correlate with good graft outcome(McLeod et al, 1983). A non-specific immunosuppressive mechanism which relates the

blood transfusion effect to the red cell breakdown has also been proposed(de Sousa, 1983), based on previous findings that ferritin suppresses T cell responses to mitogens(Matzner et al, 1979) and that there is an inverse correlation between serum ferritin levels and ratio of helper T to suppressor T cells in the blood of multi-transfused dialysis patients(Dupont et al, 1983). Similarly, Keown & Descamps(1979) have suggested that non-specific suppression of cell mediated responses could occur due to impaired function of mononuclear phagocytes caused by increased uptake of altered red cells. There are also two cellular mechanisms which have been proposed to explain the effects of blood transfusion to the immune system. First, it has been suggested that pre-transplant blood transfusion prime the recipient to donor antigens and when the renal graft is implanted, rapidly proliferating lymphocytes mount a secondary response and these are uniquely sensitive to the immunosuppressive therapy which the patient receives(Terasaki, 1984).

The graft enhancing mechanism though which is favoured by the majority of investigators is the activation or generation of suppressor cells. Since the first demonstration that the blood transfusion-induced suppression of cellular immunity is caused by a non-specific suppressor cell(Fisher et al, 1980), many workers have described the presence of both monocyte/macrophage-like cells and suppressor T cells in transfused uraemic patients. Soon

after transfusion monocytes and Ia(DR)-positive cells appear transiently in the peripheral blood and MLR activity is non-specifically suppressed(Lenhard et al, 1982a). Removal of adherent mononuclear cells restores MLR responses to normal. At later times suppressor T cell activity increases although this is not accompanied by an increase in the number of suppressor/cytotoxic T cells as measured by immunofluorescence(Smith et al, 1981, Lenhard et al, 1982b). The effect of planned blood transfusion on MLR to both donor and unrelated cells has been examined and a marked decrease in MLR activity was observed after the first blood transfusion in 38% of the patients and after the second or third blood transfusion in an additional 24% of the patients(Klatzmann et al, 1983). Other patients had only a transient decrease, but otherwise MLR were normal or even increased. In the majority of patients with decreased MLR, peripheral blood lymphocytes were able to inhibit the MLR response of autologous cells taken before blood transfusion when they were mixed together in three-cell experiments. Since these suspensions were not cytotoxic to the stimulating cells the observed inhibition was attributed to suppressor cells. Although patients with reduced alloreactive responses exhibited suppressor cells which inhibited both MLR and the generation of allospecific cytotoxic T lymphocytes, the role of blood transfusion induced suppressor cells and their exact nature is still controversial.

As suppressor T cells regulate immunoglobulin production by polyclonal B cell activator-driven B-cells one possible means of assessing the role of suppressor cells under clinical conditions would be to examine the production of immunoglobulin by peripheral blood lymphocytes in response to polyclonal B cell activators which differ in their dependence on regulation by suppressor T cells. Although both PWM and SAC depend on T cell help for full B cell differentiation(Falkoff et al, 1982), SAC induced B cell differentiation has been reported not to be regulated by suppressor cells(Pryjma et al, 1980). Therefore, one aim of this study was to assess the effect of third party blood transfusion on the in vitro immune response of prospective renal allograft recipients by measuring immunoglobulin secretion by peripheral blood lymphocytes both spontaneously and after stimulation with PWM or SAC.

IMMUNOLOGIC MONITORING IN RENAL TRANSPLANTATION

1. MECHANISMS OF ALLOGRAFT REJECTION

Transplantation of allogeneic tissue stimulates a series of immunological reactions which usually result in the rejection of the graft. The realisation that clinical transplant rejection constituted an immune response necessitated the development of immunological tests which could predict or confirm this unwanted complication. In addition, these assays are required to monitor the degree of immunosuppressive therapy.

Rejection can be defined as the process by which the host's immune system recognises, becomes sensitised against, and attempts to eliminate the antigenic differences presented by the donor organ. Rejection of renal allografts can be characterised clinically as hyperacute(within minutes or hours), accelerated(within several days), acute(within the first month), or chronic(after the first month). The rejection process is a very complex phenomenon both in the way in which antigen is recognised and in the way the host's response results in graft damage. Recent evidence indicates that while the tissue cells themselves can act as antigenic stimuli, class II MHC antigen expressing bone marrow derived dendritic cells of donor origin(passenger leukocytes) represent the most potent stimulus to rejection(Morris, 1984). In addition, recirculating macrophages and dendritic cells of host origin may also play a role in processing and

presentation of donor histocompatibility antigens to host T cells. Although the effector arm of rejection is mediated by T cells, there is still controversy about the nature of this effector T cell. In the specific cellular response the cytotoxic T cell is considered the cell which mainly produces graft damage. In addition, recent studies in T cell deprived mice and rats indicate that rejection involves a non-specific response resulting from a delayed type hypersensitivity reaction(DTH) reaction mediated by cells with the phenotype of T(helper) and T(DTH)(Loveland et al, 1981, Dallman et al, 1982). Nevertheless these studies have been criticised because cytotoxic T cell precursors may still be present in recostituted animals and cytotoxic T cell precursors may share the helper T cell phenotype. Furthermore, cytotoxic T lymphocytes(CTL) specific for Class II MHC antigens are phenotypically identical to helper T cells(Biddison et al, 1982, Ball et al, 1982, Widmer et al, 1983). Together, these studies suggest that both CTL and DTH may be involved in the effector limb of allograft rejection.

The role of antibody in the destruction of the graft has not been clearly established. The vascular lesions observed in hyperacute rejection are attributed to the direct action of complement fixing antibody , while an antibody response against donor HLA antigens has been implicated in accelerated rejection(Williams, 1984). Acute rejection can be of cellular, humoral or mixed form although the use of immunosuppressive drugs has rendered cellular acute

rejection unusual(Dunnill, 1984). Chronic rejection of allografts has also been associated with repetitive deposition of IgG on the vascular endothelium leading eventually to obliterative arteritis. In addition, lymphocytotoxic antibodies which react with donor MHC antigens have been found in the serum of patients who are rejecting or have rejected renal allografts(Morris et al, 1968, 1969, Stiller & Sinclair, 1979) and antibodies have also been found to be deposited in rejecting kidneys(Morris, 1984).

In addition to these specific components of the immune system, many non-specific effector mechanisms may also be involved in graft rejection. Macrophages and NK cells have also been implicated in graft rejection, but only limited and partly controversial data has been published about their relationship to allograft rejection. Complement has been shown to determine the effect of antibody in hyperacute rejection and is required for alloantibody induced graft damage in rats(French, 1982, Morris, 1984).

2. IMMUNOSUPPRESSIVE TREATMENT

The history of immunosuppressive treatment started when 6-Mercaptopurine(6-MP) was given to rabbits together with an antigen and it was observed that antibody formation was totally suppressed(Schwartz & Dameshek, 1959). Shortly afterwards 6-MP and particularly its imidazolyl derivative azathioprine were used in animals and in patients undergoing

kidney transplantation(Calne, 1960, Zukoski, 1960, Calne et al, 1962). Although many powerful immunosuppressive drugs have been discovered since then, azathioprine is still considered as one of the most important drugs in clinical transplantation and acts by blocking the synthesis of inosinic acid which is manifested as a decrease in DNA synthesis(Webb & Winkelstein, 1982). Azathioprine can be used on its own to suppress rejection but this seldom done since immunosuppression with azathioprine alone is not very effective and it is administered usually together with steroids.

Steroids are frequently administered in high concentrations as a prophylaxis against rejection or for treatment after it has occured. It is certain that administration of steroids depresses the number of T and B lymphocytes as well as antigen and lectin induced blastogenesis(Cupps & Fauci, 1982). However, the precise mechanisms of steroid induced immunoregulation in man have not been elucidated and it is still not certain whether their effects on the immune response are responsible for their usefulness in preventing and treating rejection or their powerful anti-inflammatory action.

In the mid 1970s a powerful cyclic polypeptide received wide attention as a new and powerful immunosuppressant. It was named cyclosporine A and it was isolated from two strains of fungi(Cylindrocarpon Lucidum Booth and Trichoderma Polysporum) by Sandoz in Basle. This new drug

was not cytotoxic for lymphocytes, had no antimitotic activity and it appeared to inhibit helper T lymphocytes by preventing the production of lymphokines and especially IL-2. Its effects on the humoral immune responses are variable and although it has been shown to suppress antibody responses to T-dependent antigens and mitogens, it is likely that this inhibitory effect is due to an effect on helper T cells(Morris, 1982).

In the present study, all the transplanted patients were treated with either azathioprine and prednisolone or cyclosporine A and prednisolone, and an attempt was made to compare the effects of these two forms of immunosuppression on production of immunoglobulin in vitro.

3. IMMUNOLOGIC MONITORING

The large number of mechanisms which may be involved in allograft rejection has been paralleled by the wide variety of assays which have been developed to assess the immune status of transplant recipients. Some of these are designed to assess specific anti-graft immunity, but the majority measure non-specific immunological parameters and each has its own limitations. The principal of these are that in vitro observations do not necessarily reflect conditions in vivo while studies of peripheral blood lymphocytes do not fully represent immune phenomena in the graft. In addition, many of the immune reactions in vivo are short-lived and frequently have no obvious clinical manifestations. Therefore the timing, number and evaluation of immunological

tests presents a difficult problem.

Under ideal circumstances, the most valid assays of immune function in graft recipients would be those which assess specific anti-graft immunity.The most commonly used donor specific tests in renal transplantation detect antibodies against donor HLA antigens as well as donor-specific cytotoxic T lymphocytes and mixed lymphocyte reactivity against donor cells. Nevertheless, assays of this type do not confirm or predict rejection episodes consistently, probably because the exact antigens initiating rejection are not known and so cannot be measured directly. For these reasons, non-specific assays were introduced and these tests have the additional advantage that host immunosuppression can be evaluated by the same means. Most of the initial studies of this nature concentrated on the enumeration of peripheral blood lymphocytes while later studies also enumerated individual T cell subpopulations. The results from these studies have been conflicting. Thus, in early work using the sheep red blood cell rosetting technique to enumerate T cells, some workers found increased numbers of T cells during rejection episodes(Bishop et al, 1975) but this was not confirmed by other groups(Kerman & Geis, 1976, Buckingham et al, 1977). When monoclonal antibodies were used to enumerate T lymphocytes and their subsets, some groups reported an increased ratio of OKT4+(helper-inducer) to OKT8+(suppressor-cytotoxic) populations during rejection episodes in patients treated

with azathioprine(Cosimi et al, 1981, Ellis et al, 1982) or cyclosporine(Kehrman et al, 1983) while others could not find similar changes(Guttman & Paulsen, 1981, Carter et al, 1983, Von Willebrand, 1983). Different doses of immunosuppressive drugs as well as differences in the evaluation of rejection episodes may account for the different results reported by different groups.

In view of the possibility that peripheral blood lymphocytes did not reflect events occuring in the graft itself, other workers studied the phenotypes of cells within the transplanted graft and attempted to correlate cell numbers with either renal allograft rejection or the effectiveness of immunosuppressive treatment. During rejection episodes in azathioprine and cyclosporin treated patients, a decreased OKT4+ to OKT8+ ratio in the graft compared to the peripheral blood was found in frozen sections of conventional core needle biopsies(CNB)(Platt et al, 1982, Hancock et al, 1983, Platt et al, 1983). The introduction of fine needle aspiration(FNA) allowed serial biopsies to be performed safely at short intervals and this technique confirmed a significant fall in the OKT4+ - OKT8+ ratio of graft T cells in cyclosporine treated patients undergoing rejection(Wood et al, 1983).

Functional studies have also been used to study rejection and immunosuppression. The initial finding that increased spontaneous blastogenesis by peripheral blood lymphocytes was associated with rejection(Hersh et al, 1971) was

confirmed later by other groups(Jonas & Uldall, 1974, Gailiunas et al, 1978) but it has been also suggested that this is usually due to systemic infections(Miller et al, 1978). Similarly, the initial report that an increased PHA response by peripheral blood lymphocytes correlated with rejection episodes(Thomas et al, 1978) has been challenged on the same grounds(Stenzel et al, 1978).

Many groups also enumerated immunoglobulin secreting cells in azathioprine treated recipients and a correlation was reported between increased numbers of these cells in the peripheral blood and rejection episodes(Horsburgh et al, 1983, Satomi et al, 1983).

Thus, most of the studies which have been performed so far are complicated by the effects of secondary infection. Furthermore, patients have often received different immunosuppressive regimes and there have been no in vitro immunological studies which compare directly the effect of these two forms of immunosuppression on the responses of peripheral blood lymphocytes. The spontaneous plaque forming cell assay reflects the status of in vivo B cell differentiation into immunoglobulin secreting cells and it was considered as a relevant and useful assay to provide information about the immune status of transplanted patients. Thus,the aim of the present study was to examine whether renal allograft rejection can be predicted by using a protein A plaque assay to enumerate immunoglobulin secreting cells in renal transplant recipients treated with

either cyclosporine or azathioprine. In addition, the effect of these agents on immunoglobulin production was examined and compared with the results obtained from groups of uraemic patients and from normal controls.

PLAQUE ASSAYS

Although polyclonal B cell activators were first used during the mid 1960's, until 1976 there were no reliable assays to measure immunoglobulin secretion by individual cells and hence terminal differentiation could not be assessed. Proliferation assays do not give this information while measuring cytoplasmic immunoglobulin by immunofluorescence(Cooper et al, 1973, Janossy & Greaves, 1975) is simple but not very accurate. In contrast those assays measuring immunoglobulin levels in the supernatants of cell cultures stimulated with B cell polyclonal activators were simple and reproducible but gave no information about the number of cells responding. Thus, Fauci & Pratt(1976) developed a system for culture and measurement of single cell antibody production by human tonsillar or peripheral blood mononuclear cells stimulated either with B cell polyclonal activators or sheep red blood cells. The method they used to measure antibody production was a modification of the plaque assay described by Jerne & Nordin(1963).

Jerne & Nordin had observed that when a mixture of sheep red blood cells and lymphoid cells from a rabbit immunised with sheep red blood cells was plated in agar and incubated at 37°C with complement, distinct plaques appeared in the agar. These consisted of circular clear areas of haemolysis with a central lymphoid cell and it was suggested that this

was due to the release of haemolysin by an antibody-forming cell at the centre of the plaque. Furthermore, the authors predicted correctly that by coating red cells with other antigenic determinants it would be possible to study other antigen-antibody systems at the single level. Dresser & Wortis(1965) subsequently showed that addition of rabbit anti-mouse immunoglobulin enhanced the number of plaque forming cells observed. They suggested that the additional plaques which appeared were due to the augmentation of the activity of a low haemolytic activity antibody, such as IgG. Thus, this "indirect" plaque assay allows measurement of both IgG and IgM secretion. Although these methods could detect cells secreting different immunoglobulin classes, they were limited to detecting cells secreting specific antibodies. In 1974, Molinaro & Dray introduced a plaque assay which detected all cells secreting immunoglobulin of a particular class by coating sheep red blood cells with antiserum specific to the various immunoglobulin classes. Using this technique, they were the first to make quantitative assessments of immunoglobulin secreting cells among circulating peripheral blood lymphocytes(Eby et al, 1975). This plaque assay was called the reverse haemolytic plaque assay(RHPA) because the antibody was coated on to sheep red blood cells rather than the antigen. However, this method had the disadvantage that if measurement of more than one class of immunoglobulin was required, each antibody had to be coupled separately to

sheep red blood cells and, in 1976, Gronowicz et al introduced a modified plaque assay which overcame these difficulties. Taking advantage of the ability of protein A from Staphylococcus Aureus to bind to the Fc portion of IgG molecules, sheep red blood cells were coated with protein A and mixed with mouse lymphocytes. The plaques were then developed by adding complement and an immunoglobulin class specific antibody(or preferably its IgG fraction), which bound to the sheep red blood cells via its Fc receptor and to the antibody secreted by the cell via its antibody binding site.

It rapidly became clear that this new plaque assay would facilitate assessment of human B cell function and Bird & Britton(1979b) modified it for use in the assay of cultures of human peripheral blood lymphocytes. Since then the protein A plaque has been used extensively in studies not only of the B cell itself but also in studies of the different cellular and factor mediated interactions required for B cell differentiation. The plaque assay can not only be used in combination with polyclonal activators to measure stimulated B cell activity, but can also be used to enumerate cells which are secreting immunoglobulin spontaneously. Furthermore, the assay can be applied to B cells isolated from peripheral blood, spleen, tonsils and lymph nodes or from biopsies and extracellular fluids. Other workers have also modified the plaque assay to detect secretion products of cells other than B cells, including

ferritin and various complement components.

In this thesis the protein A plaque assay has been used in two ways. First, I have enumerated the cells which are secreting immunoglobulin spontaneously in the peripheral blood of healthy and uraemic subjects and have studied the effect of blood transfusion, renal transplantation, immunosuppression and allograft rejection on the spontaneous secretion of immunoglobulin by peripheral blood lymphocytes obtained from uraemic patients. Secondly, I have studied whether uraemia and blood transfusion affect the ability of human B cells to proliferate and differentiate into immunoglobulin secreting cells after being stimulated with different polyclonal B cell activators.

CHAPTER II

MATERIALS AND METHODS

MONONUCLEAR CELL SEPARATION

Peripheral blood mononuclear cells were prepared from heparinised venous blood from patients and normal volunteers as described by Boyum et al(1968). Blood was collected into heparinised Evans bottles(Evans Medical Ltd, Speke, Liverpool, England) and two volumes of blood were layered over three parts of Ficoll-Hypaque(Nygaarde, England) before centrifuging at 400g for 25 minutes. Interface cells were removed using a Pasteur pipette, washed twice with RPMI 1640(GIBCO, Paisley, Scotland) and counted in a haemocytometer. All lymphocyte samples from each patient or healthy control were cryopreserved in liquid nitrogen and assayed in one experiment to avoid test to test variations.

SERUM

Control lymphocytes were cultured or pre-incubated with serum which was obtained from normal and uraemic subjects. Blood from these subjects was allowed to clot on ice, and serum was obtained and decomplemented by heating at 56°C for 30min.

DETERMINATION OF VIABILITY

In studies of viability, trypan blue(Gurr, Searle Diagnostic, Bucks, England) exclusion was used. One part trypan blue(0.2% in saline) was mixed with one part of a cell suspension containing 2 x 10^6 PBMC/ml. The percentage of peripheral blood mononuclear cells which excluded trypan blue(viable cells) was then counted. Viability of peripheral blood mononuclear cells was also examined routinely by phase contrast microscopy.

WHITE BLOOD CELL AND LYMPHOCYTE COUNTS

White blood cell counts were performed using a coulter counter(Coulter Electronics, England). Lymphocyte counts were performed after partially depleting peripheral blood mononuclear cells from plastic adherent cells by incubating $2x10^6$ PBMC/ml(in RPMI+10%FCS) for 60 minutes in plastic culture plates(Sterilin, Teddington, England) at 37°C.

FREEZING AND THAWING OF CELLS

The freezing technique was that of Tauris & Jorgensen(1982).

Before freezing, each fresh cell sample was centrifuged at 400 x g for ten minutes and resuspended at 6×10^6 cells/ml in foetal calf serum(FCS, GIBCO, Paisley, Scotland) which had been inactivated by heating at 56°C for 30min. An equal volume of a 20% dilution of dimethylsulfoxide(DMSO, Koch Laboratories, England) in RPMI was added dropwise to the cell suspension and after mixing with a vortex mixer, the cells were distributed in 1ml aliquots in freezing tubes(Sterilin, Teddington, England). The tubes were kept overnight at -70°C before transferred to the vapour phase of a liquid nitrogen freezer.

Thawing was achieved by agitating the tubes in a 37°C water bath until only a small lump of ice remained. The cells then were transferred immediately into plastic conical bottom tubes containing 9 ml of RPMI 1640 at 4°C, spun at

400 x g for five minutes, resuspended in 10 ml of RPMI 1640 and eventually washed three times in medium at 400 x g before being counted and used.

PLAQUE-FORMING CELL ASSAY

A. MATERIALS

Sheep Red Blood Cells

Sheep red blood cells(SRBC, Flow Laboratories, U.K.) were obtained fresh each week and were stored at 4°C in Alsevier's solution. Batches of SRBC were always discarded after one week.

Normal Saline

Normal Saline was obtained from the hospital's pharmacy(Steriflex, Boots Company PLC, Nottingham, England). Sterile saline prepared in the lab was not used because of occasional phosphate contamination.

Protein-A

Staphylococcal protein-A(Pharmacia, Uppsala) was reconstituted to 5mg/ml with normal saline and this stock solution was stored at 4°C and was used for a maximum of three months.

Chromic Chloride

Chromic chloride(CrCl3.6H2O, Analar, BDH Chemicals Ltd,

Poole, England) was used for coupling protein-A to SRBCs as reported by Uyas et al(1968). Fresh chromic chloride was prepared by diluting the reagent in normal saline while aged chromic chloride was prepared by dissolving 100mg of chromic chloride in 100ml saline. This latter solution was stored at room temperature and three weeks later its pH was adjusted to 5.0. The stock solution was diluted 1/10 with normal saline before being used in the coating procedure.

Antisera To Human Immunoglobulins

In all experiments rabbit antisera specific for the heavy chains of human IgA, IgG and IgM(Dako Immunoglobulins, Copenhagen, Denmark) were used. The antisera were always diluted in Hank's balanced salt solution(HBSS, Gibco, Paisley, Scotland).

Complement

SRBC absorbed guinea-pig complement(Sera-Lab, England) was used after being diluted in HBSS.

Agar

The agar mixture for the plaque assay was prepared by dissolving agar(Bacto Agar, Difco Laboratories) in a 1/10 dilution of x10 HBSS concentrate and the pH adjusted to 7.4.DEAE-Dextran(Pharmacia, Uppsala, Sweden) was then added to the agar mixture to overcome the anti-complementary properties of agar(Lefkovits & Cosenza, 1979), while polyethylene glycol 6000(PEG, Sigma, St Louis, USA) was

added to improve detection of plaques as described by Trienekens et al(1984).

B. METHODS

The protein-A plaque assay was performed as described by Gronowicz et al(1976) and modified by Bird & Britton(1979b). Several experiments were performed to standardise the conditions and technique for the assay. These are described in detail in CHAPTER III and, for simplicity only the outline of the method is given here(Fig.2.1).

1. COATING OF SRBC WITH PROTEIN-A

SRBC were washed six times in normal saline and were then coated with protein-A by incubating one volume of SRBC with one volume of protein-A and ten volumes of chromic chloride for 45min at 37°C with continuous rotation. The packed cells and protein-A were mixed first and the chromic chloride was then added dropwise while mixing with a vortex mixer. After incubation the protein-A coated SRBC were washed three times with HBSS and used in plaque assays for up to 5 days after preparation.

2. PLAQUE FORMING CELL ASSAY

0.025ml Protein-A-coated SRBC, 0.1ml of lymphocyte suspension, 0.025ml rabbit antisera specific for heavy chains of human IgA, IgG or IgM and 0.025ml of SRBC absorbed guinea pig complement all diluted in HBSS were added to 0.75 ml of agar supplemented with DEAE-dextran and PEG 6000 kept

in a 46°C water bath. After mixing in a vortex mixer, three 0.2ml drops were pipetted into a plastic 90mm Petri dish(Sterilin, Teddington, England) and a 24 mm x 32 mm coverslip(Chance Propper Limited, Warley, England) immediately placed on top of each drop. The plates were then incubated from 1 to 16 hrs at 37°C in a humidified CO2 incubator. Potential plaque forming cells were identified by naked eye as microscopic holes in the agar and the presence of a central lymphoid cell at the centre of haemolytic spots was confirmed by microscopy(Fig.2.2). These plaques were counted and the results were expressed as the mean plaque forming cell count(of triplicate determinations) per million of plated cells. True plaques were easily differentiated from small air bubbles by the sharp edged appearance of the latter. Dust particles also produced false plaques but they were easily detected by the naked eye.

PRE-INCUBATION WITH CYCLOHEXIMIDE

Peripheral blood mononuclear cells were suspended in RPMI 1640 alone or RPMI 1640 with cyclohexiimide(Sigma, Poole, England). They were then incubated at 10^6 PBMC/ml at 37°C for 30min, washed three times in cold HBSS and assayed as above.

LYMPHOCYTE CULTURES

Lymphocytes were cultured for the measurement of both proliferative and immunoglobulin responses. Once again many

experiments were required to standardise the culture systems and these are described in detail in CHAPTER IV. The outline of the assays is given below.

Proliferative Responses to Mitogens

Peripheral blood mononuclear cells were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin/streptomycin(100units/ml), fungizone(250mcg/ml) and L-glutamine(2mmol)(Gibco, Paisley, Scotland). Peripheral blood mononuclear cells were cultured in 0.1ml volumes in conical bottomed microtiter plates(Titertek, Flow Laboratories) at cell concentrations of 0.05 to 5 x 10^6 PBMC/ml. Various concentrations of purified phytohaemagglutinin(PHA, Wellcome, England) and pokeweed mitogen(PWM, Gibco, Paisley, Scotland) were added before mixing on a plate shaker. The plates were sealed with non-toxic film(Nescofilm, Nippon Shoji Kaisha LTD, Japan) and incubated at 37°C in 5% CO2 in air humidified incubator(Flow Laboratories, Irvine, Scotland) for 24 to 120hrs. Four to eighteen hours before harvesting, the plates were pulsed with 50µl of 14C-Thymidine(Phoenix Pharmaceuticals, West Germany, 0.015 µCi/well) using a Hamilton syringe(Hamilton Company, Whittier, California, USA) and, after reculture, cell bound DNA was harvested using a multi-cell harvester(Titertek Cell Harvester, Skatron, Norway). Cell extracts were placed in scintillation fluid(LKB, Scintillation Products, Loughborough, England) and radioactivity was measured in a scintillation

counter(Packard, United Tecnologies, USA). Cultures were performed in triplicate and the results are presented as the mean counts per minute(cpm) per well.

Antibody Responses to Mitogens

A. Materials

Culture Tubes

Conical bottomed plastic tubes(12x75mm, Sterilin, Teddington, Middlesex) were used for lymphocyte cultures.

Culture Medium

Cells were cultured in RPMI 1640 supplemented with 10% FCS, L-Glutamine, and antibiotics as described above.

Concavalin-A

Concavalin A(ConA, Sigma, Poole, England) was added in unstimulated and PWM and SAC cultures of control lymphocytes.

Methyl-prednisolone

Methyl-prednisolone(MP, Upjohn Ltd, Sussex, England) was added in some unstimulated and PWM and SAC induced cultures of control and uraemic lymphocytes.

Indomethacin

Indomethacin(Sigma, St Louis, USA), a cyclooxygenase inhibitor was added in unstimulated and PWM and SAC cultures of control and uraemic lymphocytes. Indomethacin was dissolved in 0.15M tris-buffer(pH=8.8). ConA, MP, and indomethacin were made up in RPMI 1640.

Control and Uraemic Serum

Serum was obtained from control and uraemic subjects as described above.

Poluclonal B Cell Activators

Lymphocytes were cultured in the presence of PWM(Gibco, Paisley, Scotland), Staphylococcus aureus Cowan I strain bacteria(SAC), or Epstein-Barr virus (EBV). SAC was prepared following the procedure described by Forsgren et al(1976). Staphylococcus aureus Cowan I strain(NCTC 8530) was cultured overnight in tryptic soy broth(Difco Laboratories, England) and washed in sterile saline before being killed by incubation in 0.5% formaldeyde for 3hr and heating at 80°C for 3 min. EBV containing supernatant of the B95-8 marmoset cell line was used as source of EBV and this preparation was supplied by Dr Ailsa Campbell(Department of Biochemistry, University of Glasgow, Glasgow).

B. Method

$0.05-5\times10^{6}$ peripheral blood mononuclear cells in one ml of medium were cultured in plastic tubes in the presence of PWM, SAC, or a combination of PWM and SAC. Exposure to EBV was performed as described by Bird & Britton(1979b) by suspending 1×10^{6} cells in 0.2 ml of virus containing supernatant and incubating the cell pellet for 60min at 37° C. The cells were then washed twice with RPMI 1640 and cultured as above.

Cultures stimulated with PWM, SAC and combinations of SAC

and PWM were incubated for 1 to 8 days, but those stimulated with EBV were only cultured for 5 days to avoid the generation of cytotoxic T lymphocytes which can occur with EBV-stimulated unfractionated cells(Bird et al, 1981).

After the end of the culture period, cells were washed twice in RPMI 1640 medium, counted in a haemocytometer, their viability assessed and then assayed for immunoglobulin secreting cells using the plaque forming cell assay as described above.In some cultures, ConA, MP, indomethacin, control or uraemic serum was added to study their effect on immunoglobulin production. When the effect of different sera was studied, peripheral blood mononuclear cells obtained from healthy subjects were either cultured(5%) or pre-incubated with test serum at 5% final concentration.

ANALYSIS OF T LYMPHOCYTE SUBCLASSES BY IMMUNOFLUORESCENCE

T cell subsets were analysed by two stage immunofluorescence using monoclonal antibodies. One million peripheral blood mononuclear cells(0.2ml) were mixed with 5 μg of one of the monoclonal antibodies, OKT3, OKT4, or OKT8(Ortho Diagnostic Systems Ltd, High Wycombe, England) and were incubated on ice for 30min. The cells were then washed twice in phosphate buffered saline(PBS) azide(0.01%) at 4°C and then were resuspended in 0.1ml of culture medium plus 5% FCS. 0.1ml of 1/30 FITC(rabbit anti-mouse immunoglobulin) was added and the cells were incubated for 30min at 0°C. After washing twice cells were resuspended in one drop of 30% v/v glycerol in PBS(pH 7.2) and immediately placed on a

glass slide for examination under ultra-violet light using a fluorescence microscope. T3, T4 and T8 positive cell counts were expressed as a proportion of the total lymphocyte count.

ANALYSIS OF B LYMPHOCYTE NUMBERS BY IMMUNOFLUORESCENCE

Measurement of Sig-positive cells often gives false results because external Ig in serum adsorbs to Fc-receptor bearing lymphocytes. In order to eliminate this artefact, PBMC were incubated at 37°C for 60 minutes and then washed three times in HBSS kept at 37°C. With this procedure cytophilic Ig is released.

A pellet of 2.5×10^6 PBMC/ml was resuspended in 25 µl of FITC-labelled ($F(ab')_2$ Fragment) sheep anti-human immunoglobulin serum(Cappel Laboratories, Dynatech, Sussex, England) diluted 1/4 in PBS. The mixture was incubated for 30 minutes at 4°C and then washed three times at 4°C with PBS containing 0.01% azide to prevent capping. The percentage of cells with surface immunoglobulin out of 200 total lymphocytes was counted in a fluorescence microscope.

STATISTICAL ANALYSIS

Statistical analysis was carried out using a variety of tests. Student's t test was used when data conformed to a normal distribution and the unpaired t test was used to test two independent sets of random samples while the paired t test was used for sets of paired observations from two populations. The chi-squared test was used to compare the distribution of a discrete variable in a sample with the distribution of a discrete variable in another sample.

When the analysed data did not conform to a normal distribution non-parametric tests were used. The Wilcoxon rank sum test was used on unpaired data and the signed rank test on paired data.

Results were expressed as the mean+1SEM unless otherwise stated.

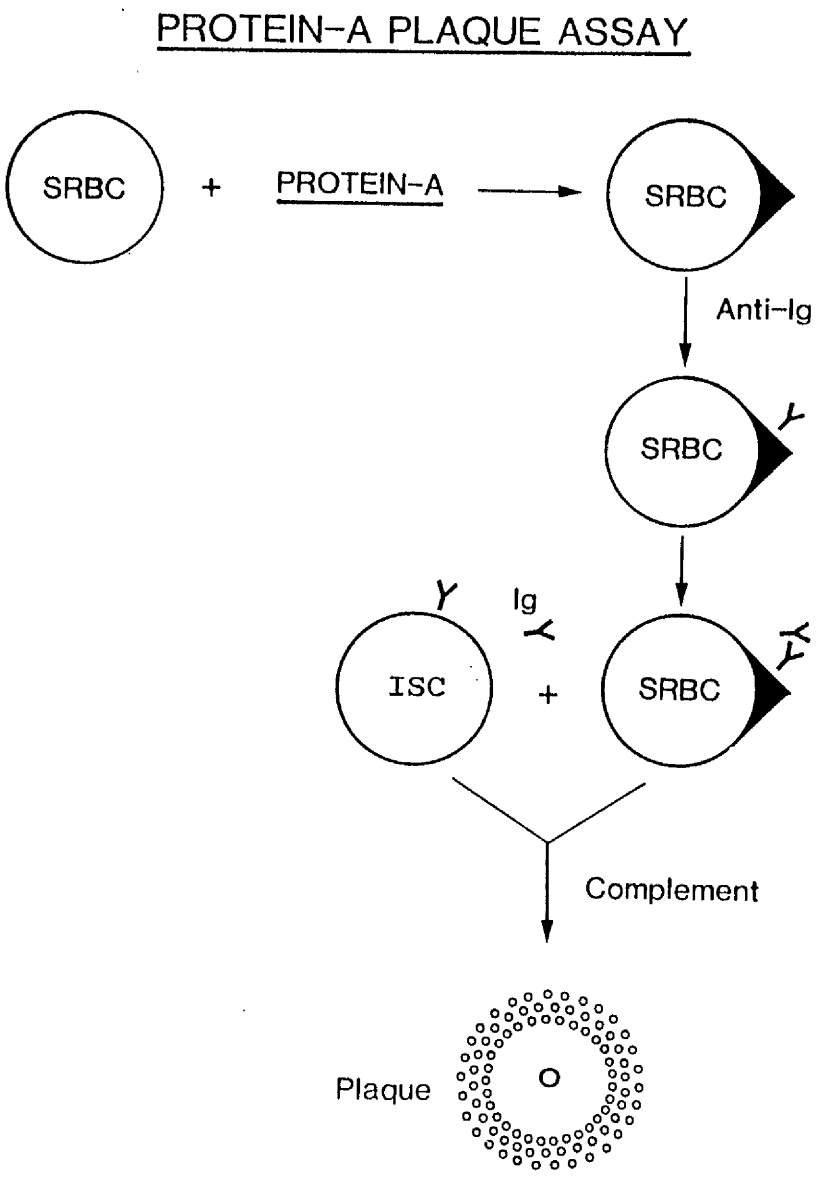


Fig.2.1

The outline of the protein-A plaque assay as described by Gronowicz et al(1976)

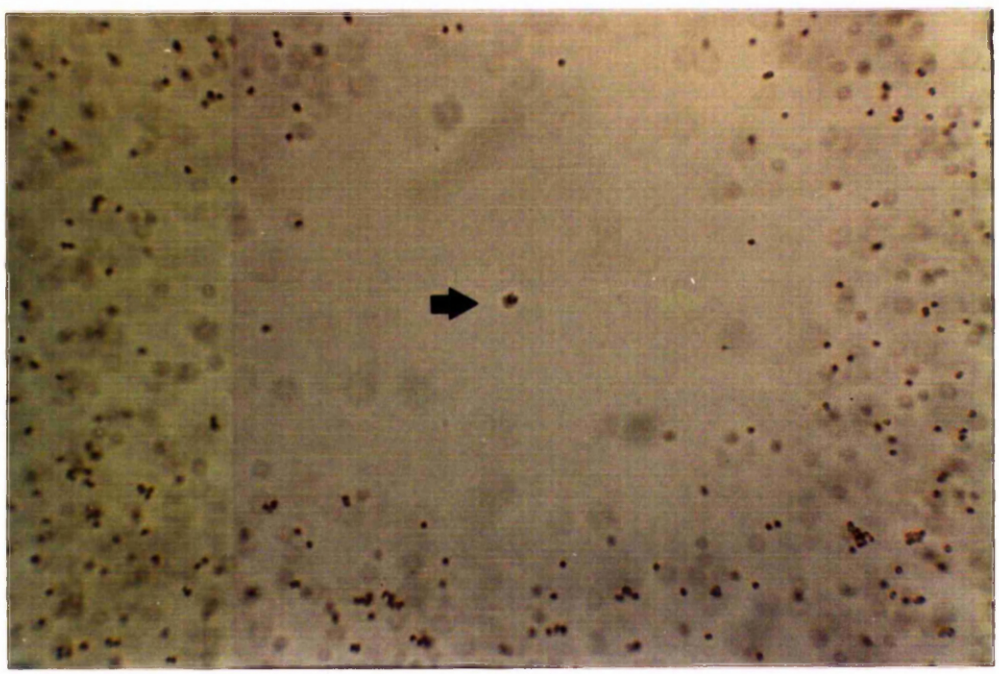


Fig.2.2

Haemolytic plaque with an immunoglobulin secreting cell at the centre

CHAPTER III

CHARACTERISATION OF THE PLAQUE-FORMING CELL ASSAY

INTRODUCTION

The clinical studies performed in this thesis used the plaque forming cell assay to assess B cell function in vitro and therefore it was essential to establish the conditions which gave optimal results. Although most workers performing plaque assays use similar reagents, it is generally agreed that considerable problems of sensitivity and reproducibility remain. One particular area of difficulty is that there are many different techniques for coating of sheep red blood cells with protein A in the presence of chromic chloride with different workers using either fresh or aged chromic chloride, different dilutions of chromic chloride and different dilutions of protein A.

The aim of the experiments in this section was to investigate the technical factors affecting spontaneous plaque formation by circulating peripheral blood mononuclear cells and to identify optimal conditions.

MATERIALS AND METHODS

Venous blood was collected from twenty five healthy subjects(Table 3.1) working in the Renal Unit and the Department of Bacteriology and Immunology and mononuclear cells were prepared as described above. All the experiments were performed on freshly isolated cells unless otherwise stated and cell viability determined by phase contrast microscope was usually approaching 100%. Frozen cells were used only when viability was exceeding 80%.

RESULTS

OPTIMAL CONDITIONS FOR COATING SHEEP RED BLOOD CELLS WITH PROTEIN A

Age of Chromic Chloride

In order to examine whether fresh or aged chromic chloride give better results in the plaque assay, experiments were performed using chromic chloride of different ages to coat sheep red blood cells. Peripheral blood mononuclear cells from one donor were isolated, cryopreserved and spontaneous plaque formation was assayed on four separate occasions using sheep red blood cells which had been coated with protein A in the presence of chromic chloride which was either freshly prepared, 1 week old or 1 month old. In these experiments, only IgA-PFC were counted because, as discussed later(CHAPTER IV), IgA-PFC counts are consistently higher than those of other isotypes in the spontaneous plaque forming cells assay. Fig.3.1 shows the numbers of IgA-PFC obtained in these four experiments and it can be seen that sheep red blood cells coated with fresh chromic chloride produce a good level of spontaneous plaque forming cell response which was consistent throughout the four experiments. In contrast, although aged chromic chloride occasionally produced higher plaque forming cells counts in individual experiments, there was also considerable variation between the responses obtained on different occasions.

Optimal Concentration of Chromic Chloride

The next experiments were designed to establish the optimal concentration of chromic chloride for coating sheep red blood cells and Fig.3.2 shows the effect of using 0.25×10^{-4} M to 25×10^{-4} M chromic chloride on IgA plaque formation. If no chromic chloride was used in the coating procedure no plaques were observed, establishing that chromic chloride was an absolute requirement for coating. Both 0.25×10^{-4} M and 2.5×10^{-4} M chromic chloride allowed plaques to develop, but 2.5×10^{-4} M was more efficient and this dilution was used in all subsequent experiments. At the highest concentration(25×10^{-4} M) lysis of sheep red blood cells occurred during the coating procedure.

Optimal Concentration of Protein A

The final variable to be studied in this section was the effect of different dilutions of protein A on plaque formation. Fig.3.3 shows IgA-PFC counts from three separate experiments in which cells from the same donor were coated with different dilutions of a 5mg/ml stock solution of protein A. The highest number of plaques were consistently obtained using 1:5 or 1:10 dilutions of this stock solution, but the 1:10 dilution was eventually preferred because it gave less variation between experiments.

It was concluded from these three groups of experiments that optimal numbers of plaque forming cells were obtained when sheep red blood cells were coated with 0.5mg/ml protein A in the presence of fresh chromic chloride at 2.5×10^{-4} M concentration.

OPTIMAL CONDITIONS FOR PLAQUE ASSAY

The next series of experiments were designed to establish the optimal conditions for the development of plaque forming cells of each immunoglobulin class.

Peripheral Blood Mononuclear Cells

The effect of varying the number of peripheral blood mononuclear cells was examined first and Fig.3.4 shows the results from three separate experiments using cells from the same healthy subject. These studies showed that the concentrations of cells used in the assay had no effect in the number of plaque forming cells obtained.

Concentration of Sheep Red Blood Cells

In the next five experiments, different concentrations of protein A coated sheep red blood cells were used in the plaque assay and the results are shown in Fig.3.5. Although the highest number of plaque forming cells of all classes were obtained using 1:12 and 1:24 dilutions of packed sheep red blood cells, these plaques were difficult to count because of the low numbers of sheep red blood cells and, indeed, using 1:48 sheep red blood cells it was impossible to determine whether there was plaque formation or not. As a 1:6 dilution of sheep red blood cells gave similar levels of plaque forming cell counts, this dilution was used subsequently because it was associated with well defined and easily counted plaques.

Optimal Dilutions of Antisera and Complement

The effects of varying the concentrations of the developing antisera and complement were then examined in five separate experiments(Fig.3.6,7). The number of plaque forming cells of all classes was directly related to the concentration of both antisera and complement, with the highest plaque forming cell responses being observed when each antiserum was diluted 1:15-1:30(Fig.3.6) and when complement was diluted 1:2-1:4(Fig.3.7). As anticipated, plaque formation was not observed in the absence of antisera or complement. In all subsequent experiments, antisera were used at 1:30 and complement at 1:4.

Use of DEAE-dextran and PEG 6000

Next, the optimal dilution of DEAE-dextran(n=3) and the effect of adding PEG 6000(n=3) to the agar mixture were studied. In the absence of DEAE-dextran, very few plaques were observed, indicating that it was essential to inhibit the anti-complementary effect of agar(Fig.3.8). These experiments also showed that the optimal response for all plaque forming cell classes was observed when DEAE-dextran was added at a final concentration of 2mg/ml. In contrast, the addition of PEG 6000 did not improve the plaque forming cell response(Fig.3.9). Nevertheless PEG made the plaques clearer and easier to count and therefore PEG 6000 was incorporated subsequently at a concentration of 1%.

Effects of Agar Concentration

A further parameter which was studied in one experiment was the concentration of agar used in the assay. Optimal numbers of IgA-PFC were obtained using 0.1 or 0.5% agar and very few plaque forming cells were found using 1 or 2% agar(Fig.3.10). Although both lower concentrations gave similar results, 0.5% agar was preferred because it was easier to handle in preparation.

Effect of Varying the Incubation Period

The effect of different incubation periods on the plaque forming cell response was the next parameter to be studied and the results are shown in Fig.3.11(n=3). Very few plaque forming cells of any class appeared during the first 1-2hours of incubation and the highest counts of plaque forming cells were obtained after 8hrs. Although a further increase was occasionally found after overnight incubation, the longer incubation period caused the plates to dry out.

Effect of Time and Temperature

It has been suggested that differences in handling effector cells before plating may account for much of the test to test variation which is frequently observed with plaque forming cells assays(Freijd & Kunori, 1980). Thus, in the following experiments the effects of storing peripheral blood mononuclear cells for different times and at different temperatures after collection were examined. Fig.3.12 shows

the results of three such experiments in which cells were held at room temperature for different times before an 8hr plaque assay was performed. It was clear that even after 1hr there was a reduction in the number of all classes of plaque forming cells and when plaque assays were performed after a 6hours delay virtually no plaque forming cells were observed. In the second series of experiments(n=3), the effect of storing peripheral blood mononuclears for 2hours at different temperatures was examined. It can be seen from Fig.3.13 that a reduction in plaque forming cell numbers occurred when separated cells were held for 2hours at either room temperature or 37°C, while incubation of cells on ice prevented the decay in plaque forming cells activity caused by storage.

Effect of Cryopreservation of Cells on Plaque Formation

As some of the clinical studies would involve assaying large numbers of serial samples, it was important to determine whether a storage method could be devised which would allow one set of samples to be tested simultaneously, thus eliminating errors due to interassay variation. Therefore, the effect of cryopreserving peripheral blood mononuclear cells on plaque formation was studied. On separate occasions, peripheral blood mononuclear cells from one donor were tested for plaque forming cell activity when freshly obtained and after cryopreservation in liquid nitrogen for periods of 1-12 weeks. It can be seen from Fig.3.14 that the ability of peripheral blood mononuclear

cells to form plaques does not deteriorate with cryopreservation over these times. Recently, other workers have reported that plaque forming cell activity remains stable during cryopreservation over even longer periods(Tauris & Jorgensen, 1983).

Quality Control of the Assay

The ability to freeze Peripheral blood mononuclear cells without losing plaque forming cells activity allowed several experiments to be performed to establish a quality control for the assay.

In the first experiment, the variability caused by using different batches of protein A coated sheep red blood cells was examined. A large sample of peripheral blood mononuclear cells was frozen in liquid nitrogen as described previously. At weekly intervals an aliquot of cells was tested for the ability to form plaques using fresh batches of protein-A coated sheep red blood cells and in Fig.3.15 can be seen that the plaque forming cell counts of all classes were identical on twelve separate occasions. Thus, the levels of plaque forming cells were not affected by the batch of protein A coated sheep red blood cells which was used. In contrast, when serial plaque forming cell assays were performed using cells which were obtained fresh from a healthy volunteer at weekly intervals, there was considerable variation in the level of all classes of plaque forming cells(Fig.3.16). To investigate whether this variation reflected changes in lymphocyte reactivity or true

test to test variation, part of each of these peripheral blood mononuclear cell samples was cryopreserved and all twelve samples were assayed on one single occasion(Fig.3.17). It can be seen that apart from a slight elevation in IgG-PFC in the 6 week sample(also seen in Fig.3.16), there was little variation when the serial samples were tested simultaneously. Thus, the variation observed when the samples were tested fresh was due to test to test variation.

These results indicated the usefulness of cryopreservation when performing studies of large numbers of serial samples and also showed that the conditions of coating sheep red blood cells with protein A have very little effect on plaque formation.

Cycloheximide

The final experiment which investigated the methodology of the spontaneous plaque forming cell assay was designed to confirm that the assay detected only those B cells which were actively secreting immunoglobulin. Therefore, peripheral blood mononuclear cells were pre-incubated with serial dilutions of cycloheximide to examine if protein synthesis was a pre-requisite for plaque formation. Fig.3.18 shows that cycloheximide produced a dose dependent inhibition of IgA-PFC numbers with a complete absence of plaque forming cells at concentrations greater than 200μg/ml. Thus, plaque formation was due to active secretion of immunoglobulin and was not merely due to passive release of cytophilic or pre-formed cytoplasmic immunoglobulin.

CONCLUSIONS

This chapter has examined several parameters which might affect the performance and reproducibility of the protein-A plaque assay. These studies established the optimal conditions for coating sheep red blood cells with protein A and for incubating peripheral blood mononuclears with sheep red blood cells. In addition, it was shown that the number of plaque forming cells was dependent on the concentrations of sheep red blood cells, antiserum, and complement used in the assay, while incorporation of PEG 6000 in the agar mixture was found to improve the quality of the plaques. In further studies, it was also shown that effector cells should be stored on ice for as short a time as possible before assaying and that the plaques should be counted after a standard incubation period because of the marked differences observed at the different times of storage. Two findings of considerable importance for future studies of serially obtained peripheral blood mononuclear cells, were that cells could be cryopreserved without loss of plaque forming cell activity and that different batches of sheep red blood cells could be used without introducing variability to the assay.

Following these studies a positive control sample like the one shown in Fig.3.15 was routinely assayed simultaneously with the test samples to provide an indication about the performance of the assay.

NORMAL CONTROLS

	AGE	SEX
1. D.T.	38	M
2. J.L.	25	F
3. M.W.	40	F
4. V.J.	33	F
5. T.D.	31	M
6. P.D.	32	M
7. B.J.	40	M
8. E.F.	55	F
9. E.G.	28	M
10. G.C.	20	M
11. A.Y.	20	M
12. C.M.	32	M
13. T.M.	29	F
14. D.D.	27	M
15. G.P.	29	M
16. Z.M.	35	M
17. M.F.	29	F
18. J.V.	32	M
19. C.T.	30	M
20. S.T.	28	M
21. A.S.	21	F
22. D.K.	27	M
23. P.T.	24	M
24. S.B.	27	M
25. D.L.	25	M
	Mean+SEM:30.2+1.5	M/F:18/7

Table 3.1

Normal controls used in all the experiments described

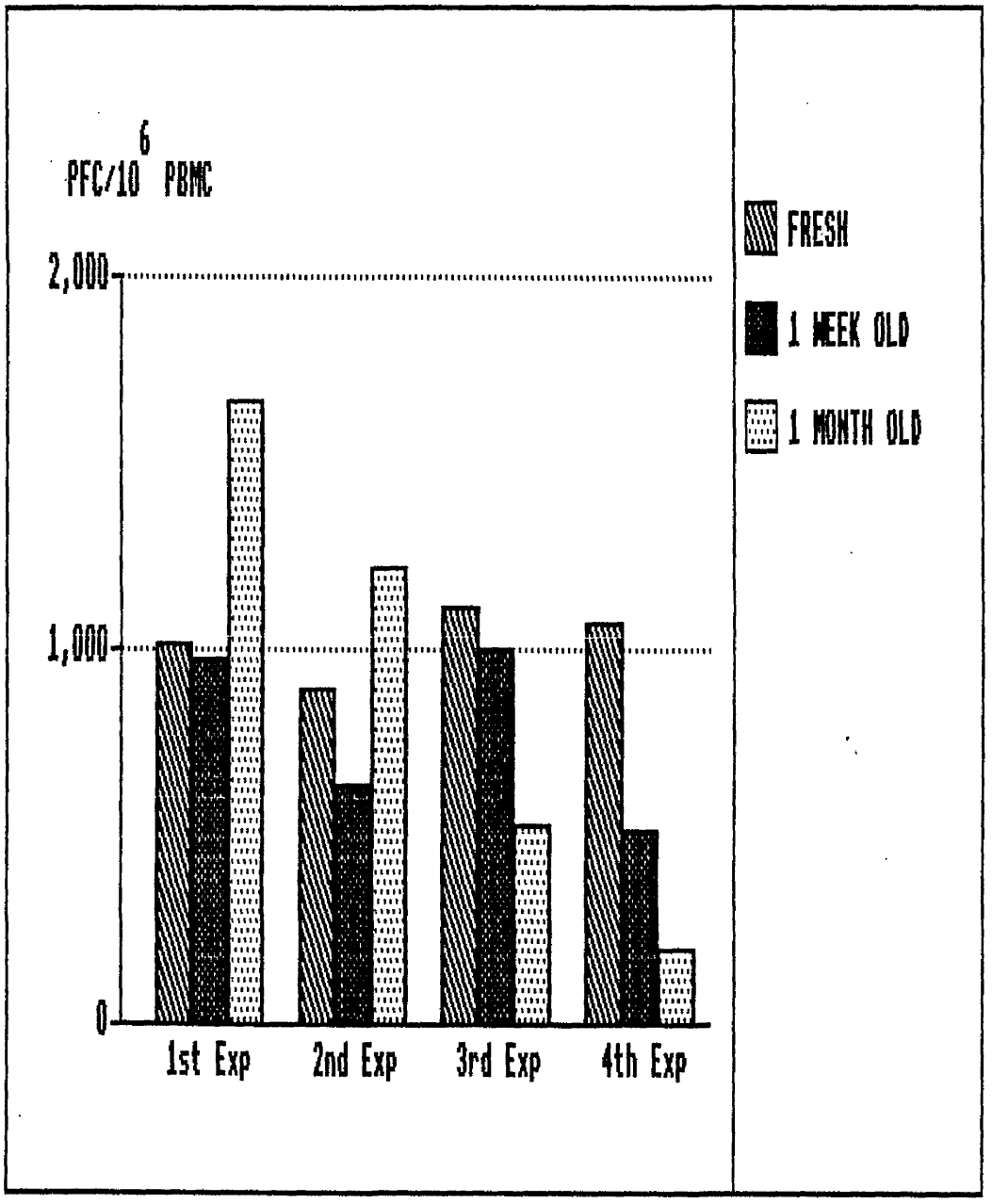


Fig.3.1

Influence of the age of Chromic Chloride on spontaneous IgA plaque formation. Bars represent the mean of triplicate determinations(PFC/10^6 cells).

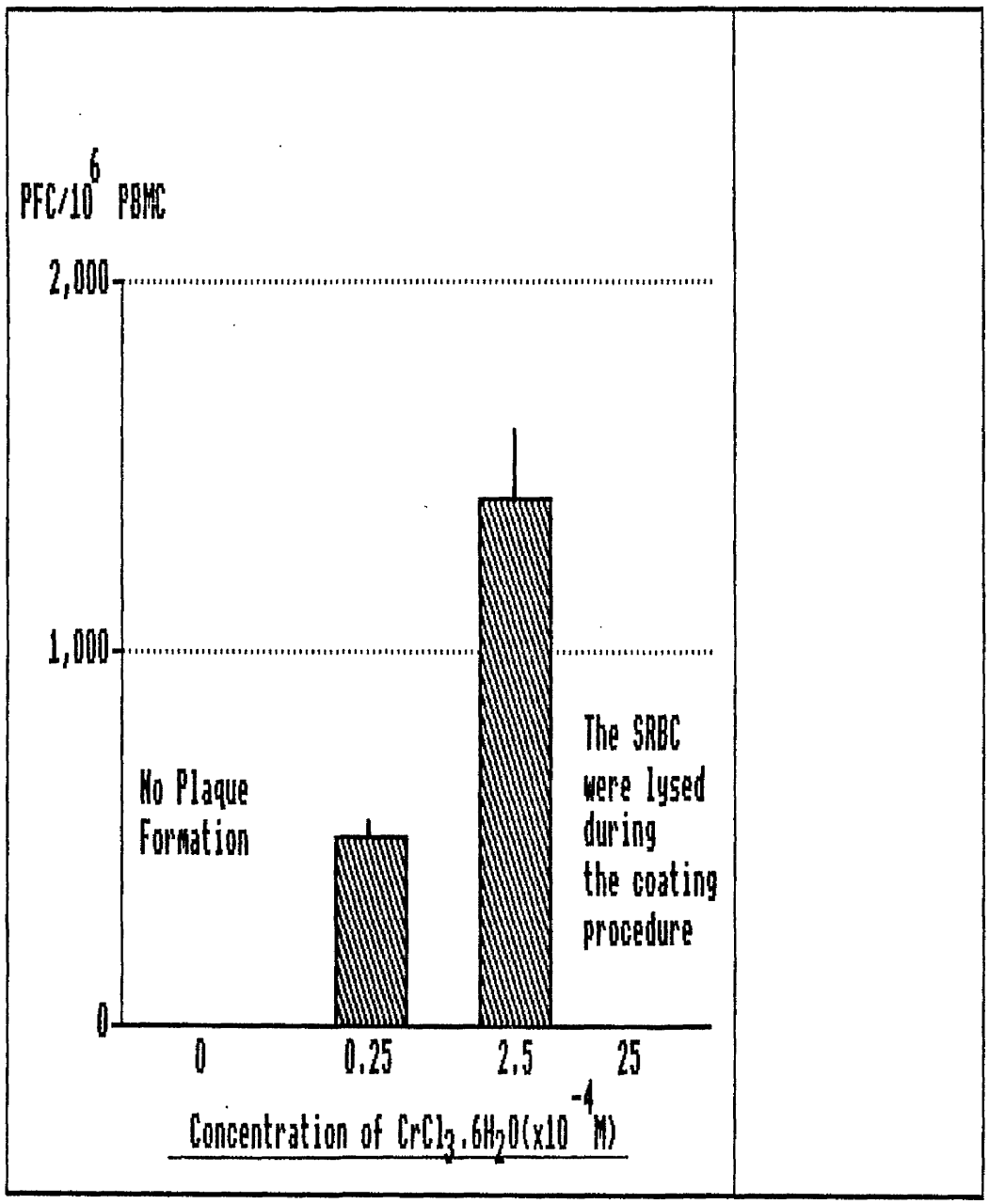


Fig.3.2

The effect of Chromic Chloride concentration on spontaneous IgA plaque formation by control peripheral blood mononuclear cells. Bars representthe mean+1SEM(PFC/10^6 cells) of three experiments.

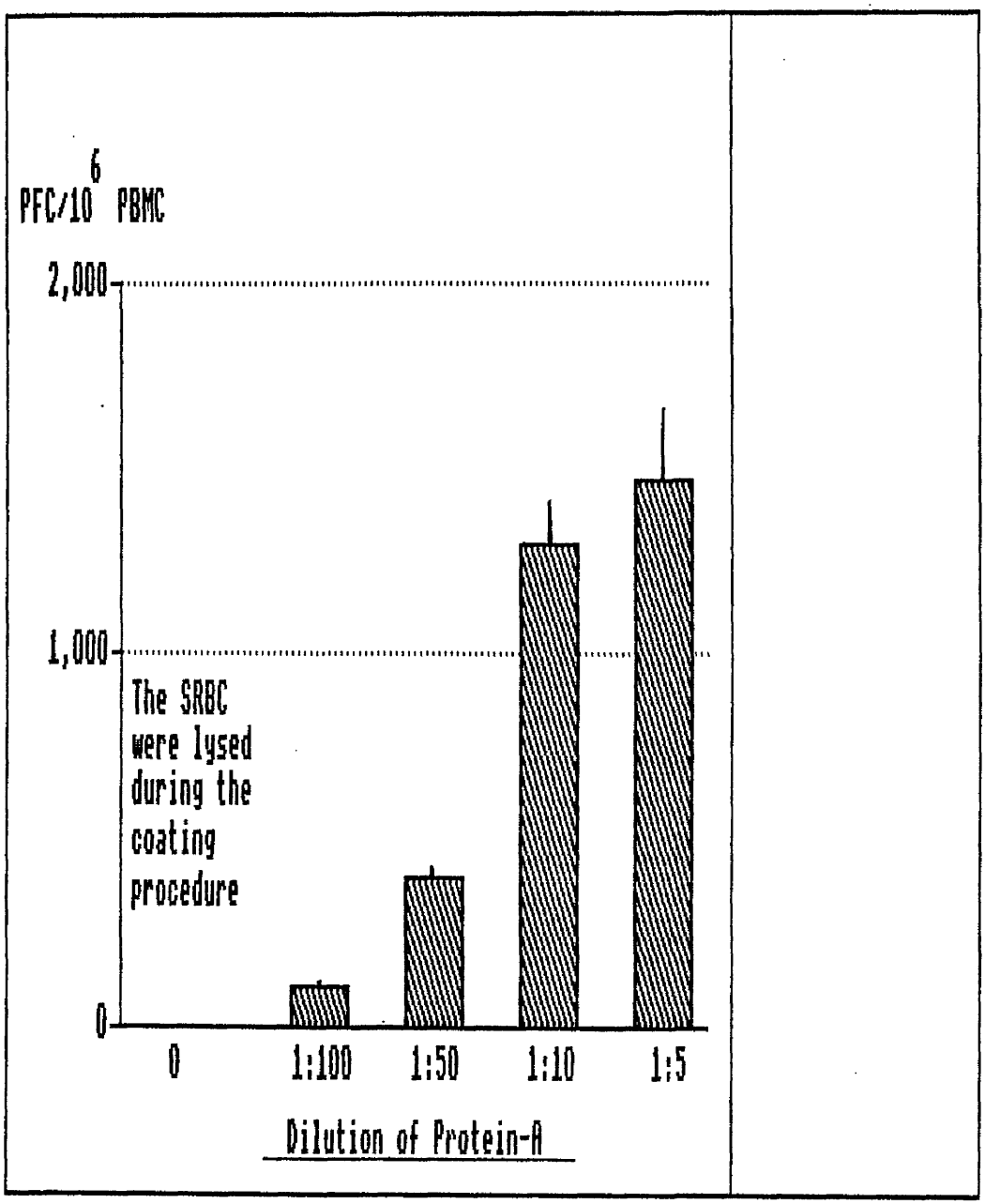


Fig.3.3

The effect of the amount of Protein-A added to the reaction mixture on spontaneous IgA plaque formation. Bars represent the mean+1SEM(PFC/10^6 cells) of three experiments.

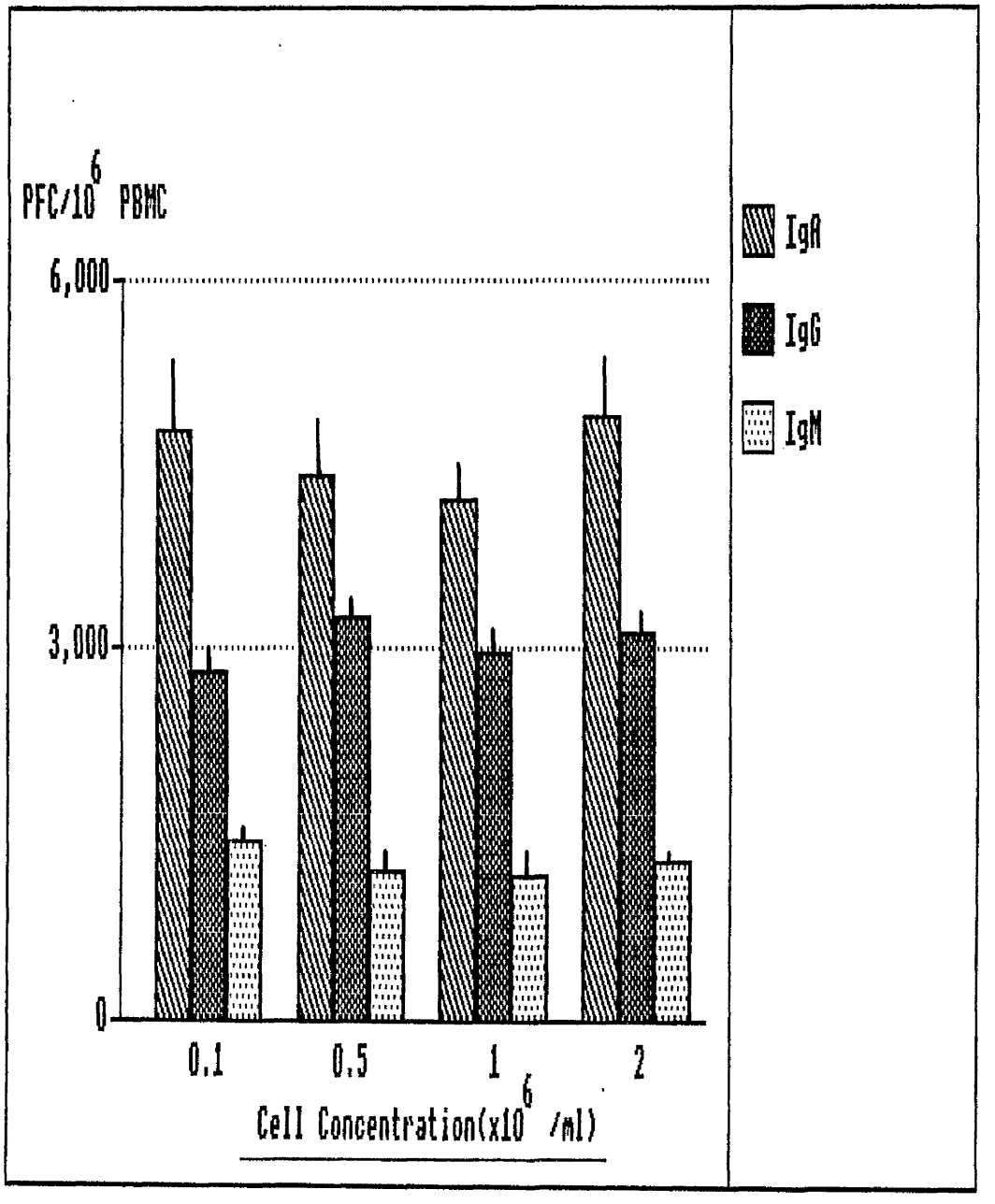


Fig.3.4

The effect of concentration of peripheral blood mononuclear cells on spontaneous IgA plaque formation.
Bars represent the mean+1SEM(PFC/10^6 cells) of three experiments.

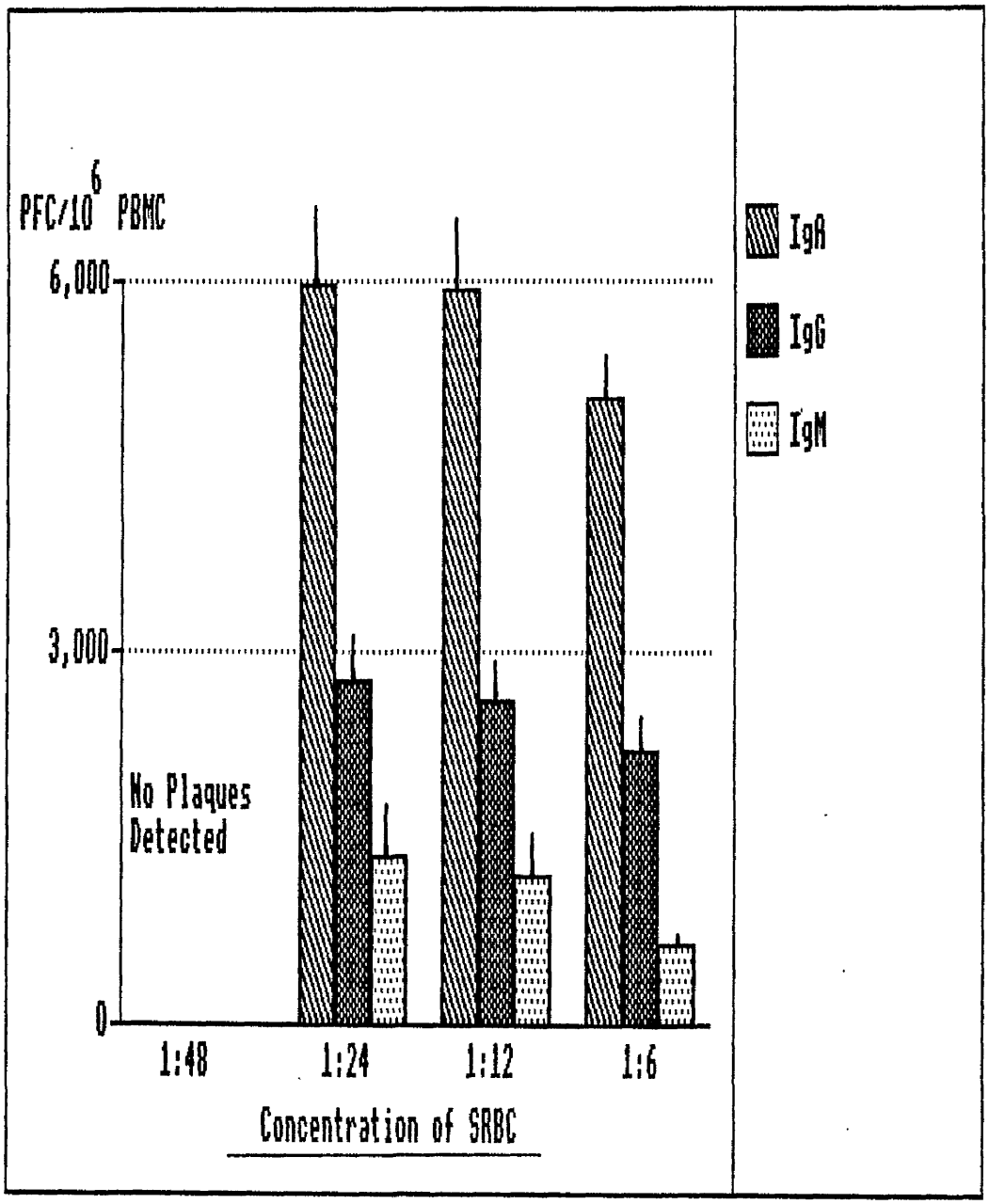


Fig.3.5

The effect of the concentration of sheep red blood cells on spontaneous plaque formation.
Bars represent the mean+1SEM(PFC/10^6 cells) of five experiments.

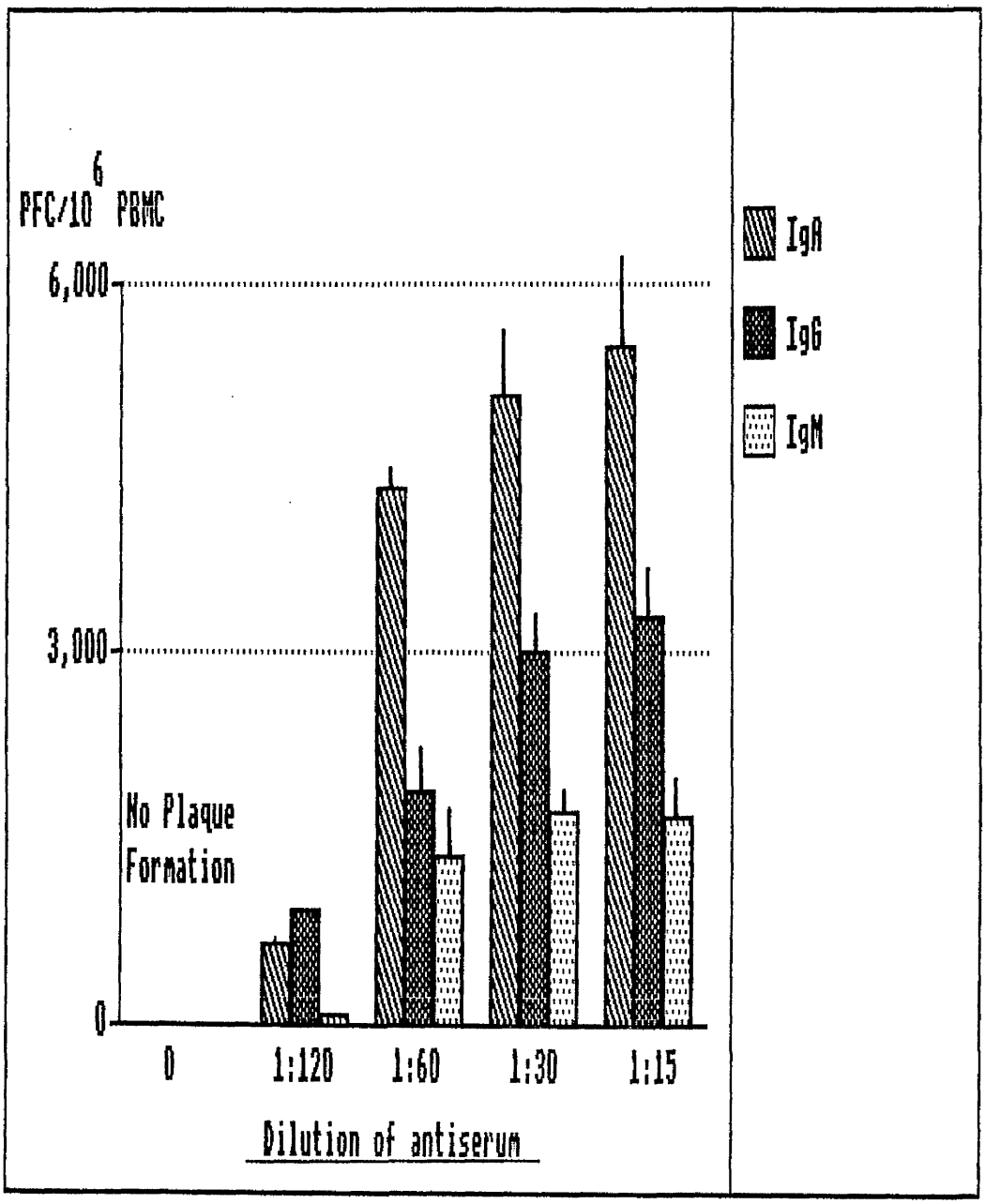


Fig.3.6

The effect of the amount of developing antisera on spontaneous plaque formation. Bars represent the mean+1SEM(PFC/10^6 cells) of five experiments.

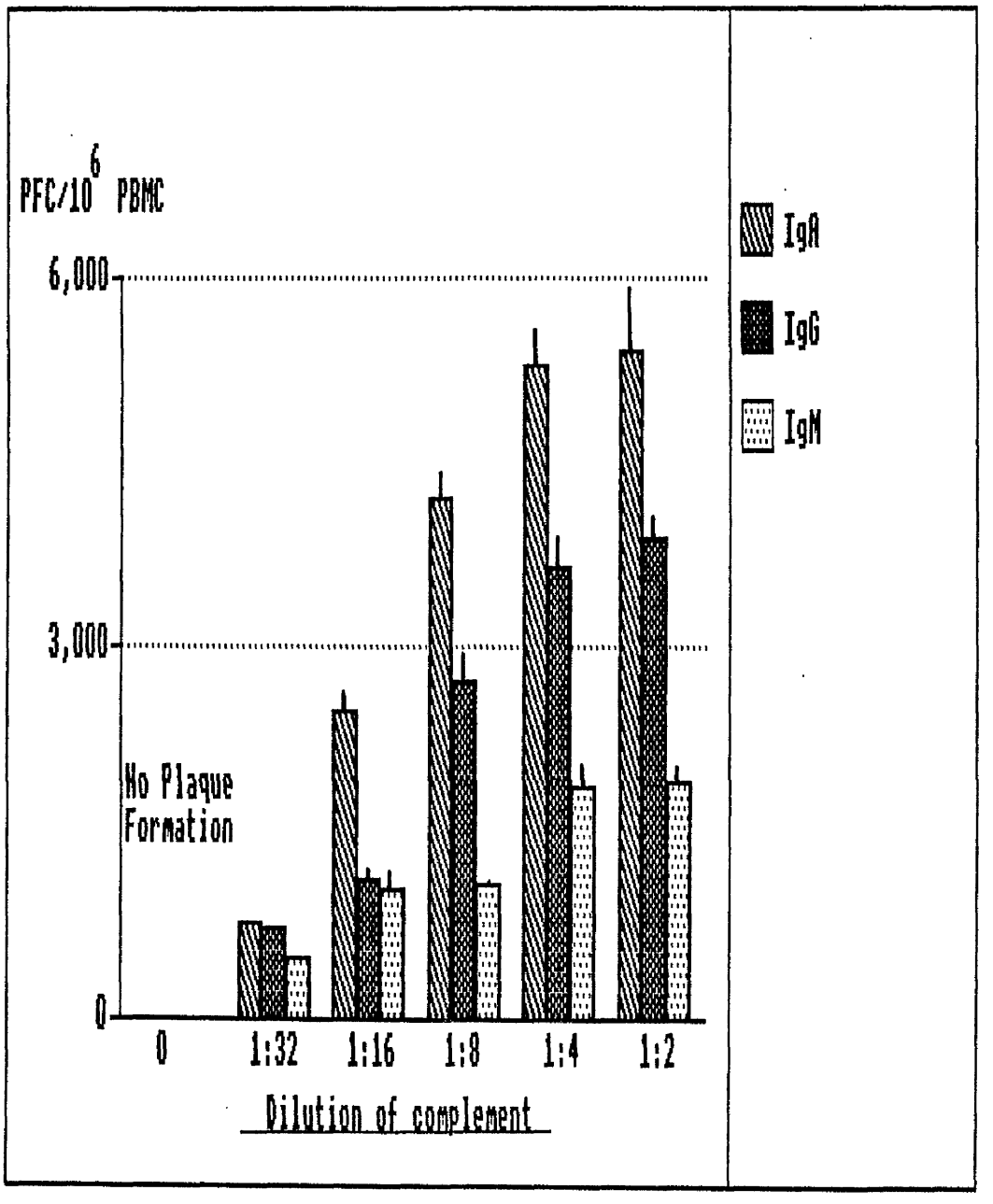


Fig.3.7

The effect of the amount of complement on spontaneous plaque formation. Bars represent the mean+1SEM(PFC/10^6 cells) of five experiments.

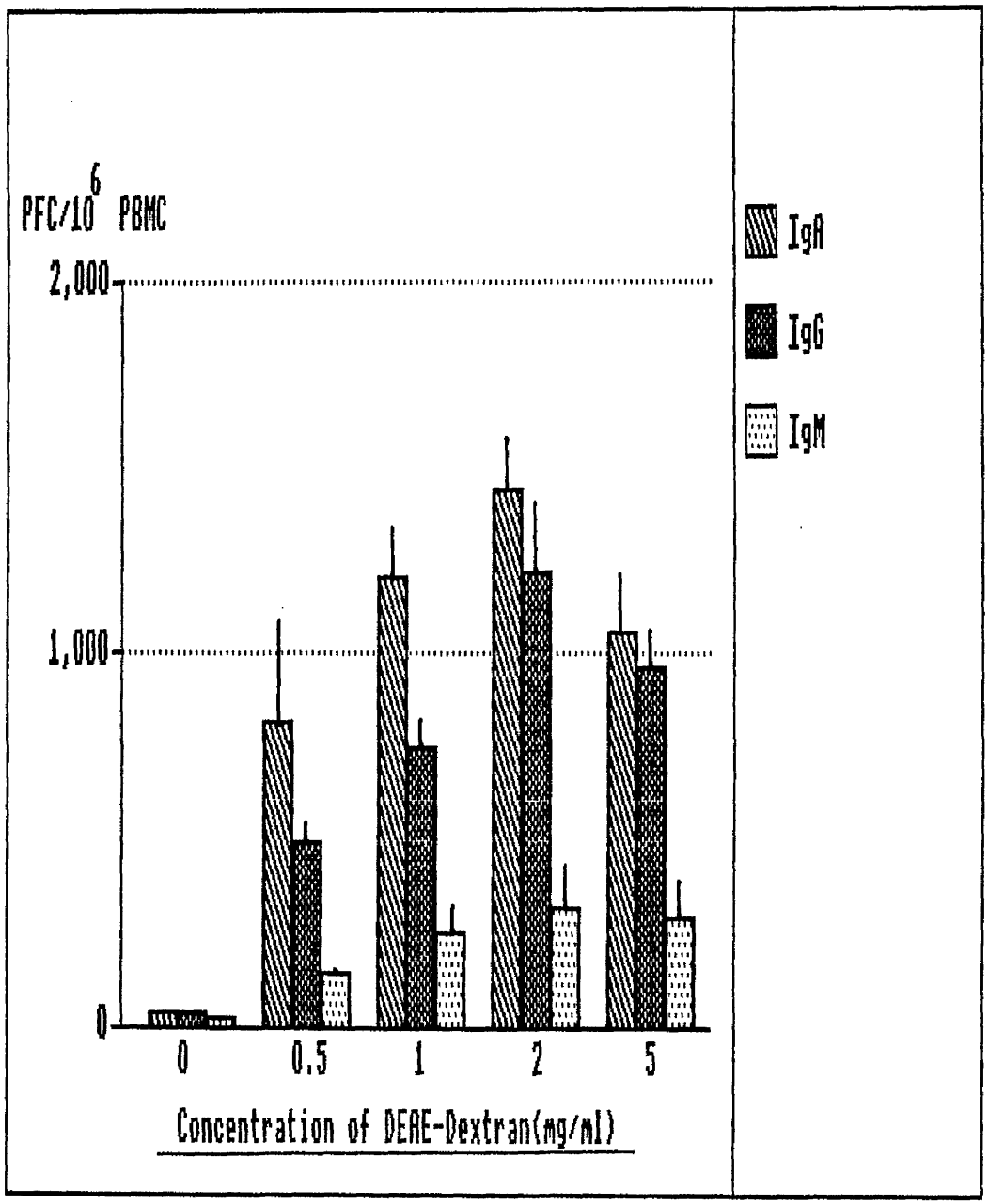


Fig.3.8

The effect of adding DEAE-dextran on spontaneous plaque formation.
Bars represent the mean+1SEM(PFC/10^6 cells) of three experiments.

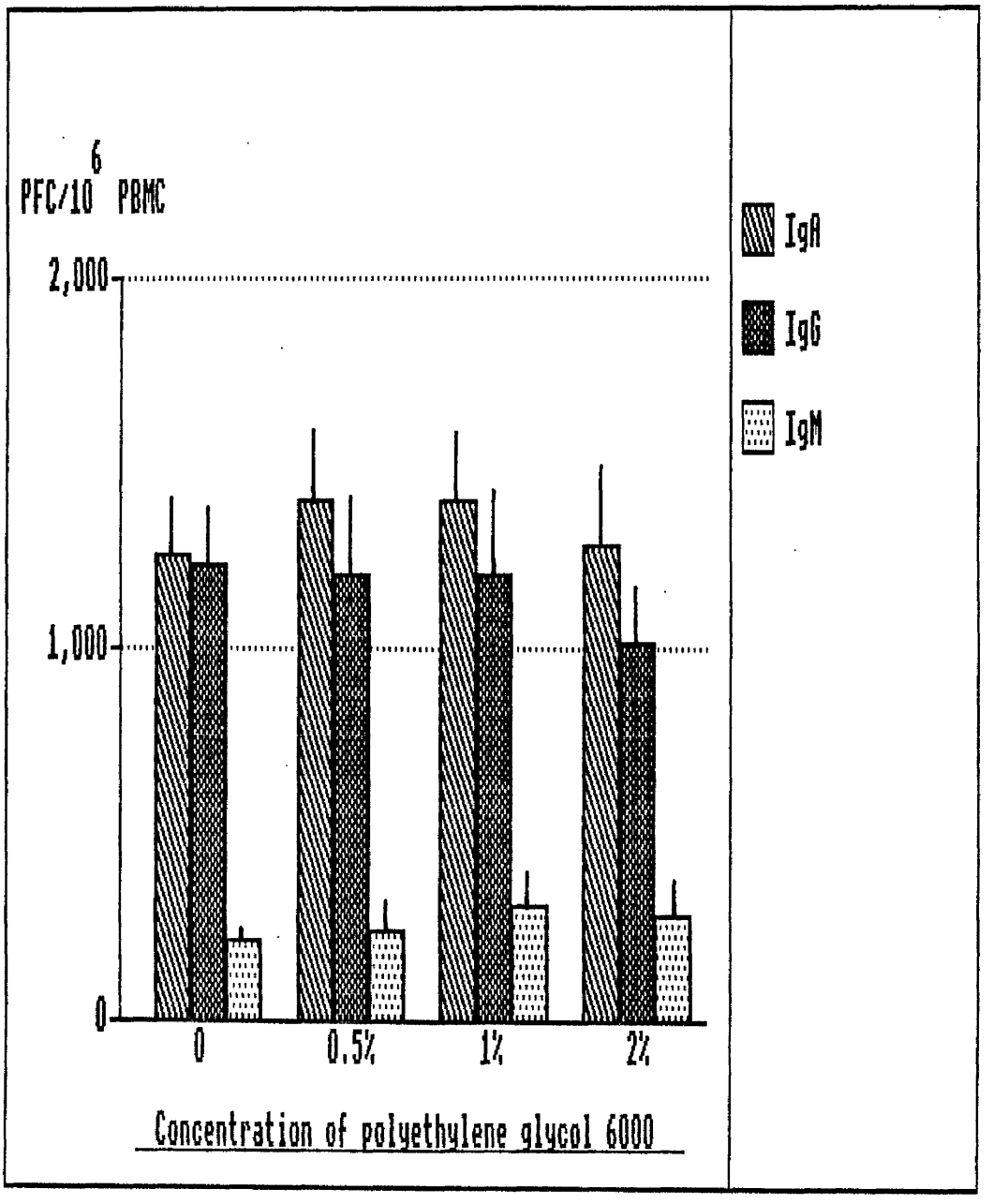


Fig.3.9

The effect of adding polyethylene glycol 6000 on spontaneous plaque formation. Bars represent the mean+1SEM(PFC/10^6 cells) of three experiments.

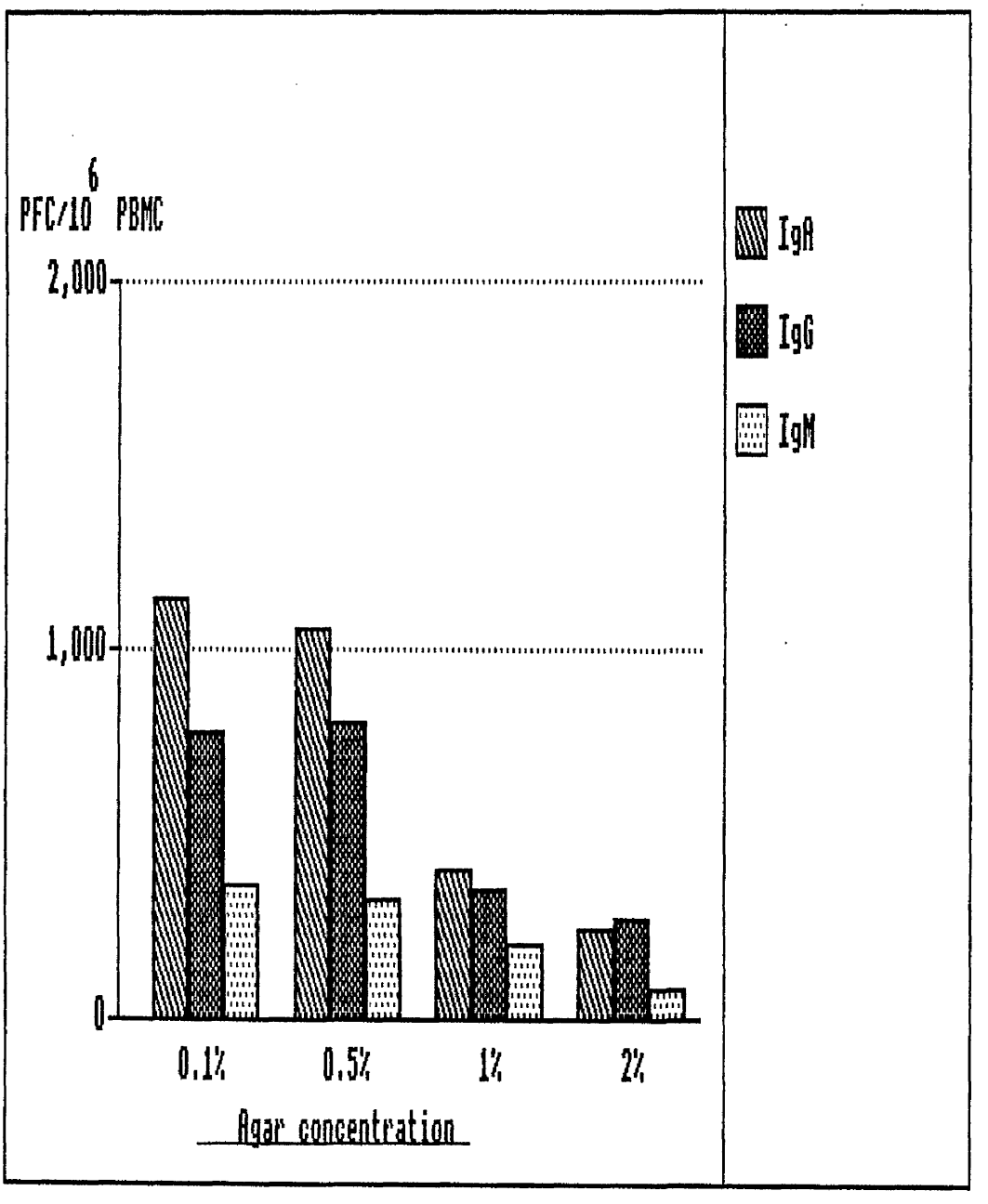


Fig.3.10

The effect of agar concentration on spontaneous plaque formation.
Bars represent the mean of triplicate deteminations(PFC/10^6 cells)

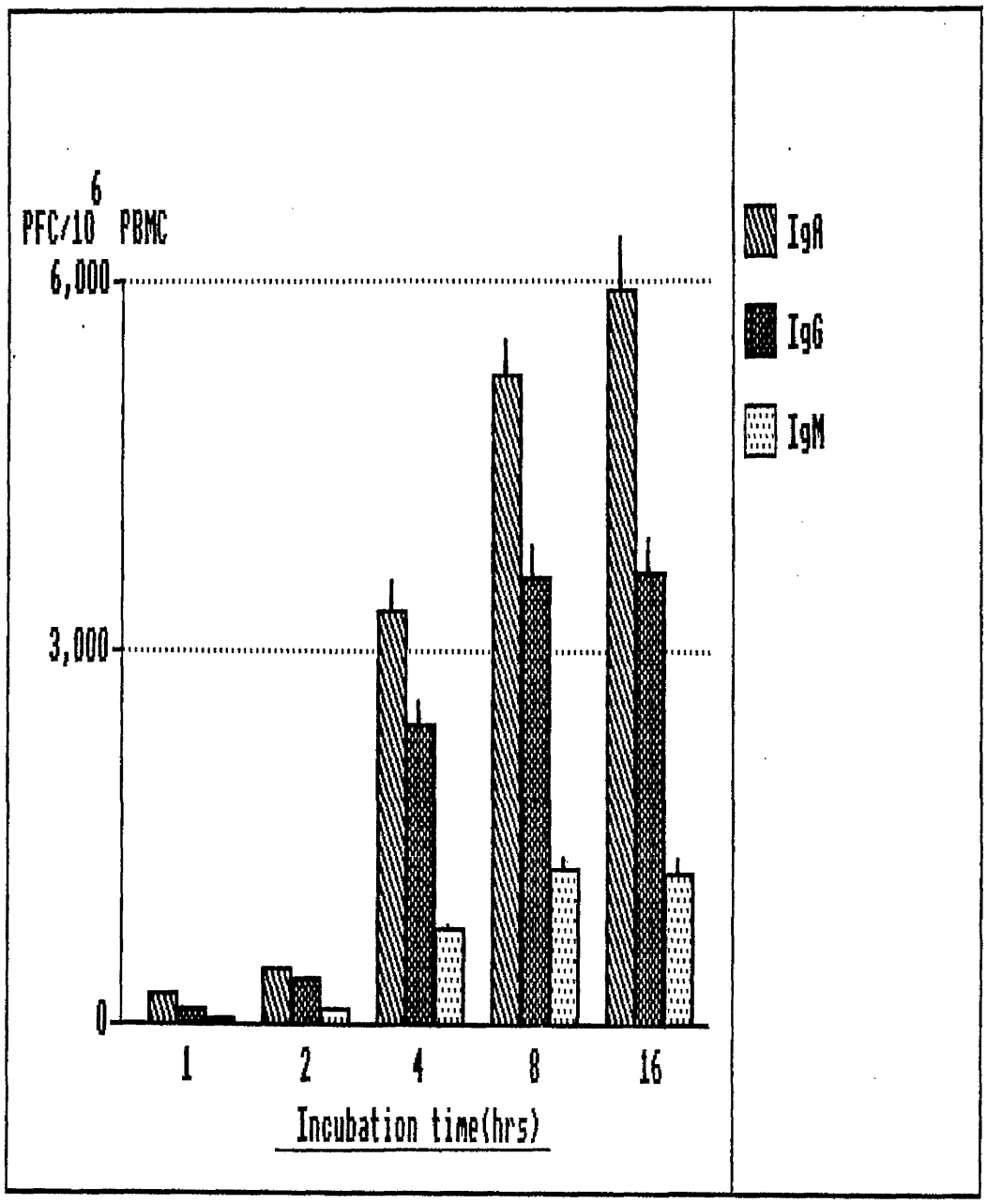


Fig.3.11

The effect of incubation time on the development of spontaneous plaques. Bars represent the mean+1SEM(PFC/10^6 cells) of four experiments.

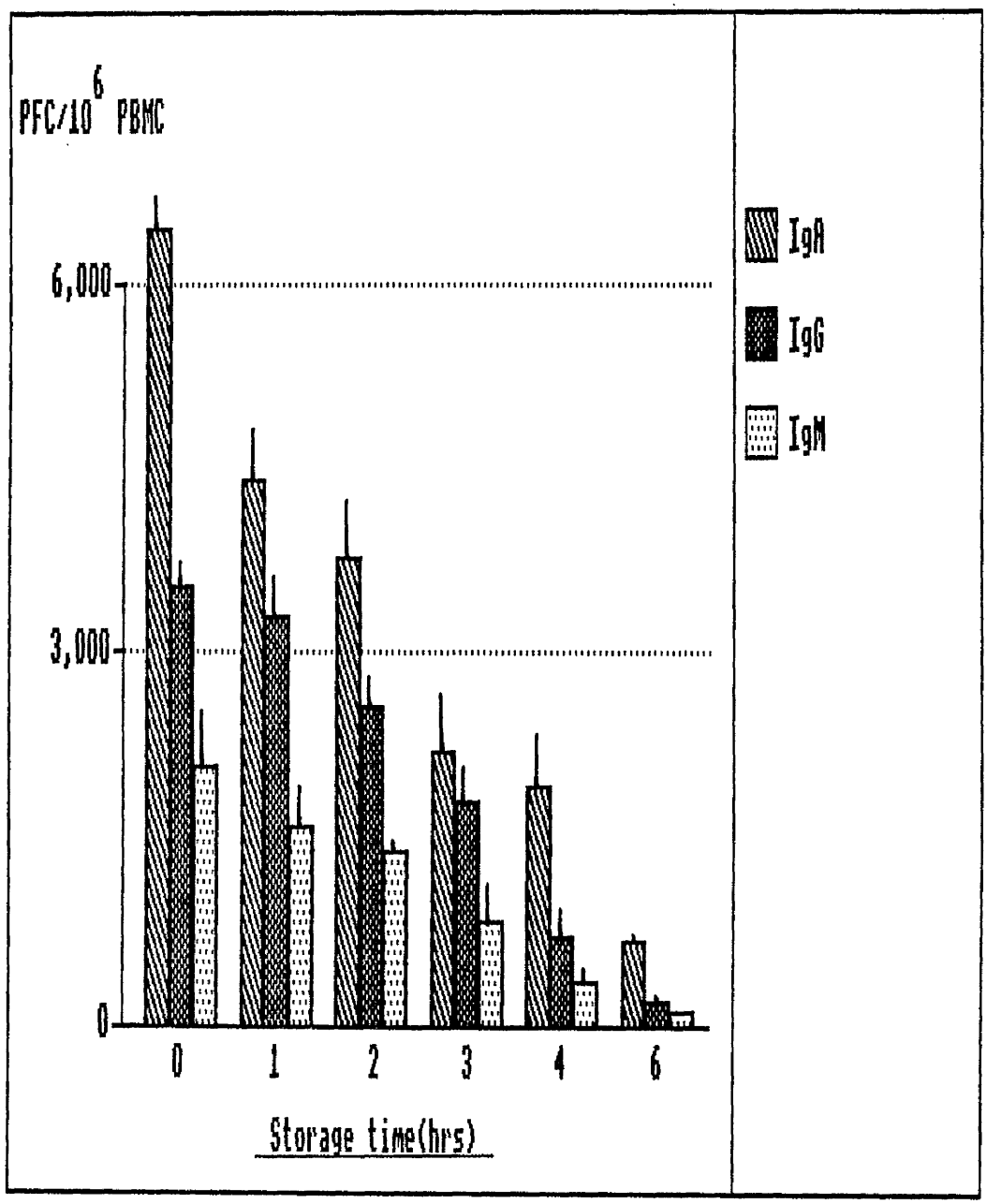


Fig.3.12

The effect of storing control peripheral blood mononuclear cells for different times on spontaneous plaque formation.
Bars represent the mean+1SEM(PFC/10^6 cells) of three experiments.

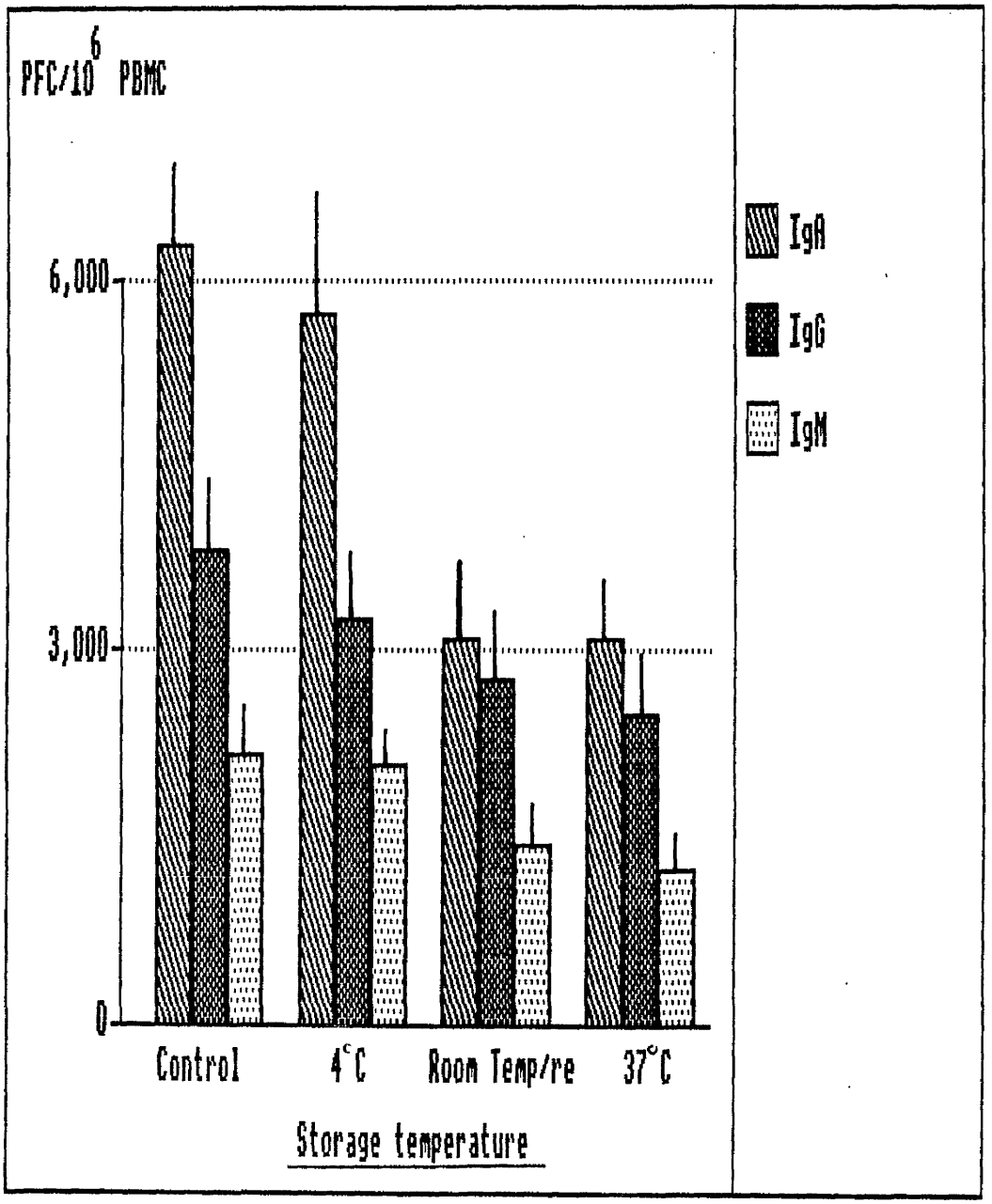


Fig.3.13

The effect of storage temperature on spontaneous plaque formation. Control cells were assayed immediately without storage while other cells were stored for 2hrs at different temperatures.
Bars represent the mean+1SEM(PFC/10^6 cells) of three experiments.

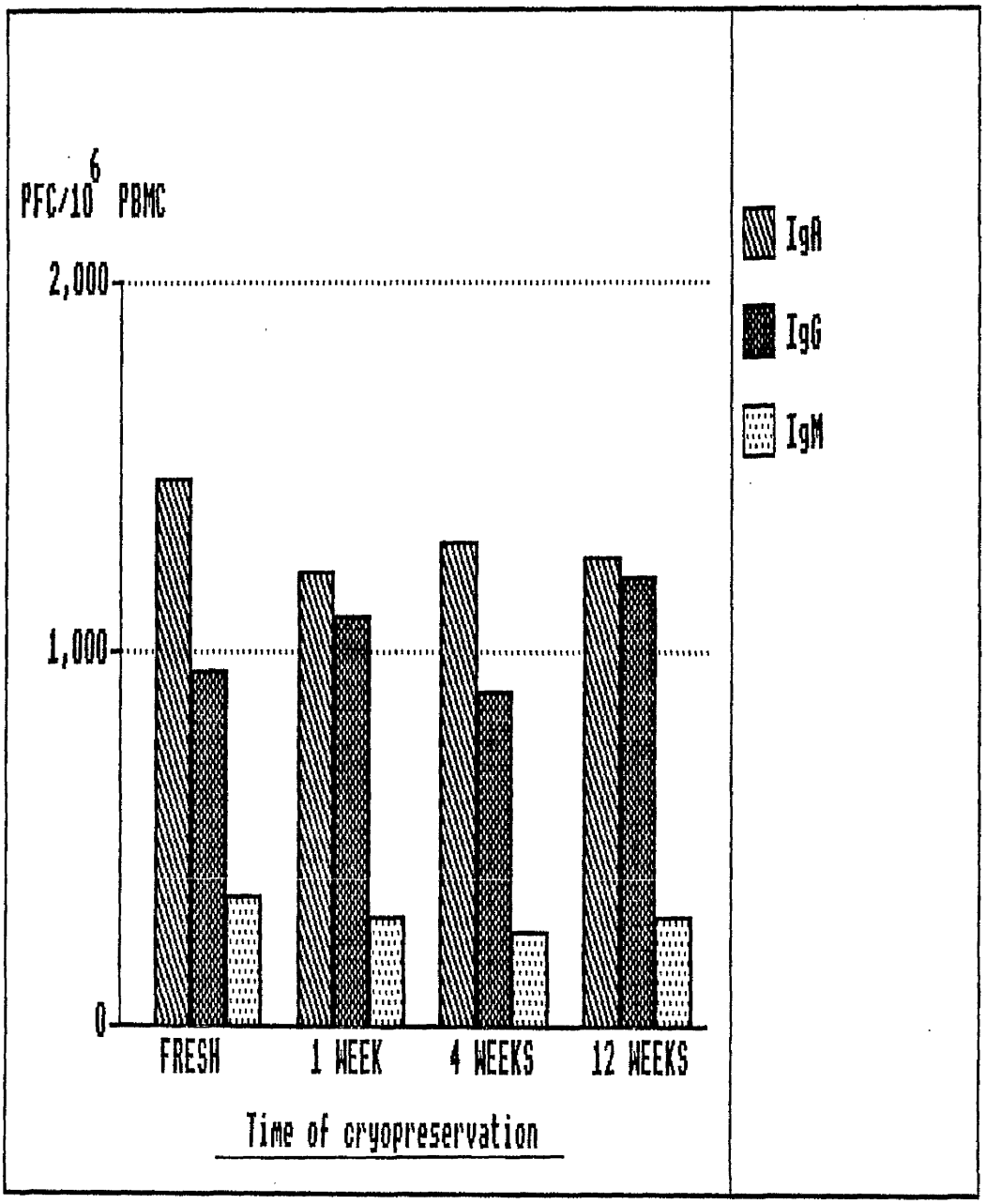


Fig.3.14

The effect of cryopreservation on spontaneous plaque formation. Peripheral blood mononuclear cells were assayed either fresh or after storage in liquid nitrogen for different times. Bars represent the mean of triplicate determinations(PFC/10^6 cells).

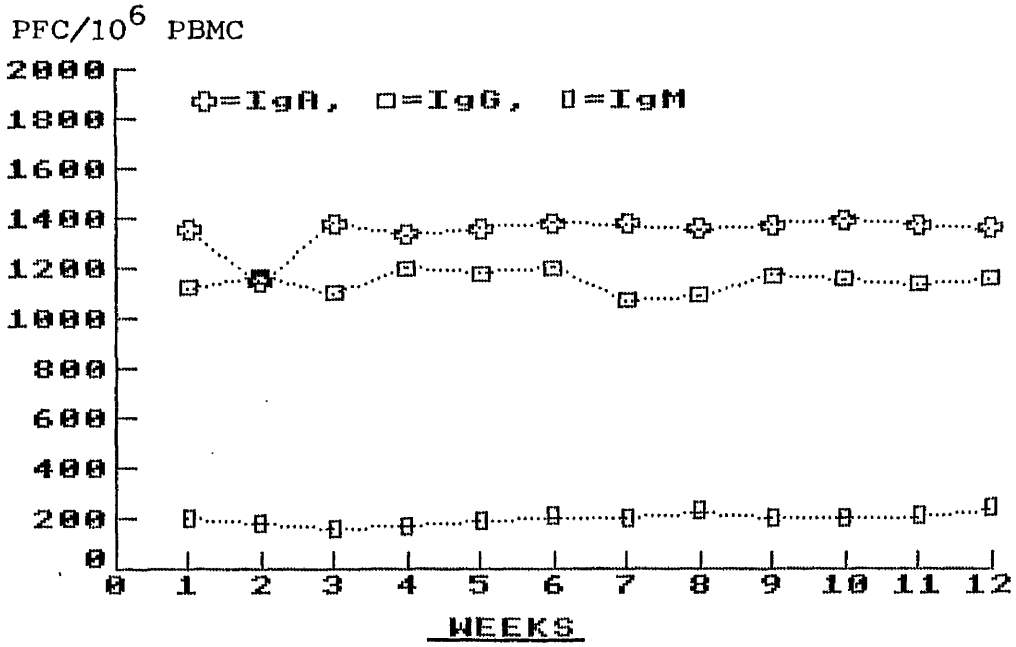


Fig.3.15

Spontaneous plaque formation by cryopreserved peripheral blood mononuclear cells obtained from the same healthy subject. One aliquot of cells was tested weekly using different batches of protein-A coated sheep red blood cells.
Graphs shown are the mean of triplicate determinations ($PFC/10^6$ cells)

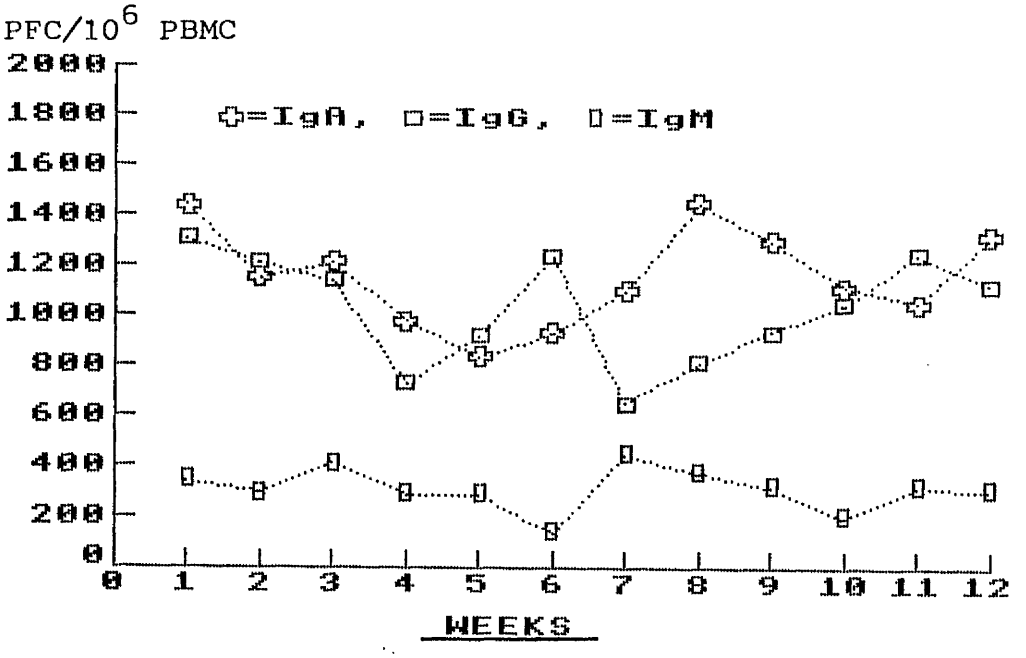


Fig.3.16

Spontaneous plaque formation by peripheral blood mononuclear cells obtained from the same healthy subject at weekly intervals. Each sample was tested for spontaneous plaque formation using different batches of protein-A coated sheep red blood cells.
Graphs shown are the mean of triplicate determinations ($PFC/10^6$ cells)

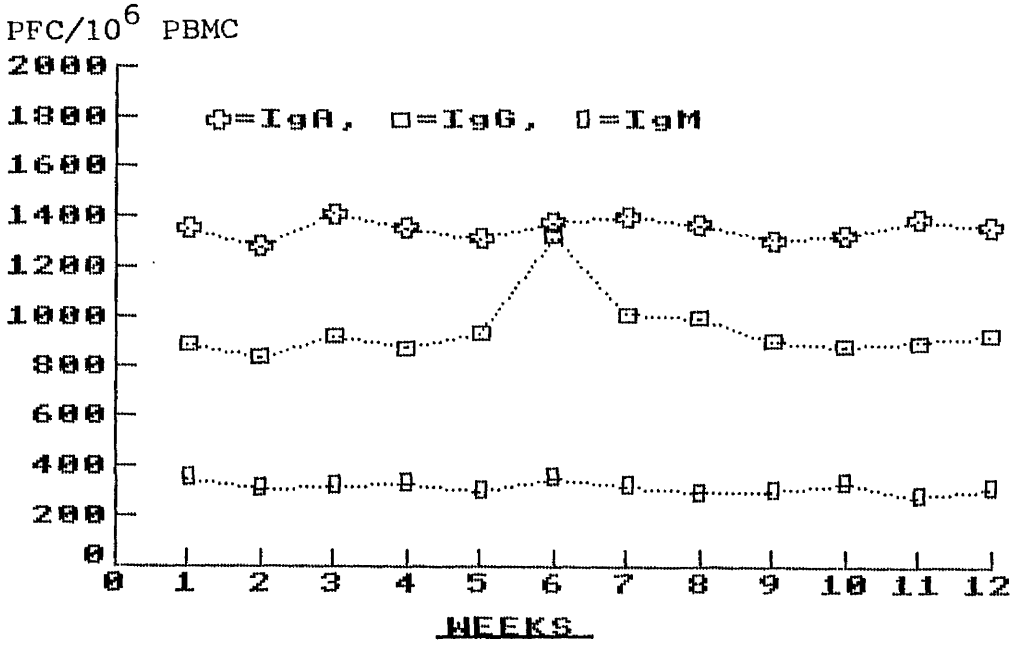


Fig.3.17

Spontaneous plaque formation by cryopreserved peripheral blood mononuclear cells obtained at weekly intervals from the control subject shown at Fig.3.16. All twelve samples were tested on the same day.
Graphs shown are the mean of triplicate determinations ($PFC/10^6$ cells)

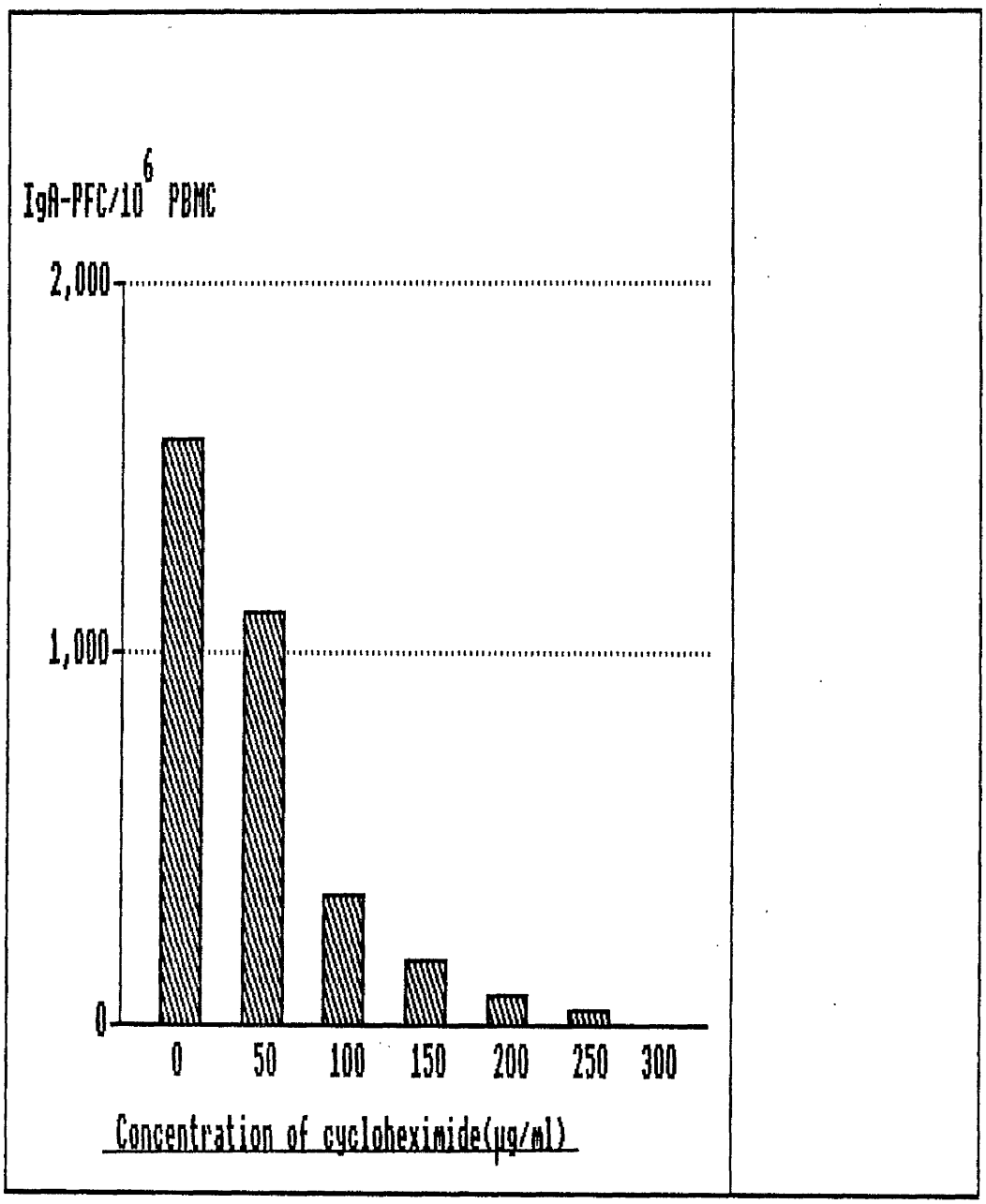


Fig.3.18

The effect of pre-incubating peripheral blood mononuclear cells with cycloheximide on spontaneous IgA plaque formation.
Bars represent the mean of triplicate determinations ($PFC/10^6$ cells)

CHAPTER IV

PROLIFERATIVE AND PLAQUE FORMING CELL RESPONSES OF PERIPHERAL BLOOD MONONUCLEAR CELLS STIMULATED WITH MITOGENS IN VITRO

INTRODUCTION

A major aspect of this thesis was to study the ability of patients's peripheral blood mononuclears cells to proliferate and secrete immunoglobulin in response to mitogens and therefore the aim of the following experiments was to study the factors which affected proliferation and immunoglobulin secretion by mitogen stimulated normal peripheral blood mononuclear cells. The proliferative responses of control peripheral blood mononuclear cells were studied by measuring DNA synthesis in response to the T cell mitogen phytohaemagglutinin(PHA) and to a mitogen which stimulates both T and B cells-pokeweed mitogen(PWM). In addition, the polyclonal B cell activators, PWM and SAC, were used alone and together to establish the optimal conditions for immunoglobulin secretion in vitro.

MATERIALS AND METHODS

Venous blood from healthy subjects(Table 3.1) was collected, mononuclear cells were prepared and cultures of peripheral blood mononuclears were set up as described previously. Proliferation was measured after pulse-labeling the cultures with 14C-thymidine and immunoglobulin secretion was measured using the protein-A plaque assay to enumerate immunoglobulin secreting cells.

RESULTS

Measurement of Proliferative Responses to PHA and PWM

In the first experiments, the response of peripheral blood mononuclear cells to mitogens was examined. Cells were cultured in medium containing FCS selected for its low mitogenic activity and the effect of different concentrations of PHA and PWM are presented in Fig.4.1 and Fig.4.2 respectively. When cells were cultured in the presence of mitogen, a dose-dependent response in thymidine-uptake was noted which was maximal with 10μg/μl of PHA and 1/500 dilution of PWM. Fig.4.3 also shows the response of peripheral blood mononuclear cells after 1-5days in culture with optimal doses of PHA and PWM respectively. It can be seen that the highest responses to both mitogens were observed on the third day of culture and thereafter the responses declined. This loss of responsiveness after three days was associated with falling viability in the cultures(data not shown), and so three days was chosen for all future experiments measuring proliferative responses to mitogens. PHA and PWM induced blastogenesis measured after 3 days of culture tends to reflect T cell proliferation(Cupps & Fauci, 1982) and since SAC is known to stimulate selectively B cells(Falkoff et al, 1982)an attempt was made to establish the optimal conditions for SAC induced blastogenesis. Four experiments were performed and although a degree of thymidine uptake was obtained(data not shown), the dose and day responses were not reproducible.

The effect of using different concentrations of peripheral blood mononuclear cells on their response to either PHA or PWM was examined next. Although, good responses were obtained when cells were cultured at concentrations between 0.5×10^{5}-50×10^{5} cells/ml, optimal proliferation was found using 5×10^{5} cells/ml, cell crowding producing significant suppression of lymphocyte transformation(Fig.4.4).

Production of Immunoglobulin in Response to Polyclonal B Cell Activators

Having established the optimal conditions for mitogen induced proliferative responses, I went on to examine the optimal conditions for immunoglobulin secretion in response to PWM and SAC. Peripheral blood mononuclear cells from different donors were cultured with different concentrations of PWM and SAC and secretion of IgA, IgG, and IgM was measured using the plaque forming cell assay. Fig.4.5 shows IgA, IgG and IgM responses obtained from four experiments using PWM and it can be seen that the most consistent responses were obtained using a 1:100 final dilution of PWM. This applied to all isotypes and this dilution was used in all subsequent experiments. Next, the optimal dose for SAC was determined and Fig.4.6 shows plaque forming cell responses from four different cultures stimulated with the same batch of SAC. It was essential to use the same batch of bacteria in each group of experiments since it was prepared in the laboratory on several different occasions and

different conditions. The 1:1000 dilution of SAC gave the highest plaque forming cell response and, despite some variation between batches of SAC, this dilution consistently gave the optimal response.

Synergistic Effects of PWM and SAC on Immunoglobulin Production

In the next series of experiments, peripheral blood mononuclear cells were cultured with both SAC and PWM to study the ability of a combination of these two activators to produce more efficient B cell activation than either alone(Saiki & Ralph, 1981b). As before, all classes of plaque forming cell response to PWM and SAC alone were optimal using 1:100 and 1:1000 dilutions respectively(Table 4.1). For each concentration of PWM, addition of all concentrations of SAC produced more plaque forming cells than seen with PWM alone, and, using lower concentrations of mitogens, the response of mixed cultures was greater than would be expected if the responses to either alone were added. This synergistic effect was most pronounced using 1:10000 SAC plus 1:1000 PWM and these concentrations were used in all subsequent experiments.

Time Course of Plaque Forming Cell Development

In view of the different proliferative responses observed at different times of culture it was important also to examine the time course of plaque formation by cultured

cells in response to polyclonal B cell activators.

In the previous series of experiments IgG-PFC counts were consistently shown to be the highest and it was this class which was subsequently studied in order to establish the optimal conditions of mitogen-induced immunoglobulin production. Thus, peripheral blood mononuclear cells were cultured with PWM, SAC, PWM+SAC and assayed for IgG secretion one to eight days later(Fig.4.7). Very few plaque forming cells were found in cultures before day 4 and in each case the optimal response was usually obtained on the sixth day, with a similar response also frequently being found on the seventh day. On the eigth day a sharp drop in plaque forming cell counts was observed and this was associated with marked loss of viability within the cultures, which was normally >90% until this time.

Effect of Cell Concentration on Immunoglobulin Production

The effect of cell concentration on PWM or SAC induced IgG-PFC was also studied and the results are shown in Fig.4.8. Peripheral blood mononuclear cells were cultured in concentrations ranging from 0.5 to 50×10^5 PBMC/ml and, as with the proliferative responses, most cell concentrations responded well in the plaque forming cell assay, but the highest plaque forming cell responses were consistently obtained when cells were cultured at either 5 or 10×10^5 PBMC/ml. The lower of these concentrations was used in subsequent experiments to minimise cell use in patients with low white blood cell count.

Reproducibility of Mitogen Stimulated Immunoglobulin Production

Reproducibility is a major concern when measuring mitogen-stimulated immunoglobulin production(Burns & Pike, 1981). Therefore, similar experiments to those performed to study the reproducibility of spontaneous plaque formation were performed. When peripheral blood mononuclears from one donor were cryopreserved for 1-12 weeks and assayed in individual experiments for PWM-induced plaque forming cell activity, the levels of all plaque forming cell classes remained stable throughout the period(Fig.4.9). When peripheral blood mononuclears were taken on 12 separate occasions from a single donor and cutured fresh with PWM, considerable variations were observed in the level of plaque forming cells over that period(Fig.4.10). As with the spontaneous plaque forming cell assay, these variations were due to test to test variability in the plaque assay itself, rather than in lymphocyte reactivity, as these differences were minimised when each blood sample was cryopreserved and assayed on the same occasion(Fig.4.11). However, it should be noted that there was generally more variability in the plaque forming cell responses after culture than with the spontaneous plaque forming cell assay, presumably reflecting the greater complexity of the culture system.

Other Factors Affecting reproducibility

It was also observed that other factors like different

materials, reagents and equipment used could affect significantly plaque forming cell activity. Thus, culture tubes of different shape, PWM or FCS from different sources and different CO2 incubators were all found to affect plaque formation(Data not shown)

CONCLUSIONS

This chapter examined the optimal conditions for both proliferation and immunoglobulin production in response to mitogenic stimulation.

Three parameters which may affect the proliferative response were studied and the optimal response was obtained when cells were cultured at a final concentration of 5×10^5 PBMC/ml in the presence of either 10µg/µl PHA or 1/500 PWM for three days. In parallel studies of immunoglobulin production in vitro the optimal plaque forming cell response was obtained when 5×10^5 PBMC/ml were cultured in the presence of 1/100 PWM, or 1/1000 SAC or 1/1000 PWM plus 1/10000 SAC for 6 days.

In addition my studies confirmed the synergistic effect of PWM and SAC on immunoglobulin production and established the optimal conditions for this synergy. Studies using cryopreserved peripheral blood mononuclear cells confirmed their suitability for use in mitogen-stimulated cultures and indicated that the reproducibility of the PWM and SAC culture systems was satisfactory. Thus, the experiments described here and in CHAPTER III have established the optimal conditions for studying B cell responses using normal peripheral blood mononuclear cells and these methods will now be applied to the study of B cell activation and immunoregulation in uraemic subjects.

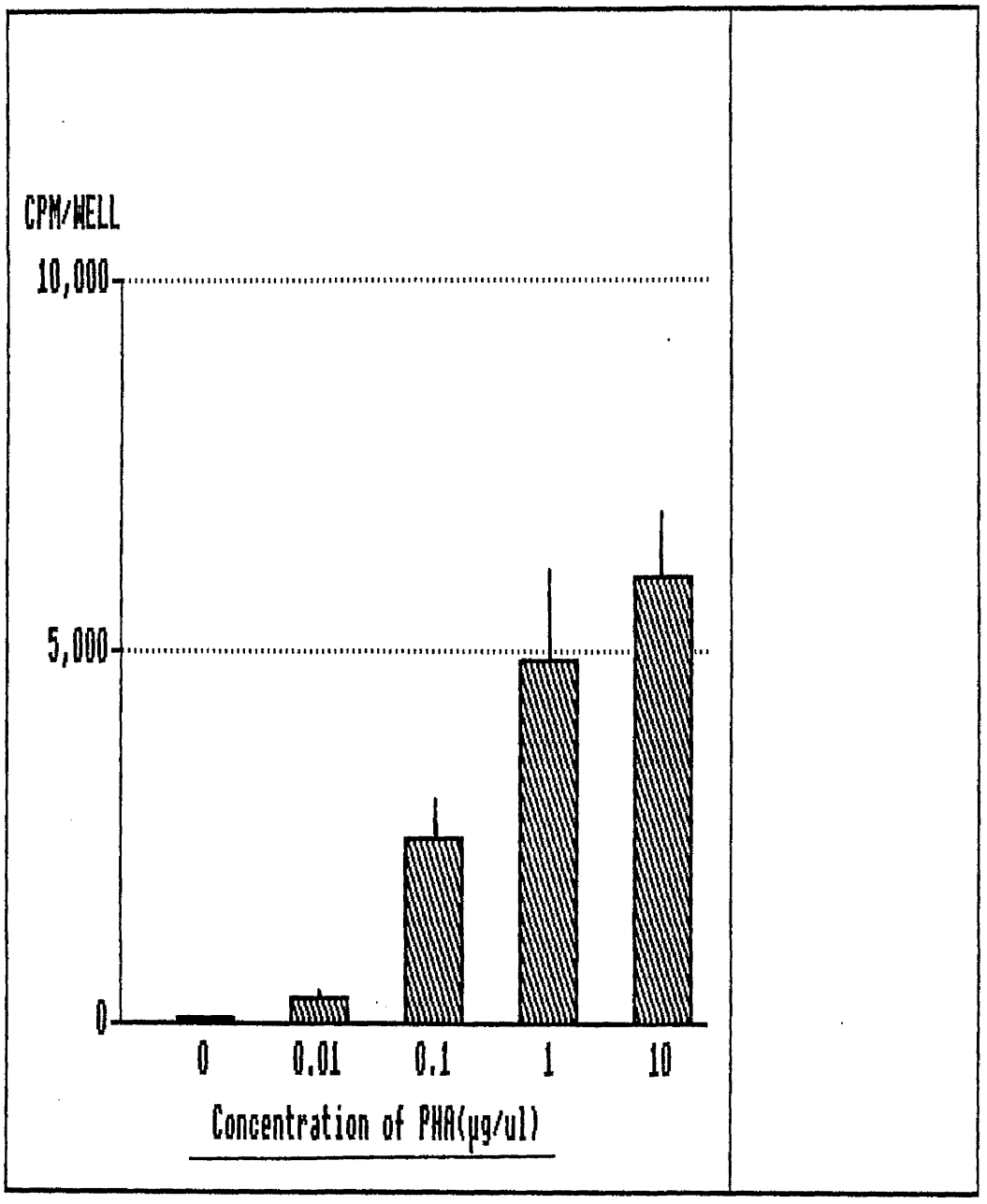


Fig.4.1

Proliferative responses of control peripheral blood mononuclear cells to PHA.
Bars represent the mean ^{14}C Thymidine uptake on the third day of culture +1SEM in 4 experiments.

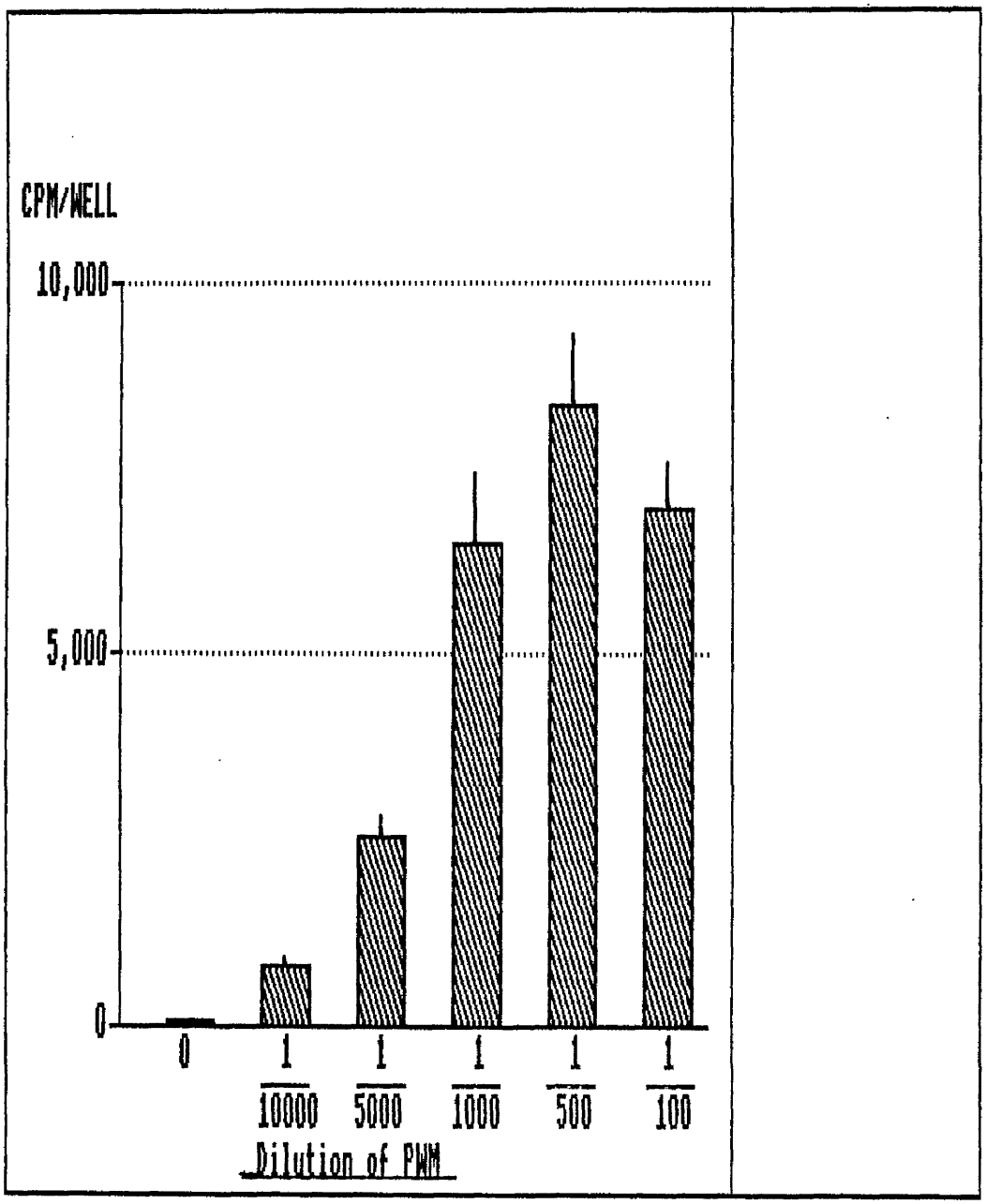


Fig.4.2

Proliferative responses of control peripheral blood mononuclear cells to PWM.
Bars represent the mean ^{14}C Thymidine uptake on the third day of culture +1SEM in 4 experiments.

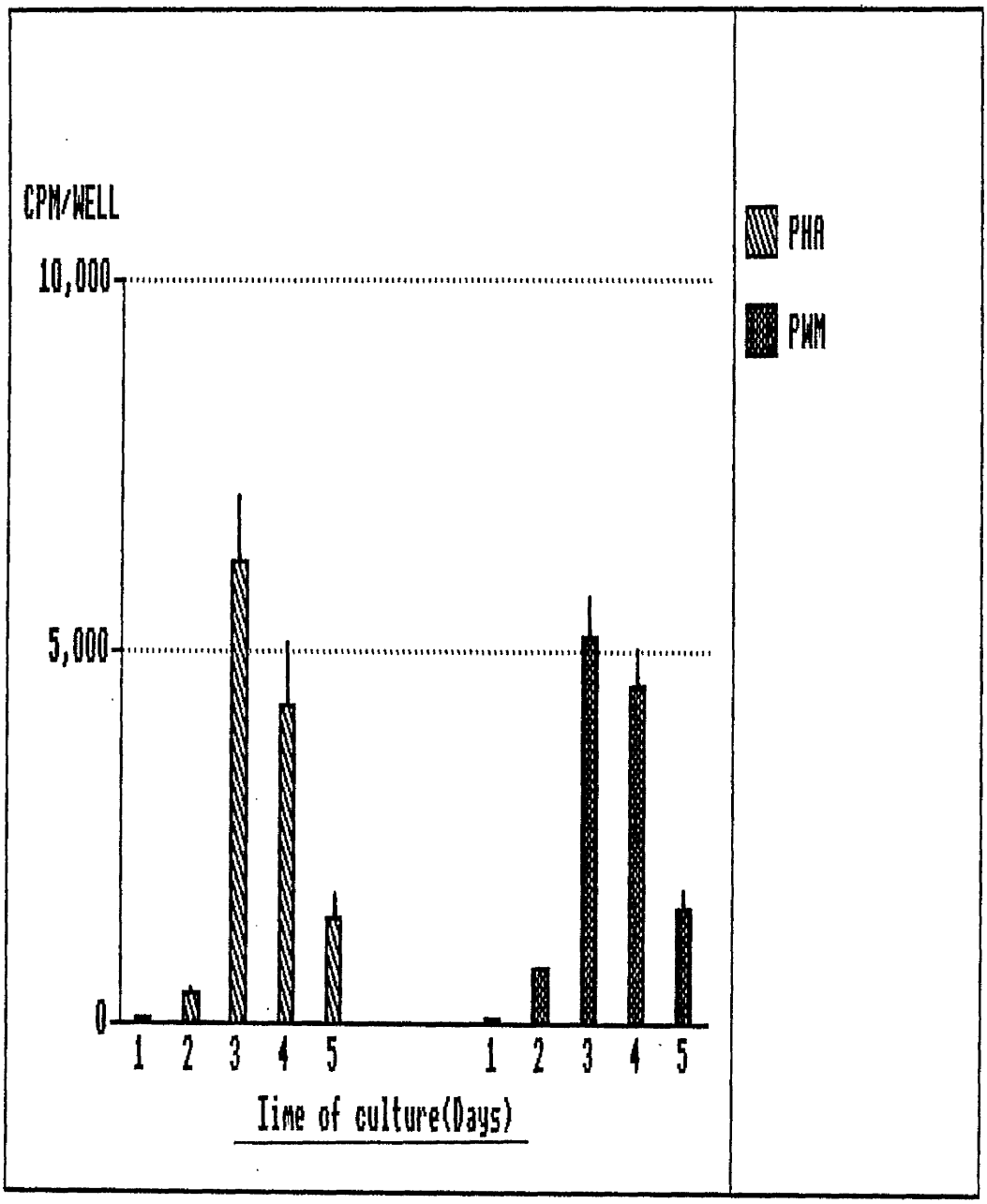


Fig.4.3

Proliferative responses of control peripheral blood mononuclear cells cultured for different times with PHA or PWM.
Bars represent the mean ^{14}C Thymidine uptake +1SEM in 4 experiments.

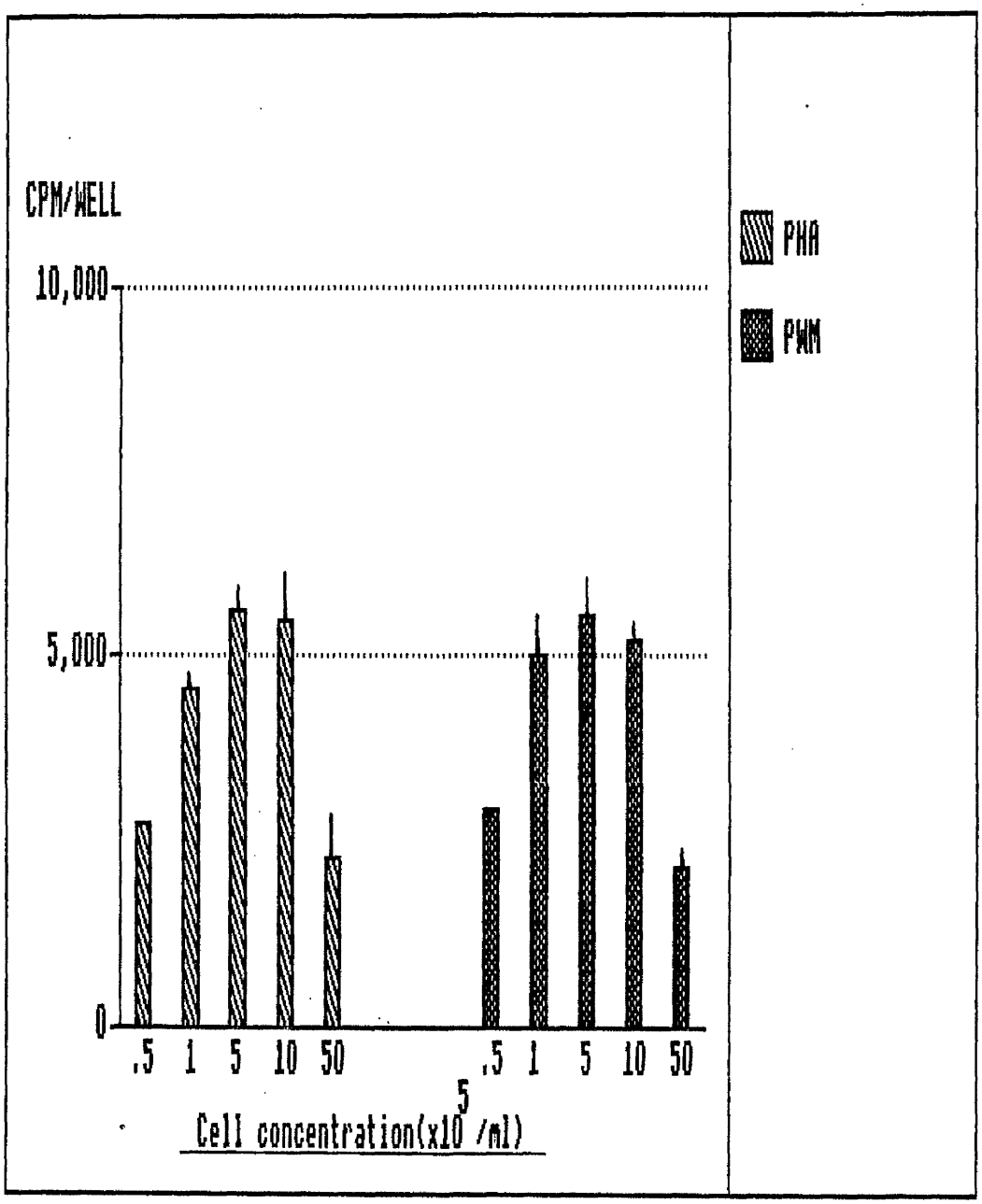


Fig.4.4

The effect of cell concentration on the 3 day proliferative responses of control peripheral blood mononuclear cells to PHA and PWM.
Bars represent the mean ^{14}C Thymidine uptake +1SEM in 4 experiments.

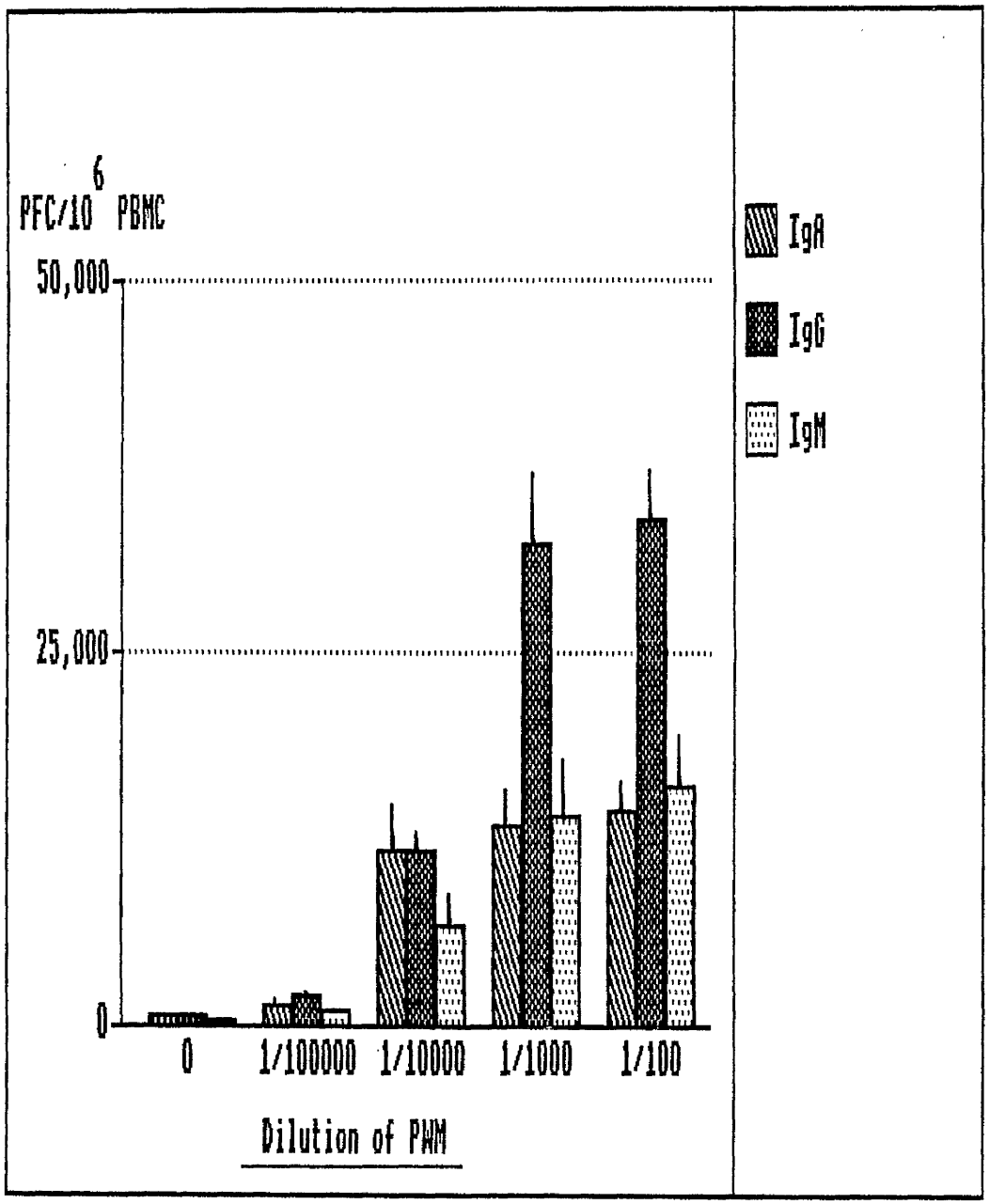


Fig.4.5
The effect of different dilutions of PWM on immunoglobulin production by control peripheral blood mononuclear cells. Bars represent the mean+1SEM(PFC/10^6 cells) of four experiments.

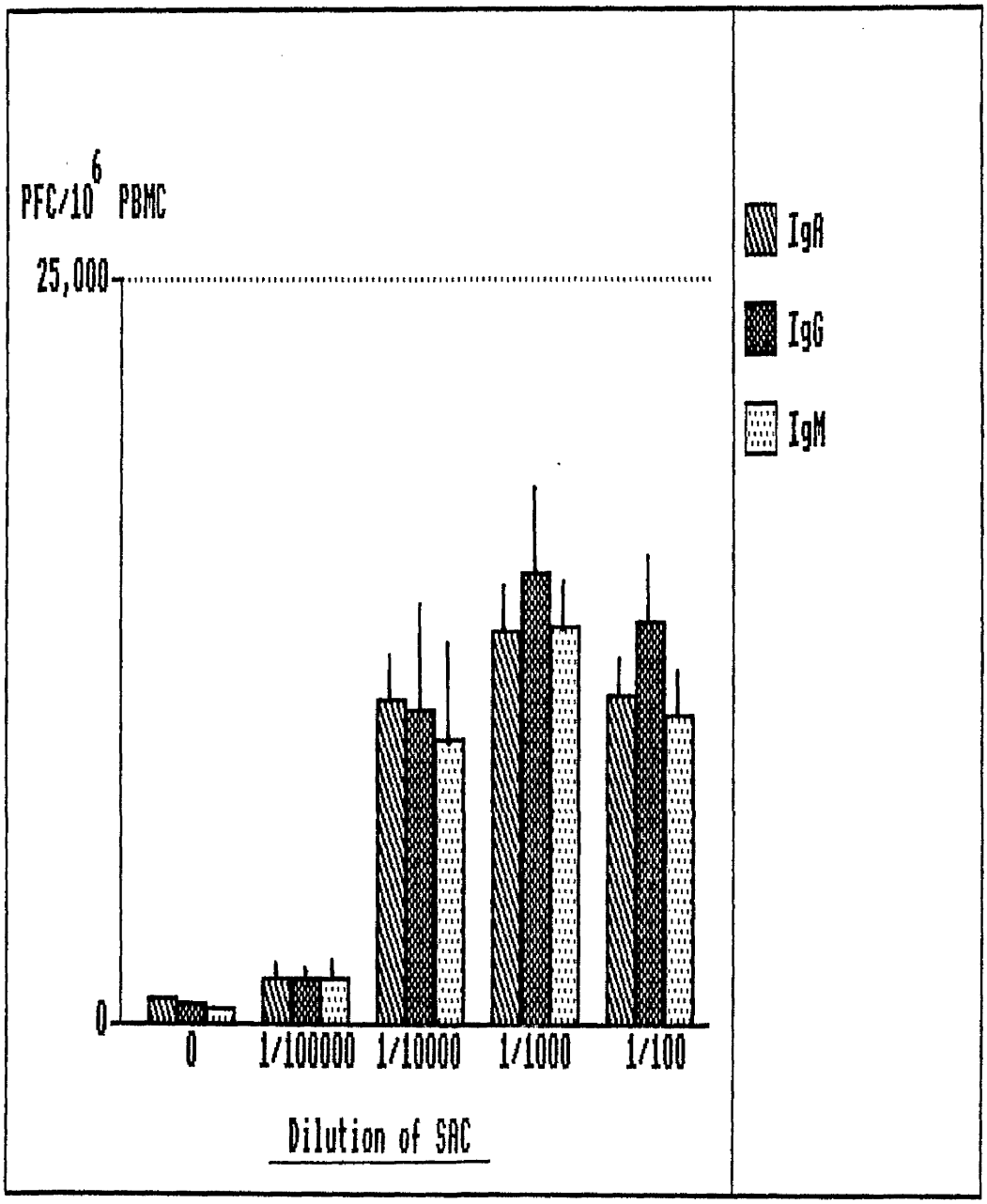


Fig.4.6

The effect of different dilutions of SAC on immunoglobulin production by control peripheral blood mononuclear cells. Bars represent the mean+1SEM(PFC/10^6 cells) of four experiments.

	DILUTION OF SAC				
DILUTION OF PWM	0	1:100	1:1000	1:10000	1:100000
		1. IgA - PFC			
0	-	14792	18416	11233	2674
1:100	32316	45768	53239	67203	54766
1:1000	24754	36398	69892	88404	72238
1:10000	6532	8934	12153	11298	5766
1:100000	1277	1398	2214	3574	2473
		2. IgG - PFC			
0	-	13732	23576	12137	1986
1:100	42566	58632	69754	62638	49574
1:1000	32738	45833	62988	97766	47532
1:10000	12165	32796	44568	52832	8574
1:100000	3267	12133	33654	36743	3476
		3. IgM - PFC			
0	-	11286	14586	8573	547
1:100	16732	28032	31517	29413	18217
1:1000	12416	18413	28507	46756	21014
1:10000	8571	17561	22832	38664	4772
1:100000	1016	14436	21677	10116	2273

Table 4.1

Synergistic effect of SAC and PWM on production of immunoglobulin. Control peripheral blood mononuclear cells were cultured in the presence of PWM, SAC or a combination of the two. Results are expressed as mean PFC/10^6 cells of triplicate determinations.

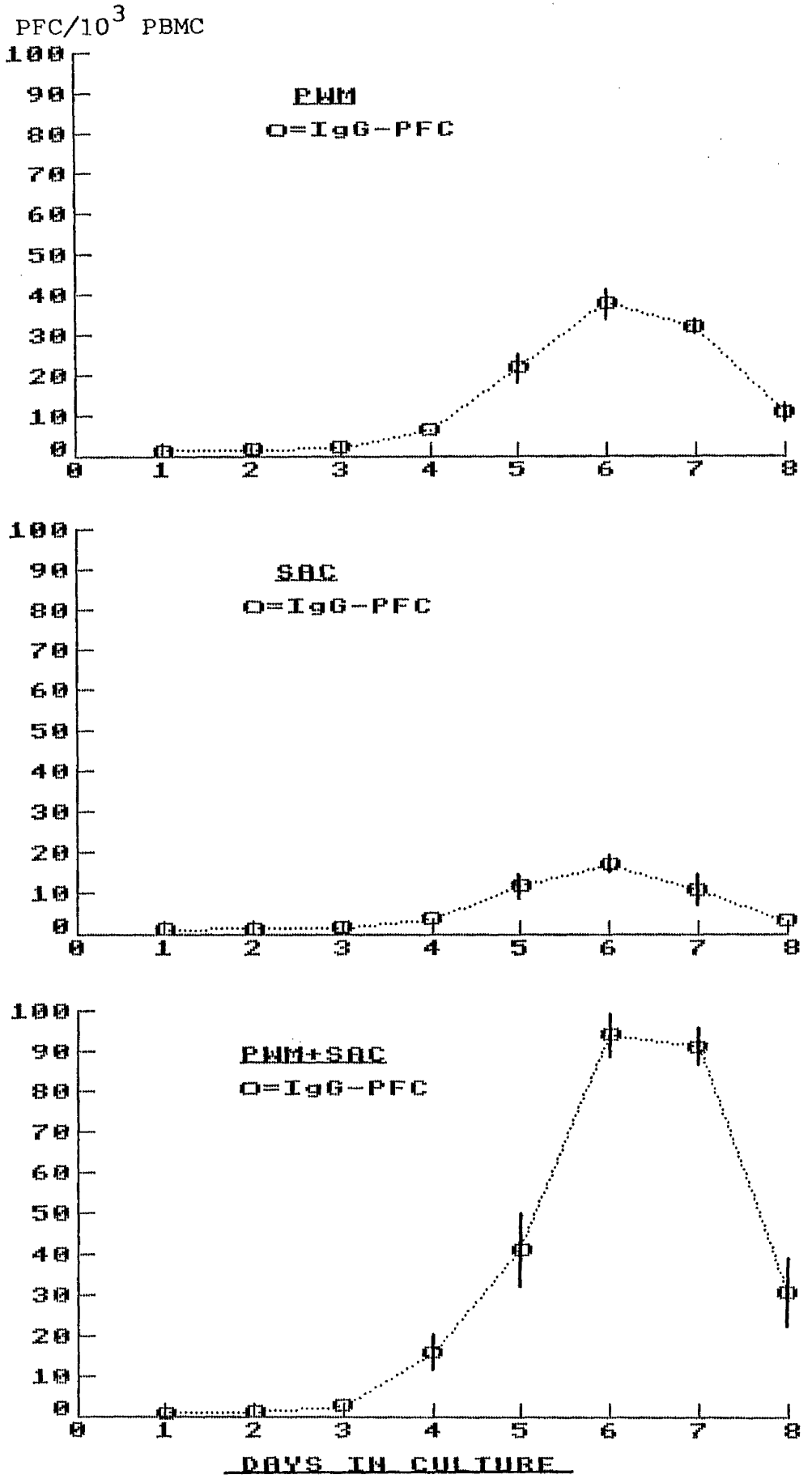


Fig.4.7

Time course of plaque forming cell response to optimal concentrations of PWM, SAC, PWM+SAC.
Graphs shown are the mean+1SEM of three experiments.

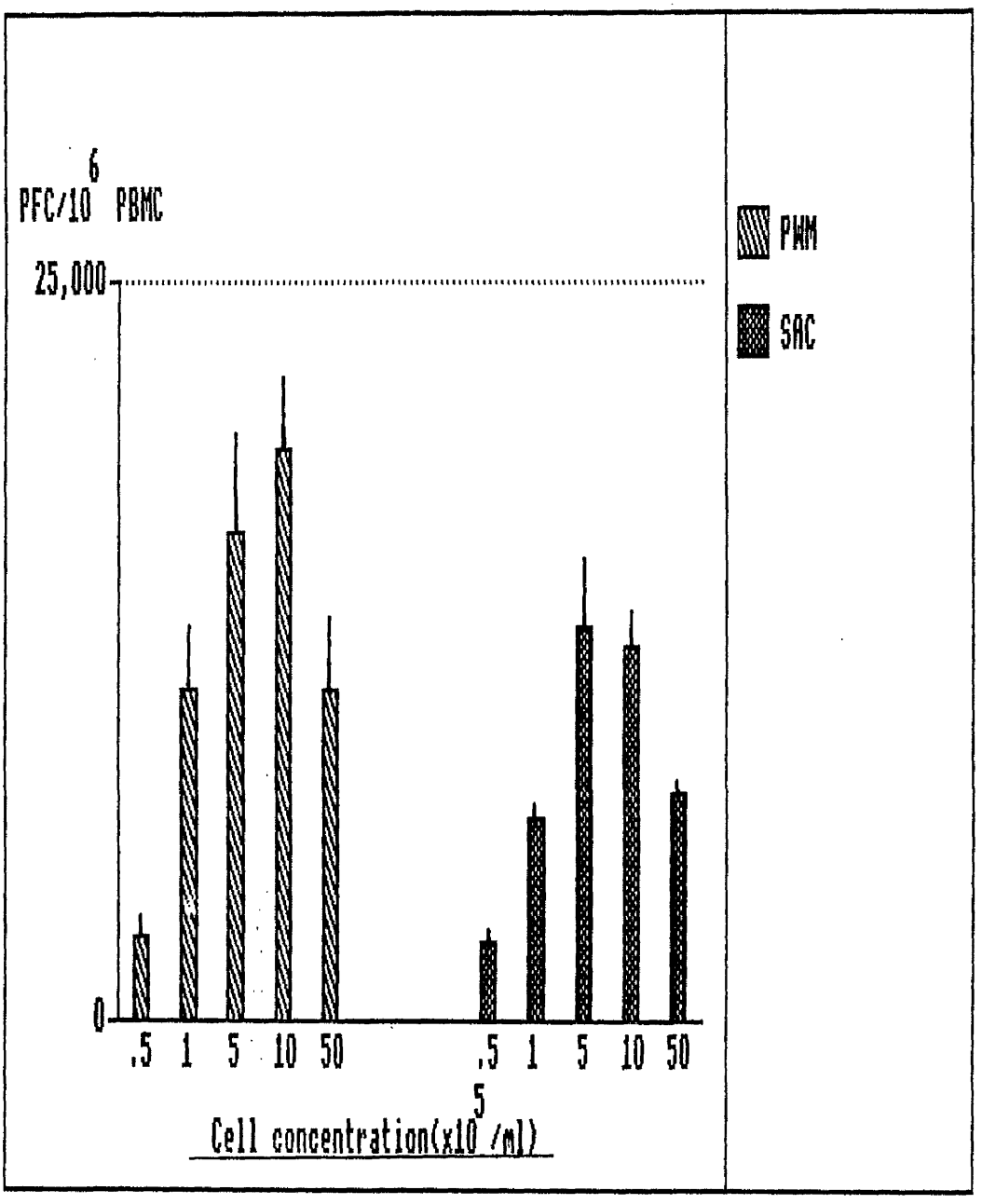


Fig.4.8
The effect of cell concentration on the 6 day immunoglobulin responses of control peripheral blood mononuclear cells to PWM and SAC. Bars represent the mean+1SEM(PFC/10^6 cells) of three experiments.

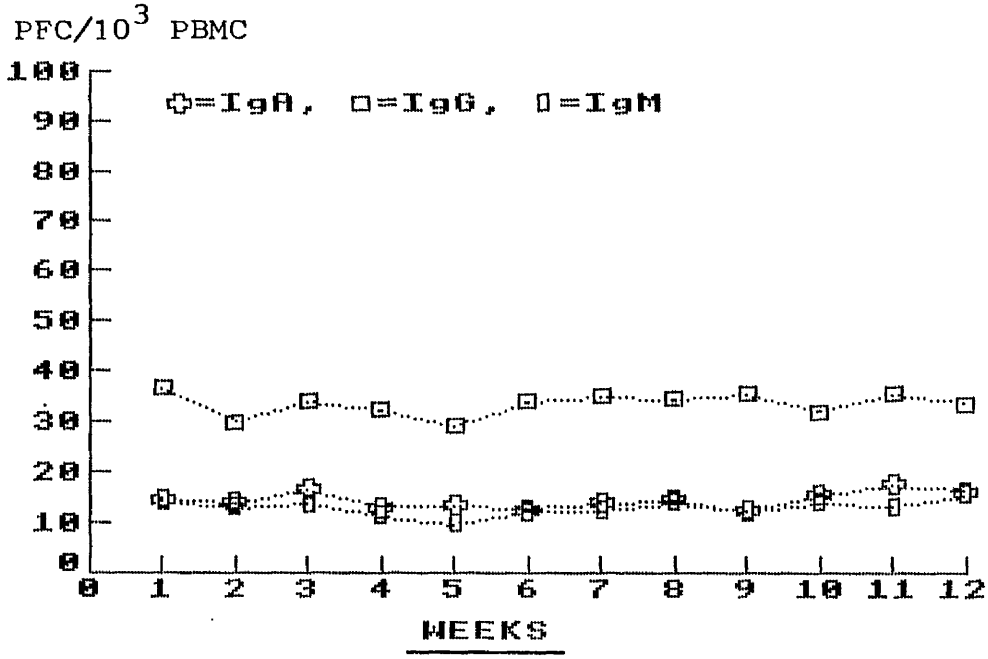


Fig.4.9

PWM induced plaque formation by cryopreserved peripheral blood mononuclear cells obtained from the same healthy subject. One aliquot of cells was tested weekly using different batches of protein-A coated sheep red blood cells.
Graphs shown are the mean of triplicate determinations (PFC/10^3 cells)

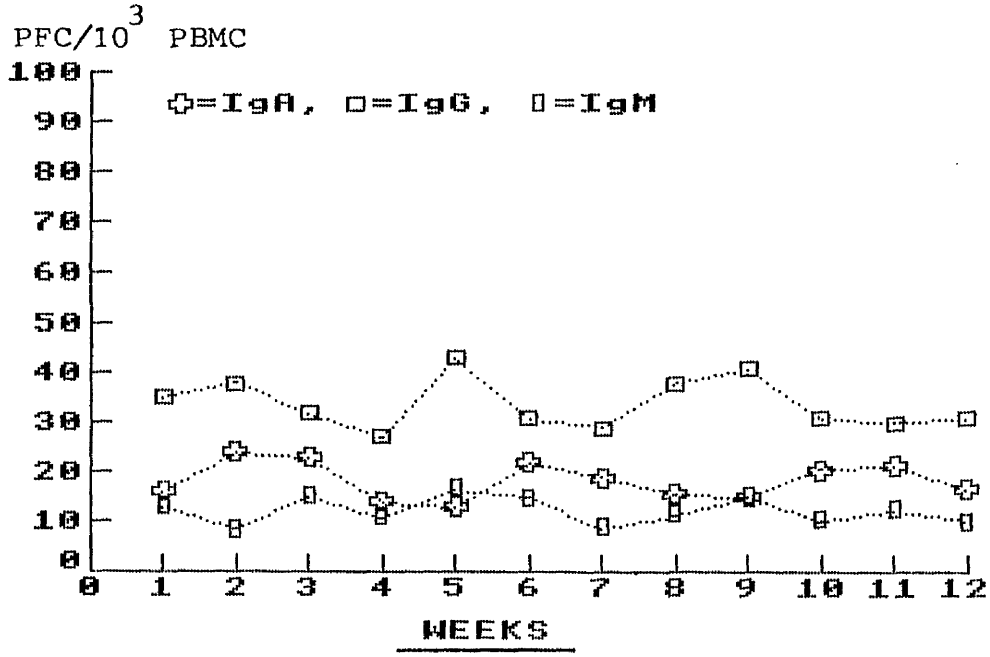


Fig.4.10

PWM induced plaque formation by peripheral blood mononuclear cells obtained from the same healthy subjects at weekly intervals. Each sample was tested using different batches of protein-A coated sheep red blood cells.
Graphs shown are the mean of triplicate determinations ($PFC/10^3$ cells)

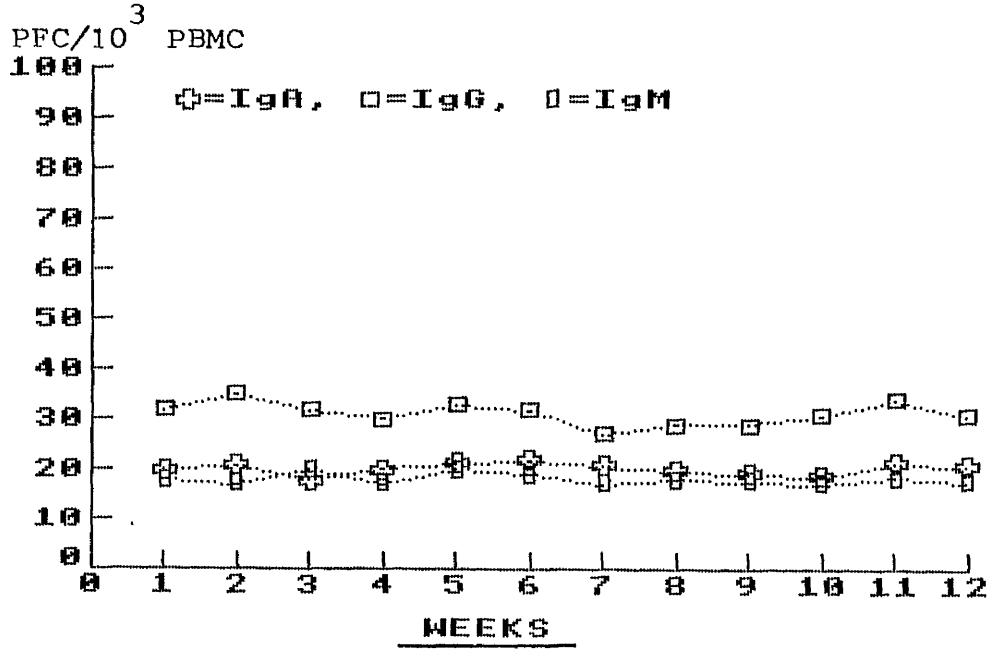


Fig.4.11

PWM induced plaque formation by cryopreserved peripheral blood mononuclear cells obtained at weekly intervals from the control subject shown at Fig.4.10. All twelve samples were tested on the same day. Graphs shown are the mean of triplicate determinations (PFC/10^3 cells).

CHAPTER V

PRODUCTION OF IMMUNOGLOBULIN IN VITRO BY HEALTHY SUBJECTS

INTRODUCTION

The results of the previous chapters established the optimal conditions for measuring immunoglobulin production by the plaque forming cell assay using freshly isolated peripheral blood mononuclear cells and also determined the culture conditions which favoured optimal proliferation and immunoglobulin synthesis in response to mitogens. The aim of the studies in this chapter was to use the protein A plaque forming cell assay to establish a normal range for production of immunoglobulins both spontaneously and in response to polyclonal B cell activators, and to investigate the regulation of immunoglobulin production in response to different activators.

Thus, peripheral blood mononuclear cells from normal volunteers were stimulated with the T dependent mitogens PWM and SAC as well as the T independent EBV. In addition, I investigated the role of suppressor T cells and monocytes in regulating immunoglobulin production. Making use of the finding that only PWM responses are sensitive to the action of suppressor T cells(Pryjma et al, 1980), ConA was added as a possible stimulator of suppressor T cell activity, while methyl-prednisolone was used to selectively inhibit suppressor T cell activity(Cupps & Fauci, 1982). Furthermore, I examined the possibility that monocytes can suppress immunoglobulin production in vitro(Knapp & Baumgartner, 1978, Gmelig-Meyling & Waldmann, 1981) via cyclooxygenase pathway products of arachidonic acid metabolism, such as prostaglandin E2(Goodwin & Webb, 1980). Thus, indomethacin, a cyclooxygenase inhibitor, was used to study the role of prostaglandins in the differentiation of B

cells into immunoglobulin secreting cells in response to PWM or SAC.

MATERIALS AND METHODS

Venous blood was obtained from the same normal adult volunteers(Table 3.1) and mononuclear cells were prepared and tested for immunoglobulin production as described above. In the ConA, methyl-prednisolone and indomethacin experiments only IgG-PFC were measured because preliminary experiments showed that IgG-PFC response was consistently higher.

RESULTS

Spontaneous Production of Immunoglobulin

Spontaneous IgA-, IgG-, and IgM-PFC counts of peripheral blood mononuclear cells from these twenty five normal adult volunteers are shown in Fig.5.1(IgA-PFC:1380+253, IgG-PFC:1137+171, IgM-PFC:342+73). Although IgA-PFC counts were higher than IgG-PFC counts in 18 of the 25 subjects, there was no significant difference between these values. However, both IgA and IgG production was significantly higher than that of IgM($p<0.001$). These findings therefore confirm those of CHAPTER III.

Production of Immunoglobulin in Response to Polyclonal B Cell Activators

When peripheral blood mononuclear cells were cultured

unstimulated for six days the plaque forming cell reponse was minimal(IgA-PFC:414+29, IgG-PFC:636+28, IgM-PFC:445+37). In contrast, when peripheral blood mononuclear cells from the same healthy volunteers were cultured in the presence of PWM, SAC or PWM plus SAC, the production of all immunoglobulin classes showed a marked increase over the spontaneous plaque forming cell responses(Fig.5.2). IgG responses were significantly higher than those of IgA and IgM($p<0.001$) which were identical, while significantly higher IgA- and IgG-PFC responses were found in response to PWM than to SAC($p<0.001$). The highest plaque forming cell responses were observed when PWM and SAC were used together to induce immunoglobulin production($p<0.001$ for all classes). A synergistic effect was arbitrary defined when the PFC counts exceeded by 25% the PFC counts induced by SAC and PWM alone. This synergistic effect was demostrated for all immunoglobulin classes and occurred in 72% of healthy subjects.

Although the amounts of EBV available prevented formal testing of the dose dependency and time course, studies with additional subjects were performed to compare EBV with PWM and SAC for its ability to induce immunoglobulin synthesis. The culture conditions for the EBV were as described by Bird et al(1979). Fig.5.3 summarises the results of seven such experiments and it can be seen that EBV stimulated higher IgM responses which were higher than those found with SAC(n.s) and similar to those with PWM. IgG responses to EBV were much smaller than with PWM($p<0.01$, Wilcoxon rank sum test) and similar to those with SAC. The IgA responses were similar in all three systems.

Effect of ConA on Immunoglobulin Production

The ability of 20μg/ml ConA to modify IgG-PFC responses to PWM or SAC was studied in six volunteers and the results are shown in Table 5.1. When cells were cultured only in the presence of ConA, no plaque forming cell response was observed(Data not shown). In all cases, the response to PWM was substantially depressed by addition of ConA(range:25-66%) with a mean suppression of 51+5.8%(p=0.05 vs controls, signed rank test). In contrast, although ConA suppressed SAC responses in 5/6 subjects this was markedly less than that seen with PWM(mean:25.6+4.9%, range:5-33%) and was not found to be significant. When the sensitivity of the two culture systems to ConA was compared, there was significantly greater suppression of PWM responses than SAC responses($p<0.05$, Wilcoxon rank sum test).

Effect of Methyl-prednisolone on Immunoglobulin Production

The effect of different doses of methyl-prednisolone on PWM and SAC induced IgG-PFC responses was examined in eight control subjects and the results are presented in Fig.5.4. When cells were cultured for six days only in the presence of 0.01 or 0.1μg/ml methyl-prednisolone, the plaque forming cell reponse was minimal and not significantly higher than the background plaque forming cell response(1057+77 and 1220+112PFC/10^6cells, respectively, vs 750+37PFC/10^6cells, background). The response to PWM was enhanced by addition of 0.01, 0.1, and 1μg/ml methyl-prednisolone with an optimal

response at 0.1μg/ml(p<0.01, signed rank test). At higher concentrations, the plaque forming cell response showed lesser increases above control levels. The effect of methyl-prednisolone was most evident in the response to PWM, although a similar, if lesser effect, was observed in the SAC system, where only 0.1μg/ml of methyl-prednisolone produced statistically significant increase(p<0.05, signed rank test). At this optimal concentration, methyl-prednisolone produced a mean increase in PWM response of 232+29% compared with 61+13% enhancement of the SAC response(p<0.01, Wilcoxon rank sum test).

Effect of Indomethacin on Immunoglobulin production

The effect of indomethacin(1μg/ml) on IgG-PFC responses to PWM and SAC was studied in six control subjects(Table 5.2). Indomethacin had no significant effect on the response to PWM(PWM:24844+2977, PWM+Indo:25206+3468) but in 5/6 cases addition of indomethacin increased the response to SAC(SAC:15393+1266, SAC+Indo:18334+1940). In the other case, indomethacin suppressed the response. When peripheral blood mononuclear cells were cultured for six days only with indomethacin, as in the Cona study, no plaque formation was observed(Data not shown).

CONCLUSIONS

Using the protein A plaque forming cell assay to measure spontaneous secretion of immunoglobulin, this study showed that the counts of IgA-PFC exceeded those of IgG-PFC in most cases(18/25) whereas IgG-PFC counts were usually highest after stimulation by polyclonal B cell activators. Studies with polyclonal B cell activators showed PWM to be the most effective, although EBV induced similar IgM-PFC counts as PWM. SAC was the least efficient inducer of IgG and IgM synthesis.

Normal peripheral blood mononuclear cells were also cultured in the presence of ConA and methyl-prednisolone in order to study the role of suppressor T cells in the production of immunoglobulin in response to different polyclonal B cell activators. Co-stimulation of cells with polyclonal B cell activators+ConA showed that while both the PWM and SAC culture systems were suppressed by ConA activity, PWM induced responses were much more sensitive to this effect and SAC responses showed little suppression. PWM stimulated production of immunoglobulin was also more readily enhanced by addition of methyl-prednisolone and together, these findings support the view that stimulation of B cells by PWM is much more susceptible to regulation by suppressor T cells than SAC responses. In contrast, preliminary studies using indomethacin to inhibit prostaglandin synthesis showed that SAC induced immunoglobulin production was affected considerably more by the addition of indomethacin, and this indicates indirectly that SAC induced immunoglobulin production maybe regulated by monocytes rather than suppressor T cells.

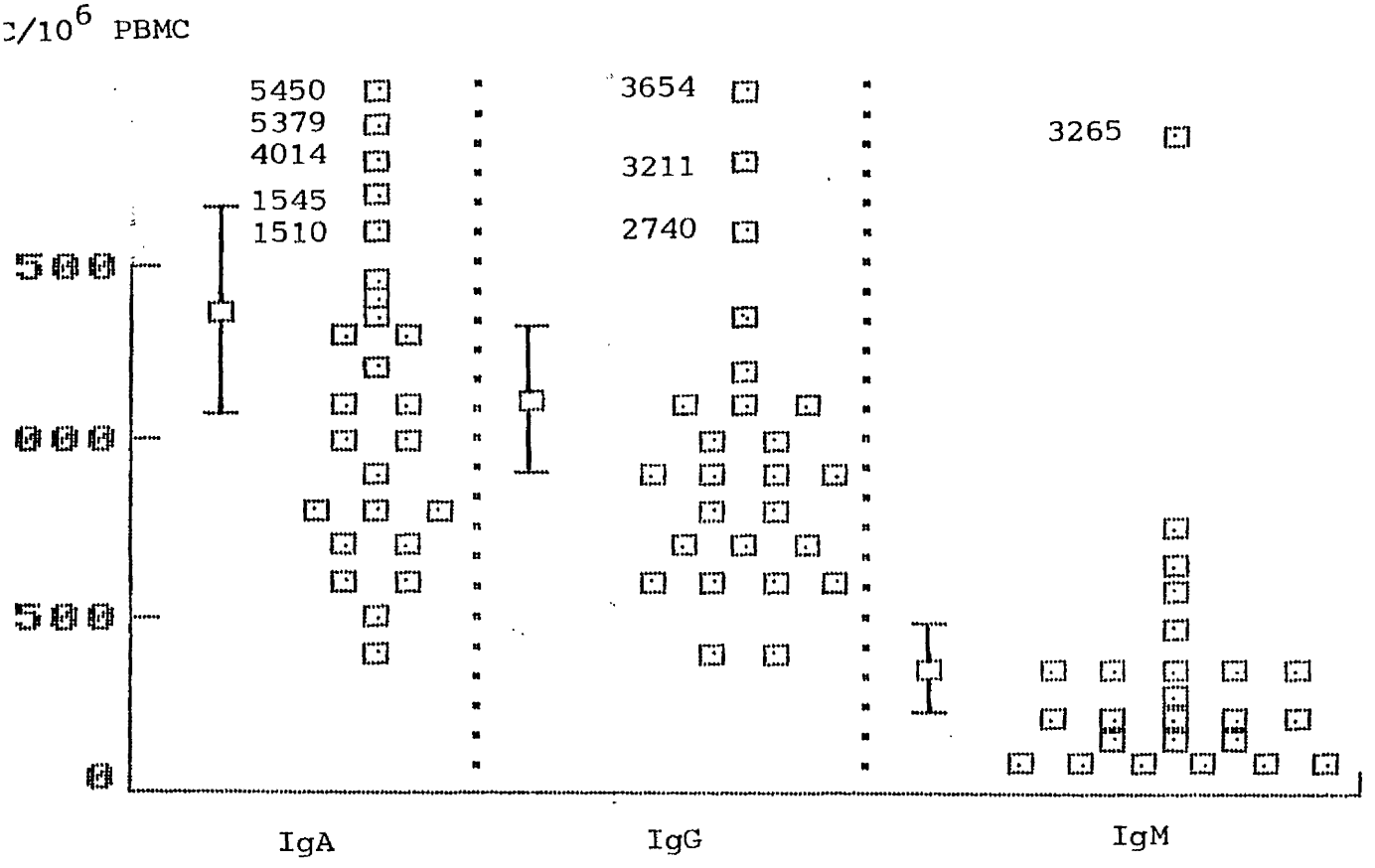


Fig.5.1

Spontaneous PFC counts in 25 healthy subjects. PFC counts shown are the mean of triplicate determinations(PFC/10^6 cells)

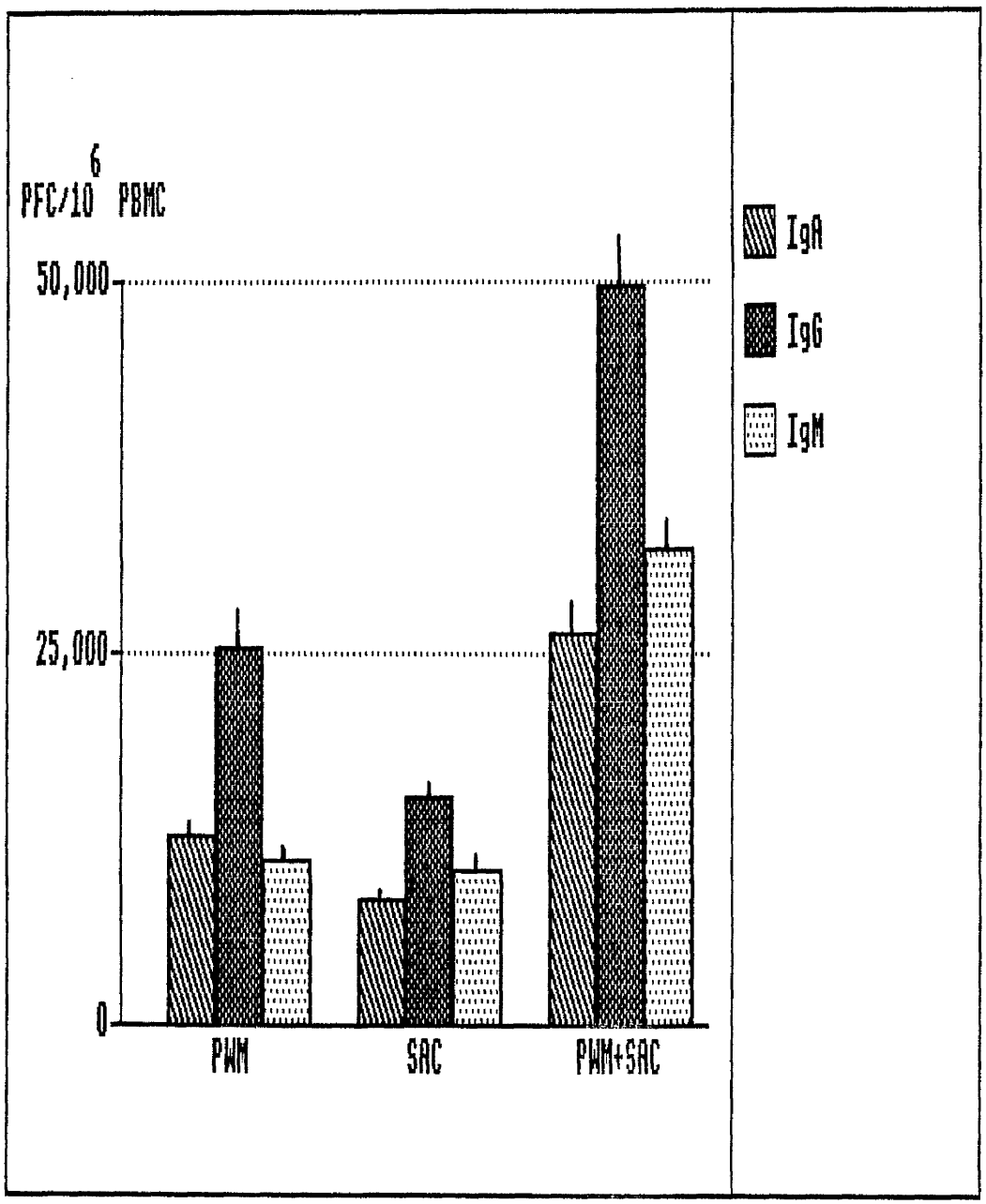


Fig.5.2

PFC responses to polyclonal B cell activators by control peripheral blood mononuclear cells. Results shown are the mean+1SEM PFC responses by 25 healthy subjects.

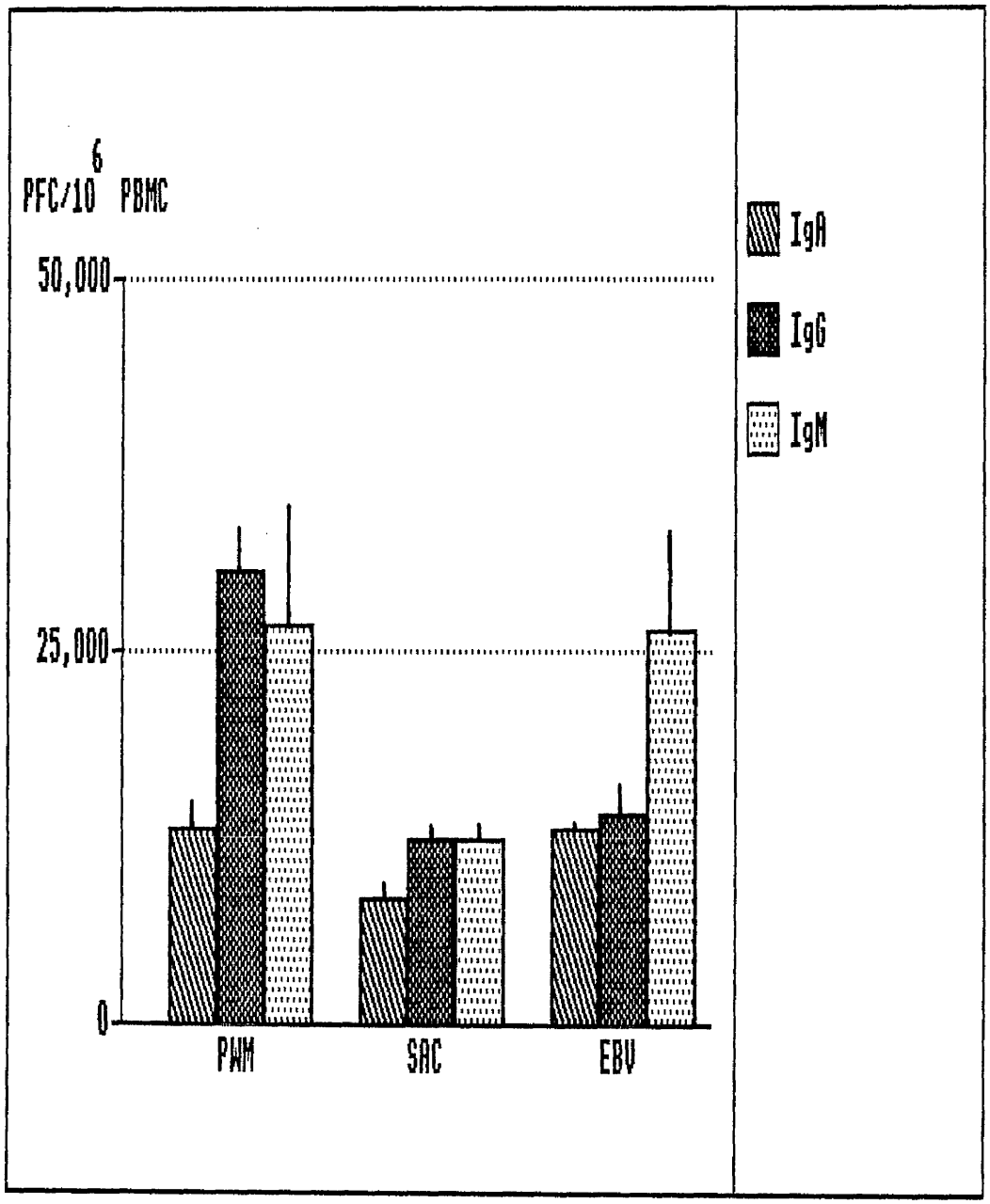


Fig.5.3

PFC responses by control peripheral blood mononuclear cells to EBV in comparison to response to PWM and SAC.
Results shown are the mean+1SEM(PFC/10^6 cells) for 7 healthy subjects.

	1.PWM			/	2.SAC		
	PWM	PWM+ConA	%		SAC	SAC+ConA	%
EXP 1	16733	12554	-25%	/	15732	11257	-28%
EXP 2	27820	9573	-66%	/	14653	9857	-33%
EXP 3	23572	11214	-53%	/	15744	11408	-28%
EXP 4	31765	14818	-53%	/	13912	15765	+13%
EXP 5	18000	7530	-58%	/	13324	12664	-5%
EXP 6	20505	9956	-51%	/	12346	8135	-34%
MEAN	23095	11137			14285	12899	
SEM	2179	1130			553	998	

Table 5.1

The effect of co-stimulating peripheral blood mononuclear cells with 20µg/ml ConA on their PFC response to PWM and SAC. The results shown are the mean IgG-PFC/10^6 cells of triplicate determinations from 6 normal controls. The percentage values represent the proportion of decrease after adding ConA.

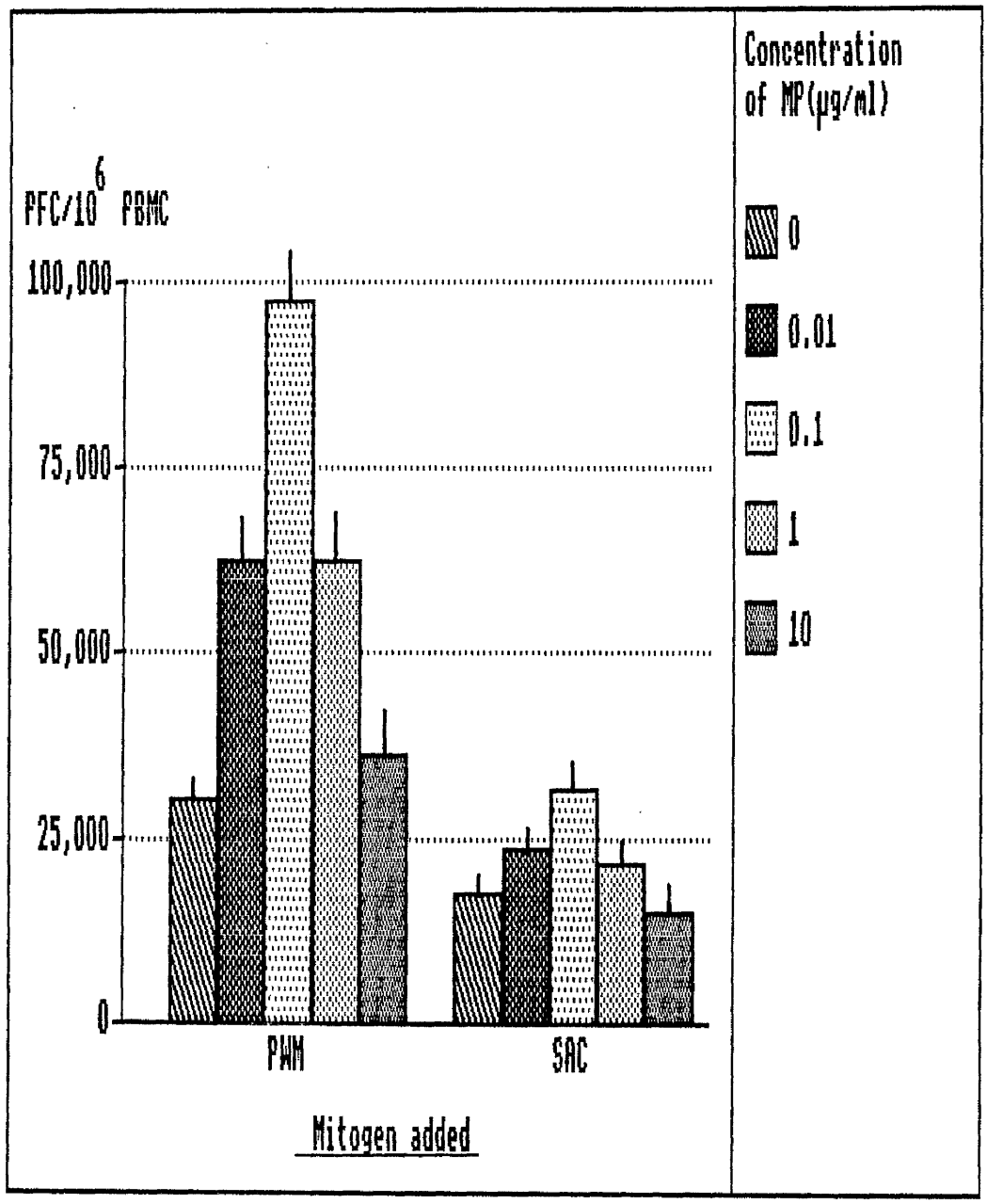


Fig.5.4

The effect of methyl-prednisolone on PFC responses to PWM and SAC by control peripheral blood mononuclear cells. Bars represent the mean+1SEM(PFC/10^6 cells) of 8 experiments.

	1.PWM				2.SAC		
	PWM	PWM+INDO	%	/	SAC	SAC+INDO	%
EXP 1	31735	32134	+1.2%	/	15614	18732	+20%
EXP 2	21051	22211	+5,5%	/	12670	17330	+36.7%
EXP 3	18766	16957	-9.6%	/	14935	12201	-19%
EXP 4	15785	14902	-5.6%	/	21337	26681	+26%
EXP 5	33151	35692	+7.7%	/	13494	16435	+22%
EXP 6	28581	29345	+2.7%	/	14313	18625	+30%
MEAN	24844	25206		/	15393	18334	
SEM	2964	3454		/	1261	1932	

Table 5.2

The effect of indomethacin(1μg/ml) on PWM and SAC cultures of peripheral blood mononuclear cells. The results shown are the mean IgG-PFC/10^6 cells of triplicate determinations from 6 normal controls. The percentage values represent the proportion of change after adding indomethacin.

CHAPTER VI

IMMUNE DEFECTS IN CHRONIC RENAL FAILURE

INTRODUCTION

The aim of this study was to use the plaque assay to examine the effect of uraemia on immunoglobulin production in vitro and to correlate this with other assays of lymphocyte number and function.

In addition to counts of total white blood cells and lymphocytes, B lymphocyte numbers were assessed by the presence of surface immunoglobulin while monoclonal antibodies to T3, T4, and T8 were used to detect T cells and their subsets. The proliferative responses of uraemic lymphocytes to PWM and PHA were studied and the effect of steroids on these responses was also studied to determine whether uraemic lymphocytes are more sensitive to steroids than control lymphocytes, as has been suggested by Langhoff & Ladefoged(1984).

In parallel, extensive studies of immunoglobulin production by uraemic peripheral blood mononuclear cells were performed by measuring secretion of immunoglobulin both spontaneously and after stimulation by either PWM, SAC, PWM plus SAC, or EBV. In addition, the possibility that uraemia altered the kinetics of immunoglobulin production was examined by investigating the response to PWM on different days and comparing this with the responses by healthy subjects. The role of active suppression of immunoglobulin production by uraemic peripheral blood mononuclear cells was examined by adding methyl-prednisolone and indomethacin to PWM and SAC stimulated cultures of

uraemic peripheral blood mononuclear cells. Finally, the effect of uraemic serum on immunoglobulin production was studied by pre-incubating normal peripheral blood mononuclear cells with uraemic serum.

MATERIALS AND METHODS

Thirty seven patients(Table 6.1) undergoing regular haemodialysis(4hr, thrice weekly) and ten patients on CAPD were studied, ranging in age from 18 to 63 years(mean:40.85+1.9 years). The mean duration on dialysis prior to the study was 2.4 months with a range from 1 to 13. Chronic renal failure was ascribed to chronic glomerulonephritis(17 patients), chronic pyelonephritis(15 patients), polycystic kidney disease(5 patients), essential hypertension (4 patients), diabetes(4 patients), and unknown aetiology(2 patients). The mean serum creatinine prior to the study was 737 μmol/l with a range from 523 to 1134(Normal Range:62-120μmol/l). None of the patients were receiving corticosteroids or other immunosuppressive drugs and none of them had evidence of systemic lupus erythematosus or other identifiable systemic immunological disease. Finally, none of the patients had ever received a blood transfusion. Control blood samples were those shown in Table 3.1 and to enable direct comparison with results in uraemic subjects, some of the results presented in CHAPTER V are also presented here. In some experiments, only the IgG plaque forming cell response is presented, because initial

experiments showed that this was consistently higher than the other immunoglobulin classes. All the cell cultures and assays were performed as described previously and results are expressed as mean+SEM unless otherwise stated.

RESULTS

White Blood Cell and Lymphocyte Counts

The mean total white blood cell count in the uraemic group(n=18) was lower than the control group(n=12, 4556+247/cmm vs 5798+487/cmm) as were the total lymphocyte counts(1151+91 vs 1430+130). However, these differences were not statistically significant. The results from B and T lymphocyte studies performed in ten patients with uraemia and twenty normal controls were expressed as percentages of the total lymphocyte number and are presented in Fig.6.1. Patients with uraemia had a normal proportion of T cells as measured by the monoclonal antibody OKT3 and the percentages of OKT8+ and OKT4+ cells were also not significantly different from those found in normal controls. Uraemic patients also had a normal proportion of B cells, as measured by the presence of surface immunoglobulin(k,l)(Fig.6.1).

Proliferative Responses of Lymphocytes

Peripheral blood mononuclear cells from fifteen normal controls and seventeen uraemic patients were stimulated with PHA and PWM and their proliferative responses assessed on day 3. It can be seen that the response to both PHA and PWM

were significantly lower in uraemic patients compared with controls(Fig.6.2, $p<0.001$).

Addition of 0.01-10μg/ml methyl-prednisolone to PHA(Fig.6.3) and PWM(Fig.6.4) stimulated cultures led to a dose dependent depression of proliferation which was similar in normal and uraemic patients. Thus, the maximally effective dose of methyl-prednisolone(10μg/ml) produced 61+9% suppression of uraemic PHA responses and 66+6% suppression of their PWM responses compared with 56+4% and 48+5% suppression of control responses to PHA and PWM respectively. However, these differences were not statistically significant and thus, the findings of Langhoff & Ladefoged(1984) were not confirmed.

Spontaneous Production of Immunoglobulin in Uraemia

The spontaneous secretion of IgA, IgG, and IgM by uraemic and control cells was investigated using the spontaneous plaque forming cell assay. As shown in Fig.6.5, uraemic patients had very low levels of all immunoglobulin classes, with IgA- and IgG-PFC numbers around 25%-30% of levels previously found in controls($p<0.001$). IgM-PFC were also suppressed(40% of control levels), although this was not as marked as with IgA and IgG($p<0.05$). Interestingly, the counts of IgG secreting cells in the uraemic group were consistently higher than the counts of IgA secreting cells whereas the opposite pattern was observed in the control group. However, the difference between IgA- and IgG-PFC counts in the uraemic group failed to reach statistical significance.

Production of Immunoglobulin in Response to Polyclonal B Cell Activators

The ability of uraemic peripheral blood mononuclear cells to produce immunoglobulin in response to polyclonal B cell activators was studied by enumerating immunoglobulin secreting cells in cultures stimulated with PWM, SAC, PWM plus SAC, or EBV.

The results of the PWM induced cultures are presented in Fig.6.6. and it can be seen that IgA, IgG, and IgM-PFC counts were not significantly different from those obtained previously in normal controls, with both groups having higher IgG responses than either IgM or IgA. In addition, uraemic subjects showed the same dose response pattern to PWM as controls, with optimal doses at 1:100(Fig.6.7) and had normal responses from 3-7 days of culture, with the peak response also being found on day 6(Fig.6.8). Furthermore the viability of 6 day cultures was the same using uraemic or control peripheral blood mononuclear cells(87+4% vs 92+5%). The PWM induced plaque forming cell responses of CAPD and haemodialysis patients were compared but not significant differences were found(data not shown).

In contrast to PWM, uraemic patients had a significantly lower response of IgA, IgG and IgM classes to SAC compared with the control group values($p<0.02$, $p<0.001$, $p<0.001$, respectively). This was most pronounced for IgG and IgM responses and, unlike control responses, the IgG responses of uraemics to SAC was not higher than IgA and IgM, with identical plaque forming cell numbers being found for all classes(Fig.6.9).

As shown earlier(CHAPTER IV and V), the combination of PWM+SAC induced IgA, IgG, and IgM responses by control cells which were significantly higher than would be expected by addition of the responses to either. Synergy was not observed for any immunoglobulin class in uraemic subjects(Fig.6.10) and indeed, the response to PWM+SAC in these patients was substantially less than that found with PWM alone and similar to that with SAC alone. This synergistic effect was observed in 13/18 healthy subjects but only in 2/27 uraemic patients(Table 6.2, $p<0.001$).

When the responses of control and uraemic peripheral blood mononuclear cells to EBV were studied, equivalent plaque forming cell responses were found in the two groups, with only IgM-PFC response being slightly but not significantly lower in uraemic subjects(Fig.6.11).

Effect of Uraemic Serum on Immunoglobulin Production

In view of the suppressed response of uraemic peripheral blood mononuclear cells to SAC and previous evidence of inhibitory factors in uraemic serum(Sengar et al, 1975, Holdsworth et al, 1978), I investigated the effect of serum from uraemic patients on immunoglobulin production by control peripheral blood mononuclear cells.

Peripheral blood mononuclear cells were pre-incubated for 2hrs with either uraemic or normal heterologous serum before culture with PWM. The 2hours incubation period was chosen as more convenient, since no differences were found when control cells were pre-incubated between 1-6hours with uraemic serum(Data not shown). The mean IgG response

was lower after treatment with uraemic serum than with control serum(Fig.6.12), but this difference failed to reach statistical significance. Interestingly, in comparison with control plaques, the plaques produced by peripheral blood mononuclear cells which had been pre-incubated in uraemic serum were always less clear and less well-defined than plaques produced by peripheral blood mononuclear cells pre-incubated with normal heterologous serum, and microscopic examination revealed incomplete lysis of sheep red blood cells near the central lymphocyte. To investigate further the effect of uraemic serum, control lymphocytes were also cultured with PWM for six days in the presence of uraemic or control serum. In five out of seven experiments, no plaque forming cells were detected after six days in culture in the presence of either normal or uraemic serum, despite normal blastogenesis measured after 72hours and normal viability after the end of the culture(Data not shown). The continuous presence of either uraemic or control serum interfered with PWM induced B cell differentiation and thus, no definitive results were obtained.

Effect of Methyl-prednisolone on Production of Immunoglobulin in vitro by Uraemic Peripheral Blood Mononuclear Cells

As described in CHAPTER V, addition of certain doses of methyl-prednisolone led to higher IgG responses by control subjects, presumably due to selective depletion of suppressor T cells. I therefore investigated the effects of

methyl-prednisolone on immunoglobulin production by uraemic peripheral blood mononuclear cells in an attempt to determine a possible role for suppressor T cells in the defective immune responsiveness in these patients.

As discussed earlier, addition of methyl-prednisolone to PWM stimulated cultures of control cells produced a significant increase in IgG-PFC counts which was maximal when 0.1μg/ml of methyl-prednisolone was used. Uraemic cells showed a normal response to PWM alone but although there was a small increase after adding methyl-prednisolone to uraemic cultures, this was minimal in comparison to those observed in control cultures(Maximal Enhancement:Controls:243+26, Uraemic Patients:46+6) and failed to reach statistical significance at any dilution of methyl-prednisolone(Fig.6.13). However, control cells were significantly more sensitive than uraemic cells to the addition of methyl-prednisolone($p<0.05$, Wilcoxon rank sum test).

Similar results were obtained in cultures stimulated with SAC where the responses of control peripheral blood mononuclear cells were significantly enhanced by the addition of 0.1μg/ml methyl-prednisolone($p<0.05$) while the very low responses of uraemic peripheral blood mononuclear cells were not improved by methyl-prednisolone(Fig.6.14).

Effect of Indomethacin on Production of Immunoglobulin in vitro by Uraemic Peripheral Blood Mononuclear Cells

The role of prostaglandins in regulating production of immunoglobulin by uraemic peripheral blood mononuclear cells

was studied by adding indomethacin to cultures stimulated with PWM or SAC(Table 6.3).

Variable results were obtained when indomethacin was added to PWM cultures with 7/11 uraemic patients showing an increased IgG-PFC response ranging from 20 to 69% increase, while in four cases a decrease was noted. However, none of these changes were statistically significant. These results are therefore comparable to those obtained with PWM stimulated control cells(Table 5.2, CHAPTER V) where it was found that indomethacin had no overall effect on PWM induced production of immunoglobulin. Interestingly, when indomethacin was added to cultures stimulated with SAC, an increased IgG response was observed in 8/11 patients and this was frequently very large(up to 219% enhancement). A decreased IgG response was observed in three cases. Although the overall effect of indomethacin failed to reach statistical significance, when the effect of indomethacin on the SAC response of uraemic peripheral blood mononuclear cells was compared with that obtained with control subjects(Table 5.2), it appeared that the response of uraemic patients was more sensitive to enhancement than that of normal controls.

CONCLUSIONS

These studies showed that while uraemia had not significant effect on the number or proportion of lymphocyte populations, the proliferative responses of uraemic peripheral blood mononuclear cells to PHA and PWM were reduced.

Studies employing the plaque assay demonstrated that EBV and PWM induced production of immunoglobulin were normal in uraemic patients. In contrast, both spontaneous and SAC induced immunoglobulin production were reduced by uraemia and a combination of PWM and SAC did not produce the synergistic effect found in control cultures.

In an attempt to identify the factors responsible for the abnormal spontaneous and SAC induced immunoglobulin responses, the effects on plaque forming cell production of uraemic serum, methyl-prednisolone, and indomethacin were investigated. Pre-incubation of control peripheral blood mononuclear cells with uraemic serum had both quantitative and qualitative effect on PWM-induced IgG plaque forming cell whereas addition of methyl-prednisolone to PWM and SAC stimulated cultures of uraemic peripheral blood mononuclear cells did not produce the increased immunoglobulin production found in controls(CHAPTER V). In contrast, the IgG response of uraemic peripheral blood mononuclear cells to SAC seemed to benefit more by the addition of indomethacin than the corresponding response of control peripheral blood mononuclear cells.

URAEMIC PATIENTS

	AGE	SEX
1. S.W.*	23	M
2. M.S.*	43	F
3. I.M.	48	F
4. J.B.*	35	F
5. C.M.	34	F
6. A.K.*	27	M
7. T.Y.*	59	M
8. J.H.	56	M
9. W.B.	57	M
10. D.H.	28	M
11. C.B.	18	F
12. J.M.	63	M
13. M.H.*	36	F
14. J.M.*	39	F
15. B.J.*	27	M
16. J.P.*	53	M
17. W.Y.	42	M
18. J.D.*	52	M
19. C.S.*	33	M
20. M.M.*	38	M
21. W.H.*	31	M
22. J.L.*	33	M
23. P.F.*	49	M
24. T.C.*	53	M
25. K.M.*	53	F
26. P.M.*	24	F
27. W.D.	57	M
28. T.S.*	49	M
29. W.G.*	22	M
30. R.H.*	27	M
31. R.F.*	27	M
32. R.B.*	53	M
33. L.A.*	59	F
34. L.M.*	55	F
35. J.G.*	20	M
36. D.M.	52	M
37. A.V.	50	F
38. R.F.	47	M
39. T.J.	50	M
40. J.H.*	52	M
41. H.M.	42	M
42. S.L.	18	F
43. E.S.	55	M
44. J.H.	35	M
45. A.H.	38	F
46. H.H.	59	M
47. R.M.	19	M
	Mean+SEM:40.85+2	M/F:33/14

Table 6.1

Uraemic patients used to examine B cell function.
*These patients were also studied as part of the blood transfusion trial, described in CHAPTER VII>

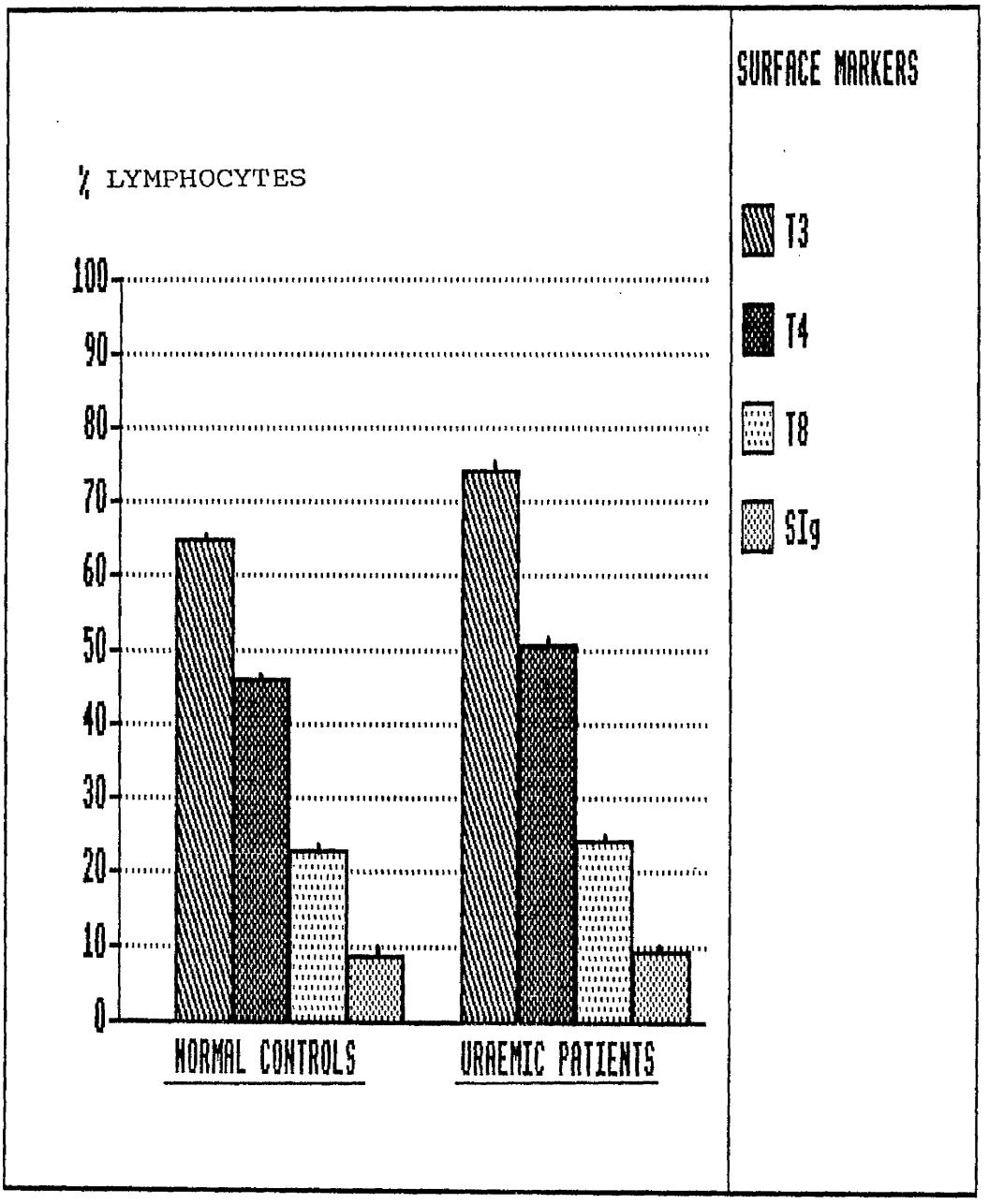


Fig.6.1

T and B lymphocyte populations in normal controls(n=20) and uraemic patients(n=10) assessed by immunofluorescence. B cells and T cells were expressed as a percentage of the lymphocyte count.

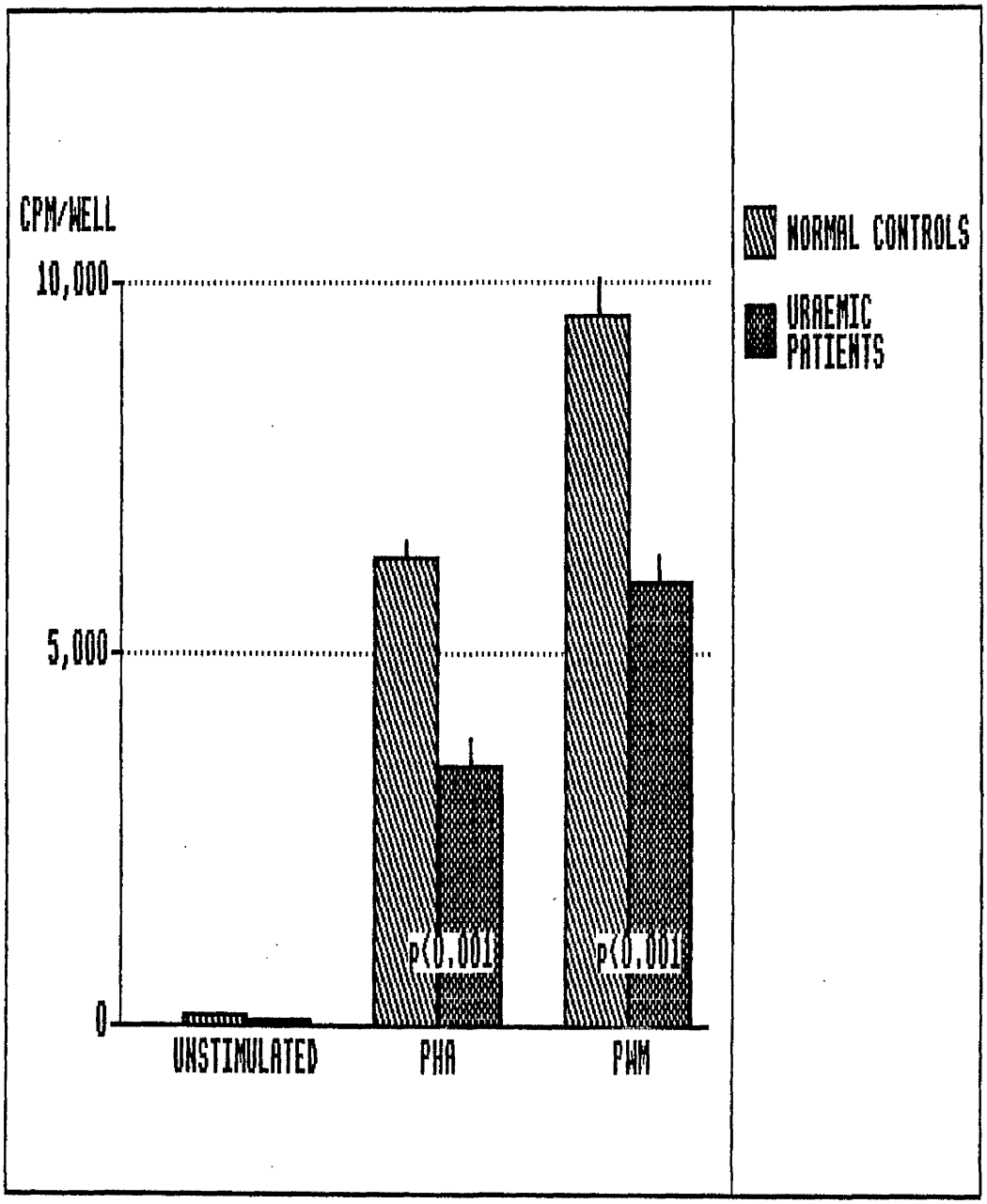


Fig.6.2

Proliferative responses to PHA and PWM of peripheral blood mononuclear cells from normal controls(n=15) and uraemic patients(n=17). Bars represent the mean ^{14}C Thymidine uptake on the third day of culture +1SEM.

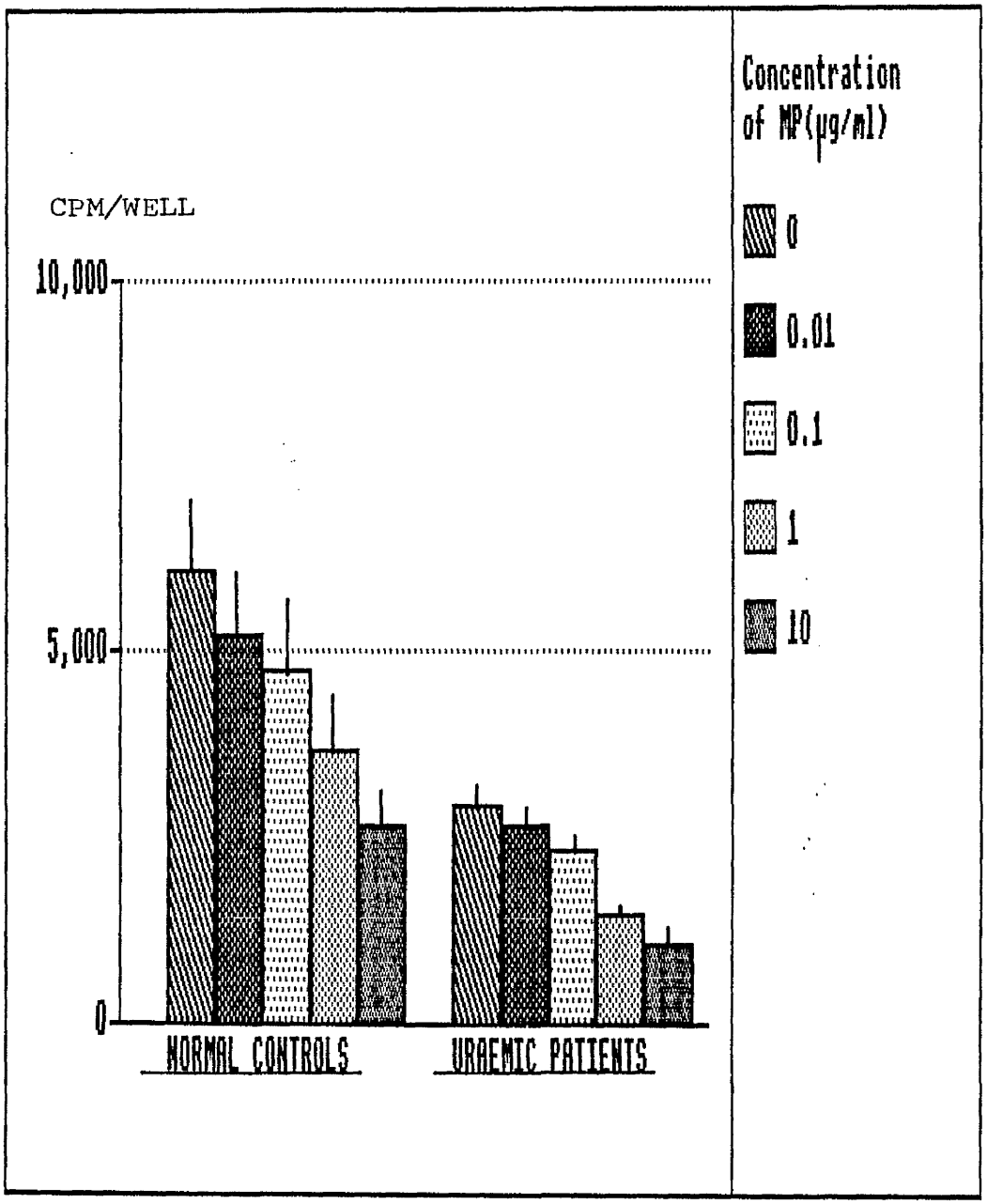


Fig.6.3

The effect of methyl-prednisolone on the proliferative responses to PHA by control and uraemic peripheral blood mononuclear cells. Bars represent the mean ^{14}C Thymidine uptake on the third day of culture +1SEM in 4 experiments.

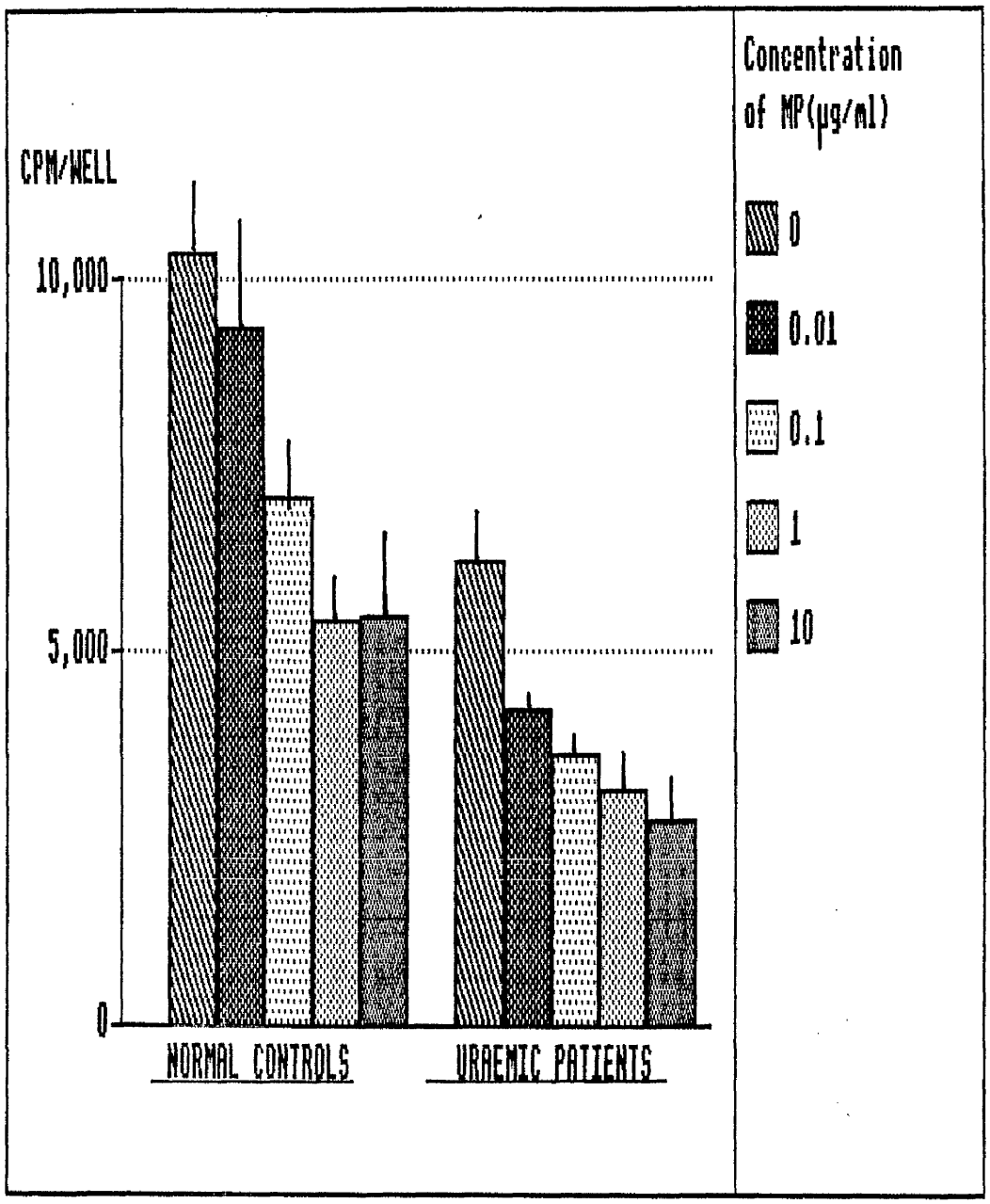


Fig.6.4

The effect of methyl-prednisolone on the proliferative responses to PWM by control and uraemic peripheral blood mononuclear cells. Bars represent the mean ^{14}C Thymidine uptake on the third day of culture +1SEM in 4 experiments.

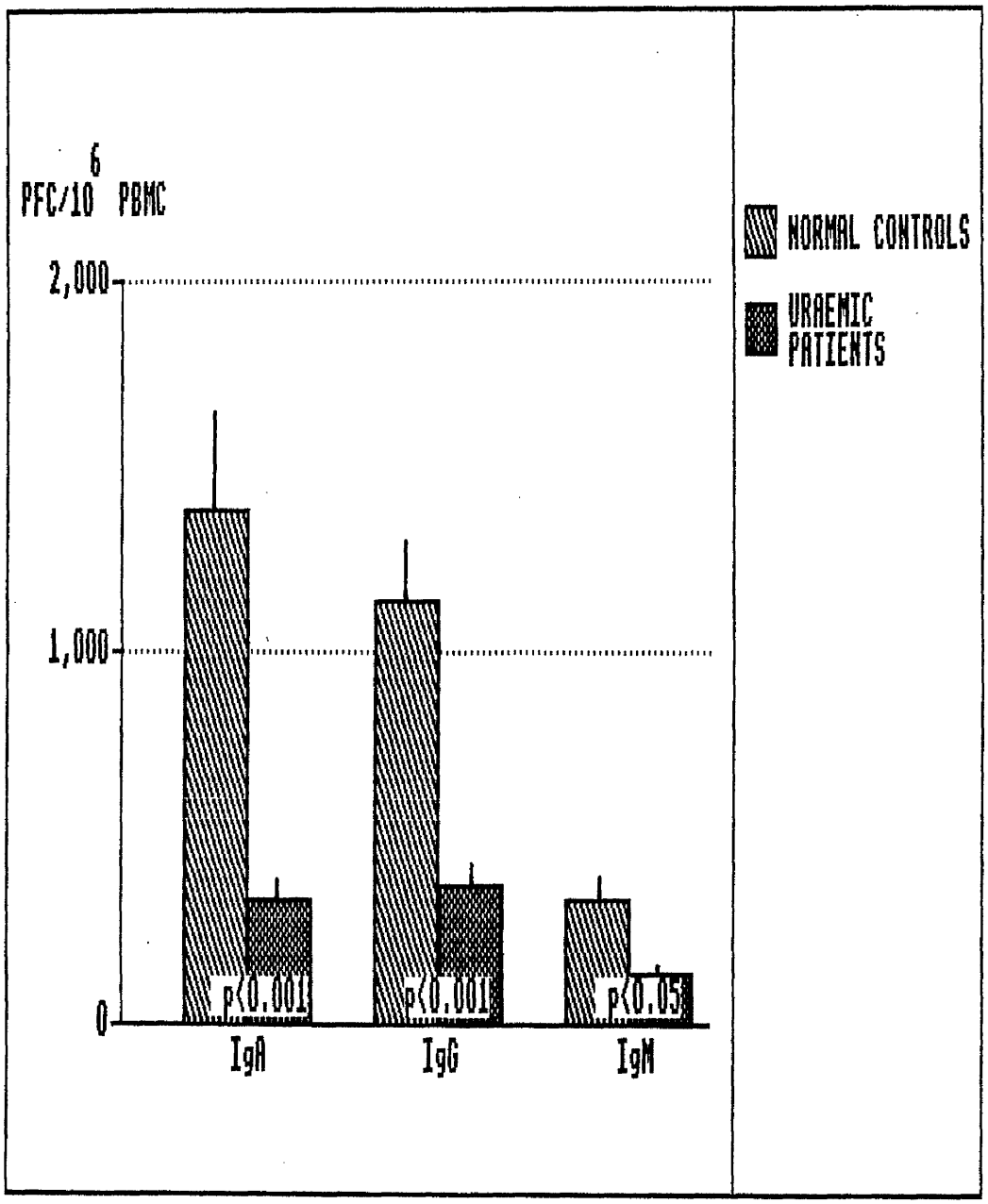


Fig.6.5

Spontaneous plaque formation by peripheral blood mononuclear cells from normal controls(n=25) and uraemic patients(IgA:n=21, IgG:n=39, IgM:n=16).
Bars represent the mean+1SEM(PFC/10^6 cells)

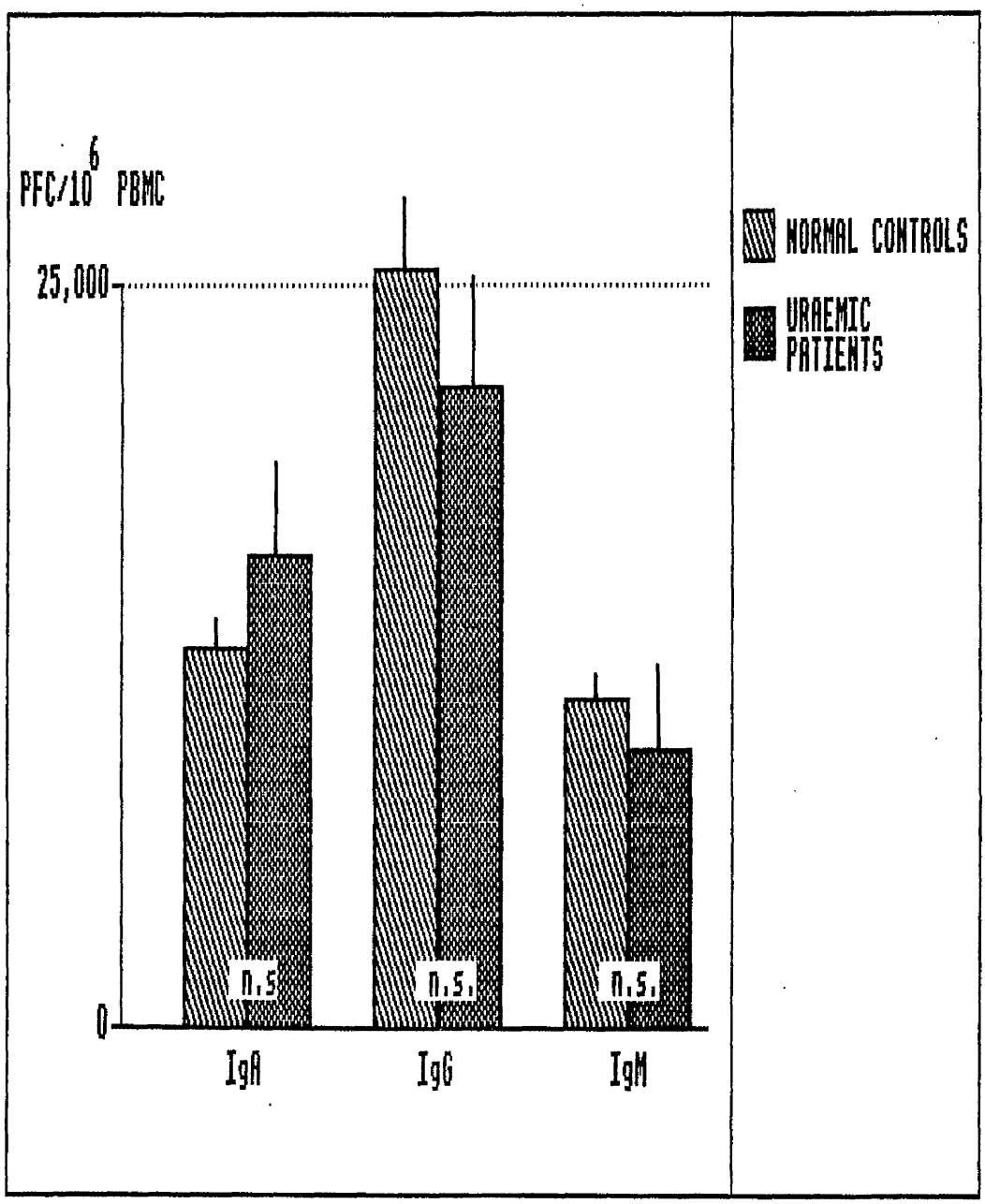


Fig.6.6

PWM induced plaque formation by peripheral blood mononuclear cells from normal controls(n=25) and uraemic patients(IgA:n=15, IgG:n=38, IgM:n=15). Bars represent the mean+1SEM(PFC/10^6 cells)

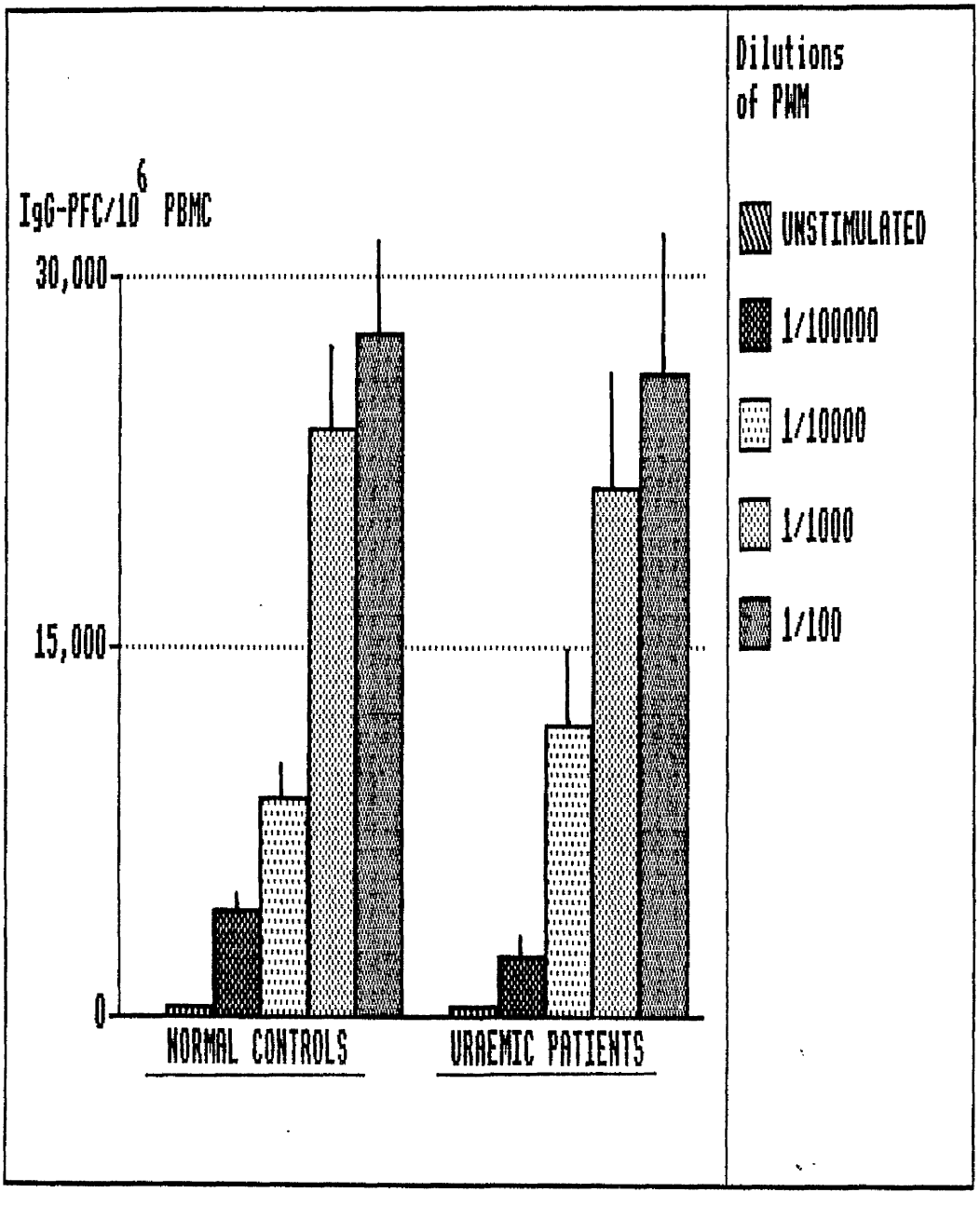


Fig.6.7

The IgG response to different dilutions of PWM of control(n=4) and uraemic(n=4) peripheral blood mononuclear cells.
Bars represent the mean+1SEM(PFC/10^6 cells)

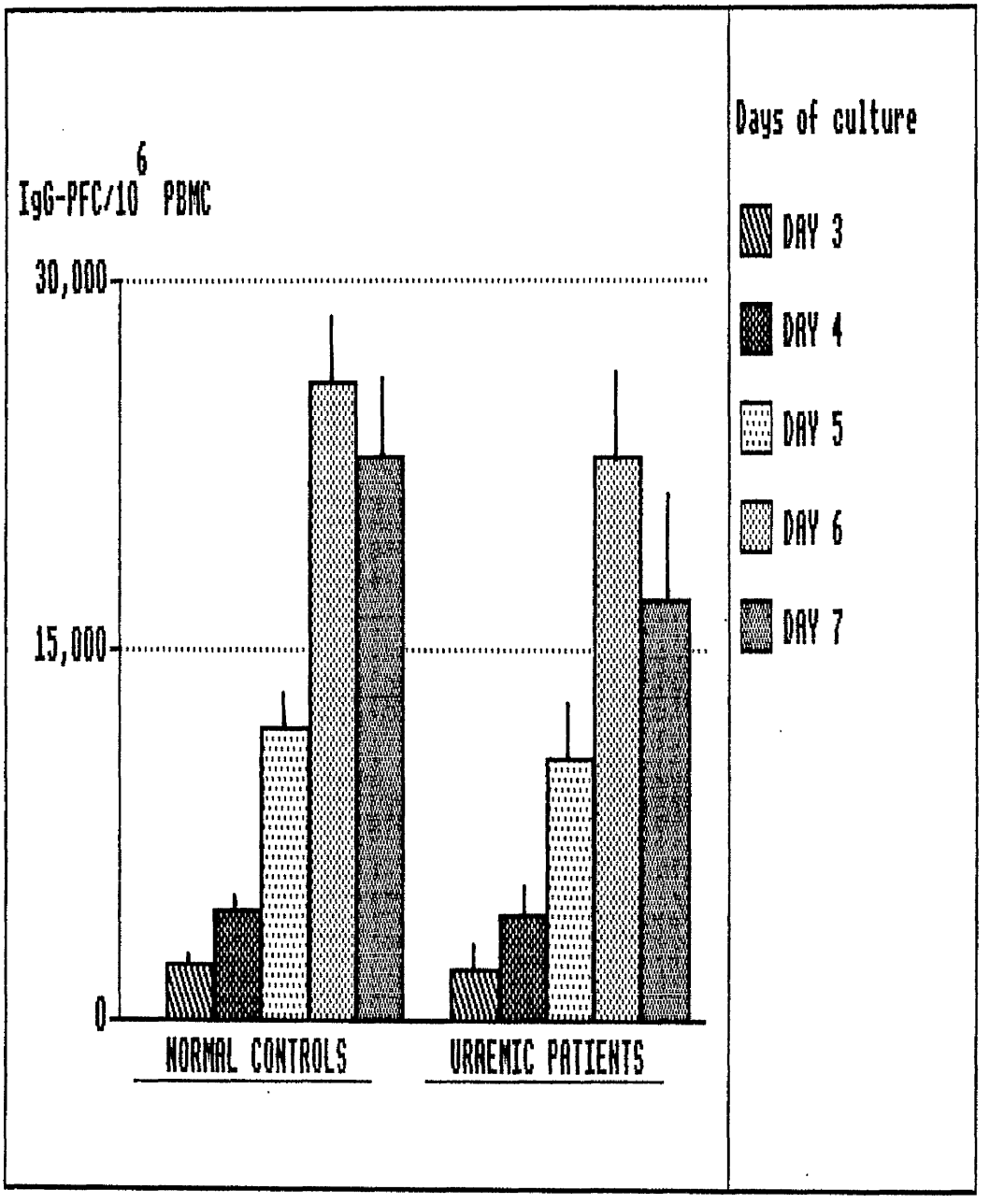


Fig.6.8

Time course of IgG production by control(n=4) and uraemic(n=4) peripheral blood mononuclear cells in response to PWM.
Bars represent the mean+1SEM(PFC/10^6 cells)

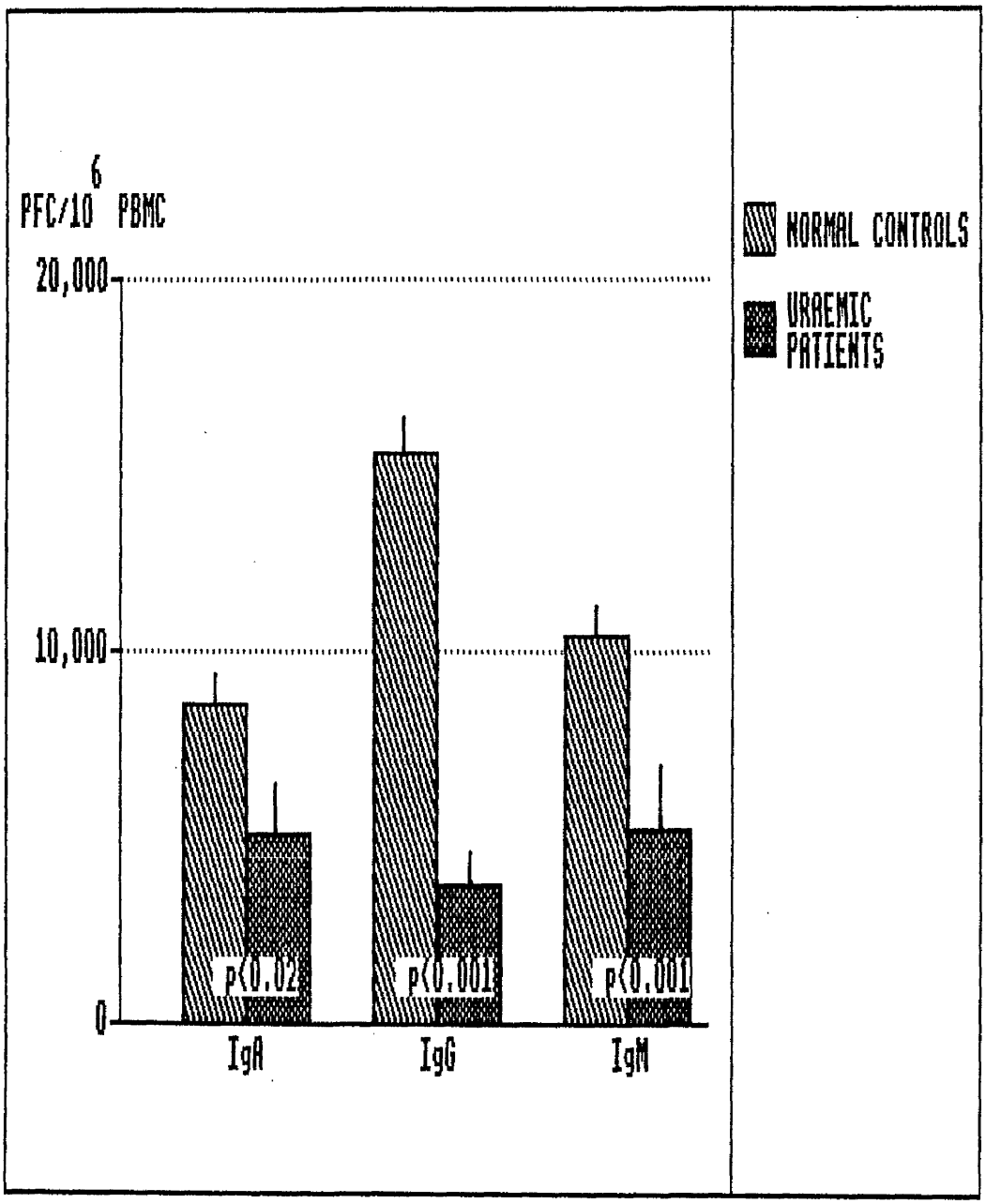


Fig.6.9

SAC induced plaque formation by peripheral blood mononuclear cells from normal controls(n=25) and uraemic patients(IgA:n=15, IgG:n=39, IgM:n=15). Bars represent the mean+1SEM(PFC/10^6 cells)

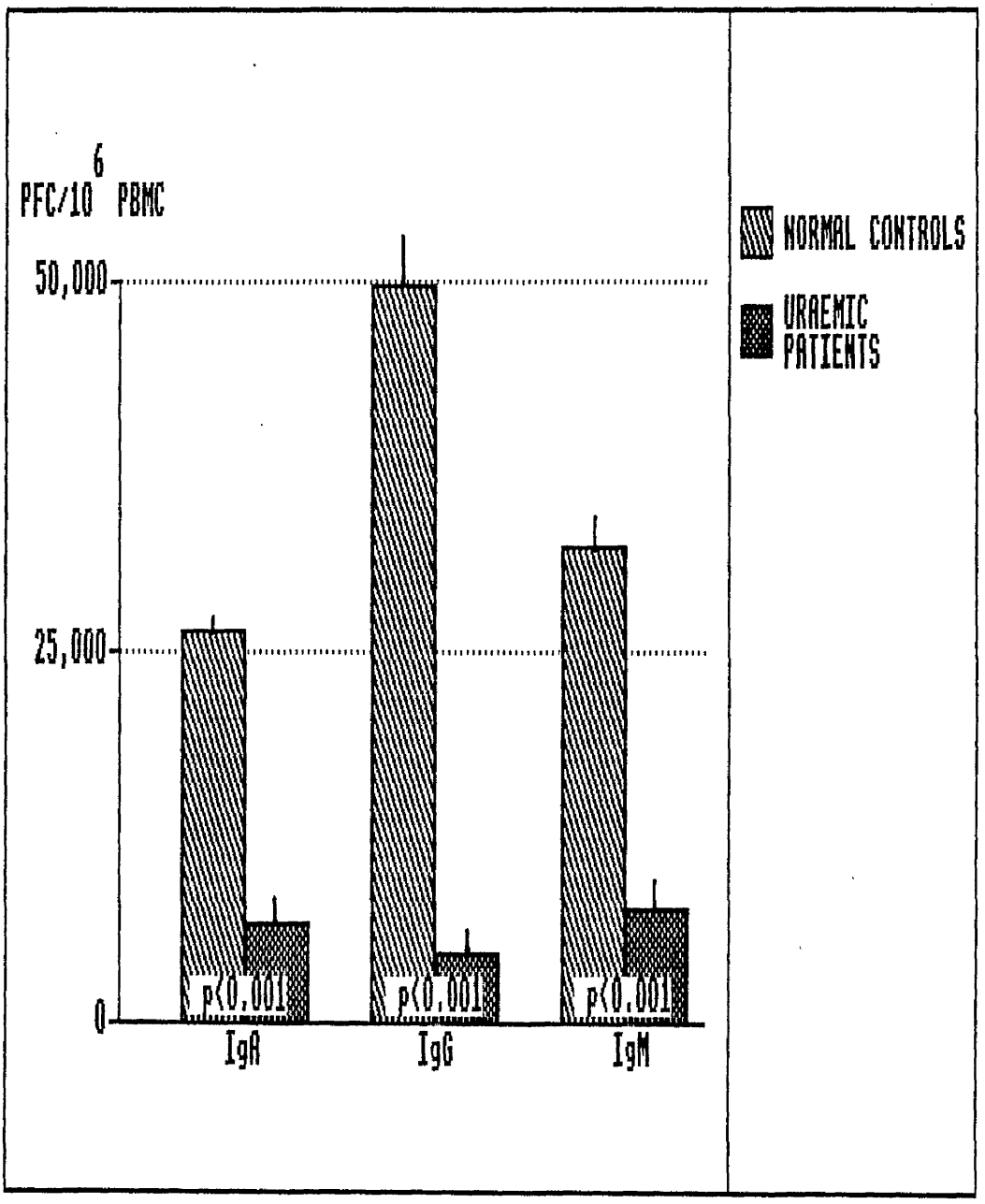


Fig.6.10

Plaque formation induced by an optimal combination of PWM and SAC in normal controls(n=18) and uraemic patients(IgA:n=15, IgG:n=27, IgM:n=15).
Bars represent the mean+1SEM(PFC/10^6 cells)

	Synergistic effect	No synergistic effect
Normal Controls	13	5
Uraemic Patients	2	25

Table 6.2

Synergistic effect between SAC and PWM in Normal Controls(n=18)and Uraemic Patients(n=27). A synergistic effect was defined when IgG-PFC counts induced by PWM+SAC were more than the sum of IgG-PFC counts induced by SAC and PWM alone plus 25%. The chi-squared test was used to analyse the results($\chi^2 = 23.40$, $p<0.001$)

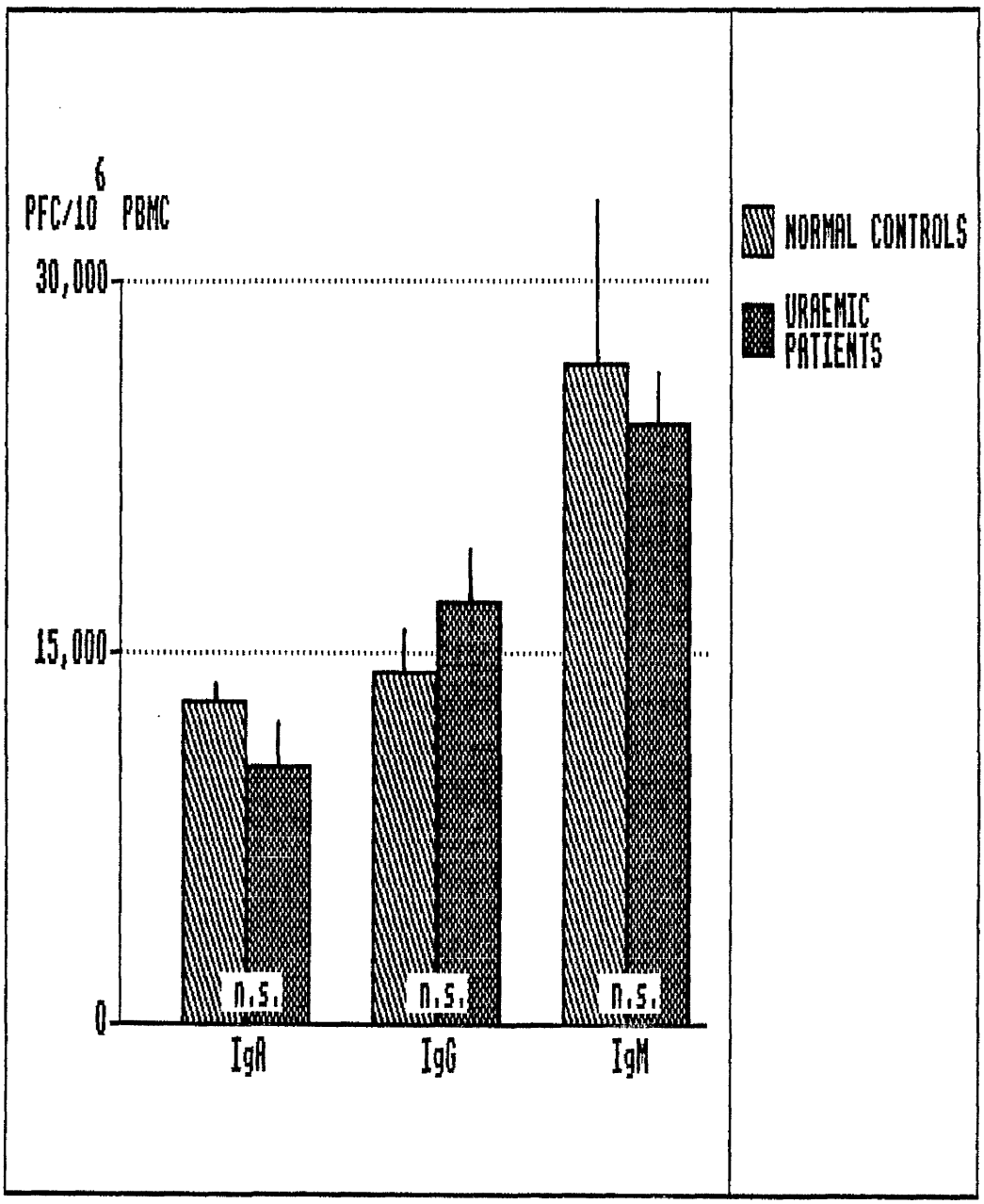


Fig.6.11

EBV induced plaque formation by peripheral blood mononuclear cells from normal controls(n=7) and uraemic patients(n=7).
Bars represent the mean+1SEM(PFC/10^6 cells)

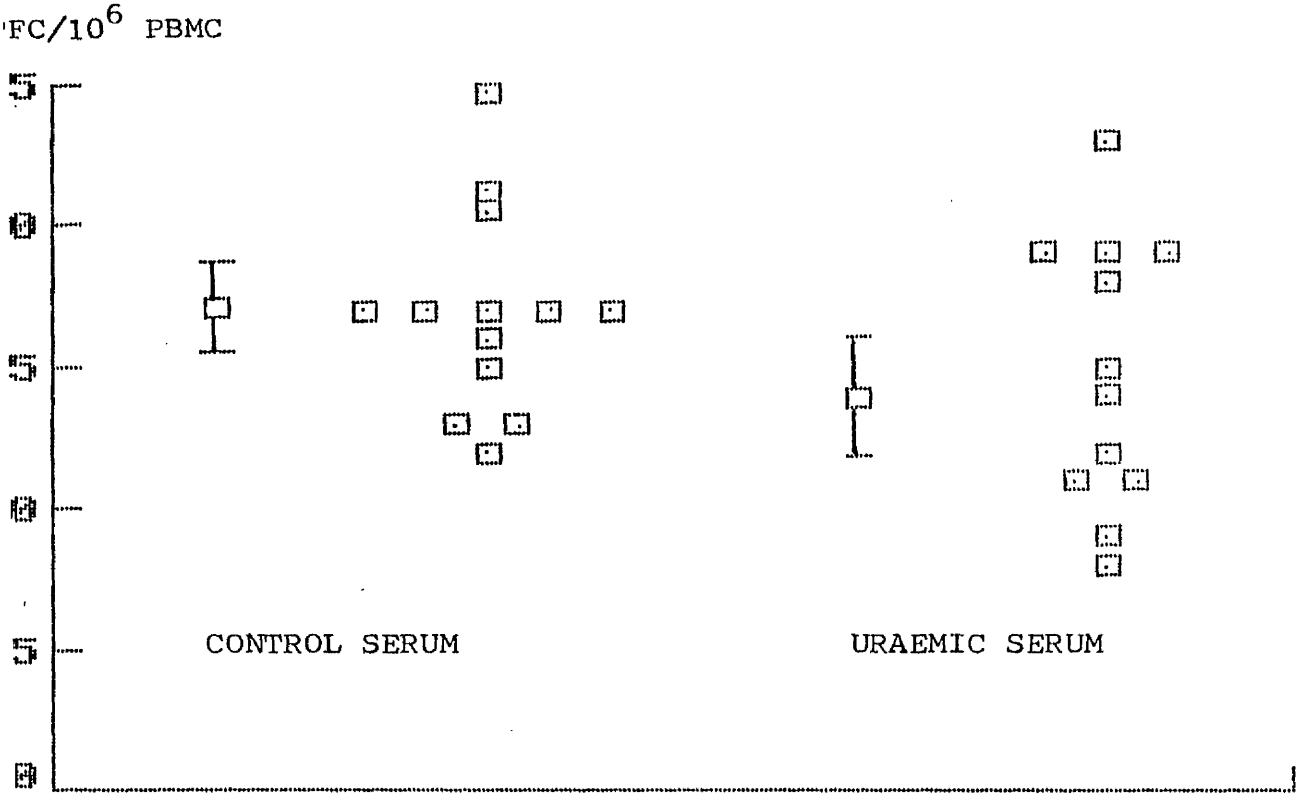


Fig.6.12

PWM induced IgG-PFC response of control peripheral blood mononuclear cells pre-incubated with either control or uraemic serum.
Results shown are the mean of triplicate determinations.

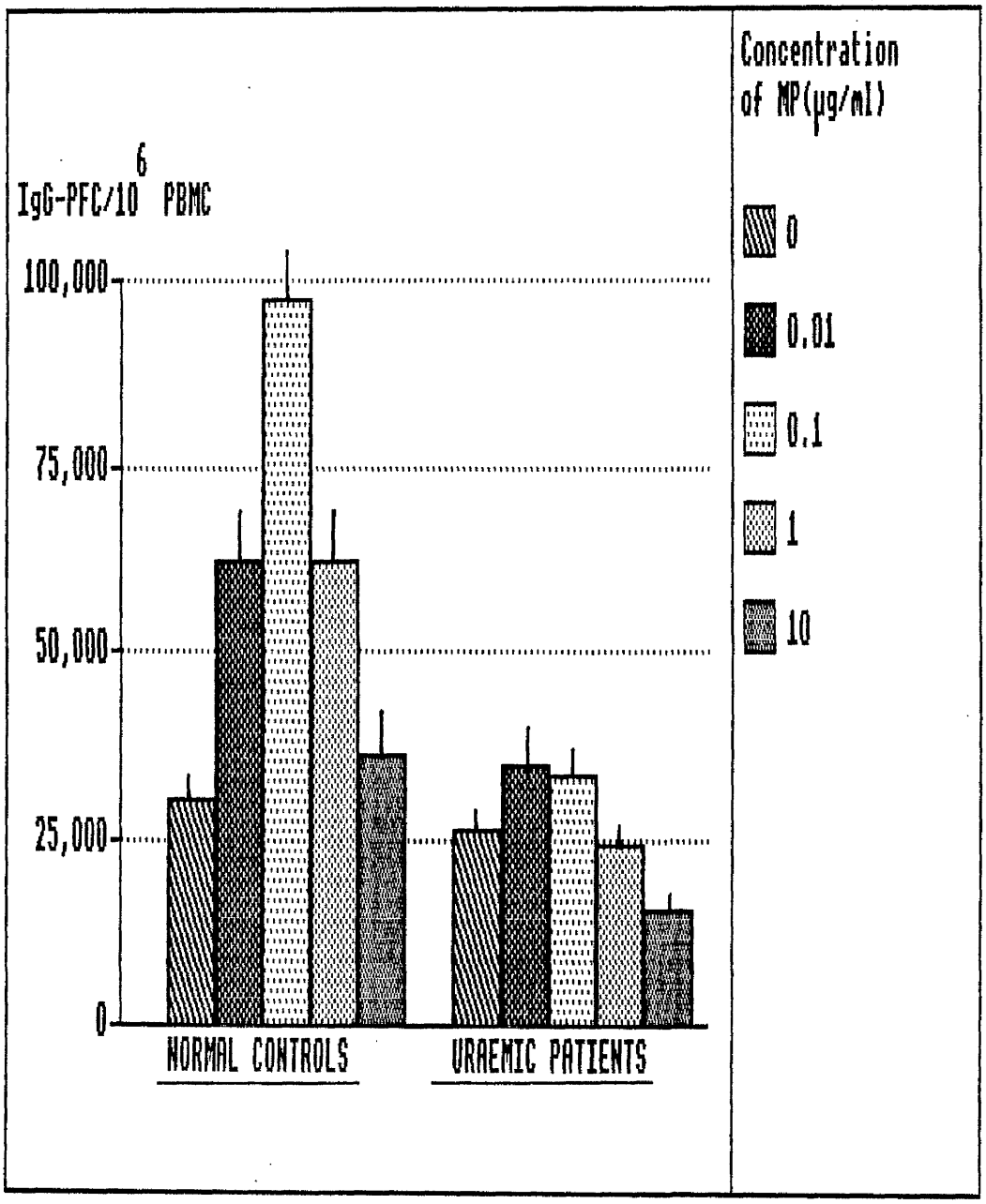


Fig.6.13

The effect of methyl-prednisolone on PWM induced plaque formation by control(n=8) and uraemic(n=8) peripheral blood mononuclear cells. Bars represent the mean+1SEM(IgG-PFC/10^6 cells)

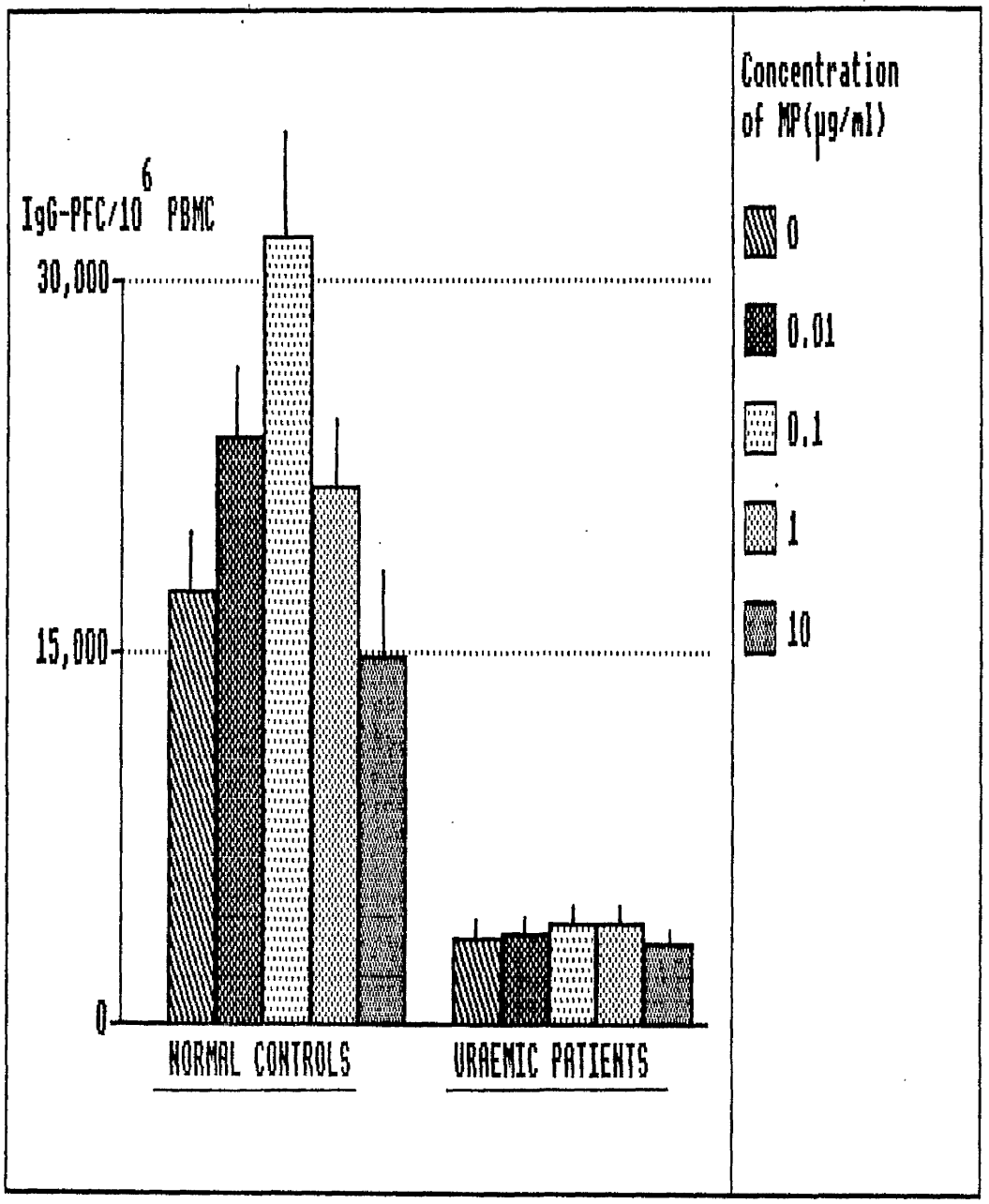


Fig.6.14

The effect of methyl-prednisolone on SAC induced plaque formation by control(n=8) and uraemic(n=8) peripheral blood mononuclear cells. Bars represent the mean+1SEM(IgG-PFC/10^6 cells)

	1.PWM				2.SAC		
	PWM	PWM+INDO	%	/	SAC	SAC+INDO	%
EXP 1	4775	5995	+25.5%	/	21506	18500	-14%
EXP 2	925	1486	+60%	/	1088	1660	+52%
EXP 3	16958	3557	-79%	/	1156	3303	+185%
EXP 4	4162	2569	-39%	/	9250	12950	+40%
EXP 5	4008	6801	+69%	/	1798	4162	+131%
EXP 6	1541	1982	+28%	/	1632	2522	+54%
EXP 7	14230	12331	-13%	/	1206	1360	+8%
EXP 8	22460	13056	-42%	/	12065	6727	-44%
EXP 9	10551	12662	+20%	/	5088	4201	-17.5%
EXP 10	40831	52505	+29%	/	3816	8174	+114%
EXP 11	30649	19955	-35%	/	2520	8055	+219%
MEAN	13735	12081		/	5556	6510	
SEM	3946	4282		/	1941	1596	

Table 6.3

The effect of indomethacin(1μg/ml) on PWM and SAC cultures of uraemi peripheral blood mononuclear cells. The results shown are the mean IgG-PFC/10^6 cells of triplicate determinations from 11 uraemic patients. The percentage values represent the proportion of change after adding indomethacin.

CHAPTER VII

EFFECT OF PRE-TRANSPLANT BLOOD TRANSFUSION ON IN VITRO IMMUNOGLOBULIN SECRETION

INTRODUCTION

The most effective treatment of uraemia is allogeneic renal transplantation, but its success is limited by the occurence of graft rejection. Recently, it has been shown that rejection of renal allografts may be prevented by pre-transplant blood transfusions and it is believed that this reflects altered immune responsiveness by the host(Watson et al, 1979, Fisher et al, 1980). As antibody production may be important in some forms of renal allograft rejection, the aim of this part of the study was to assess the effect of third party blood transfusion on immunoglobulin production by uraemic patients on dialysis awaiting renal transplantation. This was achieved by measuring immunoglobulin secretion by peripheral blood mononuclear cells both spontaneously and after stimulation with the polyclonal B cell activators PWM or SAC. In addition, as these activators differ in their sensitivity to regulation by T cells, the results should help elucidate the mechanisms by which blood transfusion alters the immune response.

MATERIALS AND METHODS

The study group consisted of 27 patients with chronic renal failure undergoing routine dialysis who had not previously been transfused(Table 7.1). All patients received

one unit of packed red cells from random third party donors every 14 days. 13 patients received 5 units of blood in total, while 13 received 5 additional units. Peripheral blood mononuclear cells were obtained before the first blood transfusion and at 14 day intervals throughout the blood transfusion programme. Samples were tested for their spontaneous secretion of IgG and for their ability to differentiate into immunoglobulin secreting cells in response to optimal concentrations of PWM and SAC, using the protein-A plaque forming cell assay. Ten normal subjects(Table 7.2) and seventeen age and sex-matched non-transfused dialysis patients(Table 7.3) were also studied sequentially as control groups. All samples of peripheral blood mononuclear cells were cryopreserved immediately after separation and each sample from the same subject was assayed on the same day to avoid test to test variarion. In this study, only IgG production was assessed because IgG-PFC counts were consistently higher and because it was technically difficult to measure all three isotypes simultaneously. Significant changes in the IgG-PFC count after blood transfusion were defined as a variation of more than 2SD from the mean response found in uraemic controls.

RESULTS

Spontaneous Production of Immunoglobulin

Tables 7.4, 7.5, 7.6 show the spontaneous IgG-PFC responses in transfused patients and in both groups of

controls, before, and at the conclusion of the blood transfusion programme. When the mean spontaneous IgG-PFC responses of the three groups are compared it can be seen that, at the beginning of the study, the response of both uraemic groups was significantly depressed compared with that of the healthy subjects($p<0.001$). The numbers of spontaneous plaque forming cells in healthy subjects and non-transfused uraemics remained constant throughout the study(Tab.7.5,7.6). Although there was no significant overall change in the spontaneous response after blood transfusion, it appeared that some patients reacted differently. Thus, in 12/27 transfused patients an increase in the spontaneous response was observed and in six of them the post-BT to pre-BT ratio ranged from 2 to 7.21 and there was a clear bimodal distribution. In eleven patients there was no change while a decrease was observed in four.

The five additional units of blood received by thirteen patients were not shown to produce a significant effect(pre-BT:537+120, post-5BT:568+73, post-10BT:675+90).

Production of Immunoglobulin in Response to PWM

When PWM was used to activate peripheral blood mononuclear cells from the three groups of subjects at the beginning of the study no differences were observed between the IgG responses of the three groups, confirming that PWM induced production of immunoglobulin is normal in uraemic patients(Table 7.4, 7.5, 7.6). The only difference between healthy subjects and uraemic patients was the greater intersubject variation observed in the two uraemic groups

which is reflected in the higher SEM values. As shown in Table 7.5, 7.6, the PWM induced IgG response remained constant throughout the study in both healthy subjects and non-transfused uraemic patients while in the transfused group a significant decrease in response to PWM was found after blood transfusion(Table 7.4, $p<0.05$). It can be seen in Fig.7.2 that this decrease occurred in eighteen of the twenty six (69%) transfused patients, compared to a decrease in the SAC induced IgG-PFC response in only five of the twenty six patients(19%, $p<0.001$). In three patients(12%), the PWM induced plaque forming cell response was unchanged while five(19%) transfused patients had increased plaque forming cell responses to PWM after the last blood transfusion. In these five patients the increase appeared after the second or third blood transfusion, and in three patients the increase in PWM induced plaque forming cell response was accompanied by a simultaneous increase in spontaneous and SAC induced plaque forming cell counts(Fig.7.1, 7.4). In the eighteen patients who showed a decrease of the PWM induced plaque forming cell response-compared to the pre-blood transfusion sample-the suppression of the response became significant($p<0.05$) after the second blood transfusion and was progressive thereafter(Fig.7.3). In the 13 patients who received five additional blood transfusions, the plaque forming cell counts remained unchanged overall between the fifth and tenth blood transfusion(Pre-BT:23879+6200, Post-5BT:18347+4896,

Post-10BT:21155+9007). In 8 of these patients a decrease in plaque forming cells had been observed after 5 blood transfusions and in these patients the 5 additional units of blood produced a further decrease of the plaque forming cell response which was statistically significant(Pre-BT:27697+8746, *Post-5BT:13142+3902, *Post-10BT:8592+2728, *p<0.05, Signed Rank Test).

Production of Immunoglobulin in Response to SAC

When peripheral blood mononuclear cells were stimulated with SAC at the beginning of the study, the IgG response of healthy subjects was significantly better than both transfused and non-transfused uraemic patients(Table 7.4, 7.5, 7.6, p<0.001). In the two control groups the counts of IgG-PFC were stable over the 12 week period(Table 7.5, 7.6) and this was also observed in all of the transfused group except for three patients whose plaque forming cell response increased and for five patients whose plaque forming cell response decreased(Fig.7.4). The five additional blood transfusions given to 13 patients did not change the overall values of SAC induced IgG-PFC(Pre-BT:4161+1273, Post-5BT:4377+1225, Post-10BT:5015+1718).

CONCLUSIONS

These studies confirmed that spontaneous and SAC induced plaque forming cell responses are reduced in uraemic patients and showed that blood transfusion had no effect on SAC responses whereas it enhanced the spontaneous response in some patients. In contrast, the response to PWM was confirmed as being normal in uraemic patients and it was reduced by the programme of blood transfusions. Therefore, as PWM driven B cell differentiation is regulated by suppressor T cells, in contrast to the response to SAC which is not greatly affected by suppressor T cell activity, these results indicate that blood transfusion exerts its immunosuppressive effect through suppressor T cells.

	AGE	SEX	BT
1. S.W.	23	M	10
2. M.S.	43	F	10
3. P.M.	24	F	5
4. J.B.	35	F	5
5. M.M.	38	M	10
6. A.K.	27	M	10
7. T.Y.	59	M	5
8. R.H.	27	M	5
9. M.H.	36	F	10
10. J.M.	39	F	10
11. B.J.	27	M	10
12. J.P.	53	M	10
13. J.D.	52	M	10
14. C.S.	33	M	10
15. W.H.	31	M	10
16. J.L.	33	M	10
17. P.F.	49	M	10
18. T.C.	53	M	5
19. K.M.	53	F	5
20. T.S.	49	M	5
21. W.G.	22	M	5
22. R.F.	27	M	5
23. R.B.	53	M	5
24. L.A.	59	F	5
25. L.M.	55	F	5
26. J.G.	20	M	5
27. J.H.	52	M	5
	Mean+SEM: 39.7+2.5	M/F: 18/9	

Table 7.1

Uraemic patients who received 5-10 blood transfusions and were studied in the blood transfusion trial. Additional information is presented in Appendix 1.

	AGE	SEX
1. D.T.	38	M
2. J.L.	25	F
3. M.W.	40	F
4. V.J.	33	F
5. T.D.	31	M
6. P.D.	32	M
7. B.J.	40	M
8. E.F.	55	F
9. E.G.	28	M
10. D.D.	27	M
	Mean+SEM:35+2.8	M/F:6/4

Table 7.2

Normal controls used in the blood transfusion study

	AGE	SEX
1. S.W.	23	M
2. M.S.	43	F
3. I.M.	48	F
4. J.B.	35	F
5. C.M.	34	F
6. A.K.	27	M
7. T.Y.	59	M
8. J.H.	56	M
9. W.B.	57	M
10. D.H.	28	M
11. C.B.	18	F
12. J.M.	63	M
13. M.H.	36	F
14. D.M.	52	M
15. A.V.	50	F
16. R.F.	47	M
17. T.J.	50	M
	Mean+SEM:42.7+3.3	M/F:10/7

Table 7.3

Uraemic patients who did not receive blood transfusions and were used as uraemic controls in the blood transfusion study

	Transfused uraemic patients n=27	
	pre-BT	post-BT
Spont	422+57	556+73
SAC	4576+1026	4016+899
PWM	*21237+4465	*14729+4488

Table 7.4

IgG-PFC counts(mean+SEM) in uraemic patients before and after 5-10 blood transfusions. PWM responses showed a significant decrease post*BT(*$p<0.05$, signed rank test), SAC responses were unaffected while spontaneous responses were increased due to the high titres observed post-BT in some patients. However, the difference between pre- and post-BT spontaneous responses was not statistically significant.

	Spontaneous		PWM-induced		SAC-induced	
	Day 0	12 weeks	Day 0	12 weeks	Day 0	12 weeks
1.	416	536	38756	32654	13406	14768
2.	1354	1463	21203	19507	13673	12596
3.	580	575	30796	28503	14457	12975
4.	417	462	22595	19760	12006	10942
5.	2740	3625	26668	25796	11892	13072
6.	3654	4532	11498	13673	12797	13308
7.	935	854	26738	23996	13798	12593
8.	710	1140	16407	19000	12504	11740
9.	950	1005	31766	36547	17066	16439
10.	1103	1267	32207	28456	22694	25485
MEAN	1285	1545	25863	24789	14429	14391
SEM	335	434	2541	2183	1020	1308

TABLE 7.5

Variation in IgG production by healthy peripheral blood mononuclear cells over a period of 12 weeks. Results shown are the mean IgG-PFC/1 cells of triplicate determinations from 10 normal controls. No significant variation in IgG production was noted.

	Spontaneous		PWM-induced		SAC-induced	
	Day 0	12 weeks	Day 0	12 weeks	Day 0	12 weeks
1.	215	163	44733	43680	18432	16958
2.	235	242	27335	28304	1353	1286
3.	210	211	20175	21053	1423	1898
4.	202	194	38156	40525	1954	2205
5.	198	194	15454	16480	1253	1454
6.	284	316	62734	67272	2574	2688
7.	92	110	72500	67502	1734	2332
8.	331	386	23173	21513	1354	1187
9.	153	177	14525	15784	1312	1297
10.	84	95	11197	12861	1385	1339
11.	205	175	32097	37578	1321	1387
12.	353	317	9750	12410	1120	956
13.	1905	2466	-	-	-	-
14.	553	704	6600	6440	1734	1653
15.	315	366	5045	5310	1985	2140
16.	347	432	4624	4312	1530	1842
17.	810	707	58142	53706	6246	6850
MEAN	381	426	27890	28420	2919	2967
SEM	104	134	5268	5107	1047	965

Table 7.6

Changes in IgG-PFC counts in uraemic controls. Results shown are the mean IgG-PFC/10^6 cells of triplicate determinations from 17 uraemic patients.

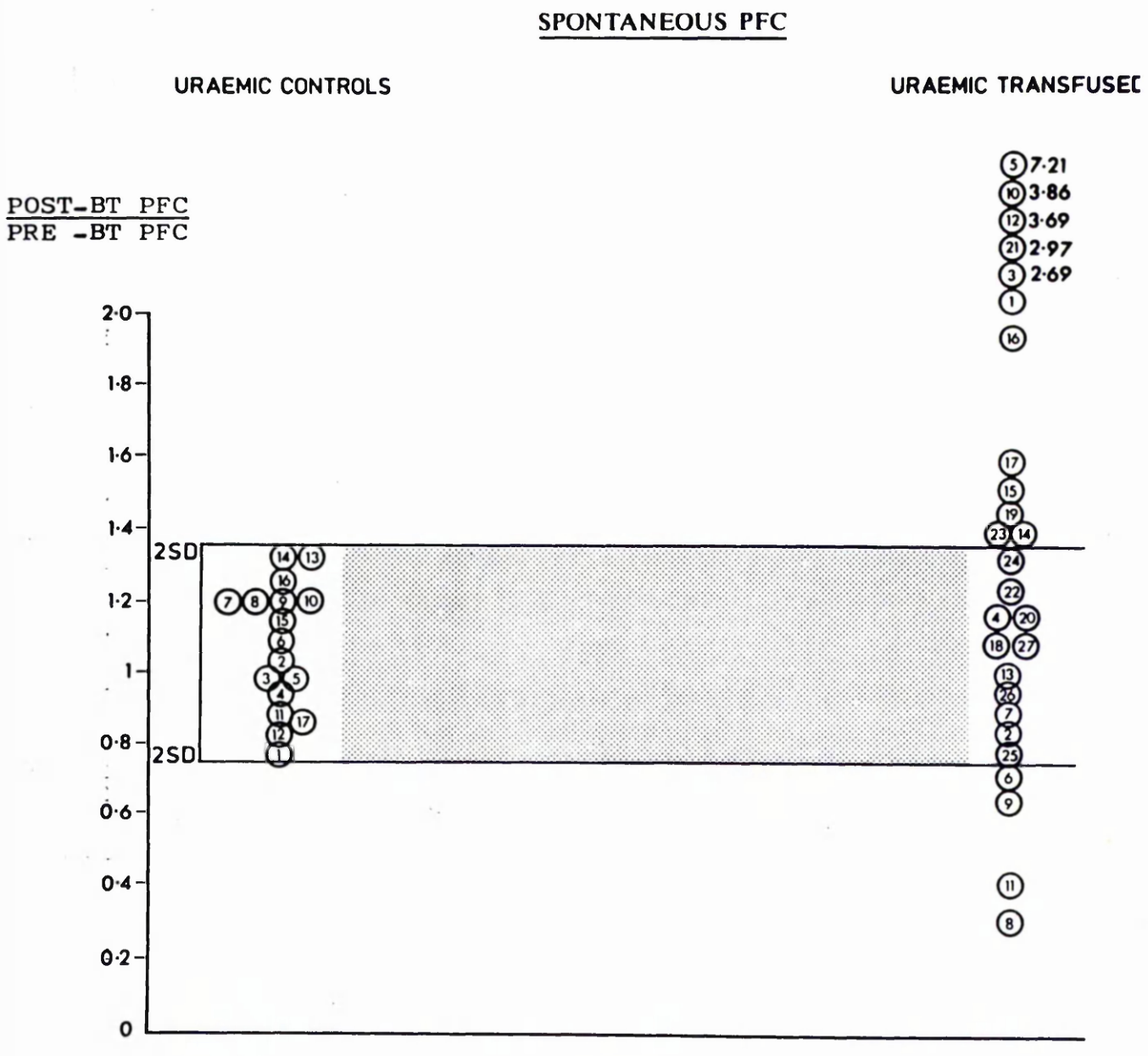


Fig.7.1

Ratio of post-BT to pre-BT spontaneous PFC in 27 transfused patients. Numbers within circles indicate the number of each individual patient as presented in Tab.7.1, 7.3. Shaded area represents the mean±2SD change in the 17 uraemic controls studied sequentially over a period of 12 weeks. Significant changes were defined as variation of more than 2SD from the mean response found in controls and a clear bimodal distribution of results is evident.

PWM INDUCED PFC

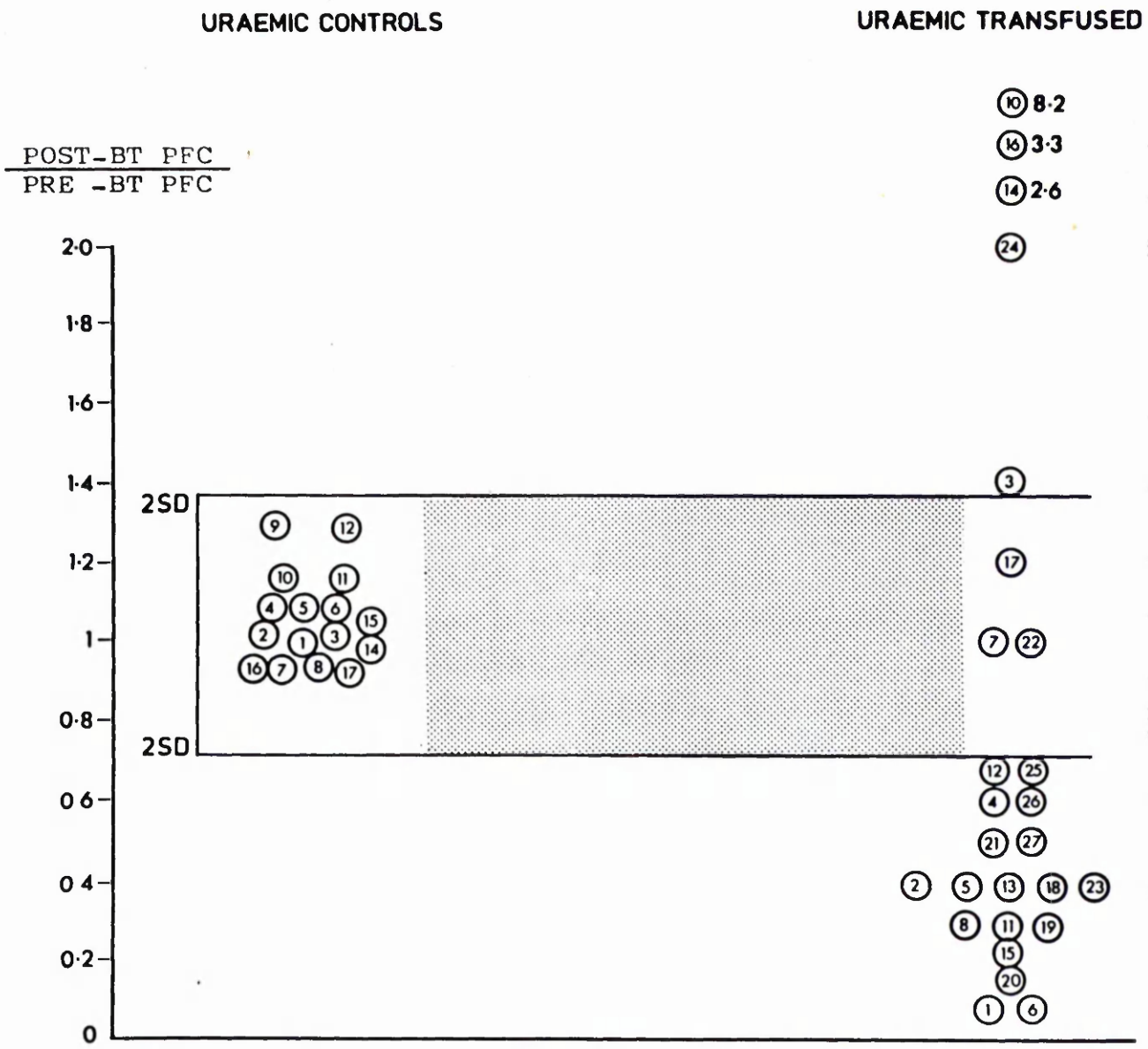


Fig.7.2

Ratio of post-BT to pre-BT PWM induced PFC in 26 transfused patients. Numbers within circles indicate the number of the patient(Table 7.1, 7.3). Shaded area represents the mean$\pm^{2SD}$ change in the 16 uraemic controls studied sequentially over a period of 12 weeks. Significant changes were defined as variation of more than 2SD from the mean response found in controls

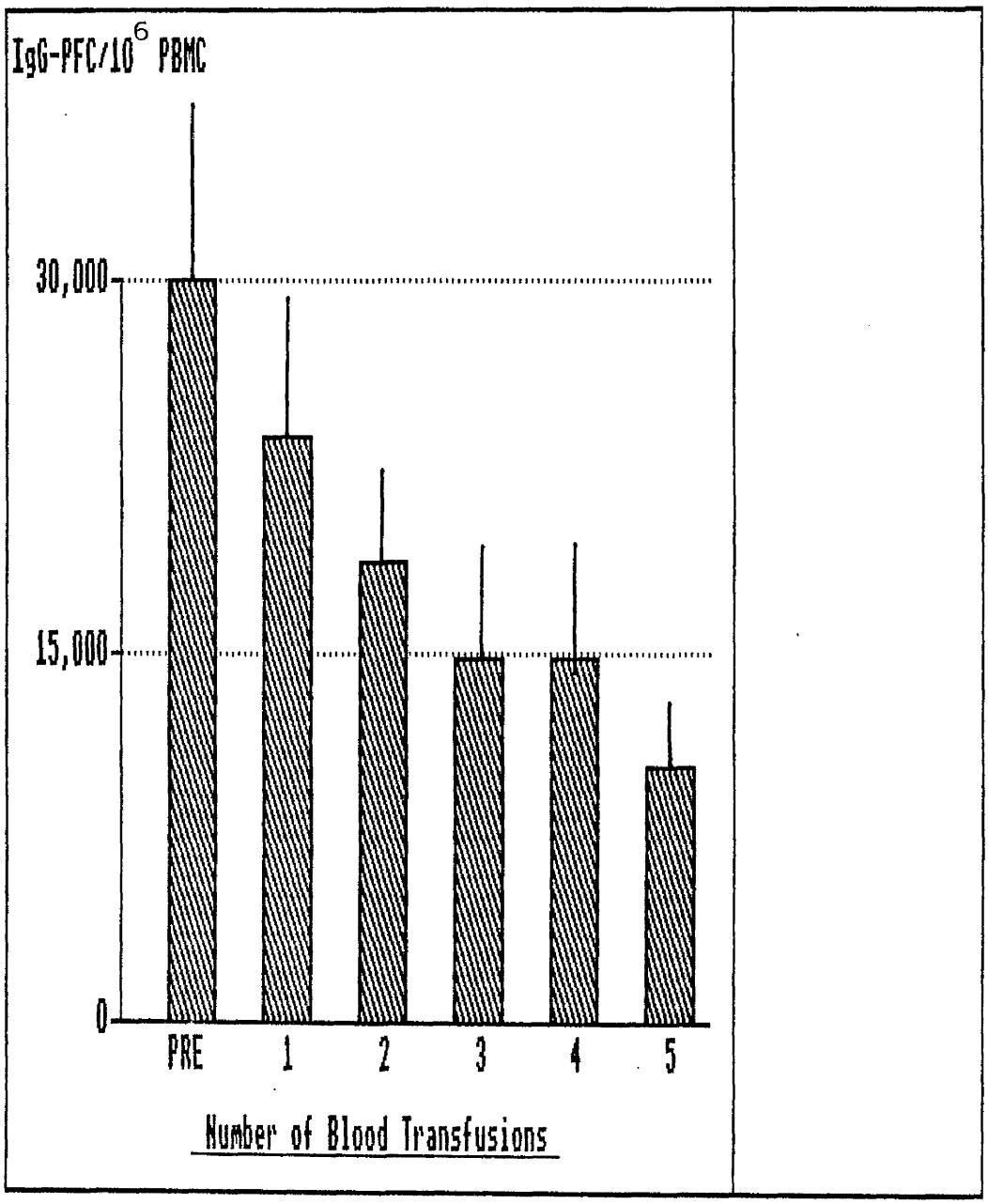


Fig.7.3

The effect of blood transfusion on PWM induced IgG production in eighteen responders. Bars represent the mean+1SEM(IgG-PFC/10^6 cells)

SAC INDUCED PFC

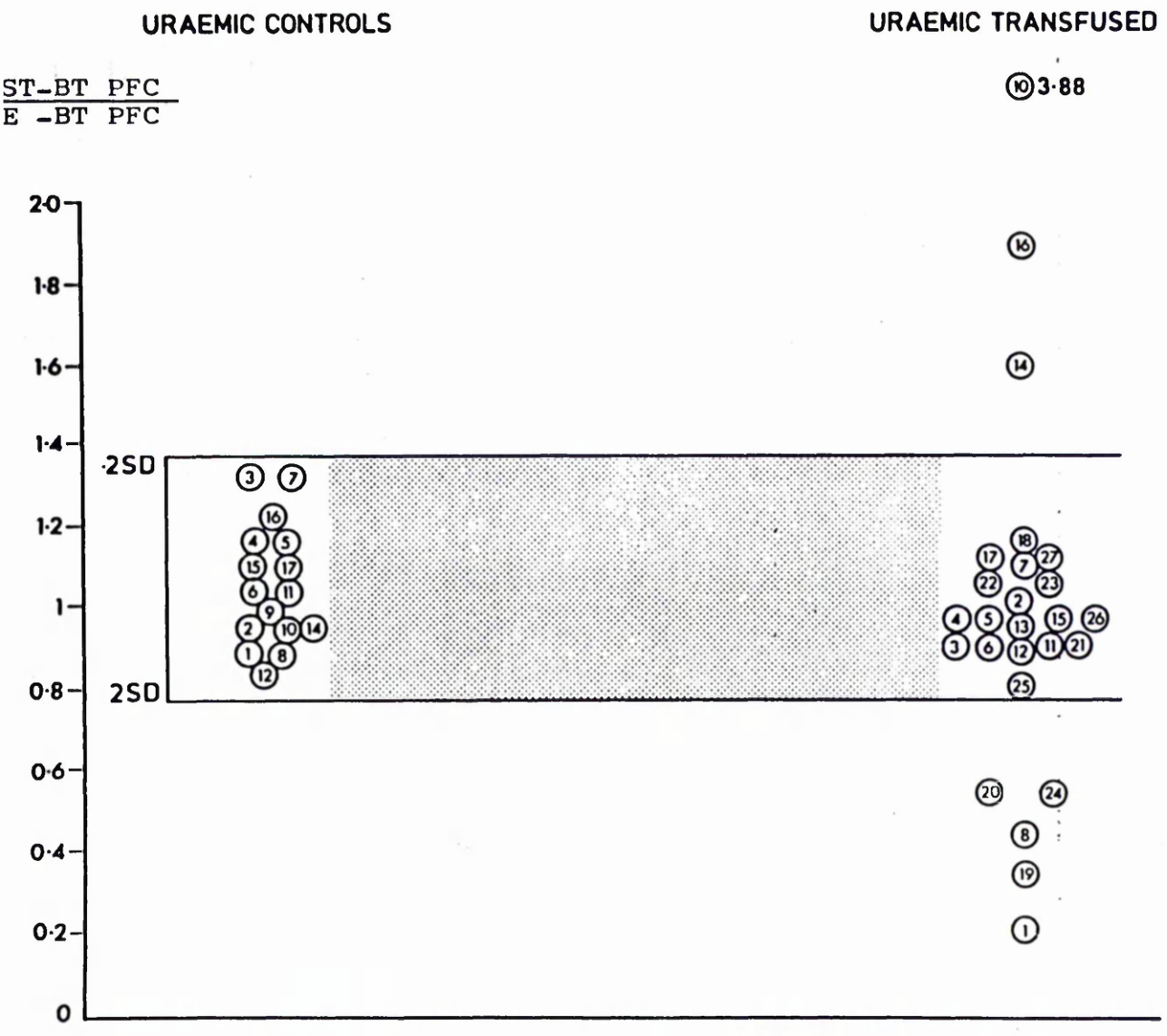


Fig.7.4

Ratio of post-BT to pre-BT SAC induced PFC in 26 transfused patients. Numbers within circles indicate the number of patient (Table 7.1, 7.3). Shaded area represents the mean±2SD change in the 16 uraemic controls studied sequentially over a period of 12 weeks. Significant changes were defined as variation of more than 2SD from the mean response found in controls.

CHAPTER VIII

STUDIES OF SPONTANEOUS PLAQUE FORMATION IN RENAL ALLOGRAFT RECIPIENTS TREATED WITH CYCLOSPORINE OR AZATHIOPRINE

INTRODUCTION

Acute rejection of renal allografts may involve an anti-donor antibody response and it would be useful to have a reliable immunological assay to establish the onset of rejection at an early stage. Thus, the principal aim of this study was to determine whether there was an association between acute rejection episodes and counts of spontaneous plaque forming cells. In addition, I examined the effects of immunosuppression with either azathioprine or cyclosporine on spontaneous immunoglobulin production by renal transplant patients within the first six weeks post transplant.

MATERIALS AND METHODS

Eleven patients treated with azathioprine and prednisolone and fourteen patients treated with cyclosporine and prednisolone were studied and the two groups were matched for age, sex, number of blood transfusions, and number of HLA-A, -B and -DR mismatches between donor and recipient(Table 8.1, 8.2). Azathioprine was administered orally at a dose of 2mg/kg/day and it was reduced accordingly when white blood cell counts were falling below 4000/cmm . The dose of cyclosporine was 17.5mg/kg/day for the first week tailing to 15mg/kg/day on the second, 10/mg/kg/day on the third, and then 4-10mg/kg/day for the first two months depending on the levels of cyclosporine in blood. Finally, prednisolone was administered orally at a dose of 20mg/day.

Spontaneous production of immunoglobulin was assessed on cryopreserved samples of peripheral blood mononuclear cells obtained 4, 7, 14, 21, 28, and 35 days after transplantation.

Rejection episodes were diagnosed clinically and were confirmed using a combination of serum creatinine levels, renal biopsy, and ultrasound. The type of rejection which occurs most frequently within the two months post-transplant is the acute form and the clinical criteria which were taken as evidence of acute rejection were fever, tenderness in the region of the transplant, reduced renal blood flow as shown by ultrasound scan, leukocytosis, reduced output of urine, gain in body weight, a rise in serum creatinine(>0.2mg/dL) and proreinuria. The diagnosis of rejection was confirmed if one or more of the following features were found on histological examination:

1. Tubulo-interstitial nephritis with oedema and cortical infiltration by lymphocytes, lymphoblasts, plasma cells and eosinophils
2. Endothelial swelling of peritubular and glomerular capillaries and arterioles
3. Fibrinoid necrosis of small arteries and arterioles
4. Platelet aggregates and thrombi in glomerular and peritubular capillaries
5. Foci of tubular necrosis with infiltration of the tubular wall with a cellular infiltrate.

Of these features, numbers 2-5 were considered to be a direct consequence of rejection due to humoral immune response.

When rejection was suspected, patients were treated by either a ten-day course of high-dose(200mg) oral prednisolone or infusions of 500mg methyl-prednisolone on two to four consecutive days.

In this study, post-transplant values of spontaneous production of immunoglobulin were also compared with plaque forming cell values of healthy controls and dialysis patients which were presented in Fig.6.5.

RESULTS

Effects of Immunosuppression on Immunoglobulin Production

To examine the effects of the two forms of immunosuppression on production of immunoglobulin, samples were taken from 1-6 weeks post-transplant and their plaque forming cell activity was compared. In addition, this was compared with the plaque forming cell response obtained in patients undergoing dialysis and in healthy subjects. The results from all individual samples were pooled and are shown in Table 8.3.

The mean IgA-, IgG- and IgM-PFC counts of dialysis patients were extremely low compared with those in healthy controls($p<0.001$, $p<0.001$, $p<0.05$, respectively) as decribed in CHAPTER VI. When transplanted patients were examined, both azathioprine(IgG) and cyclosporine(IgG,IgA,IgM) treated groups had significantly lower plaque forming cell responses

than healthy controls. In the case of cyclosporine treated patients, the plaque forming cell counts were not significantly different to the very low levels found in dialysis patients but azathioprine treated patients had significantly increased plaque forming cell numbers(IgG and IgA) compared with dialysis patients($p<0.001$). In addition, their production of IgG- and IgA-PFC was significantly higher than that found in cyclosporine treated patients. As will be discussed, these differences are likely to reflect the lower number of rejection episodes observed in cyclosporine treated patients.

A similar pattern emerged when the sequential changes in IgG-, IgA-, and IgM-PFC over the six week period were examined and the results are shown in Figs.8.1, 8.2, and 8.3 respectively. Cyclosporine treated patients showed a very stable level of IgG-PFC throughout the post-transplant period, whereas patients receiving azathioprine had increased numbers of IgG-PFC from 2-4 weeks. After an initially high level during the first week, patients on cyclosporine had a low and stable level of IgA-PFC which was significantly lower than that found in patients receiving azathioprine(Fig.8.2). IgM-PFC were generally low in all patients but azathioprine treated patients had consistently higher IgM-PFC levels after the first week, although this failed to reach statistical significance at any time(Fig.8.3).

Effects of Rejection on Immunoglobulin Production

The incidence of rejection episodes in these two groups of transplant patients was assessed in parallel with measurement of plaque forming cell activity in order to examine the relation between immunoglobulin production and rejection. In the azathioprine group 10/11 patients had at least one episode of rejection(patient No 11 was the only one who did not have a rejection episode), and as a whole, this group had a total of eighteen rejection episodes during the six weeks following transplantation. In 7/10 of these patients biopsies were performed to confirm the diagnosis and in 5/7 a large vascular component, suggesting humoral rejection, was reported.

In contrast, only three of the fourteen patients(5,10,13) in the cyclosporine group showed evidence of rejection, with a total of four episodes over the same period($p<0.001$). In two of the rejecting patients biopsies were performed and one biopsy revealed vascular features(13) while the other(10) showed interstitial changes more typical of cellular rejection. This relationship is demonstrated in the results shown in Fig.8.4 which present serial changes of plaque forming cells in one patient treated with azathioprine(6) and in one patient treated with cyclosporine(13) both of whom experienced rejection episodes. In the azathioprine treated patient, rejection was

diagnosed clinically and severe vascular rejection was confirmed histopathologically on the 10th day. This episode correlated very well with a rise in the levels of both IgG- and IgA-PFC counts at the beginning of the 2nd week. The serial plaque forming cell counts of the cyclosporine treated patient showed a similar pattern and increased IgG-PFC levels were observed during rejection episodes on the seventh day and on the twenty-eighth day. Analysis of all twenty-two rejection episodes showed that IgG-PFC were increased(>25%) in 20/22 rejection episodes and in several cases this was accompanied by a rise in IgA production. In contrast, rejection episodes were not consistently associated with changes in IgM-PFC production.

In four patients(azathioprine group:No 11, cyclosporine group:No 3,4,9), increased plaque forming cell responses were observed in the absence of rejection and in 3/4 cases(11,3,4), this was associated with systemic infection. It is important to note that these cases were the only ones in which increased IgG- and IgA-PFC counts were also accompanied by an increased IgM-PFC response. Plaque forming cell results from a representative case of this type(No 11) are shown in Fig.8.5. Elevated plaque forming cells of all classes occured from 7-21 days post-transplant and this was associated with a diagnosis of basal pneumonia on day 10.

CONCLUSIONS

These results emphasised once again that spontaneous immunoglobulin production is suppressed in dialysis patients. In addition, this study showed that immunosuppression with cyclosporine was associated with lower numbers of cells spontaneously secreting immunoglobulin compared with the response obtained in azathioprine treated patients. This difference correlated very well with a reduced number of rejection episodes in the former group. An association was also found between rejection episodes and elevation of IgG-PFC and this finding is in agreement with previous reports by other workers that an increase in the number of IgG-PFC in the first weeks post-transplant signifies rejection. Systemic infection was also found to cause a rise of IgA- and IgG-PFC and in contrast to elevation due to rejection, these were accompanied by an increase in the IgM-PFC.

				HLA MISMATCHES		
	AGE	SEX	BT	A	B	DR
1. T.G.	49	M	5	2	0	0
2. S.W.	40	M	5	2	2	0
3. E.O.	58	F	6	2	2	0
4. A.D.	42	F	5	1	2	1
5. J.O.	51	F	7	1	1	1
6. J.M.	35	M	9	2	1	2
7. W.K.	37	M	5	2	1	1
8. J.T.	32	M	5	2	1	1
9. T.K.	48	M	5	0	1	1
10. A.B.	31	M	5	1	1	1
11. S.W.	23	M	10	1	0	1
		M/F:8/3				
Mean+SEM:	40+3		6.1+0.5	1.5+0.21	1.1+0.2	0.8+0.

Table 8.1

Renal transplant patients treated with azathioprine who were used in this study

				HLA MISMATCHES		
	AGE	SEX	BT	A	B	DR
1. T.S.	49	M	5	1	1	0
2. J.A.	41	M	5	1	0	1
3. T.C.	53	M	5	2	1	1
4. W.H.	31	M	10	1	1	2
5. W.G.	22	M	5	2	2	1
6. C.S.	33	M	10	2	1	0
7. P.M.	24	F	5	1	1	1
8. M.B.	55	F	6	1	1	0
9. A.M.	53	M	5	1	0	0
10. J.P.	48	M	6	1	2	0
11. H.H.	51	F	5	0	1	-
12. M.S.	43	F	10	2	0	2
13. J.M.	39	F	10	1	2	1
14. J.G.	20	M	5	2	1	1
		M/F:9/5				
Mean+SEM:	40+12		6.6+0.5	1.3+0.2	1.0+0.2	0.8+0.

Table 8.2

Renal transplant patients treated with cyclosporine who were used in this study

Group	Number of Patients	IgG-PFC	IgA-PFC	IgM-PFC
Az-treated transplants	11	668+194	910+214	234+69
CsA-treated transplants	14	328+71	367+157	133+30
Dialysis patients	-	(n=39) 386+53	(n=21) 341+48	(n=16) 143+14
Healthy subjects	25	1137+171	1380+253	342+73

Table 8.3

Spontaneous immunoglobulin production(Mean+1SEM PFC/10^6 cells) by renal transplant patients treated with either azathioprine or cyclosporine in comparison with response in untransplanted uraemic patients and healthy subjects.
Results shown for healthy subjects and uraemic patients are the same with those presented in CHAPTER VI.

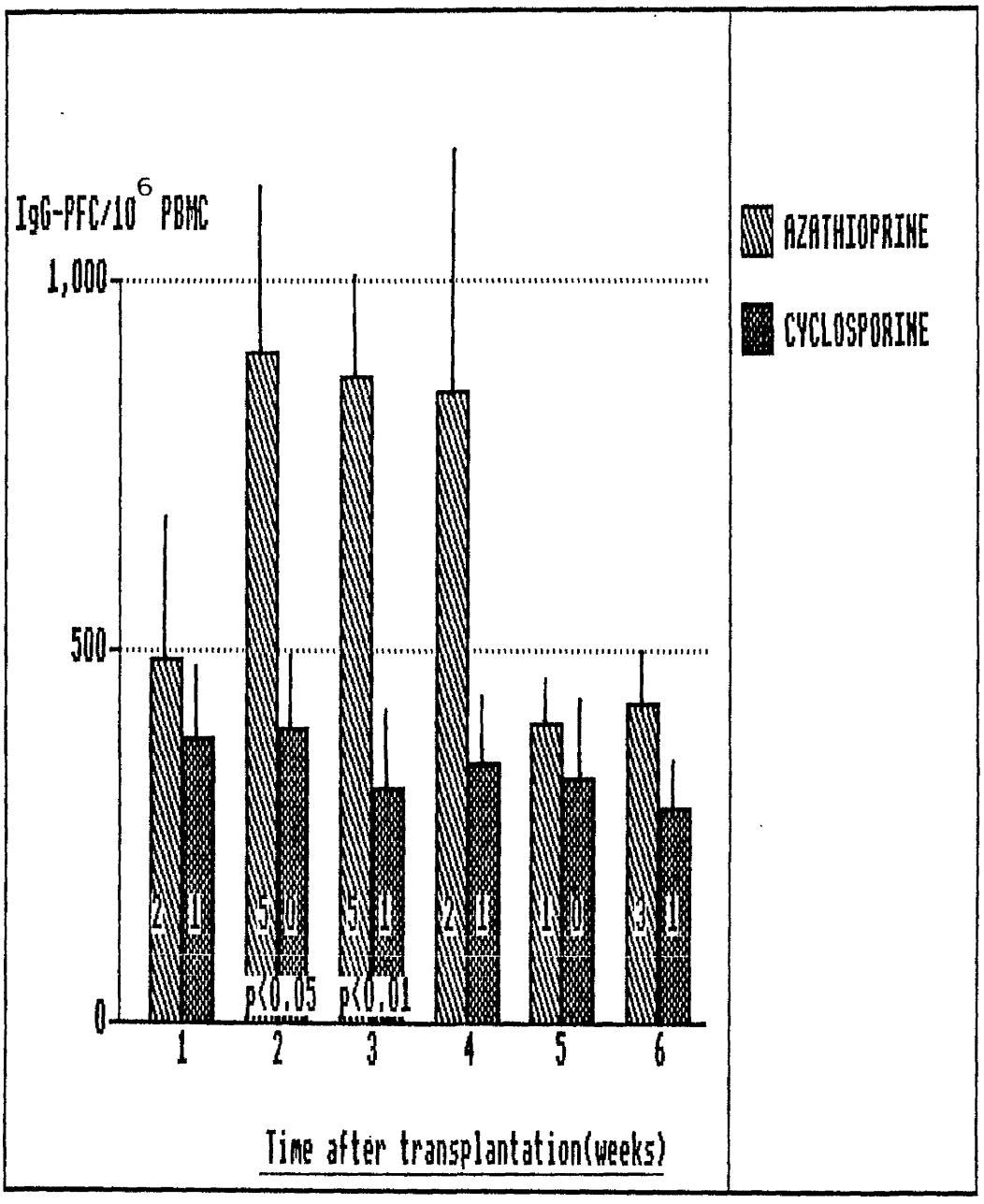


Fig.8.1

Spontaneous production of IgG during the first six weeks after renal transplantation in patients treated with azathioprine or cyclosporine.
Bars represent the mean+1SEM(IgG-PFC/10^6 cells) and figures within bars indicate the number of rejection episodes at each time.

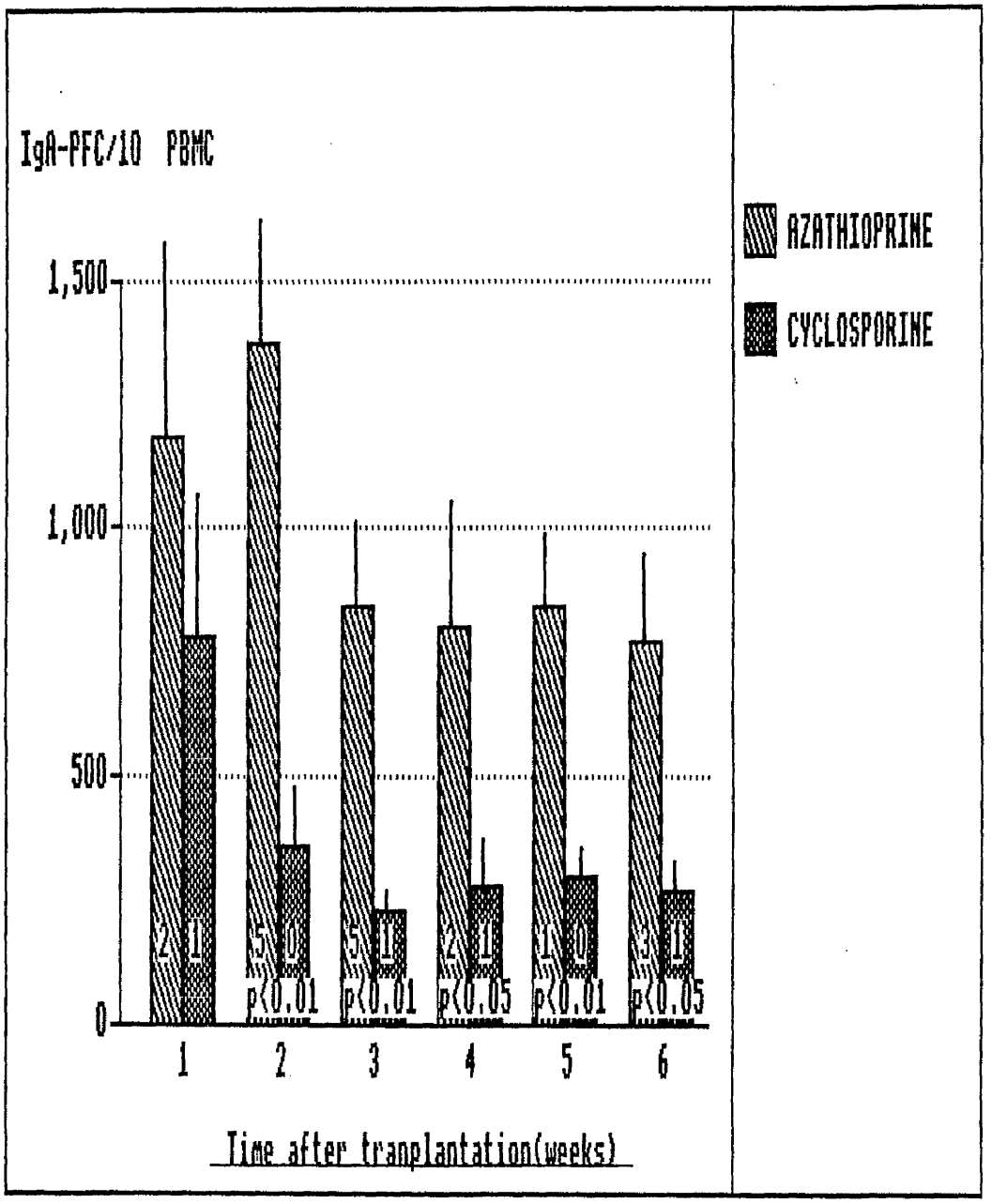


Fig.8.2

Spontaneous production of IgA during the first six weeks after renal transplantation in patients treated with azathioprine or cyclosporine. Bars represent the mean+1SEM(IgA-PFC/10^6 cells) and figures within bars indicate the number of rejection episodes at each time.

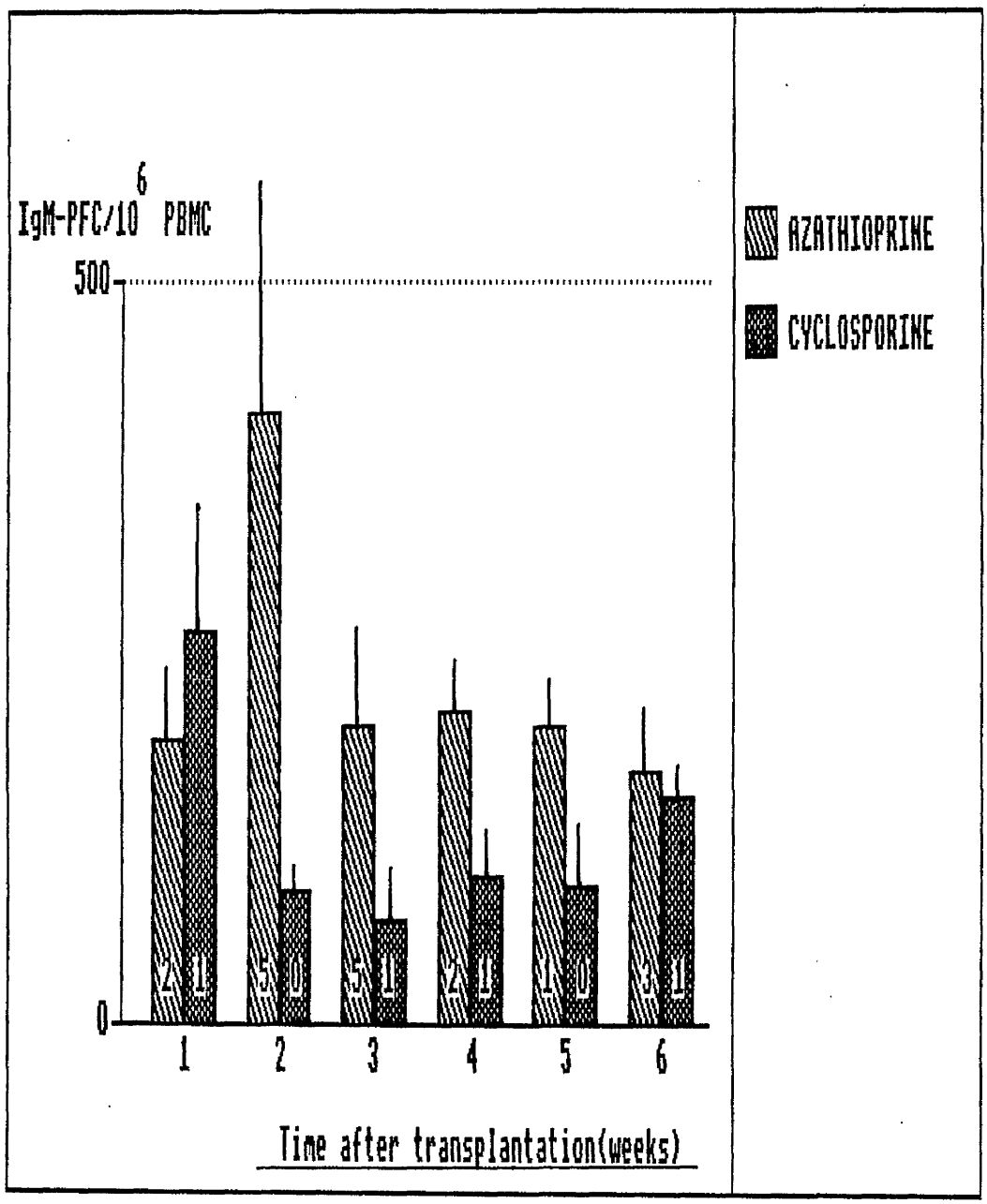
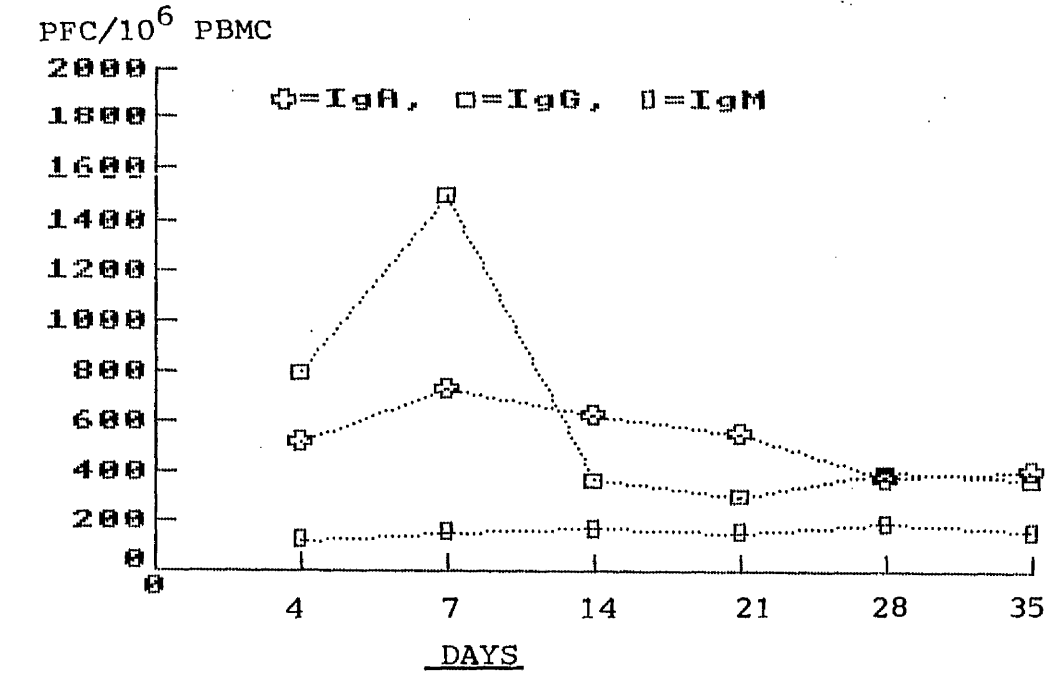


Fig.8.3

Spontaneous production of IgM during the first six weeks after renal transplantation in patients treated with azathioprine or cyclosporine. Bars represent the mean+1SEM(IgM-PFC/10^6 cells) and figures within bars indicate the number of rejection episodes at each time.



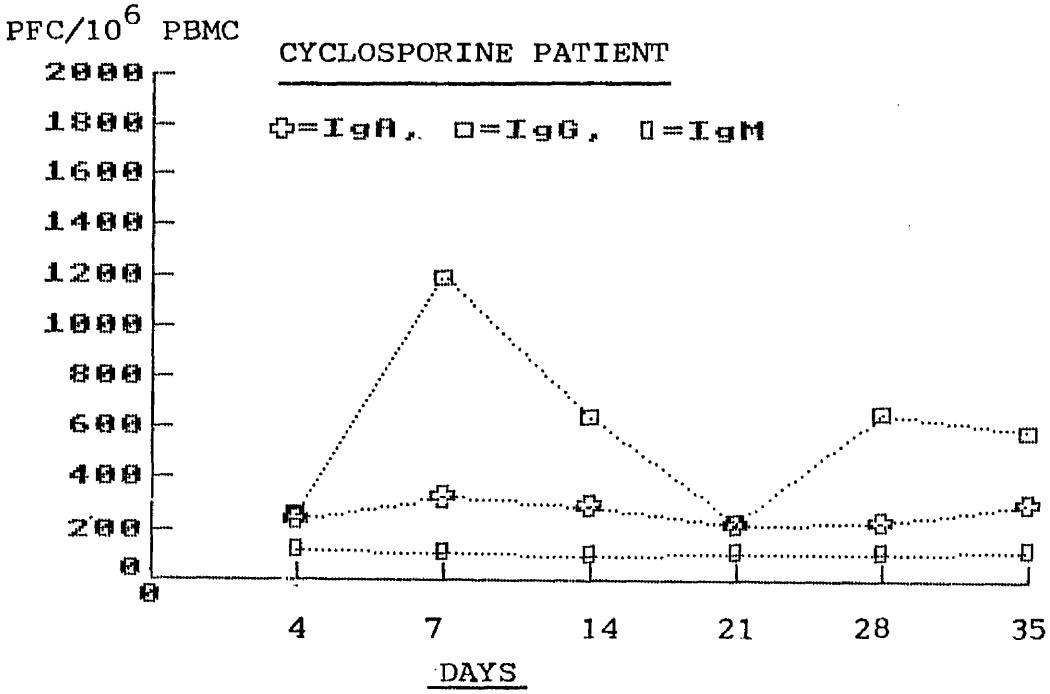


Fig.8.4

Serial changes of PFC activity in representative patients treated with azathioprine and cyclosporine.
PFC counts represent the mean of triplicate determinations(PFC/10^6 cells).

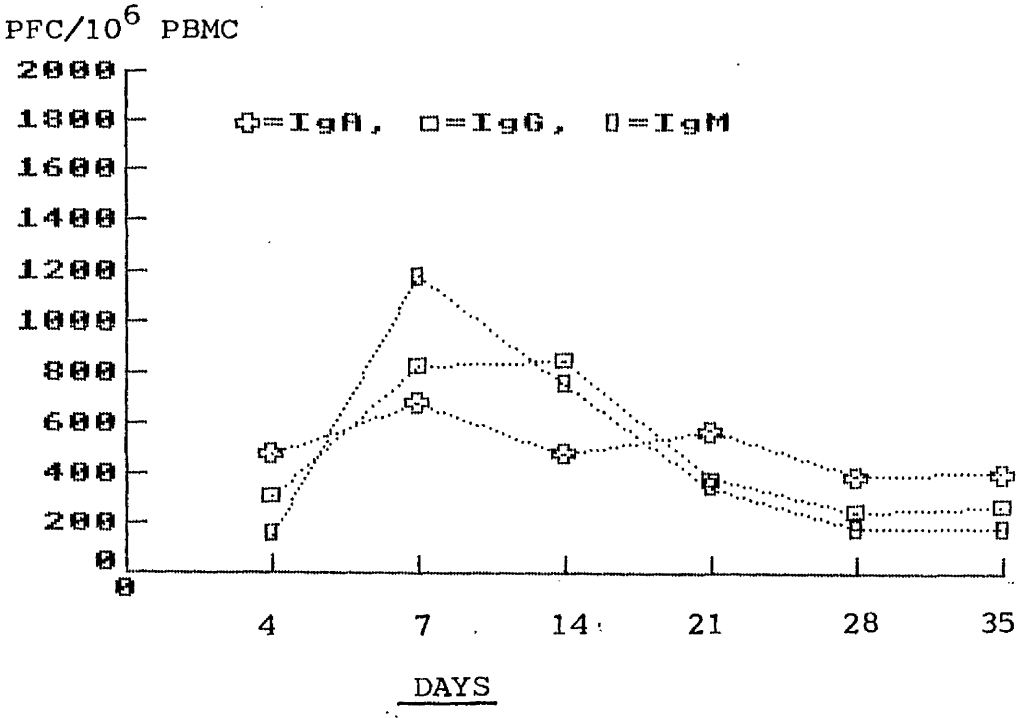


Fig.8.5

Serial changes of PFC activity in one patient with systemic infection after renal transplantation. PFC counts shown are the mean of triplicate determinations(PFC/10^6 cells)

CHAPTER IX

GENERAL DISCUSSION

It has become clear over the recent years that the mechanism of regulation of immunologic reactivity is the balance between positive and negative influences in the form of monocytes, helper T and suppressor T cells, and in the humans T and B cell function is modulated by a complex interaction by mononuclear cell populations(Stobo, 1982). The role of B cell indicator systems has also been established and B cell assays have been utilised frequently to investigate the function of monocytes, helper T, suppressor T and B cells.

The work in this thesis dealt predominantly with the haemolytic plaque forming cell assay and several culture systems have been employed both for the enumeration of immunoglobulin secreting cells and for the measurement of mitogen induced blastogenesis in healthy and uraemic subjects.

PLAQUE ASSAYS

A protein A plaque forming cell assay was used in this study to enumerate spontaneous or mitogen stimulated immunoglobulin secreting cells. Several experiments were performed using control cells to study the performance and reproducibility of the assay.

In these experiments, it was demonstrated that different handling of effector cells before plating as well as differences in the plating procedure probably account for the difficulties many workers had experienced(Truffa-Bachi &

Bordenave, 1980, Burns & Pike, 1981). The sources and optimal dilutions of the reagents I used were comparable to those used by most workers and slight modifications in dilutions and concentrations were not found to affect significantly plaque forming cell counts. It was shown that optimal reproducibility required that effector cells had to be stored on ice for as short a time as possible before plating and that the assay had to be performed as soon as possible after cell separation. In addition, the plaques had to be counted after a standard incubation period because of the differences observed in plaque forming cell counts obtained over different times. When two or more samples from the same individual were studied, I found that the cells had to be frozen down and the plaque assays performed on the same day in order to avoid significant test to test variation. This work also indicated the need for a positive control which could be used as a quality control for the assay and the one presented in CHAPTER III(Fig.3.15) proved to be reliable and simple. Alternatively other workers have used as control cells human spleen cells which were firstly cultured for a few days with PWM before being Frozen(Horsburgh, unpublished data).

When all these conditions were met, the plaque assay was a very versatile and useful method. Although other workers have suggested various major or minor modifications in order to improve reproducibility, such as storage of large batches

of protein A coated SRBC to avoid batch to batch variation(Agger et al, 1983), I found no significant variation on routine testing of each new batch of coated SRBC with its predecessor. Others have also suggested the use of IgG depleted complement to reduce variation due to different batches of complement(Van Oudenaren et al, 1981). This procedure was time consuming and, in my study, it was unnecessary, because only five different batches of complement were used, which gave similar responses when compared with each other. Trienekens et al(1984) suggested that addition of PEG 6000 in the agar mixture improves reproducibility and reliability of the assay by improving stability of immune complexes. In the present experiments, the use of PEG 6000 was also associated with the development of better defined haemolytic zones but did not produce a significant increase in the number of plaque forming cells.

My results showed clearly that the most important technical factor affecting the detection and measurement of immunoglobulin secretion by peripheral blood mononuclear cells was the handling of the cells particularly before plating. Although the amounts and sources of reagents were also important these factors were not difficult to standardise.

Spontaneous Plaque Formation

When spontaneous plaque formation was examined in normal controls, the counts of IgA-PFC usually exceeded those of

IgG-PFC in agreement with previous reports of spontaneous immunoglobulin production(Freijd & Kunori, 1980) and presence of intracellular immunoglobulin. These findings are interesting in view of the predominant role of IgA as a mucosal immunoglobulin and contrast with the low serum concentration of IgA compared to that of IgG. It has been suggested that this discrepancy may be explained partly by the short half-life of IgA in serum and by the hypothesis that the recirculation of IgA secreting cells is very short(Freijd & Kunori, 1980). The same authors also suggested that these IgA secreting cells are IgA lymphocyte precursors in Peyer's patches, which after being primed by antigen, leave the gut by the lymphatics and recirculate until settling in the lamina propria of mucous membranes of secretory glands.

Production of Immunoglobulin in Response to Polyclonal B Cell Activators

The protein A plaque assay was also used to enumerate plaque forming cells in polyclonal B cell activator induced cultures of peripheral blood mononuclear cells. Several experiments were performed to find the optimal conditions for B cell differentiation and several technical factors were examined including the time course, dose response, and cell concentration. In addition, it was found important in these studies to use the same reagents, materials and

equipment throughout, because even small changes in one or more of these parameters, had significant effects on the final result.

The IgA-, IgG-, and IgM-PFC counts obtained from PWM, SAC, PWM plus SAC, and EBV cultures were in agreement with previous studies(Fauci et al, 1980, Bird & Britton, 1979, Tauris, 1983a,b, Saiki & Ralph, 1981a). IgG-PFC counts were higher in most cases and this is the reason why only IgG-PFC were measured in the studies of various parameters such as co-stimulation with ConA etc. Other workers have reported a larger number of IgM-PFC than IgG-PFC after PWM or SAC stimulation(Hammarstrom et al, 1979). The apparent difference between these studies and my study may have resulted from the chance selection of donors or from different laboratory conditions, eg. Hammarstrom et al cultured human cells in RPMI 1640 supplemented with human AB serum instead of FCS. However, other workers also reported a predominance of IgG production in the PWM system and also found that EBV gave rise to a proportional dominance of IgM secretion(Tauris, 1983a,b, Bird & Britton, 1979b). In my study a small number of control samples were studied with EBV and it was found that IgM response was the highest and and at the same level as PWM induced IgM-PFC. The IgG-PFC response was comparable to the response induced by SAC and significantly lower than the PWM response.

When all technical parameters were standardised, these

initial studies confirmed the potential usefulness of the plaque assay as a means of assessing humoral immunity in serial samples from large groups of patients. In addition, although not studied here, the assay could be modified to investigate functions of purified lymphoid cell subpopulations and, using appropriate antisera, could also be used to enumerate cells which are secreting mediators other than immunoglobulin, including interleukins, growth and differentiation factors, and other immunoregulatory substances.

STUDIES OF LYMPHOCYTE SUBPOPULATIONS AND LYMPHOCYTE FUNCTION IN URAEMIA

Previous studies have shown that both cellular defects and immunosuppressive factors in uraemic serum contribute to the reduced immune responsiveness of uraemic subjects. In the present study of a number of immunological parameters, an emphasis was given to the investigation of immunoglobulin production in vitro. In addition, total white cell, lymphocyte and lymphocyte subpopulation counts were performed together with assays of proliferative responses of uraemic lymphocytes.

There was no difference in white cell and lymphocyte counts between normal controls and uraemic patients. Lymphopenia is not a universally accepted feature of uraemia and while my results support the report by Kauffman et al(1975), they contradict other reports which showed that

uraemic patients were lymphopenic(Jensson et al, 1958, Wilson et al, 1965, Boulton-Jones et al, 1973). It is possible that these discrepancies reflect the study of patients with different degrees of renal failure as well as differences in the use of cytotoxic drugs, blood transfusions, and time on dialysis.

My results also showed that lymphocyte subpopulations were not significantly abnormal in uraemic patients, a finding which contrasts with other recent reports that the helper T cell subpopulation is reduced in uraemia(Lortan et al, 1982, Raska et al, 1983). All the patients examined in the present study had never received a blood transfusion, and this may account for the differences between my results and those of other workers. In a recent study, Bender et al(1984) tried to determine the effects of blood transfusion on lymphocyte subsets of uraemic patients and showed that changes in peripheral blood lymphocyte subpopulations occured almost exclusively in the group who received erythrocyte transfusions. The other workers who examined white cell and lymphocyte counts in uraemics did not separate their patients in transfused and non-transfused groups.

My results indicate strongly that immunosuppression in uraemia is not merely due to reduced numbers of T and B lymphocytes but reflects a true functional abnormality.

This hypothesis is supported by the finding that proliferative responses of peripheral blood mononuclear

cells to both PHA and PWM were reduced in uraemic patients. This confirms a number of previous reports of depressed proliferative responses to T-cell mitogens and confirms that T lymphocyte function is impaired(Huber et al, 1969, Kauffman et al, 1975, Holdsworth et al, 1978, Kunori et al, 1980, Alevy & Slavin, 1981). Furthermore, I did not find that uraemic lymphocytes were more sensitive than control cells to the suppressive effects of steroids in vitro and, thus, my results are in contrast to a recent report(Langhoff & Ladefoged, 1984) and do not support the idea that immune responsiveness in uraemia is inherently normal and that immune suppression is observed merely because uraemia potentiates the activity of drugs known to suppress the immune response(Nelson et al, 1980).

The spontaneous production of immunoglobulin by uraemic peripheral blood mononuclear cells was examined, using the protein A plaque assay, and it was clearly demonstrated that spontaneous plaque formation was impaired in uraemic subjects. This impairment was more pronounced for the IgA and IgG classes although IgM was also significantly lower than normal. These differences have not been reported before, and contradict two previous reports which could not detect any abnormalities in immunoglobulin production by unstimulated peripheral blood mononuclears(Horsburgh et al, 1983, Satomi et al, 1983). Studies of serum concentrations of immunoglobulins in uraemia have shown that IgA and IgM

did not differ significantly from normal values, while serum IgG had a wide scatter of values(Cascianni et al, 1978). Nevertheless, serum immunoglobulin levels are not a reliable method of studying humoral immunity in these patients because of the possibility of abnormal renal excretion of immunoglobulins.

This result is a reliable indicator of depressed humoral immunity in uraemic patients and, since B cell numbers were normal in these patients, the present study suggests that the defective humoral immune response is due to abnormalities in B cell differentiation associated with the uraemic state.

When the PWM induced plaque forming cell responses were examined, it was found that PWM stimulated uraemic peripheral blood mononuclear cells differentiated normally into immunoglobulin secreting cells, in contrast to the defective proliferative responses to both PWM and PHA. In addition, no significant differences were found in the kinetics of PWM stimulated cultures of peripheral blood mononuclear cells obtained from healthy subjects and uraemic patients, and in both groups the peak plaque forming cell responses were observed on the 6th day using the optimal PWM dilution. These results indicate that there is a defect in the proliferative potential of uraemic T cells and also suggest that the PWM stimulated uraemic helper T cell can provide the necessary help for terminal B cell

differentiation. This apparent discrepancy is not surprising in view of the fact that T cells do not need to divide to exhibit helper activity(Keightley et al, 1976). Furthermore, these findings suggest that the overall reduction in proliferative response to PWM mainly reflects a defect on the T cell population(Cupps & Fauci, 1982) and that B cell function is relatively normal. As PWM stimulated immunoglobulin production was normal in uraemia, it is also apparent that T-B cell cooperation remains intact in uraemic patients.

EBV induced B lymphocyte activation and differentiation is independent from T cell help and it is unaffected by the presence of autologous ConA induced suppressor cells(Bird & Britton, 1979, Bird et al, 1981). These properties of EBV are advantageous in the study of human B cell function and accordingly, I studied the plaque forming cell responses of uraemic peripheral blood mononuclear cells to EBV in order to examine whether T-cell independent B cell differentiation was affected. Peripheral blood mononuclear cells were exposed to EBV and then cultured for a short period in order to avoid secondary in vitro activation of cytotoxic virus-specific T lymphocytes resulting in limitation of B cell growth(Bird et al, 1981). The EBV responses were found to be normal in uraemic patients and this finding in association with all my previous findings further support the hypothesis that reduced immunoglobulin production in

uraemia is due to a T cell rather than B cell defect.

In contrast to the results with PWM, the plaque forming cell response of uraemic cells to SAC was shown to be impaired. This confirms and extends an earlier report by Kunori et al(1980), and provides further information on the nature of the immune defect in uraemics. SAC stimulates B cells via an interaction between its cell wall protein and immunoglobulin on the B cell surface, and while the resulting proliferative response is T cell independent, full differentiation of the B cell is dependent on T cell help(Falkoff et al, 1982). Interestingly, SAC itself does not appear to stimulate T cells(Falkoff et al, 1982), and so the B cell differentiation factors which must be present to permit B cell differentiation are probably supplied by helper T cells which are either activated in culture by FCS or which are already preactivated in vivo. As I have shown, both spontaneous and SAC induced immunoglobulin production were depressed in uraemic patients whereas PWM induced immunoglobulin production was normal. As both defective responses rely on preactivated helper T cells, it is tempting to speculate that the defective immunoglobulin response in uraemic patients is due to abnormal activation of helper T cell in vivo or alternatively due to low background stimulation in culture. In contrast, when these cells were stimulated with PWM after removal from the uraemic environment, normal immunoglobulin production was demostrated, because PWM is known to activate T

cells(Greaves & Janossy, 1972). This helper T cell defect must be functional since helper T cell counts were found to be within the normal range.

One further difference in the production of immunoglobulin in response to PWM or SAC was that while the depressed response to SAC was considerably improved by indomethacin, this had no effect on the response to PWM. These results indicate that prostaglandin production by uraemic cells may contribute to suppressed SAC response. Such a dissociation between PWM and SAC responses of B cells was also observed by Thompson et al(1984) when they studied the regulation of human B cell proliferation by prostaglandin E2(PGE2). They were able to show that PGE2 inhibits B cell proliferation in response to SAC but not in response to PWM and also presented data from preliminary studies suggesting that PGE2 exerts selective regulatory effects on human B cell responses such as inhibition of production of B cell differentiation factor(BCDF) but no effect on the production of B cell growth factor(BCGF). In this study, SAC cultures of uraemic cells responded better than control cells to the addition of indomethacin and these findings are consistent with the idea that the suppressed activation of helper T cells in uraemic patients, discussed above, could be due to excessive production of PGE2. A substantial body of evidence implicates prostaglandins as potent regulators of the immune response and recently it was

reported that prostaglandins suppress IL-2 production by human lymphocytes(Rappaport & Dodge, 1982) while very recent studies showed that IL-2 receptors are expressed on SAC activated B cells(Muraguchi et al, 1985). Thus, the low spontaneous and SAC responses in this study can be either due to inhibition of IL-2 or BCDF production by prostaglandins or due to PGE2 induced suppressor T lymphocytes(Chouaib et al, 1984). The second hypothesis was not supported in this study because SAC is not greatly influenced by suppressor T cell function and, as will be discussed later, when steroids were added to inhibit selectively the suppressor T cell subset, the SAC induced plaque forming cell responses of the uraemic patients remained relatively unchanged. Prostaglandin inhibition of IL-2 production by T cells explains the low spontaneous and SAC plaque forming cell responses in uraemia, since IL-2 can have both a direct and an indirect effect on B cell differentiation. Initially, IL-2 was considered to have a central role in immunity by its capacity to sustain division of helper and cytotoxic T lymphocytes, but recently, normal B cells were found to express functional IL-2 receptors and IL-2 was found to induce significant enhancement of proliferation in SAC activated B cells(Mingari et al, 1984). Interestingly, addition of IL-2 to PHA cultures of uraemic lymphocytes has been recently shown to normalise the proliferative responses to this T cell mitogen(Langhoff et

al, 1985). My hypothesis is that uraemic helper T cells secrete a normal or reduced amount of IL-2 or other differentiation factors the production of which is inhibited by a physiological or abnormally increased secretion of PGE2. This happens when T cells are preactivated either in vivo or in vitro but it doesn't happen when the cells are activated with PWM which activates both T and B cells. It would therefore be interesting to examine the effects of prostaglandins and IL-2 on spontaneous and SAC induced plaque formation by uraemic peripheral blood mononuclear cells. In previous studies, macrophages present in the spleen, the peritoneum and the lung of uraemic rats, have been shown to suppress mitogen reactivity of spleen cells(Alevy et al, 1983 & Alevy et al, 1982). The same authors in another study showed that the suppression of PHA response in uraemic rats is mediated by an adherent and Ia-negative suppressor cell(Alevy et al, 1981). When these cells were pretreated with indomethacin which inhibits prostaglandin synthesis, they were shown to be indomethacin-insensitive, and although these findings could reflect a role for preformed PGE2 in this model of uraemia, it would be of interest to examine directly the function of adherent cells on in vitro immunoglobulin production by uraemic peripheral blood mononuclear cells.

One further explanation for the differential effects of uraemia on PWM and SAC induced immunoglobulin responses is that this may reflect functional abnormalities in separate B

cell subsets. This is supported by evidence that B cells which respond to SAC carry receptors for mouse red blood cells(MRBC+), whereas PWM responsive B cells do not(Ito & Lawton, 1984). Thus, it would be important to determine the numbers of these B cells in uraemic patients. If such an abnormality exists, the low SAC responses in uraemia could be due to low numbers of MRBC+ B cells, but this finding would not explain the low spontaneous plaque forming cell responses.

One further point of interest from my study was that, in contrast to control cells, uraemic patients did not show a synergistic plaque forming cell response to PWM+SAC in combination. Indeed, the response to the two activators was less than that with PWM alone and correlated with the low response to SAC. In conjunction with the normal response to PWM this indicates that the defective ability to respond to SAC is alone responsible for the lack of synergy. Very little is known about the B and T signal synergy in human immunoglobulin secretion. The B cells induced by PWM may be already activated in the blood, e.g., recently stimulated or memory cells, and easily activated by T cell factors(Macy & Stevens, 1980). SAC activates the remaining B cells, and a possible role of the PWM in the synergistic effect is to provide the B cell differentiation factors secreted by T cells. In this study, uraemic B cells did not respond normally to SAC, and thus were unable to differentiate into

immunoglobulin secreting cells in response to differentiation factors provided by PWM induced T cells. Therefore, the B cells which finally differentiated were those which were already activated in the peripheral blood.

The effect of corticosteroids on immunoglobulin production was examined with the aim of exploiting the selective effect of corticosteroids on suppressor T cells and to elucidate further the mechanisms of depressed immunoglobulin synthesis in uraemia.

In the present study, the immunoglobulin response to PWM was increased by addition of methyl-prednisolone, but this was more pronounced in controls than in uraemic patients. In addition, immunoglobulin responses to SAC were also increased in normal controls while they were unaffected in uraemic patients and as discussed earlier this finding supports further the hypothesis that abnormal immunoglobulin production is due to a functional helper T cell defect.

It is well known that corticosteroids suppress in vitro mitogen induced blastogenesis although some mitogen systems are more sensitive than others(Heilman, 1972, Heilman et al, 1973, Blomgren & Andersson, 1973). More specifically, these authors reported that corticosteroids suppress the proliferative responses induced by PHA and ConA whereas the responses to PWM were only slightly inhibited. In this study, PHA and PWM responses were shown to be inhibited by

methyl-prednisolone, and as discussed above, in contrast to a recent report no difference was found in this inhibition between control and uraemic lymphocytes(Langhoff & Ladefoged, 1984). It is also known that immunoglobulin production in cultures of human peripheral blood mononuclear cells is markedly increased by in vitro corticosteroids(Smith et al, 1972 & Grayson et al, 1981) and this increase was more clearly demonstrated in the PWM system(Cooper et al, 1979). Interestingly, Grayson et al(1981) showed that corticosteroids enhanced immunoglobulin production in vitro in the absence of any other stimulating factor but in this study, when control and uraemic peripheral blood mononuclear cells were cultured only in the presence of methyl-prednisolone, the plaque forming cell response was at background levels. The role of corticosteroids in production of immunoglobulin in vitro is investigated in detail in a very recent report where it is suggested that corticosteroids produce their enhancing effect on the PWM system by selectively inhibiting at pharmacological concentrations the suppressor T cell subset(Paavonen, 1985). Nevertheless, the finding of these authors that SAC induced immunoglobulin production is insensitive to the action of corticosteroids was not confirmed in this study.

In view of the normal PWM induced plaque forming cell responses in uraemic patients one would not expect to see

such a difference in lymphocyte sensitivity to methyl-prednisolone between uraemic and control subjects. The enhancement observed in the uraemic cultures failed to reach statistical significance at any dilution of methyl-prednisolone but nevertheless a dose dependent pattern was observed(Fig.6.13). This indicates that methyl-prednisolone inhibited at least one proportion of uraemic suppressor T cells but further studies are needed to investigate whether methyl-prednisolone left unaffected one proportion of uraemic suppressor T cells and whether it also impaired the function of other immunoregulatory cells or of the B cell itself.

Methylprednisolone also enhanced the immunoglobulin response of control cultures to SAC although the increase was significantly lower than that observed with PWM. This accords with the findings of Falkoff et al(1982) who used irradiation to eliminate suppressor T cells and together these results indicate that suppressor T cells normally regulate SAC-induced immunoglobulin production, although not to the same degree as PWM-induced responses. Methylprednisolone had no effect at all on the SAC response of uraemic cells, suggesting that the low SAC responses in uraemia are not due to increased suppressor T cell activity and supporting the view that the reduced SAC responses are due to defective levels of T helper activity. In addition, the finding that there is no difference in steroid

sensitivity between control and uraemic proliferative responses indicates that the differences in the SAC-plaque forming cell responses are probably not due to differences in sensitivity to methyl-prednisolone between control and uraemic suppressor T cells.

Many groups have investigated the role of uraemic serum in mediating the immunosuppression of uraemic patients(Silk, 1967, Newberry & Sanford, 1971, Raskova & Raska, 1981, Langhoff & Ladefoged, 1984) and my own results showed that pre-incubation of control peripheral blood mononuclear cells with uraemic sera suppressed slightly the PWM induced immunoglobulin production of normal peripheral blood mononuclear cells although this failed to reach statistical significance. In addition, I found that uraemic serum also affected the quality of the plaques as determined by microscopic examination.

These findings are in agreement with previous reports that uraemic serum can suppress both mitogen responses as well as antibody production in vitro(Mezzano et al, 1984). One methodological difference between my own and previous work is that in earlier studies, lymphocytes were cultured in the presence of uraemic serum, whereas I was unable to establish a reproducible system for culturing peripheral blood mononuclear cells in the presence of uraemic serum, and cells were only pre-incubated with serum before culture. However, it has been shown in the rats(Raskova et al, 1981)

that continuous presence of uraemic serum is not essential to demonstrate inhibited PHA responses and that the later stages of the response are unaffected by uraemic serum. In the only previous study investigating the effect of uraemic serum on antibody production in vitro, Mezzano et al(1984) showed that uraemic serum suppressed the antibody responses of rat spleen cells to bovine serun albumin but not to sheep red blood cells. The same authors also found that the response of lymphocytes from uraemic rats could be restored to virtually normal levels by culture in medium supplemented with normal rat serum, emphasising the importance of serum factors in determining the response of uraemic cells. It is possible that such serum factors affect the spontaneous and SAC-induced plaque forming cell responses of the uraemic lymphocytes as well as the plaque forming cell responses of control cells pre-incubated with uraemic serum.

The nature of the factor(s) responsible for this inhibitory effect has not been elucidated and the immunosuppressive effect of the uraemic serum does not correlate with the levels of urea, creatinine, calcium, and phosphorus(Modai et al, 1985). One uraemic blood constituent which has received much recent attention is a very low density lipoprotein(VLDL) which was first identified in the serum of rats with chronic renal insufficiency. This VLDL completely inhibits the response of lymphocytes to

allogeneic cells or phytohaemagglutinin A(Raska et al, 1980, Raskova & Raska, 1981), and has also been shown to induce suppressor cell activity(Raskova & Raska, 1983). In humans, uraemia is associated with hyperlipidaemia(Norbeck & Carlson, 1981) and serum lipoproteins of different types have been shown to inhibit spontaneous plaque forming cell activity(Antonaci et al, 1984). In addition, lipoproteins contain phospholipids which can bind and activate human T8+ lymphocytes to generate suppressor T cell activity(Wadee & Rabson, 1983). Although a factor like VLDL may be responsible for the observed suppressive effect of uraemic serum on PWM induced immunoglobulin production by control cells, and for the low spontaneous and SAC-induced plaque forming cell responses of uraemic cells, it is very unlikely that VLDL induced suppressor T cell activity was the causing mechanism, because in this study no evidence was found for increased suppressor T cell activity among uraemic peripheral blood mononuclear cells. Thus, it is possible that VLDL exerts its immunoinhibitory effect on immunoglobulin production by affecting the function of helper T cells or monocytes, and although a direct effect on B cell function cannot be excluded, it is not likely since the EBV-induced plaque forming cell responses were found to be normal in uraemic patients.

EFFECT OF BLOOD TRANSFUSION ON IMMUNOGLOBULIN SECRETION

One of the factors which could possibly account for the immunosuppression of uraemia is blood transfusion, as it is well known that pre-transplant blood transfusion suppresses cell mediated immunity in prospective kidney-graft recipients. The exact cellular mechanisms of this immunosuppression are unclear and, in the present study, the effects of blood transfusion and the role of T and B cells in determining the abnormal immune responsiveness were examined. This was achieved by examining the effect of blood transfusion on production of immunoglobulin both spontaneously and in response to polyclonal B cell activators which differ in their dependence on T cells.

The results showed that spontaneous production of immunoglobulin was not affected significantly by blood transfusion, with any variations which did occur being independent of blood transfusion. Although one might have expected that the spontaneous plaque forming cell response would follow alterations in the immune status described below, this did not occur in the present study, probably because the spontaneous plaque forming cell counts in uraemic patients before blood transfusion were already very low compared with control values.

This problem of inherently low plaque forming cell responses was overcome by examining immunoglobulin production after stimulating peripheral blood mononuclear

cells with PWM or SAC. In these studies of polyclonal B cell activation, PWM induced immunoglobulin production by peripheral blood mononuclear cells was depressed by blood transfusion in 18/26 patients, and this required at least 2 blood transfusions. In eight of these patients pre-BT, post-5BT and post-10BT values were compared and it was shown that the five additional blood transfusions further suppressed the PWM response. Other workers have reported increased suppressor cell activity after the first or second blood transfusion(Klatzmann et al, 1983, Smith et al, 1983). Others found a correlation between PHA and MLR activity and the amount of blood given(Fehrman et al, 1981) as well as a progressive reduction in patient's proliferative responses to suboptimal doses of ConA with increasing numbers of transfusions(Tait et al, 1984).

In contrast to PWM, SAC induced immunoglobulin production was not affected by blood transfusion even after 5 or 10 blood transfusions, indicating that blood transfusion did not affect the ability of the B cell to differentiate into immunoglobulin secreting cells in response to SAC. SAC induced B cell proliferation is T cell independent while SAC induced B cell differentiation strictly depends on helper T cells and thus, my results indicate that blood transfusion does not affect helper T or B cell function. Further studies using EBV will not only provide information about the involvement of the B cell itself but also in parallel with PWM and/or SAC studies will be a useful control since the

EBV system does not depend on helper T cells and is not affected by suppressor T cells(in short term cultures).

As PWM induced differentiation of B cells into immunoglobulin secreting cells is a process known to be influenced by suppressor T cell activity(Pryjma et al, 1980) these results indicate that the suppression of PWM induced immunoglobulin secretion is due to an effect of blood transfusion on T cells. The position with SAC induced B cell activation is more complex. Although it has been reported that SAC induced immunoglobulin production is not affected by ConA generated suppressor T cell activity(Pryjma et al, 1980), others(Falkoff et al, 1982) irradiated T cells to eliminate suppressor T cells and found that this enhanced the SAC induced plaque forming cell response in some individuals indicating that the SAC induced B cell differentiation was also influenced by suppressor T cell activity. This is in agreement with my own results(CHAPTER V) which showed that SAC induced production of immunoglobulin was moderately decreased by costimulation with ConA and slightly enhanced after addition of methyl-prednisolone. However, my study indicated that the response to SAC was less affected than the response to PWM by suppressor activity and it does appear that these polyclonal B cell activators differ in their susceptibility to regulation by suppressor T cells. Thus, the results of the present study suggest that the differential inhibitory

effects of blood transfusion on B cell differentiation into immunoglobulin secreting cells is due to an increase in suppressor T cell activity.

My results are in accord with those of these investigators who have shown an increase in non-antigen specific suppressor cell activity after blood transfusion but further studies are needed to identify the cell(s) responsible for this effect. Both monocytes and suppressor T cells have been shown to act as suppressor cells in the immediate and late post-transfusion period, respectively(Lenhard et al, 1982b), and both these cells are known to inhibit PWM induced immunoglobulin production(Fauci et al, 1980, Rosenkoetter et al, 1984). Recognition, characterisation and isolation of these immunoregulatory cells has been achieved in large part by a variety of monoclonal antibodies and the availability of these reagents can facilitate the identification of the cell(s) responsible for the observed effect of blood transfusion on PWM responses.

This is the first study to use the plaque forming cell assay to assess the effects of pre-transplant blood transfusion on immune responsiveness and my proposal that random blood transfusion acts by suppressing T cell dependent polyclonal B cell activation is supported by a recent experimental study of donor specific blood transfusion in experimental rats(Lenhard et al, 1985a).

These workers showed that transfusion of Lew rats with BN blood produced a decreased antibody response to a subsequent BN kidney graft and this effect could be transferred by spleen cells from multi-transfused recipients. These spleen cells were nylon-wool non-adherent and carried markers for the suppressor/cytotoxic T cell subset(OX8) but not of the helper T cell class(W3/25) and it was concluded that the suppression of antibody responses in this experimental model of blood transfusion was mediated by suppressor T cells. It would be of interest to use the plaque forming cell assay and different polyclonal B cell activators to examine the effect of blood transfusion on fractionated lymphocyte subpopulations.

Although it has not be proven that suppressed humoral immunity accounts for some of the beneficial effects of blood transfusion in renal transplant recipients, there is some evidence to support this view. Thus, transfusion of strongly MHC-incompatible BN and LEW rats with 15 transfusions of BN blood suppressed both the specific anti-BN response and the reactivity to third party blood and this correlated very well with prolongation of skin grafts from BN or (BNxLEW)F1. Interestingly, there was also some improvement in the survival of third party grafts(Lenhard et al, 1985b).

Further support for the role of suppressed antibody production in preventing graft rejection comes from clinical

studies which showed that patients who did not develop donor specific lymphocytotoxic antibodies during blood transfusion had a better graft survival after renal transplantation. In detailed studies of this phenomenon, Opelz et al(1973b) found that half of a group of patients receiving random blood transfusions did not develop cytotoxic antibodies even after thirty blood transfusions and in some instances complete loss of antibody activity occurred. Similarly, Ferrara et al(1974) showed that in some volunteers given repeated injections of small aliquots of blood, cytotoxic anti-HLA antibody responses declined progressively until complete unresponsiveness occurred and they suggested that blood transfusion may induce a state of humoral unresponsiveness to HLA antigens. Finally, Barsoum et al(1980) reported one case of a pre-sensitised uraemic patient who was initially considered unsuitable for transplantation due to the presence of cytotoxic antibodies but who was subsequently accepted when these disappeared following prospective multiple transfusion therapy.

For these reasons, serial assessment of PWM induced plaque forming cells could be a useful means of determining whether individual pretransplant patients are benefiting from a course of blood transfusion and will have a good outcome after renal transplantation. My study was to small to establish statistically that there may be a relationship between blood transfusion associated suppression of PWM

induced plaque forming cell response and kidney transplant outcome.

MONITORING OF PFC ACTIVITY IN RENAL TRANSPLANT PATIENTS

The changes in spontaneous plaque formation by peripheral blood mononuclear cells from renal transplant recipients on immunosuppression were not unexpected in view of the known effects of both allograft rejection and immunosuppressive therapy on lymphocyte reactivity. The first part of this study examined the relatioship between immunoglobulin production and rejection episodes in patients receiving renal allografts. IgG-PFC were increased in 20 of 22 rejection episodes and, in most cases this was accompanied by an increase in the IgA-PFC. In addition, a greater number of azathioprine treated patients had increased plaque forming cell activity compared with cyclosporine treated patients and this correlated with the number of rejection episodes in the two groups. These results are confirmed by other recent reports of an association between increased immunoglobulin production and rejection episodes.

Garovoy et al(1982a,b) were the first to report that increased numbers of immunoglobulin secreting cells was a useful indicator of rejection activity and that these fell in response to treatment. Although these findings suggest that IgG-PFC numbers might be a reliable indicator of the humoral form of rejection, it is not clear whether this is due to graft specific antibody production or to polyclonal

activation of host B cells(Horsburgh et al, 1983). In a detailed report, Satomi et al(1983) also showed that the increased plaque forming cell response associated with rejection episodes rather than B cell numbers, pre-transplant blood transfusions or HLA differences and suggested that this was due to polyclonal activation of B cells. Although they did not present direct evidence for this idea, polyclonal production of all classes of immunoglobulin occurs in human mixed lymphocyte cultures which may be an in vitro correlate of graft rejection(Kunori & Ringden, 1979,1980). However, it has been suggested that the increased immunoglobulin production during rejection episodes reflects production of a range of alloantibodies and autoantibodies rather than true polyclonal activation of B cells (Soulillou et al, 1981) and it would be of interest to examine directly the specificity of antibodies during rejection episodes. It is also not known whether the increase in spontaneous production of immunoglobulin during rejection episodes is due to an increase in the absolute number of B cells but a recent study has suggested that an increased number of B cells in the first three weeks after renal transplantation correlates very well with rejection episodes, especially if there is also an increased proportion of T4+ cells(Green et al, 1985). A further important finding from my study was that although there was no increase in IgM-PFC during rejection episodes four patients without clinical signs of rejection had an increase

in both IgG-PFC and IgM-PFC which coincided with a systemic infection. The 20% incidence of increased IgG-PFC in the absence of clinical or histopathological evidence of rejection which I found confirms another recent report(McNaughton & White, 1985). Interestingly, these authors suggested that the plaque forming cell assay was of limited diagnostic value because elevated plaque forming cell responses occurred during infections but also suggested that it might be possible to distinguish rejection and infection by the class of immunoglobulin which was induced. My results confirm this possibility by indicating that in the presence of infection, an increase in IgG production does not suggest rejection if the IgM response is also increased and I would suggest that in further studies, IgM-PFC can be used to discriminate between rejection and systemic infection in renal transplant patients.

The second part of this study used the plaque forming cell assay to assess the immune status of transplant recipients who were immunosuppressed with either azathioprine or cyclosporine. Not unexpectedly, the results indicated that immunoglobuin production was depressed by both immunosuppressive drugs although not to the same degree. As noted above, cyclosporine treated patients had lower IgG-PFC responses than azathioprine treated patients and this correlated with a lower incidence of rejection episodes in cyclosporine treated patients. Although it is possible that this reflects stimulation of immunoglobulin

production secondary to graft rejection, as discussed earlier, it is also possible that the lower incidence of acute rejection in cyclosporine treated patients reflects a more potent immunosuppressive effect of cyclosporine on humoral immunity. In addition to its profound effect on T cell mediated immunity , there is also evidence that cyclosporine can also affect antibody production. Thus, T4+ cells from cyclosporine treated patients exhibit an impaired ability to help anti-SRBC antibody production in vitro while the same was not observed in azathioprine treated patients(Van Buren et al, 1982). In addition, several workers have reported that cyclosporine inhibits T dependent B cell proliferation and differentiation in vitro(Paavonen & Hayry, 1980, Berger et al, 1983). The question of whether cyclosporine also has a direct effect on B cell function is more controversial. Although it has been suggested that cyclosporine inhibits both T dependent and T independent immunoglobulin synthesis(Paavonen & Hayry, 1980), this work used SAC as a T independent mitogen and it is now known that SAC requires T cell help for the final differentiation of the B cell into an immunoglobulin secreting cell. In addition it has been shown that while T cell independent B cell proliferation induced by anti-IgM antibodies and by SAC is sensitive to cyclosporine(Berger et al, 1983), activation of human B cells by EBV is insensitive to the action of cyclosporine. Thus, it was suggested that certain B cell activation mechanisms are sensitive to cyclosporine while

others remain unaffected. These findings indicate that the inhibition of plaque forming cell response I observed in cyclosporine treated patients could be due to both a direct action of cyclosporine on B cells or through inhibition of helper T cells. It would be of interest to examine this by looking at PWM, SAC and EBV responses in cyclosporine treated patients.

In the present study, azathioprine treatment proved less successful than cyclosporine both in suppressing spontaneous immunoglobulin production and in preventing acute rejection episodes. The mechanism of azathioprine induced immunosuppression is mainly by decreasing DNA synthesis although there is evidence of other modes of action. Thus, depressed K cell activity has been found in long term azathioprine tre ated patients and it has also been suggested that it may interact specifically with T cells(Webb & Winkelstein, 1982). In addition, animal studies have shown that azathioprine does not interfere with the generation of antibody molecules controlling an anti-SRBC plaque forming cell response(Medzihradsky et al, 1982). It is tempting to speculate that the less potent anti-rejection activity of azathioprine reflects the fact that this agent only inhibits B cell differentiation secondary to its effects on other cells. In view of the significance of this hypothesis it would be interesting to set up in vitro correlates to compare azathioprine and cyclosporine in T-dependent and T-independent culture systems for production

of immunoglobulin.

The results presented here show that studies of B cell function in renal transplant patients can contribute considerably to understanding the mechanisms of immunosuppression as well as the acute phase of allograft rejection. In the last few years cyclosporine therapy has reduced dramatically the incidence of rejection within the first YEAR after transplantation and chronic rejection now constitutes the major threat for the transplant patient. As antibody mediated mechanisms are also important in this process(Williams, 1984, Soulillou et al, 1981), it would have been interesting to extend my study to investigate plaque forming cell responses in patients with chronic graft rejection. This idea is supported by a recent report that PWM induced immunoglobulin secretion is increased in patients undergoing chronic rejection of renal allografts(Landsberg et al, 1985). In addition, studies of this nature could also provide useful information about the involvement of B cells in chronic rejection and about the efficiency of the anti-rejection treatment under these circumstances.

CONCLUSIONS

In this thesis, a sensitive reverse plaque assay system was used in combination with various polyclonal B cell activators to investigate human lymphocyte behaviour in uraemia.

These B cell indicator systems have been used before to investigate health and disease states and have been proven very important analytical tools in studies of monocyte, T and B cell function. However, they have never been employed-at least at the extent used in this thesis-in investigations of endogenous immunosuppression in uraemia and in studies of the effect of third party blood transfusion on the immune response of uraemic patients.

The results obtained from these studies are suggesting that these systems can provide a new investigative approach to the studies of the immunosuppression associated with uraemia and blood transfusion, particularly in association with the techniques using monoclonal antibodies to obtain purified mononuclear cell subpopulations. Thus, these B cell systems will be most appropriate in studies for the identification and functional characterisation of the blood transfusion induced suppressor cell or for confirming my hypothesis that helper T cell function is defective in uraemic patients.

The large number of publications about the reproducibility of these assay systems and the technical difficulties encountered by many workers has discouraged investigators of the immune status in uraemia from using

them in their studies. I have shown that although these systems are indeed technically demanding, they are very versatile, sensitive and reproducible for in vitro studies of immune reactivity and can be significantly more informative compared with more conventional functional assays such as mitogen transformation.

Finally, I must emphasise that throughout this thesis a rather broad approach has been taken and I strongly feel that all areas described should be looked into more in depth that can be done by one person for all the problems which I have presented.

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APPENDIX 1

TABLE 7.1

Patient details

Patient No	Age	Sex	*Creatinine (uumol/1)	Original Disease	*Albumin (g/dl)	Blood Transfusions
1. S.W.	23	M	1106	GN	0.4	10
2. M.S.	43	F	642	PN	0.9	10
3. P.M.	24	F	1170	Membranous GN	1.7	5
4. J.B.	35	F	637	GN	2.9	5
5. M.M.	38	M	990	GN	2.5	10
6. A.K.	27	M	935	GN	1.6	10
7. T.Y.	59	M	670	DN	0.4	5
8. R.H.	27	M	1050	PN	2.1	5
9. M.H.	36	F	-	Unknown	-	10
10.J.M.	39	F	725	GN	2.7	10
11.B.J.	27	M	916	PN	1.0	10
12.J.P.	53	M	980	PN	2.3	10
13.J.D.	52	M	1150	HN	1.9	10
14.C.S.	33	M	857	Mesangiocapillary GN	2.0	10
15.W.H.	31	M	852	Unknown	3.7	10
16.J.L.	33	M	805	Focal GN	0.9	10
17.P.F.	49	M	980	HN	0.4	10
18.T.C.	53	M	775	PN	2.1	5
19.K.M.	53	F	973	GN	0.9	5
20.T.S.	49	M	1169	PK	2.9	5
21.W.G.	22	M	637	DN	0.9	5
22.R.F.	27	M	1134	PN	0.6	5
23.R.B.	53	M	736	PK	0.4	5
24.L.A.	59	F	990	PK	1.7	5
25.L.M.	55	F	654	PN	0.2	5
26.J.G.	20	M	732	GN	0.4	5
27.J.H.	52	M	1070	HN	1.9	5

Clinical and Biochemical data for patients studied in the BT trial. Plasma creatinine is presented as an indication of kidney function(Normal range:60-120) and plasma albumin as an indication of nutritional state (Normal range:3.5-5.5).
ABBREVIATIONS:GN:Chronic Glomerulonephritis, PN:Chronic Pyelonephritis, DN:Diabetic Nephropathy, HN:Hypertensive Nephrosclerosis, PK:Polycystic Kidneys.
*The results for creatinine and albumin are baseline measurements.