Antibody mediated feedback suppression of immunoglobulin synthesis in rheumatoid arthritis

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Award date:
1988

Awarding institution:
University of Bath

ANTIBODY MEDIATED FEEDBACK SUPPRESSION OF IMMUNOGLOBULIN SYNTHESIS IN RHEUMATOID ARTHRITIS

Submitted by

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for the Degree of Doctor of Philosophy

at the University of Bath

1988

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To my parents

ACKNOWLEDGEMENTS

I would like to thank Dr Nick Hall for his kindness, patience and understanding.

My grateful thanks also to Dr Nick Goulding for his advice on computing and statistical analysis.

I would like to express my heartfelt gratitude to all laboratory and hospital personnel for their generous donations of blood and their tremendous support throughout the duration of this project.

A special 'thank you' to Martin and Tim for helping with the illustrations and to Judith Elswood for her expert handling of the manuscript.

Last, but not least, thanks to all members of my family, especially Tarvinder and Harjit, for their love and support.

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ABBREVIATIONS

AA	Arachidonic acid
ADCC	Antibody-dependent cell-mediated cytotoxicity
AET	2-aminoethylisothiouronium bromide
AS	Ankylosing spondylitis
BCDF	B cell differentiation factor
BPB	Bromophenol blue
BRBC	Burro red blood cells
BSA	Bovine serum albumin
C'	Complement
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CMFSS	Calcium and magnesium free salt solution
cRBC	Calf red blood cells
CRI	Cross-reactive idiotype
CRP	C-reactive protein
EA	Extra-articular
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FcR	Fc receptor
γ-IFN	Gamma interferon
Hagg	Heat aggregated IgG
HI	Helper inducer
5HPETE	5-hydroperoxyeicosatetraenoic acid
IBF	Immunoglobulin binding factor
ICs	Immune complexes

id	Idiotype
IEP	Immunoelectrophoresis
Ig	Immunoglobulin
IL-1	Interleukin 1
IL-2	Interleukin 2
INTQR	Interquartile range
K cell	Killer cell
LGL	Large granular lymphocyte
LPS	Lipopolysaccharide
LT	Leukotriene
McAb	Monoclonal antibody
2ME	2-mercaptoethanol
MNC	Mononuclear cell
MLR	Mixed lymphocyte reaction
NK	Natural killer
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
ON	Old normal
PBMNC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween
PFC	Plaque forming cell
PG	Prostaglandin
PMN	Polymorphonuclear leucocyte
PVC	Polyvinyl chloride
PWM	Pokeweed mitogen
RA	Rheumatoid arthritis
RANA	Rheumatoid arthritis nuclear antigen
RBC	Red blood cell

RhF	Rheumatoid factor
Ros	Reactive oxygen species
SA	Staphylococcus aureus
SBF	Suppressor B cell factor
s-cRBC	Sensitised calf red blood cells
SF	Synovial fluid
SFL	Synovial fluid lymphocyte
SI	Suppressor inducer
sIg	Surface immunoglobulin
SM	Synovial membrane
SRBC	Sheep red blood cells
SRS-A	Slow reacting substance of anaphylaxis
STL	Synovial tissue lymphocyte
T_H	T helper cell
TNS	Tumour necrosis serum
TRF	T cell replacing factor
$T_{S/C}$	T suppressor/cytotoxic cell

ABSTRACT

Antibody-mediated feedback suppression of immunoglobulin production was investigated in early RA using the Facb fragment of rabbit IgG. Suppression of the PWM-stimulated IgG response in early seropositive and palindromic RA patients was similar to that observed in normal controls, but impaired suppression was observed in established RA subjects. There are also indications that early seropositive and seronegative disease may differ from each other with regard to this control mechanism. The degree of Facb suppression produced was found not to correlate with any clinical indices of disease activity. Data also suggest that FacbR expression may be a marker for cell activation rather than an indicator of the ability to suppress.

The effect of Facb on IgM RhF production was also examined. The kinetics of suppression were different from those observed with IgG. The possibility that the Facb behaved as an antigen rather than an FcR modulating ligand cannot be excluded.

Preliminary studies using the multivalent ligand, Hagg, as an FcR ligand were also carried out and IgG and IgM RhF responses examined. Again, defective feedback suppression was observed in established RA patients compared to controls.

The relevance of the above observations to the immunopathogenesis of RA is discussed.

CHAPTER 1

INTRODUCTION

The work presented in this thesis concerns the in vitro modulation of the humoral immune response by mononuclear FcR^+ cells in rheumatoid arthritis. In particular, $FacbR^+$ cells have been investigated with regard to their immunoregulatory role in early disease. This chapter is therefore divided into three sections:-

1. Rheumatoid arthritis - its clinical course, pathology, pathogenesis, aetiology and an assessment of immunoregulatory pathways.

2. Antibody-mediated suppression and FcR^+ cells.

3. $FacbR^+$ cells in RA; aims of the project.

SECTION 1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown aetiology which affects primarily the synovial joints. It is characterised by synovial proliferation and juxta-articular erosions which lead to progressive joint damage and disability. The disease is systemic in nature and in severe cases all major systems of the body are affected, giving rise to extra-articular features such as vasculitis, nodules, sicca syndrome and lymphadenopathy. In a small number of patients, where splenomegaly and neutropenia are associated features, the disease is termed 'Felty's syndrome'.

Some 2% of the population is affected by RA, the incidence being three times greater in women than in men (Currey, 1983). It has a worldwide distribution and although onset may occur at any age, the disease generally develops between the ages of 30 - 50 years. The onset may be acute, insidious or palindromic. Patients present with joint pain, stiffness and swelling. Prodromal features include low grade fever, weight loss and fatigue. With acute onset joint symptoms appear within a few days or weeks, affecting many joints in a symmetrical fashion. Most patients, however, have an insidious onset where symptoms emerge over several months, gradually involving more joints in a roughly bilateral distribution. With palindromic onset, symptoms flit between joints, lasting for only a few hours or days each time over a period of several weeks or months before becoming persistent in nature. The disease follows a characteristic remitting-relapsing course and although in some patients it may remit spontaneously, in most there is a gradual deterioration, with 50% of patients significantly disabled 10 years after onset (Hardin Jr (1986).

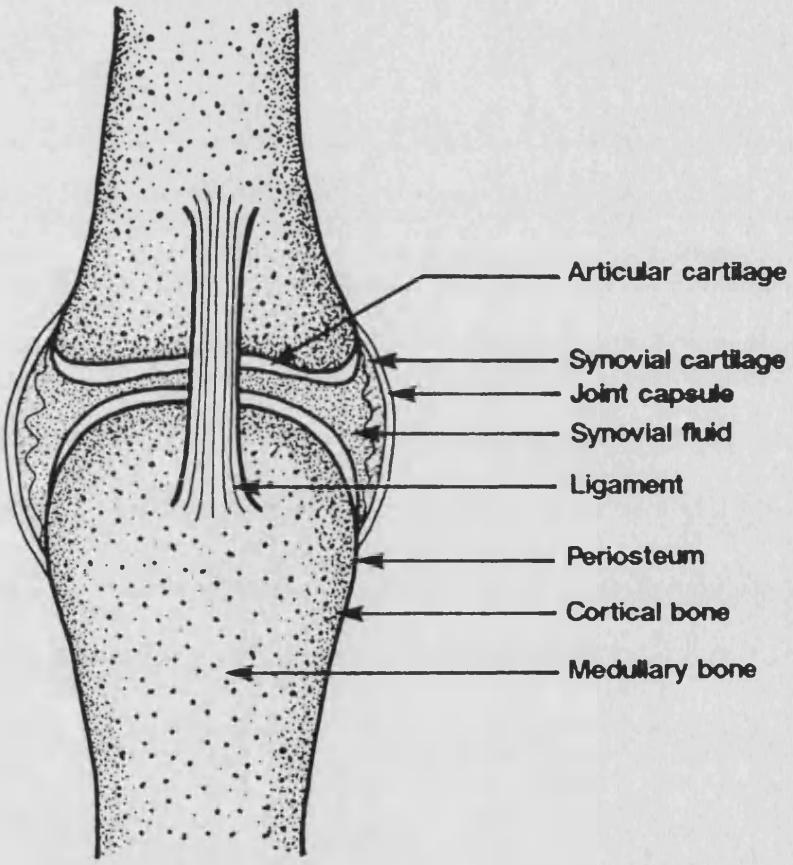
The first joints affected are usually the small joints of the hands, wrists and feet and also the knees. As the disease progresses large joints become involved, including the elbows, shoulders, hips, ankles, subtalar and sternoclavicular joints. Spinal involvement is limited to the upper cervical region. The changes which occur in the joint during the disease process are described below, following the description of the normal joint.

1. Structure of the normal joint

The synovial joint consists of two articulating ends of bone covered by hyaline cartilage and enclosed in a joint capsule (Figure 1). The latter is composed of dense fibrous tissue and contains numerous nerve fibres. The capsule is lined by a synovial membrane (synovium), the innermost (intimal) layer of which consists of two main types of cells, or synoviocytes. These form a discontinuous layer one to three cells thick. Type A cells are phagocytic and involved in the removal of material from the joint; type B cells produce protein and hyaluronate. It is thought that these two types of synoviocyte represent different functional states of the same cell (Ghadially, 1983). An intermediate type C cell has also been observed. The intimal layer lies on a layer of loose vascular, acellular connective tissue, called the subsynovium, which merges with the periosteum of the bone. The synovium does not cover the hyaline cartilage.

The hyaline cartilage appears macroscopically as a smooth, glistening structure which covers the bone. It consists of cells called chondrocytes which synthesise the other two components of cartilage - the fibres and the matrix. The fibres are made of type II

FIG 1 Structure of the normal synovial joint



collagen which is composed of three coiled polypeptide chains arranged into a triple helix configuration. These molecules are quarter staggered to each other to form fibrils which in turn form the fibres. The latter vary in thickness and distribution within the cartilage (Zvaifler, 1977). The matrix consists of proteoglycans, which comprise repeating disaccharide subunits linked covalently to a protein core (Zvaifler, 1977) and contain mainly chondratin sulphate A and C and keratan sulphate. These are attached via a link protein to hyaluronate forming large aggregates which are trapped in the collagen network. The proteoglycans are viscous and hydrophilic. They absorb water causing the collagen network to swell, thus giving cartilage its firmness and elasticity. The chondrocytes have lost much of their ability to undergo mitotic division, so regeneration is limited. Though metabolically active, the hyaline cartilage contains no blood vessels or nerves. Nutrients are derived from the synovial fluid by diffusion.

The joint cavity is normally filled with a small amount of fluid which is clear, colourless and does not clot, due to the absence of fibrinogen. This is the synovial fluid (SF). It is often described as a dialysate of plasma (Sandson and Hamerman, 1962) but which contains some 2% of hyaluronate, giving it its characteristic viscosity. SF lubricates the load-bearing surfaces, allowing them to move more easily over each other. It also allows transport of nutrients to the cartilage, as mentioned above, as well as carrying material resulting from the daily wear and tear within the joint to type A cells for removal by phagocytosis. The SF contains very few cells. Those that are present are thought to be derived from the lining.

2. Pathology of the rheumatoid joint

In RA, the earliest lesions observed are vascular. This comes from the work of Kulka et al (1955) and Schumacher and Kitridou (1972) who analysed biopsies taken from patients with disease of only six weeks' duration. These authors found signs of an increased blood flow and enhanced vascular permeability producing oedema. All samples exhibited microvascular injury as evidenced by extravasation of red blood cells (RBC), vascular occlusion, the presence of gaps between endothelial cells and endothelial cell damage. Lymphocytes and polymorphonuclear cells (PMN) were found infiltrating the superficial synovial membrane and also observed perivascularly. No plasma cells were seen. Mild proliferation of synoviocytes was also observed, with both type A and B cells being affected. There was also some evidence of phagocytosis. Mononuclear cells (MNC) were found to be the predominant cell in the SF, although some PMN were also present. All changes were focal so that depending on where the biopsy was taken, some areas appeared normal.

Whilst reports of early changes in RA are scarce because of the difficulty in accurately diagnosing the disease at this stage, descriptions of established disease are abundant. Most authors (Sokoloff, 1979; Harris Jr, 1985; Zvaifler and Silver, 1985) agree on the following course of events.

Progressive disease produces a highly characteristic oedematous and hypertrophied synovial lining. The synoviocytes are present in a layer which may well reach a depth of some 10 - 20 cells and the lining cells may exhibit hyperplasia. Multinucleate cells, thought to be derived from type A cells (Ghadially and Roy, 1969), may also be seen in the intimal layer. More common, however, are the interdigitating cells which occur in significantly greater numbers in

the RA synovium compared to those from normal or non-RA inflammatory joints (Janossy et al, 1981). These cells, which have long branching processes, lack macrophage, lymphocyte or fibroblast characteristics, but are strongly DR^+. Just beneath the lining there is a rich capillary network. Many of these blood vessels have fenestrations such as those observed in glomeruli. As well as the formation of new blood vessels, vascular lesions observed in early disease are exaggerated. Thus, venous distension, capillary occlusion, arterial wall infiltration by neutrophils, areas of thrombosis and perivascular haemorrhage are regular findings in established disease.

Most typical of established RA, however, is the intense infiltration of the subsynovium by MNC, mainly lymphocytes and plasma cells. These collect in aggregates or follicles, especially around small blood vessels (Gardner, 1965). True germinal centres, however, are not frequently seen and when present usually occur in longstanding RA (Sokoloff, 1979).

Immunofluorescence studies have shown that the principal infiltrating cell is the T lymphocyte (Abrahamsen, 1975; Van Boxel and Paget, 1975), although B cells are also present but in lower percentages than those observed in the corresponding blood (Abrahamsen et al, 1975; Van Boxel and Paget, 1975). Three types of distribution of the lymphoid cells may be observed: lymphocyte rich areas, plasma cell rich regions and transitional areas where macrophages, lymphocytes and plasma cells are intermixed (Ishikawa and Ziff, 1976). T cells are found perivascularly and B cells are located in centres of lymphoid follicles (Konttinen et al, 1981). In some patients the various lymphoid cells are scattered throughout the synovial tissue.

In contrast to early disease the predominant cell in the SF is the PMN, with cell numbers ranging from a few thousands to tens of thousands per mm^3 in severe active disease (Harris Jr, 1985). This high cellularity gives synovial fluid its turbid appearance macroscopically. MNC are also present but these are in the minority. As the inflammation progresses the connective tissue cells and vasculature of the synovial stroma undergo a marked proliferation to produce an aggressive granulation tissue known as 'pannus'. This grows from the joint margins to cover and digest the cartilage. The greatest amount of articular destruction occurs at the periphery of the cartilage. It is thought that the earliest process is brought about by immature synovial cells (before pannus formation) which arise from the recesses at the margin of the joint and creep across the surface of the cartilage. These cells appear to digest the proteoglycans enzymically and insert themselves between the collagen fibres. Loss of ground substance is also observed in areas away from the advancing margins of the proliferating synovium, indicating the importance of soluble factors.

Cartilage destruction appears to occur in waves, each followed by maturation of the granulation tissue. Kobayashi and Ziff (1975) described three types of pannus: the first resembles the synovium and contains proliferating blood vessels and perivascular mononuclear cells; the second resembles granulation tissue and consists of monocytic cells and fibroblasts; the third variety consists of dense avascular, acellular fibrous tissue which attaches strongly to the cartilage. It is not known whether each kind progresses to the next or whether they develop separately. Coverage of the cartilage by the pannus may also promote damage through interference with the nutrition of the former.

Bone destruction appears first as a marginal or juxta-articular erosion, where the bone is not covered by cartilage. This gives the articulating end a 'chewed-out' appearance radiologically. Further destruction occurs with the pannus burrowing through the disintegrating cartilage to the bone. Osteoclasts may be involved. The synovial fluid is greatly increased in volume and quite watery and therefore no longer able to provide adequate lubrication of the articulating surfaces. This also results in a high intra-articular pressure which may be instrumental in producing damage to the cartilage and bone. Furthermore, it is believed to be responsible for extrusion of the joint cavity into the neighbouring soft tissues, causing much of the pain experienced by the patient upon movement. The fibrous collagenisation which occurs with pannus maturation leads to the shrinking of the capsule and the increasing approximation of the ends of opposing bones. Subluxation is observed in some cases where the bone ends impinge upon each other with complete elimination of the joint space. Weakening of capsular and ligamentous supports by inflammation and tendon contracture and rupture are also thought to be important in producing joint deformities.

All these changes lead to progressive loss of joint function and in turn are believed to be responsible for muscle atrophy and contracture (Maini, 1977). The patient is thus left highly disabled and in pain and probably has complications of extra-articular (EA) disease as described below.

3. Extra-articular manifestations of RA

These are found mainly in seropositive disease which tends to be more severe. Seropositivity refers to the presence of an IgM autoantibody known as 'rheumatoid factor' (RhF) in the patients'

sera. This is directed against determinants on the Fc portion of IgG and was originally thought to occur only in RA, hence the nomenclature. This, however, is not true since rheumatoid factors have been demonstrated in a variety of other diseases and also occur in a small percentage of the normal population (reviewed Koopman and Schrohenloher, 1985). Anti-IgG activity has also been demonstrated in other classes of immunoglobulin, except IgD. These RhF, however, are not routinely measured in the laboratory.

Table 1 lists the various forms which extra-articular disease may take. Nearly all systems of the body may become involved, but only in a very small percentage of patients do life-threatening conditions develop. Seventy five percent of RA patients are believed to exhibit one or more manifestations of EA disease (Dieppe et al, 1985). Among the commonest features of systemic disease are rheumatoid nodules and vasculitis. The latter is in fact thought to be responsible for many of the other manifestations of EA disease. However, of particular relevance to the current work is the occurrence of Felty's syndrome in about 1% of RA patients (Short et al, 1957). This variant of chronic RA was first described in 1924 and represents a very severe form of rheumatoid disease which is associated with splenomegaly and neutropenia (Felty, 1924). Skin hyperpigmentation, leg ulcers, anaemia, thrombocytopenia and generalised lymphadenopathy may also be accompanying features (Zvaifler, 1977). The arthritis is usually at an advanced stage with numerous joint deformities and appears frequently as a burnt-out disease. It may also present as severely active disease. High RhF titres are often observed in these patients.

TABLE 1 **Complications of RA**

Complication	Comments
Vasculitis	Bland endarteritis; necrotizing arteritis, serious; rheumatoid nodules
Heart	Mainly pericarditis
Lung	Pleurisy; fibrosis
Ischaemic neuropathy	Symmetrical sensory, mild; patchy motor and sensory, serious.
Eye	Dryness (Sjogren's syndrome), common; scleritis rare, ominous
Lymphadenopathy	Not rare; local or general
Spleen	Rare; with leucopenia = Felty's syndrome
Kidney	Mainly amyloid, or drug induced

4. Pathogenesis of RA

Much of the damage seen in RA is immunologically mediated. This is evident not only from the joint pathology, where all manner of lymphoid cells and their products have been demonstrated, but also from *in vitro* and clinical studies. For example, Stastny et al (1975) reported that explants of rheumatoid synovium were capable of generating both antibody and lymphokines *in vitro*, supporting the description of the rheumatoid synovium as an ectopic lymphoid organ (Zvaifler, 1973). Clinical studies have shown that removal of circulating lymphocytes via the thoracic duct produces symptomatic relief in patients with active disease, whilst injecting these cells back into the subject exacerbates the disease process (Pearson et al, 1975).

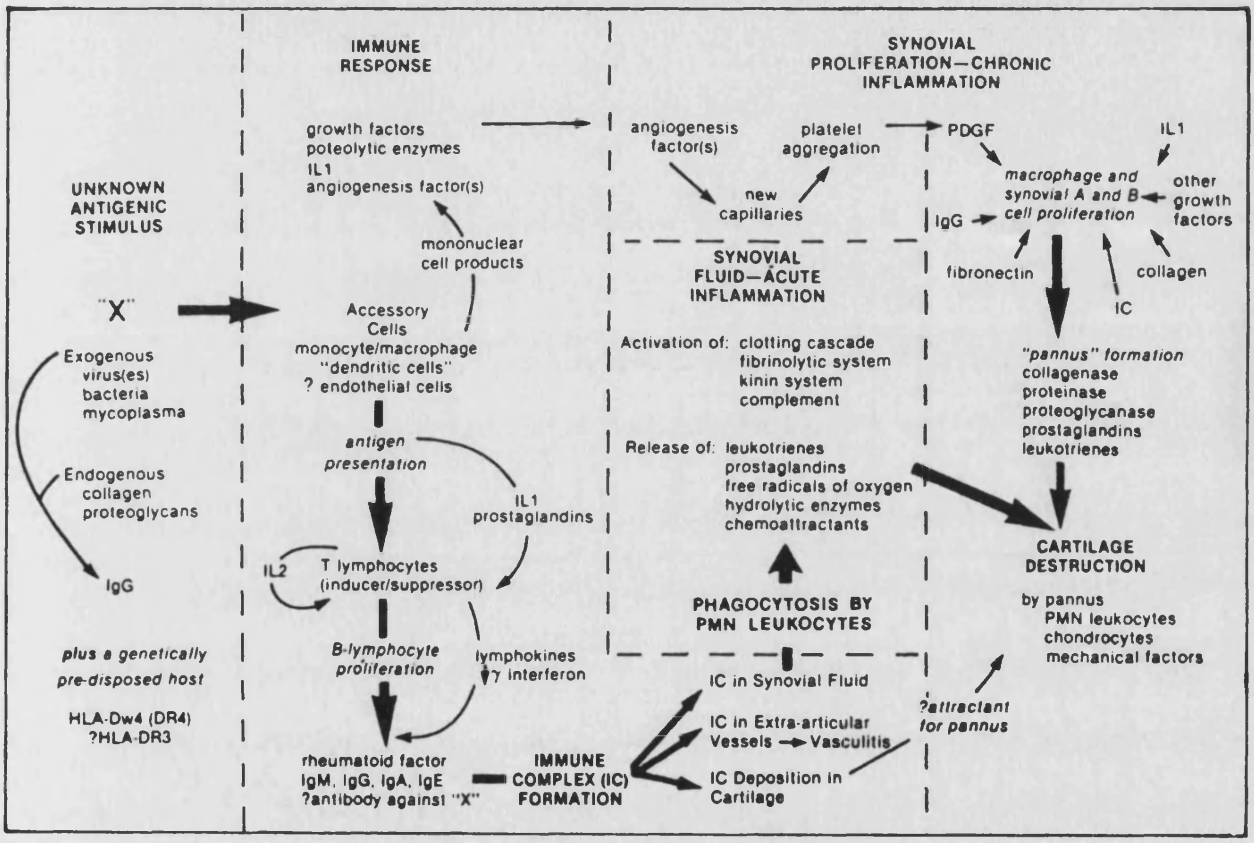
Figure 2 summarises the sequence of events which is thought to be responsible for the various pathological changes within the joint and which leads ultimately to its destruction.

The main destructive elements in rheumatoid disease are lysosomal enzymes and oxygen free radicals. Lysosomal enzymes are released from PMN and macrophages either during the process of phagocytosis or following cell death. They contain elements which are able to degrade both components of cartilage, ie the proteoglycans and the collagen framework. The earliest cartilage damage observed is the loss of ground substance. This is seen histologically as an absence of metachromatic staining. The aggregates of proteoglycan are broken down by the PMN serine neutral proteases elastase and cathepsin G, which act on the protein core and the link protein. The soluble components are then degraded intracellularly by the acidic cathepsin B and D. RA patients are also found to possess

LEGEND TO FIGURE 2

It is currently believed that the initiating antigen may become localised in the articular cavity, where local macrophage/dendritic cells process and present the antigen to T cells. There is release of IL-1 from the macrophages, activating a subpopulation of T cells to generate IL-2. This lymphokine produces proliferation and differentiation of other T cell populations. Thus inducer T cells are generated which through the medium of helper factors stimulate B cells to immunoglobulin synthesis. Immune complexes are formed and complement fixed, producing a number of anaphylactic and chemotactic factors. These increase the local blood flow and vascular permeability, leading to an influx of PMN and large molecular weight proteins such as fibrinogen and kininogen. The latter are activated, generating factors which further enhance the inflammatory process. Phagocytosis by both micro and macrophages results not only in the extracellular release of lysosomal enzymes capable of digesting articular tissues, but also in the production of pro-inflammatory arachidonic acid metabolites and oxygen free radicals. These, together with cell activation products, set up a series of highly complex, inter-related positive feedback networks which lead ultimately to the destruction of the joint.

FIG 2* Pathogenesis of RA



* from Harris Jr (1985)

high levels of enzymes which are capable of digesting the polysaccharide side chains of chondrotin sulphate and keratan sulphate (Harris Jr, 1985). Once the proteoglycan is removed, not only is the cartilage less able to resist a deforming force and therefore at a greater risk of mechanical disruption, but its infrastructure is also now open to proteolytic attack (Zvaifler, 1977). Elastase and cathepsin G solubilize the collagen by attacking the non-helical regions of the molecule and breaking down the interchain cross linkages. At 37°C the polypeptide chains unwind and are now susceptible to proteolytic attack by specific collagenase. This enzyme is elaborated by both the macrophages and the dendritic cells of the pannus (Mizel et al, 1980) and by the chondrocytes (Gowan et al, 1984) in response to interleukin 1 (IL-1). So collagen breakdown occurs from above as well as from within the cartilage. Collagenase cleaves the polypeptide chains into two fragments which are further degraded to gelatin by a variety of natural proteases including specific 'gelatinases' (Harris Jr, 1985). In severe aggressive synovitis, fibrils may be phagocytised whole by activated macrophages.

Although protease inhibitors such as α_2 macroglobulin and α-1 antitrypsin are present within the joint, it is probable that these are saturated, since free collagenase has been demonstrated in RA effusions (Harris Jr, 1985). Although this enzyme is released in an inactive form (Werb et al, 1977), the synovial fluid contains factors which are able to activate it. These include proteolytic enzymes and plasmin. Plasmin is generated from plasminogen in SF by plasminogen activator. This is secreted by rheumatoid synovial cells (Werb et al, 1977) as well as the endothelial cells of small blood vessels in the pannus.

Oxygen free radicals (Ros) further enhance proteolytic attack by inactivating protein enzyme inhibitors (Carp and Janoff, 1980). These radicals are generated by phagocytic cells following a respiratory burst which can be stimulated either by phagocytic activity or when immune complexes (ICs) or chemotactic factors are bound to the cell surface. Perturbation of the cell membrane by such stimuli leads to an increase in oxygen consumption, enhanced activity of the hexose monophosphate shunt and the generation of highly reactive species of oxygen. These include superoxide anion, hydrogen peroxide and hydroxyl radicals. The latter are amongst the most reactive chemical species known and are generated in an iron-catalysed reaction from superoxide anion and hydrogen peroxide (Halliwell, 1978). This is known as the Haber-Weiss reaction and may be enhanced in the rheumatoid joint since increased levels of iron are available following micro-haemorrhage in the synovial tissue (Harris Jr, 1985).

Large quantities of these oxygen derivatives are released extracellularly (Rosen and Klebanoff, 1979) where they have the potential for much tissue damage. For example, H_2O_2 is reported to be directly toxic to cells including endothelial cells of blood vessels (Weiss et al, 1981) and OH radicals are able to disrupt cell membranes by lipid peroxidation and the breakdown of fatty acids. Oxygen free radicals also have the ability to degrade hyaluronic acid (McCord, 1974), thereby reducing the viscosity of the synovial fluid and diminishing its lubricating properties. These data come largely from <u>in vitro</u> studies since direct evidence of Ros involvement is difficult to obtain due to the rapid disappearance of these radicals from the joint. However, Lunec et al (1981) demonstrated increased levels of free radical induced lipid breakdown products in RA

synovial fluid compared to OA effusions. In vitro studies by Jasin (1983) have shown that Ros damage IgG molecules producing structural changes which are similar to those observed in immunoglobulins obtained from rheumatoid samples (Wickens and Dormandy, 1982). Such damage may expose new antigenic sites or lead to protein aggregation.

The potential for oxidative damage in the RA joint is enhanced by an absence of enzymes such as superoxide dismutase and catalase (Blake et al, 1981), which protect against free radical damage, and the depressed levels of extracellular scavengers such as SH groups (Hall et al, 1984). The latter are thought to be oxidised by chloramines which are generated by PMN chlorination of endogenous amines in the presence of H_2O_2 and myeloperoxidase. SH groups are also important for the activity of certain proteins and for normal accessory cell function (McKeown et al, 1984).

As well as their destructive effects on the rheumatoid joint, lysosomal enzymes and oxygen free radicals are also able to promote the inflammatory reaction. For instance, the former are able to cleave components of complement (Goldstein and Weissman, 1974) whilst the latter through non-specific cell damage activate the membrane enzyme phospholipase A_2. This enzyme is found in a variety of cell types and activation by a wide range of stimuli, which include bacterial cell products, immune complexes and chemotactic peptides, leads to the release of fatty acids from the plasma membrane. Of these, arachidonic acid (AA) is the most important. This can be metabolised by two enzymatic pathways, generating two main classes of inflammatory mediators - the prostaglandins (PGs) and the leukotrienes (LTs). PGs are generated via the cyclo-oxygenase pathway, where AA is converted first to PGG_2 and then PGH_2. From the latter are produced the stable prostaglandins PGE_2, PGE_2 and D_2

(Pelus and Strausser, 1977). In platelets PGH_2 is converted to thromboxane and in vascular endothelial cells to prostacyclin. These mediators cause vasodilation and potentiate the pain produced by other inflammatory agents such as bradykinin and histamine (Hall, 1986). PGE_2 is also known to promote bone resorption (Robinson et al, 1975), a process involving osteoclasts stimulated by IL-1. PGs, in particular those of the E series, have been shown to possess immunomodulatory properties (Goodwin et al, 1984). This is to be discussed in greater detail in a later section.

The lipoxygenase pathway, which has been best investigated in neutrophils, gives rise to leukotrienes. These arise from the conversion of AA to 5HPETE which can be processed to 5HETE or to the leukotriene LTA_4. The latter may be converted to LTB_4 or to LTC_4, LTD_4 or LTE_4, which together form the slow reacting substance of anaphylaxis (SRS-A). Not only is LTB_4 a powerful chemotactic factor for PMN (Ford-Hutchinson et al, 1980) but it also prolongs the increased vascular permeability produced by other mediators (eg C5a). It has also been found to stimulate the release of oxidative products from activated neutrophils (Goetzl and Pickett, 1980). This, together with raised levels of LTB_4 reported by Rae et al (1982) probably serves to enhance the inflammatory response within the RA joint.

Phagocytic cells which are the source of these destructive and inflammatory mediators (lysosomal enzymes, AA metabolites, Ros) are lured to the site of inflammation by chemotactic factors. Here, their entry is facilitated by anaphylotoxins which enhance the vascular permeability. They also cause vasodilation thus increasing the number of cells gaining entry to the tissues. Most of these effector molecules are generated during the process of complement

fixation and arise mainly from the cleavage of complement (C') components C3 and C5. Thus, C3a and C5a behave as anaphylotoxins. These, through direct action (C5a) or indirectly through mast cell activation (C3a, C5a) - which causes release of the vasoactive amines histamine and serotonin - bring about the vascular changes described above. C5a is also cast in the role of main PMN chemotactant. It is a potent substance which can increase PMN adhesiveness to the endothelium of blood vessels as well as incite these cells to oxidative metabolism, lysosomal enzyme release and the generation of AA derivatives. Other chemotactants include those derived from mast cells, and the activated complex $\overline{C5b67}$. The latter is also involved in 'bystander attack' (Lachman, 1980). Although most of this complex is membrane bound, some may be found free in the interstitial fluid. Here it binds to 'innocent cells' in the vicinity and completes the complement cascade by fixing the remaining C8 and C9 components of C'. This results in cell lysis due to osmotic shock. It is believed that many of the vasculitic lesions produced within the rheumatoid joint and elsewhere in the body are due to the deposition of ICs in the blood vessels, which then bind C' with all its attendant effects. RA has in fact been described as an 'extravascular immune complex disease' by Zvaifler (1974).

Both alternate and classical pathways of complement are activated in RA. This is indicated by significantly lower concentrations of C4, C2 and C3, and factors B and P in RA effusions compared to those obtained from subjects with non-RA disease (Ruddy and Austen, 1970; Ruddy et al, 1975). This decrease is greater in seropositive disease, probably due to a more aggressive disease. Serum C' levels, however, are within the normal range or increased (Schur, 1985). A high local consumption of C' is probably due to the

large quantities of ICs which are present in the RA joint. These have been demonstrated in the SF and in the menisci and the cartilage of RA patients using immunofluorescence techniques (Cooke et al, 1975). Entrapment in the latter joint tissues may be due to charge differences between the macromolecules of cartilage and immunoglobulins (Harris Jr, 1985). Analysis of immune complexes have shown that although ICs containing anti nucleic, anti collagen and anti fibrinogen antibodies are present, most of the ICs within the rheumatoid joint contain rheumatoid factor (Zvaifler, 1977). These may be complexes of IgM RhF and IgG or intermediate sized complexes formed from molecules of self-associating IgG RhF. Much of this RhF is believed to be generated locally, since up to 90% of the plasma cells present in the rheumatoid synovium are reported to be committed to anti-IgG antibody production (Munthe and Natvig, 1972; Youinou et al, 1984a). Interestingly, the synovia of seronegative RA patients have also been shown to be engaged in rheumatoid factor formation (Munthe and Natvig, 1972). Although IgM RhF is more frequently found in the synovial tissues of seropositive patients compared to seronegative subjects, IgG RhF is found to be the predominant class generated (Natvig and Munthe, 1975). This exuberant synthesis of autoantibody is probably greatly aided by the presence of large numbers of Class II positive antigen presenting cells and T helper cells with which they are in close association (Janossy et al, 1981). Immunofluorescence has revealed that many of these T cells are activated. This is probably a consequence of IL-1 activity which also activates B cells. This interleukin has been demonstrated in the synovial fluids of RA patients (Fontana et al, 1982; Wood et al, 1983). Its production by both circulating and synovial RA cells in vitro is reported to be significantly higher compared to that

observed with normal PBMNC (Nouri et al, 1985). It probably has a profound effect on the inflammatory process in RA since it has been reported to stimulate synoviocytes to secrete collagenase and PGs. It induces fibroblast proliferation and activates chondrocytes to induce cartilage destruction and stimulates bone resorption. Neutrophilia, pyrogenicity and the acute phase response (hence the fever observed in the patient and the raised CRP etc of the clinical profile) are also attributed to this cytokine (reviewed Talal, 1985). What initiates the immune response in the joint is not clear, although several possibilities exist. These are described below.

5. Aetiology

Despite intensive research the cause of RA remains unknown. However, possibilities include an infectious aetiology, genetic predisposition to the disease and abnormalities of immunoregulation.

That RA may have an infectious aetiology has a basis in the observation that some infectious diseases in both man and animals may produce arthritis. Organisms such as Streptococci, Diphtheria bacilli, Mycoplasma and slow acting viruses have all been implicated at various times (reviewed by Harris Jr, 1981). More recently, Lyme disease in man has been described. This produces synovial changes similar to those observed in RA and is thought to be caused by a spirochaete (Burgdorfer et al, 1982). In swine, the Erysiopelothrix organisms produce arthritis which closely resembles the human disease and classically, subacute bacterial endocarditis is accompanied by RhF production, levels of which fall off once the disease is resolved. Johnson et al (1985) have suggested that the anti-IgG activity may arise as a result of cross-reactivity with bacterial antigens. More recently, Mouritsen (1986) has suggested that the

autoantibody is anti-idiotypic for virus induced anti-Fc antibody.

Viral infection, in particular by EBV, has recently featured prominently in the aetiology of RA. Alspaugh and co-workers (1976, 1978) showed that some 67% of patients with RA carried antibodies to EBV associated nuclear antigens (RANAs), whilst only 8% of normal subjects were positive. Furthermore, EBV is also known to be a polyclonal activator of human B cells (Rosen et al, 1977) and can induce rheumatoid factor production in vitro (Slaughter et al, 1978). This would explain the B cell hyperactivity observed in RA. However, anti-RANA antibodies also occur in other diseases and therefore data are inconclusive regarding the importance of EBV in RA.

Although some of the above agents (eg bacterial cell wall peptidoglycans and mycoplasma containing complete Freund's adjuvant) have been used successfully in generating models of RA in laboratory animals, attempts to isolate these organisms from the synovial tissues of RA patients have proved unfruitful (Harris Jr, 1981). At present, there is insufficient evidence to cast any of the above micro-organisms in an aetiological role.

However, given that such a wide variety of agents may produce arthritis, it is obvious that other factors must be involved in allowing the full expression of rheumatoid disease.

In 1973, Brewerton et al showed that a large percentage of patients with ankylosing spondylitis carried the HLA-B27 haplotype, suggesting that possession of certain histocompatibility antigens may be linked with susceptibility to particular diseases. Since then a whole series of associations between disease types and HLA antigens has been described, including the link between RA and HLA-DRw4 (Stastny, 1978). DR antigens are similar to murine Ia antigens which are encoded for by the immune response genes. These control T-B cell

co-operation, suppression of the immune response and antigen-induced antibody production (Silver and Zvaifler, 1985). This led to the proposal that RA might result from an abnormal immune response in a genetically susceptible individual to a perfectly normal pathogen. However, it has been shown that the increased incidence of HLA-DR4 is only observed in seropositive disease and that patients with seronegative RA do not differ from normal healthy controls (Alarcon et al, 1982). Thus, possession of the DR4 haplotype is thought to be related only to the degree of disease severity and not to the individual's susceptibility to RA (Jones et al, 1983). This is supported by the observation that some 95% of Felty's subjects are HLA-DR4 positive (Alarcon, 1986).

Abnormalities of immunoregulation have long been considered important in the aetiopathogenesis of RA. Whilst these may not initiate the disease, certainly their importance in allowing the establishment and the perpetuation of the disease process cannot be understated. Inflammation is usually a self-limiting process but it is clear from the sections above that the immune system in RA is unable to contain the various inflammatory processes occurring in the joint and elsewhere in the body. This is either because the immune system is unable to effectively eliminate the initiating antigen or to adequately regulate a normal immune response to the causative agent. In both situations chronic inflammation is produced. Furthermore, autoimmune phenomena are highly characteristic of RA, where some 70% of patients are seropositive (Zvaifler, 1977). In normal subjects, response to self-antigen is suppressed or eliminated by regulatory pathways.

In view of the above, it is probable that RA has a multi-factorial aetiology which in subjects with defective immunoregulation leads to the development of a common pathway of inflammation which we term 'rheumatoid disease'. This concept has led to the examination of immunoregulatory pathways in RA. These are reviewed in Section 7.

6. Treatment of RA patients

RA occurs not as a single entity but as a spectrum of disease activities, ranging from inactive or mild to severely active erosive disease. This necessitates the use of individual treatment regimes in each patient depending on the severity of illness. The chemotherapy may modify the disease process or control specific manifestations of it, but it is not curative, although in some patients the disease may remit.

The drugs used in RA may be divided into the following categories:-

1. Non-steroidal anti-inflammatory drugs (NSAIDs)
2. Corticosteroids
3. Second line or disease modifying drugs
4. Third line or cytotoxic drugs.

Agents within each group may be used singly or more usually in combination with those from other groups. Initial therapy may begin with NSAIDs (aspirin, phenylbutazone, indomethacin and phenylalkanoic acids eg ibuprofen and naproxen) to alleviate pain, fever and joint swelling. These manifestations of RA are caused by inflammatory mediators such as PGs, kinins, acute phase proteins and by increased vascular permeability and enhanced blood flow. NSAIDs act by inhibiting PG synthesis which *in vitro* occurs by suppression of cyclo-oxygenase activity (Vane, 1971). The use of such agents in RA

is questionable in the light of some PG data (see Section 1(7.B)) which suggest that they may suppress the immune response in the joint. However, not all NSAIDs are found to be equally effective in suppressing cyclo-oxygenase activity in vitro, although clinically they are equally potent. Clearly, other mechanisms are involved and these are thought to centre on the inhibition of PMN function. Both lysosomal enzyme release (Smith and Iden, 1980) and oxidative metabolism (Smolen and Weismann, 1980) have thus been shown to be suppressed in vitro by NSAIDs. The main problems with using NSAIDs are those of aspirin hypersensitivity and, more importantly, the toxic side-effects these drugs have on the GI tract (reviewed, Ward and Samuelson, 1981).

Patients examined in the current study were either receiving no medication at the time of investigation or only NSAIDs therapy. Other drug regimes are thus not directly relevant to the present investigation and therefore are only briefly discussed. Their effects on the immune system are summarised in Table 2.

In subjects whose disease activity is not adequately controlled by NSAIDs, a more potent class of anti-inflammatory drug is employed. These are the corticosteroids, which inhibit the generation of all arachidonic acid derivatives by preventing activation of phospholipase A_2. This is thought to occur through the generation of an anti-phospholipase A_2 protein called lipocortin,which is believed to either block the active site on the enzyme or reduce availability of its substrate. Other effects of steroids on the immune system have also been reported (see Table 2)

Where disease activity remains unchecked by anti-inflammatory agents or NSAIDs, second line therapy is introduced. These drugs are often referred to as slow acting or disease modifying agents. This

TABLE 2 TREATMENT REGIMES IN RA, EXCLUDING NSAID THERAPY

THERAPY	EFFECT (most data derived from in vitro studies)	ADVERSE REACTION
Corticosteroids: eg hydrocortisone, prednisolone	Strongly anti-inflammatory; ↓ phospholipase activity; ↓ T cells and monocyte numbers ↓ T cells and monocyte numbers	Peptic ulcers; Adrenal suppression; Thin and fragile skin, delayed wound healing
Second line drugs		
i. Gold eg sodium thiomalate, auranofin	↓ phagocytic activity; ↓ chemotactic response; ↓ PMN function; ↓ complement cascade ↓ in vitro lymphocyte responses in vivo lymphocyte responses normal	Proteinuria; Thrombotytopenia; Type 1 hypersensitivity
ii. D-penicillamine	↓ T_H activity in presence of $CuSO_4$ Free radical scavenging; Inhibits formation of IgG aggregates; Normalises accessory cell function	Thrombocytopenia; Nephrotic syndrome; Erythematous rashes
iii. Antimalarials eg chloroquine hydroxychloroquine	Lysosomatropic effects; ↓ Collagen degradation; ↓ IL-1 production	Retinopathy
iv. Sulphasalazine	↓ acute phase response - possible action on IL-1 production	Colon ulceration
Third line drugs: Cytotoxic drugs eg chlorambucil, cyclophosphamide, azathioprine, methotrexate	Inhibit DNA replication Cell deprivation of DNA precursor	Active against all actively dividing cells in the body eg in GI tract, gonads and bone marrow

is because firstly their effects may not be apparent until after 4 - 6 months of continued therapy. Secondly, these drugs appear to be able to halt or slow down the tissue destruction by altering the immune reactivity. Improvement in clinical status can be assessed radiologically and by clinical (eg Ritchie Index) and laboratory parameters (eg ESR, CRP and RhF levels). This group includes drugs such as gold compounds, the SH-containing drug D-penicillamine, the antimalarials - chloroquine and hydroxychloroquine, and sulpha-salazine. Gold and D-penicillamine are most commonly used in treating RA. Of the various effects these drugs may have on the immune system, one of the most interesting observations in recent years is that mixtures of $CuSO_4$ and D-penicillamine are found to inhibit T helper cell activity in vitro. This is thought to occur indirectly through the generation of hydrogen peroxide. This may explain the reduction of the serum immunoglobulin (Ig) and RhF levels seen in patients treated with this agent.

Where disease activity is not curbed by the use of even second line drugs, third line agents are employed as a final attempt to pacify the aggressive disease process. These drugs are cytotoxic and produce their effects by preventing dividing cells from replicating their DNA or by depleting the cell of DNA percursors. These drugs are not selective for activated lymphocytes but act against all actively dividing cells in the body, namely those of the GI tract, bone marrow and gonads. The seriousness of the risks of such treatment is obvious.

Alongside the chemotherapy, RA management involves physiotherapy and programmes of rest and exercise. Where erosive disease has produced destruction of the joint, surgery may improve joint function by replacement therapy, eg hip replacement.

7. Immunoregulation in RA

A. T cell subsets in RA

The earliest attempts at investigating immune function in RA involved only the very basic analysis of T and B cell responses to skin sensitising agents and mitogens. It was found that T cell responses were impaired whilst B cells were highly activated (reviewed Hall and Bacon, 1981). The latter was indicated by spontaneous Ig production by B cells in the peripheral blood and the synovial tissues (Al-Balaghi et al, 1982) as well as by enhanced ^{3}H-thymidine incorporation (Horwitz et al, 1970; Stratton and Peter, 1978). More recently a decrease in the number of mouse RBC rosetting cells has been reported (Youinou et al, 1984b), a further indication of B cell activation. The absence of T lymphocyte responses was attributed to prior committment of these cells to an ongoing immune response. Enumeration of total T and B cells by rosetting techniques and immunofluorescent labelling for surface Ig respectively showed that the relative percentages of these cells in the peripheral blood of patients were within the normal range.

In the early 1970's, it became apparent that the immune system is regulated by subsets of T helper and suppressor cells. Clearly, an overproduction of Ig may occur either through excessive T cell help or a lack of suppressor influences. The latter may also allow the development of autoimmune phenomena.

T helper and suppressor cells each carry specific antigens which are used as markers for the particular subset. These can be detected by monoclonal antibodies (McAbs). T helper cells (T_H) thus express the $CD4^+$ antigen and T suppressor/cytotoxic ($T_{S/C}$) cells, $CD8^+$. This delineation, however, is not strictly accurate since these populations are heterogeneous in function.

Assessment of T cell immunoregulatory pathways in RA has involved both phenotypic and functional analysis of these cell types.

i) **Phenotypic analysis of T cell subsets**

a) Peripheral blood

Analysis of T cell subsets in RA peripheral blood has shown the percentage of T_H and $T_{S/C}$ cells to be within the normal range (Burmester et al, 1981; Nilsson and Biberfield, 1982; Egeland et al, 1983). Some authors have found the proportions of $CD4^+$ cells significantly increased and those of $CD8^+$ cells significantly reduced compared to normal peripheral blood cells (Fox et al, 1982; Verdickt et al, 1983). This discrepancy is probably related to disease activity. For example, it is reported (Veys et al, 1982a; Duclos et al, 1982; Duke et al, 1983) that only patients whose disease is active are found to have reduced percentages of $CD8^+$ cells, whilst those with inactive disease do not differ from normal healthy controls. The former patient group may also exhibit a reduction in absolute numbers of $CD8^+$ cells (Fox et al, 1982; Veys et al, 1982a; Duke et al, 1983). Total T cell number in RA peripheral blood, however, remains unchanged. The deficit left by the decrease in the $CD8^+$ populations described above may be made up by an increase in third population cells as described by Sharpin and Wilson (1977).

Another possibility comes from the recent observation that the $CD4^+$ population is further subdivided into helper inducer (HI) and suppressor inducer (SI) subsets (Reinherz et al, 1982). These are detected by McAbs, anti-4B4 and anti-2H4 respectively. Thus, *in vitro* $CD4^+4B4^+$ cells are reported to augment Ig production (Morimoto et al, 1985a) whilst $CD4^+2H4^+$ induce $CD8^+$

cells (Morimoto et al, 1985b). Analysis of these subsets in RA has shown there to be a reduction in SI cells compared to normal healthy controls and patients with other articular diseases (Emery et al, 1987). This might explain the deficit of $CD8^+$ cells reported by some of the above authors.

In vivo activation of peripheral blood T cells in RA has been reported to be normal (Forre et al, 1982a; Duke et al, 1983) or increased (Yu et al, 1980; Burmester et al, 1981) as indicated by the expression of Class II antigens. Burmester et al (1981) reported that the activated cells belonged predominantly to the $CD8^+$ population. Using a decrease in lymphocyte density as an indicator for cell activation, Papadimitriou et al (1982) found that increased numbers of low density lymphocytes occurred only in patients with active synovitis but not in those with active extra-articular disease.

b) Synovial fluid

Most authors agree that the percentage of $CD8^+$ cells is enhanced in the SF of RA patients (Burmester et al, 1981; Duclos et al, 1982; Fox et al, 1982; Veys et al, 1982b), whilst that of $CD4^+$ cells may be normal (Duke et al, 1983) or reduced (Burmester et al, 1981; Duclos et al, 1982; Fox et al, 1982; Veys et al, 1982b) in comparison to peripheral blood. Lydyard et al (1982) correlated this increase in $CD8^+$ cells with disease activity.

The observations in SF are surprising in view of the markedly depressed levels of $CD4^+2H4^+$ cells in the SF (Emery et al, 1987). Fox et al (1982) have suggested that the increase in $CD8^+$ cells in the SF and the relative lack of this phenotype in the peripheral blood is probably due to the migration of

peripheral blood $CD8^+$ cells to the SF where they become activated. Many of the cells in the synovial fluid are reported to be activated (Galili et al, 1981; Burmester et al, 1981; Fox et al, 1982; Duke et al, 1983). Duke et al (1983) reported that though both $CD4^+$ and $CD8^+$ cells bore Class II antigens, the majority belonged to the former subset. This contrasts with others (Burmester et al, 1981; Fox et al, 1982) who reported that most of the activated cells carried the $T_{S/C}$ phenotype.

c) Synovial membrane (SM)

Reports of the proportions of T cell subsets in the SM have been conflicting. Whilst most investigators have demonstrated a preponderance of $CD4^+$ cells in the SM, with ratios of $CD4^+$:$CD8^+$ ranging from 4:1 to 14:1 (reviewed Zvaifler and Silver, 1985), reports of normal proportions (Forre et al, 1982b) or reduced numbers (Egeland et al, 1983) of these cells are also to be found. This is probably due to variations in sampling, as indicated by studies conducted by Kurosaka and Ziff (1983). Using an immunoelectron microscopic technique these authors reported that in lymphocyte rich areas $CD4^+$ cells were predominant, whilst in transitional areas $CD8^+$ cells were in the majority. Here these $CD8^+$ cells had the appearance of blast cells and were reported to be in close contact with macrophage-like cells. The latter were also found in $CD4^+$ areas but in smaller numbers.

Preliminary studies by Salmon et al (1987) have reported a reduction in the numbers of suppressor-inducer cells in the RA synovium, which might in turn explain the diminished levels of $CD8^+$ cells in this tissue. They also report that the principal interleukin-2 (IL-2) producers are $CD4^+$ $2H4^+$ cells.

The interleukins Il-1 and Il-2 may also be important in determining the percentages of cells expressing the $CD4^+$ and $CD8^+$ phenotypes. Il-1 is reported to activate mainly $CD4^+$ cells, which in turn generate IL-2, causing stimulation of $CD8^+$ cells (Alarcon-Segovia et al, 1985). Whilst production of the former is reported to elevated in RA (Nouri et al, 1985), that of the latter is found to be reduced (Alcocer-Varela et al, 1984; Miyasaka et al, 1984; Combe et al, 1985). Furthermore, T cells from the rheumatoid synovium are found not to respond to exogenous IL-2 (Miyasaka et al, 1984; Combe et al, 1985). This has been explained by prior activation. An average of 40% of T cells in the RA synovium are activated (reviewed Zvaifler and Silver, 1985). Whilst some authors reported $CD4^+$ cells as HLA DR^+ (Meijer et al, 1982a), others found $CD8^+$ T cells to be carrying Class II antigens (Burmester et al, 1981). This again may be due to sampling differences or as suggested by the findings of Pincus et al (1985) that T cells can express both phenotypes following activation.

Whatever the explanations for the differences in the $CD4^+$ and $CD8^+$ cell representation in the synovium, it has clear implications for the immunological activity in the RA joint. For example, Meijer et al (1982b) found that in synovial samples where $T_{S/C}$ cells were present in similar proportions to those of T_H cells, follicular arrangements were rare and the different cell types were distributed diffusely throughout the synovium. Only in samples where $T_{S/C}$ numbers were low was there a gathering of cells into follicles.

Phenotypic analysis, however, forms only an initial assessment of the regulatory pathways. It is more important to determine whether or not the cells function in their phenotypic role.

ii) Functional analysis of T cell subsets in RA

In view of B cell hyperactivity in RA, much emphasis has been placed on the measurement of suppressor T cell function, since it is generally believed the deficit is more likely to be here.

a) Suppressor cell function in RA

Both spontaneous and Con A-induced suppressor activities have been investigated in RA.

Peripheral blood. Suppressor activity in RA peripheral blood has been found to be normal (Patel et al, 1982; Romain et al, 1982) or depressed (Abdou et al, 1981a; Sakane et al, 1982) compared to that observed in normal blood. For example, Patel et al (1982) found no difference in Con A-induced suppressor activity between RA and control peripheral blood cells. However, Sakane et al (1982) who divided their RA patients into various disease groups, discovered that there were distinct differences between these subgroups. They found that in patients with early active disease (< 3 months duration), it was not possible to generate Con A suppressor activity and this correlated wiuth a reduction in the number of circulating $T_{S/C}$ cells. Anti-T lymphocyte antibodies were detected in the sera of these patients and these were found to be directed against $CD8^+$ cells. Furthermore, cells obtained from patients with early active disease were not responsive to signals generated by normal Con A-induced suppressor cells. Similar results were obtained using PBMNC from patients with chronic active RA,

although peripheral blood cells from these subjects could be induced by Con A to exhibit suppressor activity. Patients with inactive disease behaved as normal controls. Similar data were obtained by Abdou et al (1981) who examined suppression of Ig synthesis and its secretion. These authors, however, reported B cell function in both early and chronic active RA to be normal.

T cells from RA patients have frequently been found to be unresponsive to Con A, so it is possible that this is one reason why Con A-induced suppression may be defective in patients with RA. However, antigen specific suppression has also been found to be significantly reduced in these individuals (Keystone et al, 1980; Tosako et al, 1981).

Synovial joint. In the joint it is generally accepted that unfractionated T suppressor cell function is defective. Thus Chattopadhyay et al (1979 a and b) reported the inability of synovial tissue lymphocytes (STL) to suppress proliferation and immunoglobulin synthesis in Con A-induced and spontaneous suppressor cell assays respectively. In both assay systems STL were found to enhance the immune response. Similar data were obtained by Romain et al (1982) who examined the ability of mainly SF lymphocytes (SFL) to suppress differentiation of autologous peripheral blood cells. Deeming the lack of suppression to be due to inadequate helper activity, these authors co-cultured SFL with peripheral blood T cells (which incidentally behaved as normal cells) only to find that Ig synthesis was enhanced. This was surprising since most of the SFL expressed the $CD8^+$ phenotype. Clearly, there was a discrepancy between phenotype and function. Romain et al (1982) referred to this enhancement as 'helper augmentation' and suggested that synovial

$CD8^+$ cells may be analogous to the murine contra-suppressor cells described by Gershon et al (1981). Egeland et al (1983) obtained similar data using a purified $CD8^+$ Leu 3a and B cell population.

However, in studies where isolated $CD8^+$ cells have been examined, these cells have been reported to be very efficient suppressors (Egeland et al, 1983; Nilsson et al, 1986b). Thus, Nilsson et al (1986b) found that SF $CD8^+$ cells could suppress PWM-induced Ig synthesis as well as control peripheral blood $CD8^+$ cells. However, these authors found that choice of mitogen may affect the outcome of these functional assays since SF and blood $CD8^+$ cells were less able to suppress PPD-stimulated cultures compared to those stimulated by PWM. They suggested that this was because PWM was a much more efficient generator of suppressor activity (Nilsson et al, 1986a).

b) Helper activity in RA

Experiments analysing helper function in RA subjects have likewise produced conflicting data. Thus in peripheral blood T helper function has been reported to be defective (Bellamy et al, 1983; Egeland et al, 1983), normal (Romain et al, 1982) or increased (Kluins-Neleman et al, 1984). Patel and Panayi (1984) examined spontaneous generation of IgM RhF production in vitro and reported that T cells from RA patients produced enhanced help for autoantibody production which was not HLA restricted. Thus normal as well as RA B cells could be induced to generate RhF when co-cultured with RA T cells.

In the joint, Petersen et al (1986) reported that SF T cells had a low ability to co-operate with PWM-induced RhF

secretion. Chattopadhay et al (1979a), examining Ig production, found that synovial tissue lymphocytes did not differ from normal peripheral blood lymphocytes in helper activity, whilst Romain et al (1982) found this to be impaired compared to autologous peripheral blood T cells, which did not differ from normals. In studies examining isolated $CD4^+$ cells, contradictory data have been obtained. Whilst Egeland et al (1983) found Leu $3a^+$ cells to be weak augmentors of the antibody response, Nilsson et al (1986) reported that SF $CD4^+$ cells alone were better augmentors of PWM-induced help than the corresponding unfractionated T cells. This, they explained, was due to suppression by PWM-stimulated $CD8^+$ of $CD4^+$ function in the unfractionated preparation.

The functional assays have clearly produced much conflicting data. This is probably because investigators have tried to analyse a complex regulatory system with relatively simple fractionation or culture systems. More and more evidence is accumulating regarding the heterogeneity of the two main $CD4^+$ and $CD8^+$ T cell subsets. Ballieux and Heijnen (1983) who recently re-evaluated the usefulness of FcR on T cells as subset markers in conjunction with McAb analysis, have indicated a much greater degree of heterogeneity than previously appreciated in, for instance, the $CD8^+$ subset. For example, they postulate the existence of suppressor precursor T cells, suppressor activator and effector T cells. They also suggest that suppressor inducer activity does not exist in unprimed T cells (Heijnen et al, 1982) whilst Thomas et al (1981) believe it does. These differences may be due to the former group using an antigen to examine such interactions, whilst the latter used PWM.

Until T cell populations are more clearly defined and the various inter-relationships better established, it is probable that contradicting data will continue to emerge from various laboratories examining T cell regulatory pathways in RA. Whilst T cells are frequently considered to be the main proponents of immunoregulation, there exist other cells and mediators which are also important in maintaining the homeostasis of the body's defence system. These include prostaglandins, anti-idiotypic (anti-id) antibodies, natural killer (NK) cells and the immunoglobulin molecules themselves. The immunomodulatory actions of these various components on immune function are considered below.

B. Prostaglandins

Prostaglandins are a family of 20 carbon aliphatic unsaturated fatty acids, which are generated by every tissue in the body except for the red blood cells (Goodwin et al, 1984). They were originally described as smooth muscle stimulants (Pelus and Strausser, 1977) but have since been found to be potent immunoregulators, especially of cellular immunity. PGs thus inhibit T cell mitogenesis in response to PHA and Con A (Goodwin et al, 1977). They inhibit E rosette formation (Venza-Teti et al, 1980), the generation of cytotoxic cells in MLR (Darrow et al, 1980) and the formation of T cell colonies *in vitro* (Bockman and Rothschold, 1977). Gamma IFN production is also reported to be inhibited by prostaglandins, and this in turn is thought to be responsible for the continued growth of EBV transformed cells from RA patients rather than defects of suppressor cell activity (Hasler et al, 1983).

A number of investigators believe that some of the above effects may be achieved through PG action on IL-2 synthesising cells, since

they are reported to be inhibited by PGE_2 in both animals (Gordon et al, 1976; Bray et al, 1978; Baker et al, 1981) and humans (Rappaport and Dodge, 1982; Tilden and Balch, 1982). Furthermore, PG may also prevent cells from responding to IL-2. For instance, Tilden and Balch (1982) found that it inhibited the proliferation of IL-2-dependent T cell lines in response to exogenous IL-2. Although the inhibitory effects of PG and IL-2 production are believed to occur through direct action by some authors (Baker et al, 1981; Tilden and Balch, 1982), others (Chouaib and Fradelizi, 1982) report that IL-2 production is suppressed indirectly through the induction of a T suppressor cell. Tilden and Balch (1982) suggest that the absence or presence of a suppressor cell observed by various workers may depend on the concentrations of PG used in the experiments.

PGE_2 has also been reported to decrease NK and antibody dependent cell mediated cytotoxicity (ADCC) activity (Droller et al, 1978; Brunda et al, 1980). This is interesting in view of the report by Burchiel and Warner (1980) who found that PGE_1 increased FcR expression. NK activity is reported to be diminished in RA (Silver et al, 1982; Combe et al, 1984) and PGE_2 activity may be one of the contributory factors.

Modulation of humoral response by prostaglandins has produced variable results. In man, the proliferative response to PWM is not affected by PG (Goodwin et al, 1977; Staite and Panayi, 1982; Thompson et al, 1984) at physiological concentrations, whilst that induced by Staphylococcus aureus (SA) was found to be suppressed. This suggests that different B cell subsets may vary in their susceptibility to PG-mediated regulation.

Effects of PG on antibody responses have also been studied and produced some conflicting data. Jelinek et al (1985) reported that

PGE_2 could cause dose-dependent inhibition of Ig secreting cells in PWM- and Staphylococcus aureus-stimulated B cell cultures. The PGE_2 was found not only to suppress the production of B cell differentiation factor (BCDF) from mitogen-stimulated T cells, but it also inhibited the capacity of B lymphocytes to respond to BCDF.

In contrast, other authors (Staite and Panayi, 1982) have found that the use of PG-synthetase inhibitors suppressed the antibody response of PBMNC in man. Staite and Panayi (1982) demonstrated that this suppression could be reversed by the addition of low concentrations of PGE_2 to the cultures. They also found that the IgM response was not fully reconstituted by PG and suggested that other cyclo-oxygenase products may be more important in modulating this class of response.

Experiments by Ceuppens and Goodwin (1982) showed that PGE_2 did not act on B cells or monocytes directly. It was probable that the effects of PGE_2 on B cell responses were mediated through action on T helper or suppressor cells. These authors suggested that suppressor cells which inhibit Ig production are themselves suppressed by PGE_2. Thus a lack of PGE_2 through indomethacin administration releases the inhibitory influence on T suppressor cells, which can then suppress an ongoing antibody response. These PG-sensitive cells are reported also to be sensitive to irradiation and mitomycin C.

Support for the above hypothesis comes from earlier work by Goodwin et al (1979) who found that T suppressor cells defined by Fc R expression carried large numbers of PGE_2 receptors. Later Ceuppens and Goodwin (1982), using the $CD8^+$ marker, found that removal of these cells decreased the amount of suppression of Ig synthesis observed with indomethacin but it did not reverse it completely. These authors suggest that this is because some $CD4^+$

cells can be induced to become suppressor cells without undergoing any phenotypic changes in the process by other $CD4^+$ cells as reported by Thomas et al (1981).

In reference to RA, where large quantities of PGE_2 have been reported to be present in the SF of these patients (Morley, 1974), it is clear that this fatty acid may have profound effects on the immune function of the synovial tissue. Firstly, it would explain the depressed T cell responses seen in the rheumatoid synovium. Secondly, it also provides an explanation for the exuberant antibody response in these tissues. Ceuppens et al (1982) reported that cyclo-oxygenase inhibitors decreased IgM RhF production in normals, old subjects and RA patients. They suggested that the high level of RhF in the RA joint may be due to the suppression of T cells, which in normals suppressed RhF production.

Although it is believed that PG may mediate its effects on cellular and humoral responses by altering the intracellular CAMP and CGMP levels, it should be noted that changes in the concentration of these nucleotides and those occurring in cell function do not always correlate (Stobo et al, 1979; Tilden and Balch, 1982).

C. Anti-idiotypic antibodies

The idiotype network theory is one of the classic hypotheses in the field of immunoregulation. In 1974, Jerne suggested that the immune system is regulated through a network of idiotype-anti-idiotype interactions. This concept is based on the observation that antibody, as well as recognising antigen, can itself be perceived as an immunogen. This is due to the presence of antigenic determinants known as 'idiotypes' which are unique to the antigen binding site of that antibody and more often than not are located in the hyper-

variable regions of the Ig molecule. Idiotypes (ids) are thus to be found on surface Ig (ie antigen receptor) of B cells as well as on secreted Ig. They appear also to be present on T cell antigen receptors (Stevenson, 1986) and have been demonstrated on T helper and T suppressor subsets (Woodland and Cantor, 1978; Owen et al, 1978). Thus idiotypic regulation can occur at both cellular and humoral levels.

An antigen elicits the production of antibody, Ab_1, which gives rise to Ab_2 (anti-idiotype) and this then results in the formation of Ab_3 (anti-anti-idiotype) and so on, establishing a network. Each antibody either up regulates or down regulates the activity of the preceding component. This immunoregulatory ability is reported to be dependent on the dose and isotype used. For example, low doses of anti-id can greatly enhance the expression of the appropriate idiotype in response to a given antigen, whilst higher doses produce suppression (Roitt, 1984).

Early studies showed that whilst families of Ab_1 expressed similar but not identical antigen binding sites (reviewed Klinman and Steinberg, 1986), they could all be bound by one anti-idiotypic antiserum. This suggested that these Ab_1 carried a 'shared', 'public' or 'cross-reactive' idiotype (CRI). This would also explain why Ab_3 is often found not to express the same antigen binding specificity as Ab_1, although it may carry the same idiotype as Ab_1. The occurrence of CRIs is found to depend on the nature of the immunising antigen and the genetic make-up of the individual (Klinman and Steinberg, 1986).

It is found that autoantibodies frequently express CRIs. Kunkel et al (1973) were the first to demonstrate the restricted nature of idiotypes on rheumatoid factor. They found that the majority of IgM

anti-IgG antibodies carried the Wa idiotype. More recently, Forre et al (1979) have reported similarities between some IgG and IgM RhFs. Such similarity may be explained by the recent observation in mice (Manheimer, 1986) that autoantibodies utilise only a limited number of V_H gene families.

The presence of CRIs is particularly pertinent to autoimmunity since some authors believe that a loss of self-tolerance may result from such antigenic determinants. Furthermore, there exists much evidence suggesting that responses where CRI are prevalent are particularly susceptible to idiotypic regulation. These two aspects of idiotypy are discussed below.

i) Induction of autoimmunity

It has been suggested (Cooke et al, 1983) that an invading micro-organism may elicit an antibody response, where the latter carries an idiotype which is shared with autoreactive lymphocytes. Induction of T_H specific for the idiotype could then provide help for the autoantibody response.

Klinman and Steinberg (1986) postulate another mechanism. They suggest that during a normal immune response, immature B cells are generated, some of which are autoreactive. The induction of tolerance requires high affinity binding of immature B cells to antigen (Teale and Klinman, 1980). Low affinity binding inhibits tolerance. Klinman and Steinberg (1986) suggest that antibody which cross reacts with (ie is anti-idiotypic for) antigen receptors on autoreactive B cells, does so with low affinity and therefore could inhibit the tolerisation of these B cells and thus lead to the production of autoimmune phenomena.

Clearly, in both hypotheses a defect in the immunoregulatory T cell circuit is required for autoimmunity to arise.

Another mechanism whereby idiotypy may cause loss of self-tolerance is through the occurrence of 'internal image'. Anti-idiotype and idiotype are mutually inducible, ie Ab_2 can induce Ab_1. This occurs because Ab_2 is believed to carry the 'internal image' of the antigen, which gives rise to the formation of Ab_1. This idea has been used successfully in infectious diseases, where individuals can be vaccinated against antigens which are highly infectious or not readily available, by using Ab_2 (Stevenson, 1986).

It has been suggested that autoimmunity may arise if the immune response generates anti-id antibodies, which carry the 'internal image' of an autoantigen. Recently, Fong et al (1984) carried out experiments using an anti-id antibody which bore the internal image of human IgG Fc. This was raised using IgM RhF from patients and was used to show that internal image anti-id antibodies could behave like antigen. Thus a proliferative (but not secretory) response was obtained from patients with RA but not from a normal individual. Furthermore, the anti-id blocked RhF synthesis by PBMNC but had no effect on the total IgM response following PWM stimulation.

Such idiotypes are potentially powerful tools for analysing antigenic structures.

ii) Regulation of the autoimmune response

A number of investigators have reported the occurrence of anti-idiotype antibodies in patients' sera coincident with clinical improvement. Thus, Abdou et al (1981b) reported that

the appearance of anti-id antibodies to autologous anti-DNA was associated with decreased disease activity in SLE patients. In RA, Koopman et al (1983) reported *in vitro* suppression of monoclonal RhF synthesis by the corresponding anti-idiotype antibody and similarly, *in vivo*, Abe et al (1984) reported decreased RhF activity in the serum of a female patient with monoclonal IgM gammopathy following the appearance of an anti-idiotypic antibody.

Observations such as these, and the presence of CRI, clearly indicate an opportunity to manipulate autoimmune behaviour. Although anti-idiotypic therapy has been used with some success in treating human B cell lymphoma, the use of such agents has not proved very fruitful in autoimmunity. This is because suppression of idiotype positive clones leads, after a short latency period, to the emergence of idiotype negative antibodies of the same specificity. Furthermore, side-effects such as serum sickness and immunosuppression have to be considered.

D. Natural killer cells

Over the last decade practically all major cell types have been considered to be involved in immunoregulation to a lesser or greater degree. One recent contender is the natural killer cell. These cells are large granular lymphocytes which share some characteristics with T cell surface receptors and antigens. They have been reported to modulate B cell differentiation (Arai et al, 1983) and immunoglobulin production (Nabel et al, 1982; Abruzzo and Rowley, 1983).

There appears to be some dispute over the exact mechanism whereby NK cells mediate their suppressive effects on B cells since T_H cells (Arai et al, 1983), antigen exposed accessory cells (Abruzzo and Rowley, 1983) and B cells (Nabel et al, 1982) have all been described as the target cells.

In the RA synovium there appears to be a lack of NK cell (Silver et al, 1982; Combe et al, 1984) activity. This is not surprising since NK function is highly dependent on activation by IL-2 (Kuribayashi et al, 1981). In view of its effect on antibody synthesis, an absence of NK cell activity in a synovium already deficient in T suppressor function would serve to further enhance the B cell response.

E. Immunoglobulin molecules

Immunoregulation by immunoglobulin molecules has long been investigated. This mode of regulation forms the subject of this thesis and will be considered in detail in Section 2.

SECTION 2 ANTIBODY MEDIATED NEGATIVE FEEDBACK SUPPRESSION AND FcR^+ CELLS

1. Antibody-mediated feedback suppression

The mechanisms whereby the immune response is regulated have been the subject of intense investigation in recent years. One area which has received considerable attention is that of immunoregulation by antibody molecules. This concept is by no means new. One of the earliest known observations comes from the work of T Smith (1909) who found that by injecting varying amounts of Diphtheria toxin and anti-toxin, the immune response could be enhanced or suppressed.

Subsequent studies revealed that passive administration of antibody was able to suppress specifically the response to a given antigen (reviewed by Uhr and Moller, 1968). For instance, Moller (1963) found that when mice were injected with cells from animals of an H-2 incompatible strain, together with antibodies directed against these cells, the immune response was suppressed. Furthermore, when antiserum directed at only some antigenic determinants was used, the response to these particular determinants was suppressed, whilst the rest of the antigens on the injected cells were able to elicit antibody synthesis.

The response to soluble antigens such as DNP-BGG (Chang et al, 1969) and Diphtheria toxin (Uhr and Bauman, 1961) has similarly been shown to be suppressed with corresponding antisera.

These effects, which were demonstrated both in vivo (Cerottini et al, 1969; Sinclair et al, 1970) and in vitro (Feldman and Diener, 1972; Kappler et al, 1973; Lees and Sinclair, 1973), were thought to be due to the masking of antigenic determinants by the antibody - an

a) The $F(ab')_2$ fragment was just as effective in suppressing the immune response as its 7S counterpart (TaO and Uhr, 1966; Feldman and Diener, 1972) and that

b) High affinity antibodies were much more effective than those of low affinity (Walker and Siskind, 1968).

However, there existed evidence that other mechanisms might be involved. First, Uhr and Bauman (1961) reported that antibody injected five days after immunisation was still capable of suppressing the immune response, suggesting that a feedback mechanism operated. Second, experiments by Sinclair (1969) using 7SIgG and $F(ab')_2$ fragment found that the latter was 100 - 1000 fold less inhibitory than the intact IgG. It was concluded that this difference lay in the absence of the Fc portion. This was further emphasised by Kappler et al (1973) who examined the suppressive effects of rabbit 7S anti-SRBC IgG, its $F(ab')_2$ fragment and chicken 7S anti-SRBC IgG on the humoral response of mouse spleen cells to SRBC. Inhibition was observed with all three at high concentrations, but only rabbit 7SIgG was capable of suppressing the immune response at low concentrations. Thus it appeared that inhibition due to antibody operated at two levels (Hoffman and Kappler (1978). At high concentrations the antibody did mask antigenic determinants leading to suppression, but at low concentrations where antibody was present in insufficient amounts the suppression was mediated by the Fc portion. Chicken IgG was not able to inhibit the immune response because it could not bind to mouse spleen cell Fc receptor (FcR)

2. Mechanisms of Fc-mediated suppression

At present there are two main schools of thought concerning the mechanisms whereby Fc-mediated suppression takes place. The first

comes from the work of Hoffman and associates (1973; 1978). These authors, using TNP-SRBC as antigen, showed that anti-SRBC antibody could suppress the anti-TNP response when TNP was linked to SRBC but not when the hapten was attached to burro red blood cells (BRBC), even when the responding cells were preincubated with TNP-SRBC and anti-TNP immune complexes. SRBC primed T cells could stimulate production of antibody in response to both SRBC and BRBC (Hoffman and Kappler, 1973). It was found that whilst anti-SRBC suppressed the humoral response to SRBC, it did not prevent the SRBC primed T cells from helping the anti-BRBC antibody response. Thus, Hoffman and Kappler (1978) concluded that antibody induced suppression was not mediated via a central effect on the lymphocytes, since neither B nor T cells were directly blocked. It resulted instead from an interference between T-B cell co-operation. They suggested that this interference could be reversed by the introduction of factors which replaced T cell help - a prediction which was proved true by the addition of tumour necrosis serum (TNS) which is obtained from BCG infected mice 2 hours after injection of lipopolysaccharide (LPS) and has been shown to provide 'T cell help' (Hoffman et al, 1976).

Furthermore, it was suggested that the immune response to soluble antigens would not require the Fc piece for suppression to occur. Evidence for this comes from the work of Feldman and Diener (1972) who found that <u>in vitro</u> response to polymerised flagellin was suppressed equally well by the $F(ab')_2$ fragment and the 7S IgG antibody.

Support for Hoffman's hypothesis also comes from cell transfer experiments where lymphoid cells obtained from recipients treated with suppressive doses of antibody were able to initiate an immune

response against the corresponding antigen after transfer to incompetent hosts (reviewed Uhr and Moller, 1968).

The above findings, however, are in direct contrast to the observations of Sinclair (1982) and Kolsch et al (1980), who advocate direct blockage of B cells.

In 1971 Sinclair and co-workers proposed the tripartite model of Fc mediated feedback suppression. Here, antigen in the immune complexes bound to the antigen receptor on the B cell, imparting specificity to the suppressive process, and the Fc bound to the Fc receptor. Cross-linking of the two receptors was thought to inactivate the B cells, a concept also supported by Kolsch et al (1980). These authors, like Kappler et al (1973), used the TNP-SRBC model to investigate feedback suppression. They found that the degree of suppression depended on the concentration of antibody in the complex and that suppression was specific to the antigen in the complex and did not prevent the immune response to other antigens. Furthermore, this suppression could not be over-ridden by T cell replacing factor (TRF) - obtained from Con A-stimulated cultures, nor by the B cell mitogen, LPS. A positive response, ie antibody synthesis, was only obtained if TRF interacted with B cells before the immune complexes did. Schimpl et al (1977) have shown that TRF is able to bind to B cell FcR. Inhibition of the immune response, Kolsch et al (1980) suggested, occurs by immune complexes blocking the differentiation of B cells into plasma cells. The B cells were instead diverted to memory cell production. This might explain the observations of workers in the last century (reviewed by Uhr and Moller, 1968) who found that by injecting animals with an antibody-antigen mixture, the primary response was suppressed, but subsequent

challenge with antigen led to a full-blown secondary response. More recently, Safford and Tokunda (1971) have also found that whilst passive administration of antibody suppresses plaque cell formation, it does not suppress priming of B cells for a secondary response. Klaus (1978) has in fact demonstrated that priming with immune complexes was a hundred times more effective than antigen alone.

At present, the conflict between these two schools of thought (interference with T-B cell co-operation or B cell blockade) remains unresolved. It is possible that these concepts may not be mutually exclusive.

3. Factors affecting feedback suppression

The nature of Fc-mediated suppression is further complicated by a variety of other factors. For example, it has been found that the class or subclass used may affect the ability of the antibody to suppress. Henry and Jerne (1968) reported that IgM anti-SRBC antibody potentiated the plaque forming cell (PFC) response to SRBC, whilst 7S IgG was suppressive. Similarly, Collisson et al (1983) found anti TNP SRBC responses to be enhanced by IgM and where suppression has been observed with this class of immunoglobulin, it is usually reported to be much lower than that found with IgG (reviewed Uhr and Moller, 1968). In mice, IgG_1 was found to be suppressive at all doses tested, whilst suppression of the SRBC response by IgG_2 was only seen at high concentrations (Murgita and Vas, 1972). Low doses of IgG_2 were found to enhance the response. Thus the concentration of antibody may also influence the feedback process.

Interestingly, anti-idiotypic antibody has also been shown to require the Fc portion for suppression to occur (Weigle and Berman, 1979). Like other antibodies, it also appears to be subject to variations produced by different subclasses of immunoglobulin. For example, Eichman (1974) reported that the immune response in mice was suppressed by guinea pig anti-idiotypic antibody belonging to the IgG_2 class, whilst the use of IgG_1 resulted in enhancement.

The mechanisms whereby the immune response is enhanced have not been as well researched as those producing suppression. However, enhancement has been observed with both particulate and soluble antigens, often occurring at low concentrations of antibody (Walker and Siskind, 1968; Murgita and Vas, 1972). The Fc portion appears to be involved. Some authors suggest that it is the relative concentrations of antigen and antibody in the complex which dictate whether suppression or enhancement occurs (reviewed Uhr and Moller, 1968; Weigle and Berman, 1979). Furthermore, the immune status of the individual may play a determining role (Weigle and Berman, 1979). In mice immunized with Fab fragment of human IgG in complete Freund's adjuvant and their cells challenged with antigen 6 weeks later, it was found that the anti-Fab response was weak but this was enhanced by the addition of Fc fragment. In another group of immunised mice, which was boosted 7 days before testing, it was found that a strong anti-Fab response was obtained which was markedly suppressed by Fc fragment.

As well as an immunoregulatory role, antibody in the form of immune complexes or Fc fragment has been shown to induce proliferation in both mouse and human cells (reviewed Weigle and Berman, 1979), which again is affected by class/subclass variations. Morgan and Weigle (1983) reported that Fc fragment is also able to

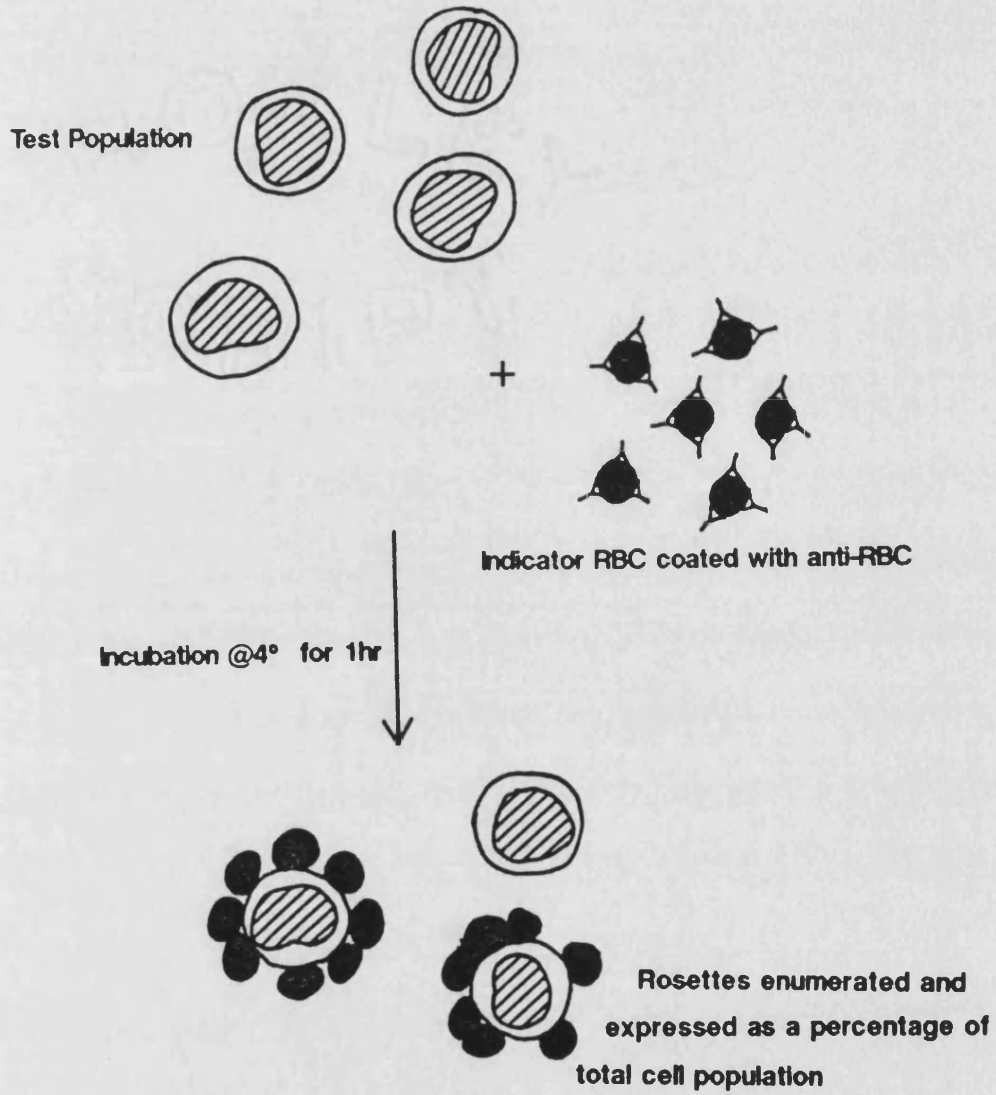
act as an adjuvant in the immune response, an effect not mediated by intact IgG. The adjuvanticity resides in a 14,000 dalton subfragment derived from the Fc fragment by macrophages.

4. Occurrence of FcR

In view of the effects that antibody has on the immune response, especially since the Fc portion appears necessary for the mediation of these effects, it is hardly surprising that many of the cells of the immune system express receptors for the Fc portion of immunoglobulin. These include B cells (Dickler and Kunkel, 1972), T cells (Lee and Paraskevas, 1972), monocytes (Lawrence et al, 1977), macrophages (Berken and Benacerraf, 1966), PMN (Gale and Ziegelbaum, 1975) and platelets (Becker and Henson, 1973). Fc receptors are also found in placental syncytiotrophoblasts, yolk sac endodermal cells and neonatal entereocytes (reviewed Fornusek and Vetvicka, 1984) as well as on connective tissue and epithelial tumours (Kerbel and Davis, 1974). The ubiquitous FcR has also been demonstrated on Staphylococci and Streptococci (Langone, 1982). In the former group, the strain Staphylococcus aureus is used as a source of protein A, which has a high IgG binding capacity and is employed in affinity chromatography.

The term 'Fc receptor' usually refers to the Fc receptor for IgG. However, FcRs have been described for all five classes of immunoglobulins (Moretta et al, 1976; Lum et al, 1979; Yodoi and Ishisaka, 1979; Sjoberg 1980). The assay usually employed is a rosetting technique where the cell population being examined is mixed with indicator red blood cells coated with specific antibody of the relevant class. This is illustrated in Figure 3. Immune complexes or aggregated immunoglobulins carrying either fluorescent

FIG 3 Rosette assay for detecting FcRs on cell populations



or radioactive tags have also been used. This methodology has been excellently reviewed by Andersson et al (1981). Monoclonal antibodies have also been used to demonstrate FcR (reviewed Anderson and Looney, 1986).

The relative distribution of FcRs on different lymphoid populations varies from report to report (reviewed Dickler, 1976; Unkeless et al, 1981). This is probably due to the varying techniques used and the Fc receptors exhibiting different binding affinities for different classes or subclasses of immunoglobulin. For example, in man, IgG_1 and IgG_3 subclasses are more strongly bound than IgG_2 and IgG_4. FcRs also vary in their ability to bind aggregated or monomeric Ig (see next section).

Despite such difficulties, estimates of the distribution of FcR on various cell populations do exist. A large percentage (80 - 95%) of B cells in peripheral blood bear FcR (reviewed by Fornusek and Vetvicka, 1984), whilst all active killer (K) cells, some null cells and a smaller percentage ($\backsimeq$ 30%) of T cells are FcR^+. This distribution changes with alteration of the activity of the lymphocyte. For example, whilst immature B cells are FcR^+ (Moller, 1974), antibody forming cells lose their Fc receptors (Basten et al, 1972). With T cells, activation is found to increase the percentage of FcR^+ cells. However, thoracic duct activated T lymphocytes do not bear FcR (Kramer et al, 1975).

5. Characterisation of FcR

Fc receptors are a group of glycoproteins which vary in molecular weight and Ig subclass specificity. Three main receptors have been described in man for IgG. These are FcRI, FcRII and FcRIII (or FcR_{10}) (see Table 3). These receptors vary in their ability to

bind IgG. Thus FcRI is of a high enough affinity to bind monomeric IgG, whilst aggregated or complexed immunoglobulin is required for detecting FcRII and FcR_{10}. Cells may express one or more receptor type. Thus macrophages may express all three FcRs whilst B cells and platelets may express only one, eg FcRII. The same receptor type may appear on different populations. Further heterogeneity is observed when enzyme sensitivity is examined: FcRI is found to be trypsin sensitive and FcRII trypsin resistant. Colombatti et al (1981) demonstrated that Fc_{γ} R on T and B cells were protease sensitive whilst those on accessory cells and PMN were not. Physical properties of these receptors are summarised in Table 3.

6. Functions of FcR^+ cells

FcR^+ cells are involved in antibody-mediated cell cytotoxicity (ADCC), Type 1 hypersensitivity reactions involving mast cells and FcR for IgE, placental transport of maternal IgG, phagocytosis, transport of biliary IgA by a specialised Fc receptor called secretory piece and, of course, immunoregulation.

FcR expression in RA has been reported to be increased in comparison to that observed in normal healthy controls by some authors (Sharpin and Wilson, 1977; Wooley and Panayi, 1978; Bach et al, 1970; Hall et al, 1980). This is found to be more pronounced in patients with systemic disease (Carter et al, 1984). Other authors, however, have observed no change (Burmester et al, 1978; Horwitz and Juul-Nielsen, 1977).

Similarly, assessment of FcR function in RA has produced conflicting data. FcR^+ function in the RA joint as measured by ADCC activity has been reported to be impaired compared to corresponding

TABLE 3 Characteristics of human IgG Fc receptors*

Characteristic	FcRI	FcRII	FcR_{lo}[a]
Molecule	72 kDa	40 kDa	50-70 kDa
Affinity for IgG monomer	$K_a=10^8-10^9 M^{-1}$	monomer binding undetectable	monomer binding undetectable
Sites/Cell	$1-4x10^4$		
Cells	monocytes (HL60, U937)	monocytes, neutrophils, eosinophils, platelets, B cells (U937, HL60, K562, Daudi, Raji)	neutrophils, eosinophils, macrophages, NK, K, LGL, T
Specificity, human	$IgG_1=IgG_3>IgG_4$; IgG_2 no	$IgG_1=IgG_3>IgG_2$ and IgG_4	$IgG_1=IgG_3$

[a] lo = low affinity

* derived from Anderson and Looney (1986)

PBMNCs (Carrigall and Panayi, 1978; Abrahamson et al, 1977; Diaz-Jouanen et al, 1976). This is believed to be due to the blocking of FcRs by immune complexes (Diaz-Jouanen et al, 1976). The other possibility is the loss of FcR from the cell surface following attachment of immune complexes, as suggested by Moretta et al (1978). These explanations may also suffice for depressed phagocytic activity observed in RA peripheral blood and in SF (Turner et al, 1973; Attia et al, 1982). Other workers, however, have reported increased (Jessop et al, 1973) or normal (Kavai et al, 1979) phagocytic activity. It is probable that these different observations result from variations in patient groups, disease activity and the techniques used. As far FcR function is concerned, one should ascertain that FcR-mediated phagocytosis is being measured, since C3b-mediated phagocytic activity also occurs. This is called immune adherence and is reported to be defective in RA (Hurst et al, 1983).

Although the role of FcR in immunoregulation has already been discussed, T cells with receptors for IgM and IgG deserve a special mention. These cells were originally thought to correspond to helper and suppressor cells respectively. This, however, was found not to be true since:

i) Tμ cells could produce suppression and $T\gamma$ cells were observed to provide help for the immune response;

ii) the same cell could express both $Fc\mu R$ and $Fc\gamma R$ and

ii) on incubation at 37°C Tμ cells could switch to $Fc\gamma R$ expression.

Pichler and Broder (1981) suggest that these receptors are markers for activation like the class II MHC antigens. Thus $Fc\mu R$ expression on T cells represents the resting state, whilst $Fc\gamma R$ the activated counterpart.

A product of activated T_γ cells is reported to be important in feedback suppression. It is found that when these cells are incubated with immune complexes, they release soluble factors called immunoglobulin binding factors (IBF) (Fridman and Golstein, 1974). These bind to the Fc portion of the IgG molecule and are able to insert themselves into the cell membranes of B lymphocytes (Fridman et al, 1981). IBF have been shown to participate in suppression of antibody synthesis in vitro (Gisler and Fridman, 1975). Thus while a T cell product - TRF - can interrupt antibody feedback suppression, IBF can enhance it.

It is clear from the above that in order to examine immunomodulation of the immune response by antibody, one needs strictly defined conditions and reagents. For this reason it was decided that antibody-mediated feedback suppression in RA should be carried out using a defined FcR-bearing population. The present work is concerned mainly with feedback suppression mediated by MNC carrying a receptor for the Facb fragment of IgG. These cells were first described in our laboratory using a modified rosette assay. Work carried out by previous workers is reviewed in Section 3, together with the aims of the present study.

SECTION 3 FacbR$^+$ CELLS IN RA; AIMS OF THE PROJECT

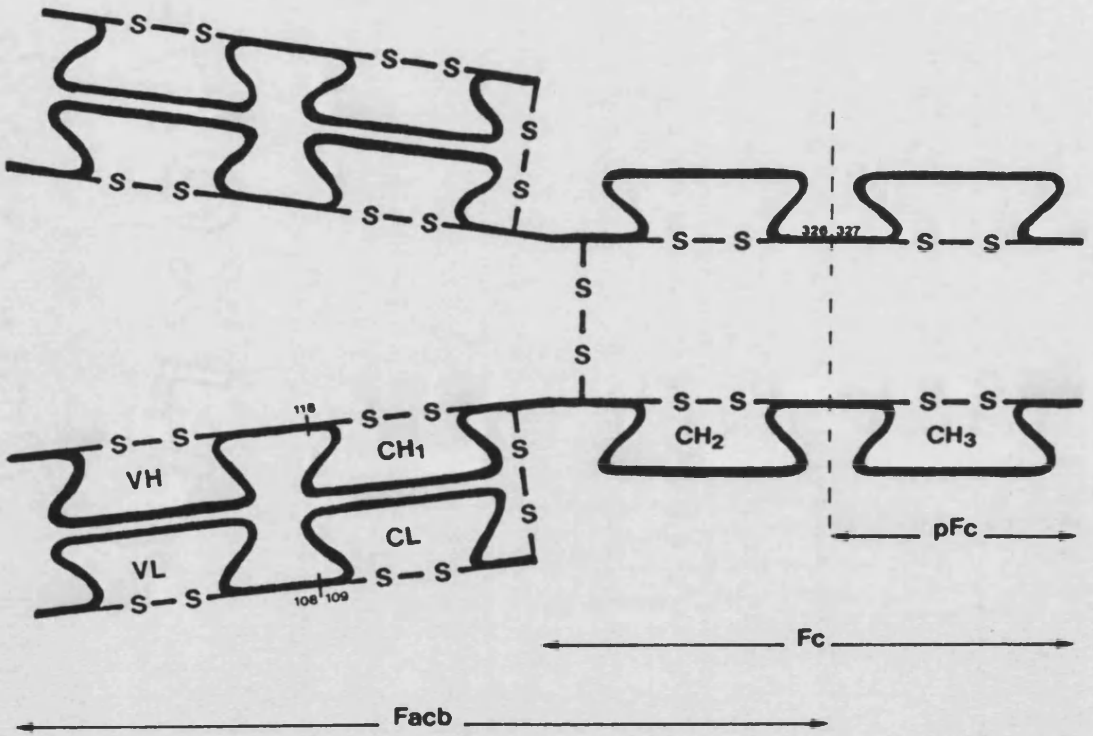
Recently, a subpopulation of MNCs has been described which carries receptors for the Facb fragment of IgG (Hall et al, 1980). Facb is essentially the IgG molecule minus the CH_3 region, as shown in Figure 4. It is enzymatically produced through the use of plasmin.

Indications for an unusual FcR came from earlier studies examining leucocyte migration inhibition in patients with RA (Hall, 1978). It was found that Facb was a much more effective inhibitor in the migration inhibition test than native IgG, suggesting an increased reactivity of RA cells to antigenic determinants in the CH_2 region. An abnormal cellular response to IgG had already been demonstrated by previous workers (Froland and Gaarder, 1971; Brostoff et al, 1973; Weisbart et al, 1975).

Using a modified rosette assay, where Facb instead of the whole IgG molecule was used to coat indicator RBC, Hall et al (1980) found that patients with RA had significantly higher numbers of FacbR$^+$ cells compared to normal healthy controls. Furthermore, patients with OA or ankylosing spondylitis did not differ from controls, although the mean percentage value of FacbR$^+$ cells in the latter was intermediate between those obtained for normal individuals and patients with RA. These results suggested that the increase in FacbR$^+$ cells was not due to old age and mechanical wear and tear but to the rheumatoid disease process. However, no correlation was found between disease activity, disease duration and levels of IgG and IgM RhF and the numbers of FacbR$^+$ cells in these patients. These findings, however, were in agreement with other authors who had also reported increased numbers of FcR$^+$ cells in RA (Wooley and Panayi,

FIG 4 **Illustrating the Facb region of the IgG molecule**

The constant (CH_{1-3}; CL) and variable (V_H and V_L) domains are shown, as are inter- and intra-chain disulphide bonds (-s—s-). ----- denotes the point of cleavage by plasmin.



1978; Bach et al, 1970; Sharpin and Wilson, 1977).

Preliminary experiments showed that these receptors were not expressed on mature monocytes since incubation with carbonyl iron did not affect the numbers of $FacbR^+$ cells present in the peripheral blood.

Thus further studies were undertaken to define the phenotypic, morphological and functional characteristics of $FacbR^+$ cells.

1. Phenotypic analysis

Monoclonal antibodies and a variety of rosetting and lymphocyte fractionation techniques were used to investigate the surface phenotype of $FacbR^+$ cells.

Winrow et al (1985) showed that $FacbR^+$ cells did not belong to the T cell population. They did not form E rosettes with AET treated sheep RBC, nor did they express CD3, a T cell specific marker. Human T cells are known to exhibit a receptor for the helix pomatia lectin following neuraminidase treatment. $FacbR^+$ cells were found to express no such receptors.

Neither did these cells carry any B cell markers. There was no sIg as indicated by direct immunofluorescence, FACS analysis and anti-light chain rosette formation. Nor was there any evidence of C3 receptors.

Thus, $FacbR^+$ cells appeared to be similar to the 'third population', 'L' or 'null' cells described by various workers (Froland et al, 1974; Horwitz and Juul-Nielsen, 1977; Lobo, 1981).

However, $FacbR^+$ cells were found to bind to the monocyte monoclonal antibody OKMI and to a lesser extent OKIa1, which defined HLA-DR antigens. Further analysis showed that these cells were able to bind to the monoclonal reagent 7.2 but not to 17.5. The former

detects an HLA-DR framework antigen present on both lymphocytes and monocytes, whilst the latter reacts only with an antigen expressed by lymphocytes carrying certain HLA-DR haplotypes.

FacbR$^+$ cells therefore have a myelo-monocytic lineage, but are not mature monocytes since they have been shown not to phagocytose carbonyl iron (Hall et al, 1980). These cells are thus different from L cells which are believed to be lymphocytes. This would explain why L cells were not found in increased numbers in RA peripheral blood (Horwitz and Juul-Nielson, 1977) whilst FacbR$^+$ cells were (Hall et al, 1980).

2. Morphology of FacbR$^+$ cells

Expression of both monocytic characteristics and class II MHC antigens suggested that FacbR$^+$ cells may be associated with accessory cell function. Electron microscope studies showed that these cells did indeed share morphological similarities with other accessory cells such as dendritic and Langerhans cells. They possessed a deeply cleaved nucleus and the cytoplasm contained numerous ribosomes. Mitochondria were also present, being more numerous in cells from RA patients. The latter also contained polysomes and well developed Golgi apparatus. Cytoplasmic fibrillar structures reminiscent of myeloid cells were also present (Eales, PhD thesis, 1982).

FacbR$^+$ cells were smaller than monocytes (10.9 μm) but RA FacbR$^+$ (8.2 μm) were significantly larger than those obtained from healthy controls (5.1 μm). This was due to the presence of a greater amount of cytoplasm in the former, as indicated by a smaller cell:nucleus diameter compared to healthy controls. Comparison of cell densities showed that FacbR$^+$ cells from patients with active disease were less

dense compared to those obtained from normal healthy controls and patients with inactive disease (Eales, PhD thesis, 1982).

3. Functional characteristics of FacbR$^+$ cells

The similarity of FacbR$^+$ cells to L cells led to the examination of various functions attributed to the latter, namely natural killer and antibody mediated cellular cytotoxicity (Kay and Horwitz, 1980; Ozer et al, 1979; Horwitz and Garrett, 1977) and immunoregulatory effects on lymphocyte activity (Horwitz and Garrett, 1977; Carvalho and Horwitz, 1980; Caraux et al, 1982; Lobo, 1981; Tilden et al, 1983). Functional analysis was carried out by Eales et al (1984 and 1985).

i) FacbR$^+$ cells and NK and ADCC activity

NK and ADCC activities were examined using K562 erythroleukaemic line cells and IgG-coated Chang cells respectively. Release of ^{51}Cr from lysed cells was used as an indicator of cytotoxicity. Removal of FacbR$^+$ cells from RA and normal PBMNC preparations did not alter the NK activity measured at two lymphocyte:target cell ratios. Nor was ADCC affected. However, depletion of all FcR$^+$ cells using EA rosettes abrogated all ADCC activity.

ii) FacbR$^+$ cells and lymphocyte proliferation

Various authors have reported that L cells promote T cell proliferation (Horwitz and Garrett, 1977; Carvaux et al, 1982). Eales et al (1985) examined the role of FacbR$^+$ cells in lymphocyte proliferation in response to ConA, PHA and PWM stimulation. Depletion of FacbR$^+$ cells was not found to produce a significant effect on lymphocyte proliferation as assessed by ^3H-thymidine incorporation. Nor did addition of Facb fragment to PBMNC

cultures alter the proliferative response.

Thus, FacbR$^+$ cells are not important in T cell proliferative responses, data which correlate with observations in mice showing that FacbR$^+$ cells did not participate in DTH reactions (Eales et al, 1984).

iii) FacbR$^+$ cells and immunoglobulin synthesis

L cells have been reported to have both enhancing and suppressing effects on antibody production (Lobo, 1981). The role of FacbR$^+$ cells in modulating the immunoglobulin synthesis was thus examined. Initial experiments were carried out in mice, where the primary and secondary PFC responses to sheep RBC in the spleen were investigated. It was found that while the primary response to SRBC produced only direct PFCs, the secondary response resulted not only in an indirect and direct PFC response but also produced a marked increase in the number of FacbR$^+$ cells which peaked on Day 3 of the response. A similar response was obtained when a cross-reacting antigen, goat RBC, was used, but no secondary response was observed with a non-cross-reacting antigen (chicken RBC).

These results suggested that this increase in FacbR$^+$ cells was in some way connected with a secondary response. These data correlate with previous work (Eales et al, 1982) where an increase in FacbR$^+$ cells was observed in the circulation of Mantoux test positive subjects but not in Mantoux test negative individuals after challenge with tuberculin. So the rise in FacbR$^+$ cells was related either to the generation of memory cells or to the synthesis of IgG.

Studies with lipopolysaccharide showed that a similar increase in FacbR$^+$ cells was produced during the primary and

secondary responses to this antigen. LPS is a T-independent antigen and thus does not generate memory cells. This therefore lead to the conclusion that the increase in FacbR$^+$ cells was related in some way to IgG synthesis.

Modulation of $Fc_\gamma R$ on L cells by immune complexes has been shown to suppress the antibody response (Lobo, 1981). Experiments were set up to investigate the effect of using different fragments of IgG as an antigen-specific response.

Mice sensitised with calf RBC (cRBC) were challenged with cRBC alone, cRBC plus anti-cRBC IgG, cRBC plus anti-cRBC Facb or cRBC plus anti-cRBC $F(ab')_2$ fragment. These experiments showed that Facb was able to suppress the PFC response to the same extent as the whole IgG molecule whereas the $F(ab')_2$ fragment had no effect at all. This suppression was highly specific and could not be reproduced with a cross-reacting antigen such as SRBC. Thus FacbR$^+$ cells were involved in negative feedback suppression.

So the increase in the numbers of FacbR$^+$ cells seen in mice during the secondary response and in patients with RA may well reflect an attempt by the immune system to limit the production of IgG. But in view of the B cell hyperactivity and auto-antibody production just how receptive are the RA cells to this suppression?

4. FacbR$^+$ cells and negative feedback suppression in normal healthy controls and RA patients

Eales et al (1985) examined Facb suppression of IgG production in vitro using PBMNC from normal healthy controls and patients with RA. A dose-dependent suppression of the IgG response was produced in

both control subjects and patients. However, the latter were found to be significantly less suppressed compared to the normal healthy controls.

This suppression could be due either to the activation of a suppressor mechanism or to blocking of accessory cell function. Depletion of $FacbR^+$ cells resulted in a dramatic fall in IgG synthesis. This was not altered in any way by addition of Facb fragment. Furthermore, the lack of an IgG response was not due to a loss of monocytes, since these were still present, shown by non-specific esterase staining.

Removal of suppressor function would have resulted in an enhanced IgG response so the results above correlate with a blocking of accessory function by Facb fragment. These data are clearly in agreement with the phenotypic characteristics of the $FacbR^+$ cell. Furthermore, these results support Hoffman's postulate (1980) that antibody-mediated feedback suppression occurs through interference with T and B cell co-operation.

Aims of the project

In a chronic disease such as RA it is usually not clear whether defects in immunoregulation are secondary to the disease process or actually serve to initiate it (or at least allow its establishment). One way to answer this is to examine patients with early disease. However, diagnosis of RA at this stage is notoriously difficult. The availability of a technique which clearly differentiates patients with RA from others with non-RA arthropathies would be of immense benefit to both the clinician and the patient. Early accurate diagnosis would mean early treatment, which might reduce the risk of severe, disabling disease.

The aims of the present investigation were twofold:- first, to examine Facb-mediated negative feedback suppression of antibody synthesis in patients with early RA; second, to evaluate the potential of the assay system as a tool for determining disease specificity.

Patients with early seropositive, seronegative or palindromic RA were investigated and compared to individuals with established disease. A very small number of Felty's patients were also investigated. Control groups included normal healthy controls as well as patients with ankylosing spondylitis (AS) and osteoarthritis (OA).

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

Materials	Suppliers
All chemicals except where otherwise stated	British Drug Houses Ltd Poole, Dorset
All reagents for media and tissue culture plastics	GIBCO Biocult Ltd Paisley, Scotland
All antisera and reagents for the ELISA technique except where otherwise stated	Sigma Chemical Co Poole, Dorset
All enzymes: Pepsin (from hog stomach 1:60,000 2 x crystallised and lyophilised) Plasmin (from human plasma, lyophilised)	Sigma Chemical Co Poole, Dorset
γ-IFN	Biogen Research Corp, Cambridge, Mass. USA
Flexible, polyvinyl chloride flat bottom plates, M29	Dynatech Laboratories Ltd, Billinghurst, Sussex

Rigid polystyrene M129A plates	``
Rigid polystyrene M129B plates	``
Rigid polystyrene Nunc Immunoplate	GIBCO Biocult Ltd Paisley, Scotland
Pokeweed mitogen (lyophilised; crude preparation from phylolacca americana)	GIBCO Biocult Ltd Paisley, Scotland
Concanavalin A (lyophilised from Jack bean Canavalia ensiformis)	Pharmacia (GB) Ltd Milton Keynes, UK
DEAE Sephadex A50	Pharmacia (GB) Ltd Milton Keynes, UK
Ficoll paque (density 1.077 +/- 0.001 g/ml)	Pharmacia (BGB) Ltd Milton Keynes, UK
Protein A Sepharose 4B	Pharmacia (GB) Ltd Milton Keynes, UK
Sephadex G100 (fractionation range 4,000 - 150,000 daltons)	Pharmacia (GB) Ltd Milton Keynes, UK
Agarose (Type 1 low EEO)	Sigma Chemical Co Poole, Dorset

Bovine serum albumin (<1% immunoglobulin)	Sigma Chemical Co Poole, Dorset
Casein	Sigma Chemical Co Poole, Dorset
Calf RBC in Alsevers solution	Tissue Culture Services Slough, Berks
'Tago' affinity purified antihuman IgM alkaline phosphatase conjugate	Tissue Culture Services Slough, Berks
Heparin sodium (mucous) 'monopurin' 1100 units/ml	Weddell Pharmaceutical Ltd Wrexham, UK
Phytohaemagglutinin (purified from Phaeseolus supp. lyophilised)	Wellcome Foundation Ltd Dartford, Kent
Rheuma-Wellcotest	Wellcome Foundation Ltd Dartford, Kent

EQUIPMENT

Equipment	Supplier
Amicon stirred cells and Diaflo filtration membranes	Amicon Ltd Stonehouse, Glos
Rotary turntable	Baird and Tatlock PO Box 1, Romford, Essex
Dynatech automatic microELISA reader Mk 580	Dynatech Instruments California, USA
Laborlux 12 microscope	E Leitz Ltd Luton, UK
LKB 6520 recorder	LKB Instruments Ltd South Croydon, Surrey
LKB Bromma 7000 Ultrorac fraction collector	LKB Instruments Ltd South Croydon, Surrey
LKB Bromma 8300 Uvicord II detector unit	LKB Instruments Ltd South Croydon, Surrey
LKB 12000 Varioperpex peristaltic pump	LKB Instruments Ltd South Croydon, Surrey

Power pack and electrophoresis tank	Shandon Southern Products Ltd, Astmoor, Runcorn, Cheshire

ROUTINE BUFFERS AND MEDIA

A. CELL CULTURE

i) Calcium and magnesium-free salt solution (CMFSS)

	g/l
Sodium chloride	8.00
Potassium chloride	0.40
Glucose	1.60
Potassium dihydrogen phosphate	0.06
Disodium hydrogen phosphate	0.048

The above were dissolved in triple distilled water, the pH adjusted to 7.3 with 1M NaOH and the volume made up to 1 litre. The solution was then filter sterilised and stored at 4°C until use.

ii) Cell culture medium RPMI 1640 (+10% FCS)

RPMI 1640 (x 10 strength)	100 ml
Heat inactivated foetal calf serum	100 ml
Penicillin and streptomycin solution (5000 units/ml)	20 ml
200 mM L-Glutamine	20 ml
7.5% sodium bicarbonate solution	27 ml

The above reagents were diluted to 1 litre with sterile triple-distilled water and the pH adjusted to 7.3 using sterile 3M NaOH. The medium was filter sterilised and stored at 4°C until required.

iii) Cell counting fluid

Glacial acetic acid	3 ml
Methylene blue	0.1 g
Distilled water	97 ml

The above constituents were mixed and stored at room temperature.

B. COLUMN CHROMATOGRAPHY

i) 10/150 mM phosphate buffer, pH 7.4

0.1M Na_2HPO_4	(17.8 g/l)	A
0.1M KH_2PO_4	(13.6 g/l)	B

Solutions A and B were mixed with stirring to give a buffer of pH 7.4. This was diluted 10-fold in distilled water. To each litre of dilute solution was added 8.77g sodium chloride.

ii) 0.1M acetate buffer, pH 4.5

Stock solutions containing 0.82g sodium acetate in 100 ml distilled water (solution A) and 1.15 ml glacial acetic acid in 200 ml distilled water (solution B) were prepared. Solution B was then added to solution A until the pH was 4.5. This buffer was then used to prepare a 6M urea solution for use in the preparation of Facb.

iii) 0.01M phosphate buffer, pH 6.5

0.01M Na_2HPO_4	(1.78 g/l)	A
0.01M KH_2PO_4	(1.36 g/l)	B

Solutions A and B were mixed to give a buffer of pH 6.5

C. ELISA

i) Coating buffer, pH 9.6

	g/l
Sodium carbonate	1.59
Sodium bicarbonate	2.93
Sodium azide	0.20

The above were dissolved in 1 litre of distilled water, the pH checked and the solution stirred at 4°C.

ii) Phosphate buffered saline (PBS), pH 7.4

	g/l
Sodium chloride	8.0
Potassium dihydrogen chloride	0.2
Disodium hydrogen phosphate dodecahydrate	2.9
Potassium chloride	0.2
Sodium azide	0.2

The above were dissolved in 1 litre of distilled water and stored at room temperature until use.

iii) PBS-Tween (PBST)

To 1 litre of PBS was added 0.5 ml Tween 20 (polyoxyethylene sorbitan monolaurate). The solution was stored at room temperature until required.

iv) PBS-1% BSA

1g of bovine serum albumin (BSA) was dissolved in 100 ml of PBS. This solution was prepared fresh and diluted 1 in 2 with PBS to give

PBS-0.5% BSA which was then used for diluting test samples for IgG and IgM ELISA.

v) Diethanolamine buffer, pH 9.6

Diethanolamine	97 ml
Distilled water	800 ml
Sodium azide	0.20 g
Magnesium chloride hexahydrate	0.10 g

The above were mixed and the pH adjusted to 9.6 with 1M hydrochloric acid. The final volume was made up to 1 litre and the solution stored in an amber bottle at room temperature.

D. IMMUNOELECTROPHORESIS

i) Barbitone buffer, pH 8.4 - 8.6

	g/l
Barbitone	4.14
Barbitone sodium	26.30

The above were dissolved in 1.5 litres of water, heated to aid dissolution. The buffer cooled, 0.4 g of sodium azide was added and the volume made up to 2 litres and stored at 4°C until required.

ii) 1% Agarose in barbitone buffer

1g of agarose (Type 1; low EEO) was placed in a conical flask containing 90 ml of barbitone buffer. The mixture was boiled until the agarose lumps dissolved completely. The solution was then made up to 100 ml with barbitone buffer and 10 ml aliquots were dispensed

into glass bottles. The gel was allowed to cool and stored at 4°C until required.

iii) Coomassie blue

Coomassie blue	1.25 g
Glacial acetic acid	50 ml
Distilled water	185 ml

Destainer

This is the same as the above solution but minus the Coomassie blue.

E. MODIFIED LOWRY METHOD FOR PROTEIN ESTIMATION

Stock solutions

i) Copper tartrate

50 mg of $CuSO_4$ was dissolved in 10 ml of 1% w/v sodium potassium tartrate. This solution was then added to 100 ml of 10% w/v sodium carbonate in 0.5M NaOH.

ii) Folin and Ciocalteau phenol reagent

This is available commercially and was diluted 1 in 10 in distilled water immediately prior to use.

iii) Standard protein

20 mg of high grade purity BSA were dissolved in 100 ml of distilled water to give a 200 ug/ml solution.

1. PREPARATION OF HUMAN IgG AND ITS FRAGMENTS

a) Preparation of IgG

Two sources of human IgG were used: one, a pool of normal human serum and the other, the serum of an IgG1 class myeloma patient. Ion exchange chromatography using DEAE-Sephadex A50 was employed for both purifications.

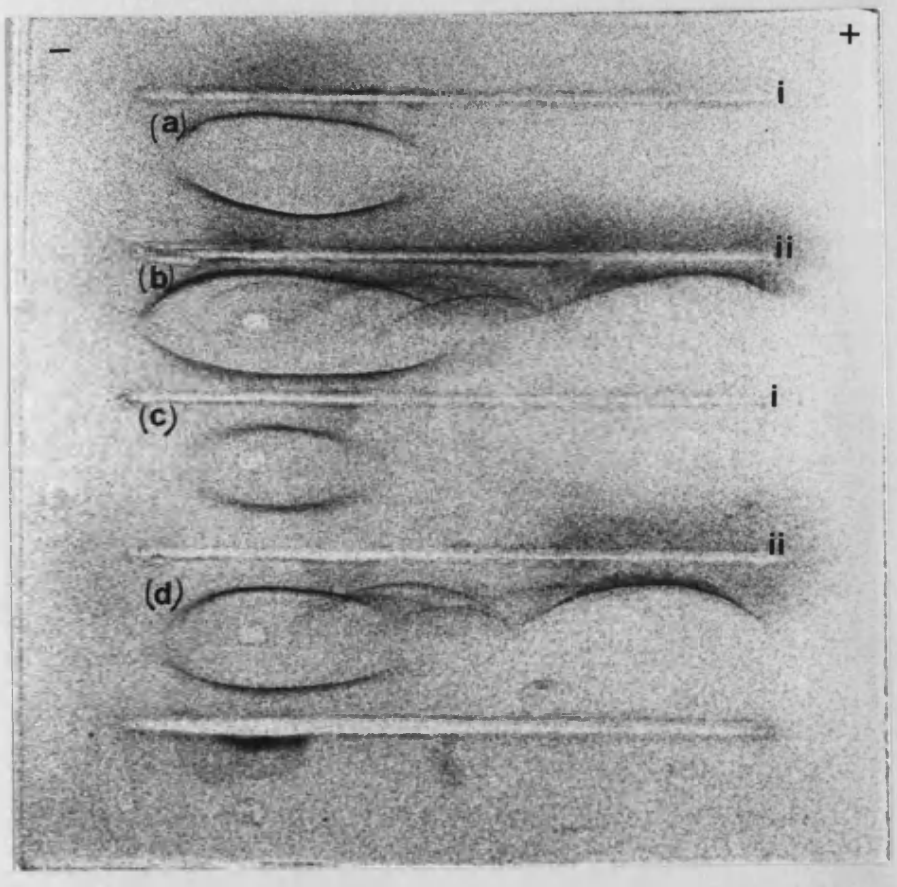
i) Purification of normal human IgG

A pool of normal serum was diluted x 10 in PBS and saturated ammonium sulphate solution added dropwise until 50% saturation was achieved. The serum-ammonium sulphate mixture was left stirring for one hour at room temperature to equilibrate before the immunoglobulin-rich precipitate was harvested by centrifugation at 1000g for 15 minutes. This was then dissolved in 0.01M phosphate buffer, pH 6.5 and dialysed first against distilled water and then against several changes of 0.01M phosphate buffer at 4°C until the pH and the conductivity matched that of the buffer. The dialysate was centrifuged to remove any precipitate formed during dialysis and mixed with DEAE-Sephadex A50 equilibrated with 0.01M phosphate buffer, pH 6.5, for batch preparation of IgG.

The gel was left mixing for one hour and then washed with several aliquots of 0.01M phosphate buffer on a Buchner funnel, until the $O.D_{280}$ of the eluant, which was collected into a flask by suction, fell to near zero. The contents of the flask were concentrated by vacuum dialysis using the Amicon stirred cell ultrafiltration system. The protein content was then assessed by a modified Lowry assay. The IgG was shown to be pure by immunoelectrophoresis (IEP) (see Figure 1).

FIG 1 Immunoelectrophoresis of purified normal human IgG

A single immunoprecipitin arc **(a)** was obtained when IgG purified from a normal human serum pool was analysed by IEP. **(b)** shows the precipitin lines obtained when normal human serum was examined. **(c)** and **(d)** are the same as **(a)** and **(b)** respectively, but at 1:2 dilution.



i=Anti-Human IgG anti-serum

ii=Anti Whole Human Serum antiserum

The purified normal human IgG was aliquoted and stored at -20°C until required. This preparation was used in the rheumatoid factor assay.

ii) Purification of human myeloma IgG_1

30 ml of myeloma serum were extensively dialysed against 0.01M phosphate buffer, pH 6.5, centrifuged and applied to a 2.5 x 30 cm column of DEAE-Sephadex A50 which had been previously equilibrated with 0.01M phosphate buffer, pH 6.5. 10 ml fractions were collected. A single peak was obtained and this was concentrated by ultrafiltration over an Amicon Diaflo XM50 membrane (exclusion limit 50,000). Protein content and purity were assessed as above. Results of the IEP appear in Figure 2. The preparation was filter-sterilised using a 0.22 um filter, aliquoted and stored at -90°C until use.

Purified myeloma IgG_1 was used in experiments employing heat aggregated IgG (Hagg) and also for preparation of the IgG fragment $F(ab')_2$.

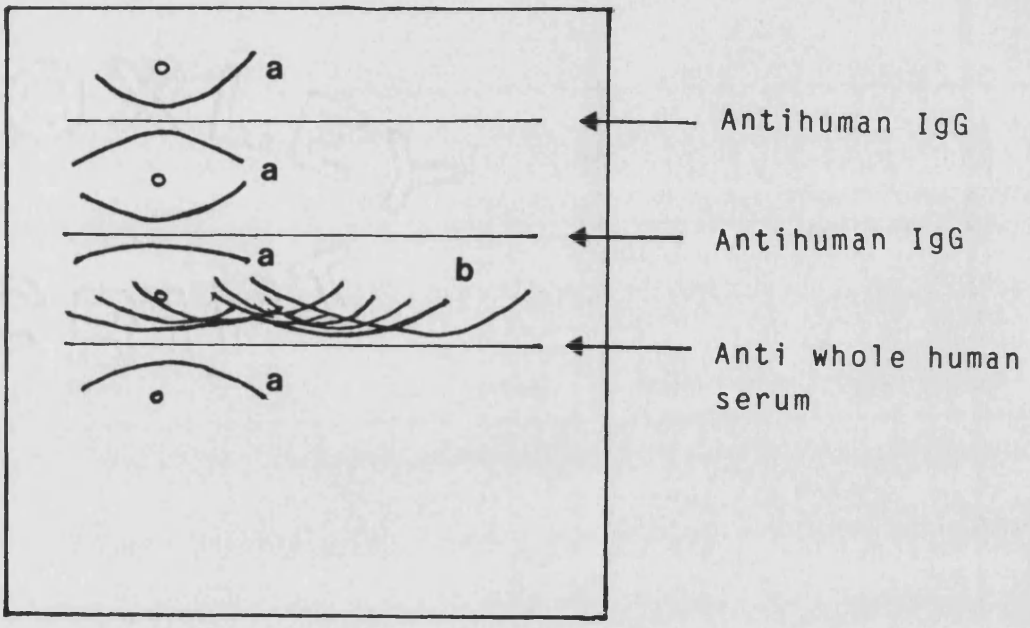
b) Preparation of $F(ab')_2$

This was prepared from purified IgG_1 using the method described by Nisonoff et al (1961).

IgG_1 was dialysed against 0.01M acetate buffer, pH 4.5, for 4 hours at 4°C. A solution of pepsin was prepared (1 mg/ml in 0.01M acetate buffer) and added to the IgG_1 dialysate to give a final concentration of 1:100. The solution was incubated at 37°C for 22 hours. The enzymic reaction was stopped by the addition of solid Tris (hydroxymethyl) methylamine to produce a pH of 8. The digest was then applied to a 1.5 x 100 cm Sephadex G-100 column equilibrated

FIG 2 Immunoelectrophoresis of purified human myeloma IgG_1

Purity of the isolated IgG_1 is indicated by the single immunoprecipitin arc **(a)** produced when the immunoglobulin was analysed by IEP. **(b)** shows the precipitin lines obtained with normal human serum.



with 10/150 mM phosphate buffer, pH 7.4. Undigested IgG and $F(ab)'_2$ both appear in the first peak, which was concentrated and applied to a protein A-Sepharose 4B column. The $F(ab)'_2$ was eluted off in the first peak using 10/150 mM phosphate buffer and the undigested IgG eluted in the second peak using 1M acetic acid. The $F(ab)'_2$ was concentrated and assayed for protein content as above. The preparation was filter-sterilised, aliquoted and then stored at -90°C until use.

2. HEAT AGGREGATED HUMAN IgG

Heat aggregated IgG (Hagg) was produced by incubating a 1 ml volume of purified IgG_1 (concentration 11.5 mg/ml) at 63°C for half an hour. This was prepared fresh for each experiment, just prior to use.

3. PREPARATION OF Facb FRAGMENT FROM RABBIT IgG

Facb fragment was prepared from a rabbit anti-calf RBC serum (a gift from Dr Hunneyball, Boots, Nottingham) using a modification of the method described by Stewart et al (1973).

The hyperimmune rabbit serum was applied to a 1 x 15 cm column of Protein A-Sepharose 4B equilibrated with 10/150 mM phosphate buffer, pH 7.4. Unbound material appeared in the first peak (Figure 3, peak (a)) and IgG was eluted in the second peak (Figure 3, peak (b)) using 1M acetic acid. The immunoglobulin fraction was concentrated by ultrafiltration and incubated for one hour at 37°C. The pH of the concentrate was then adjusted to pH 7 using 1M NaOH and the plasmin added (3 units/100 mg IgG). The mixture was incubated for 4 hours at 37°C with periodic mixing. After this period the digest was applied to a 1.5 x 100 cm Sephadex G-100 column,

equilibrated with 6M urea in 0.1M acetate buffer, pH 4.5. Using a flow rate of 8 ml/hr, 4 ml fractions were collected. Two peaks were obtained; undigested IgG and Facb appearing in the first peak (Figure 3, peak (c)), which was concentrated and dialysed against two changes of 10/150 mM phosphate buffer for 4 hours at 4°C. Peak (d) (Figure 3) contained the digested C_H3 domain and other smaller peptides. The IgG-Facb containing dialysate was then applied to Protein A-Sepharose 4B to effect separation of the Facb from undigested IgG. Facb is unable to bind to Protein A and thus appears in the first peak (Figure 3, peak (e)). Peak (f) (Figure 3) contains the undigested IgG. The Facb preparation was concentrated, the protein content determined immediately, and the solution was aliquoted and stored at -90°C until required.

All procedures were carried out at 4°C unless otherwise stated.

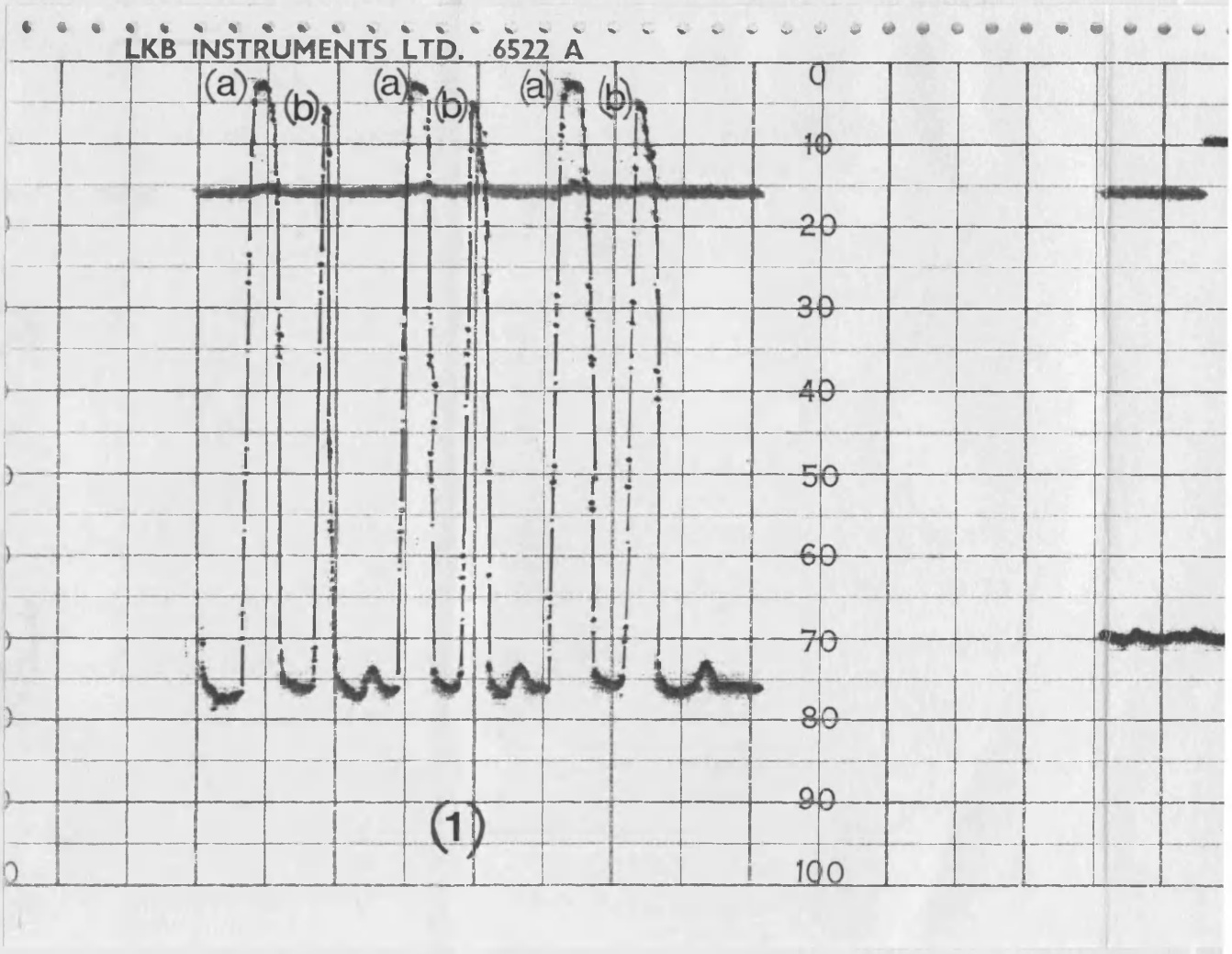
4. CELL CULTURE

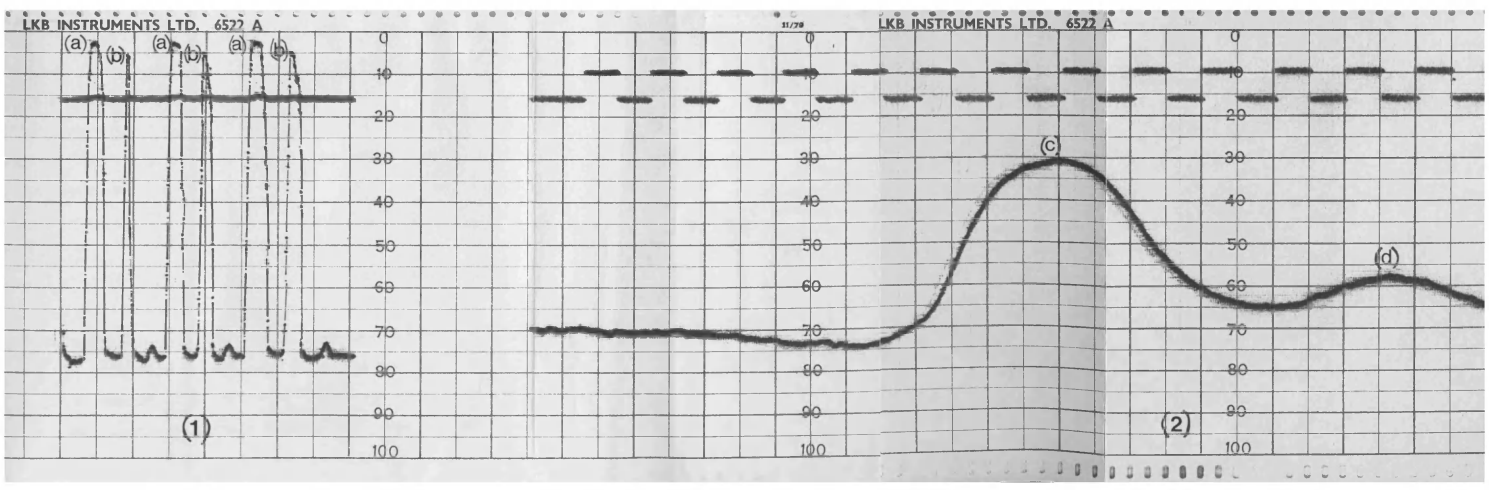
a) Preparation of peripheral blood mononuclear cells

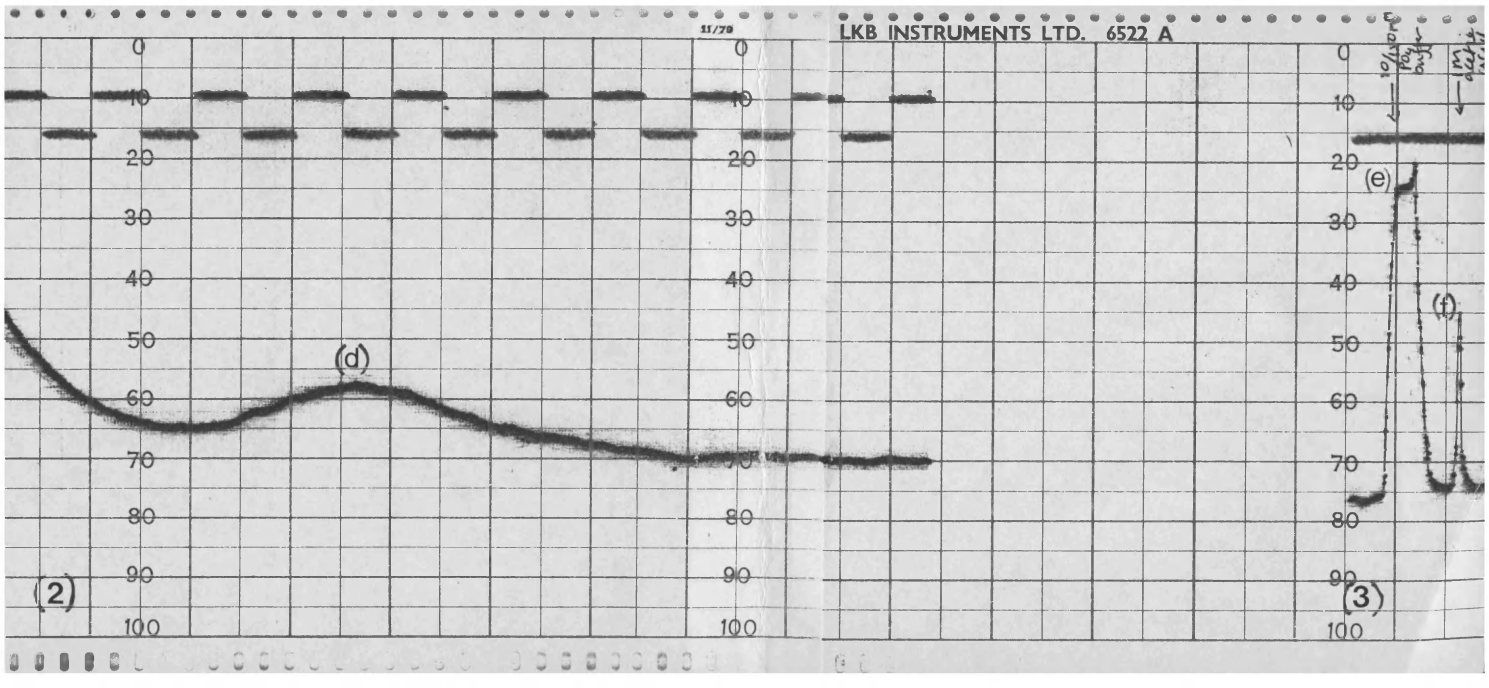
Peripheral blood mononuclear cells (PBMNCs) were isolated from heparinised (10 IU/ml blood) venous blood by density centrifugation as described by Boyum (1968). The blood was diluted 1 in 2 with CMFSS and approximately 7 ml layered onto 3 ml of Ficoll-paque in a sterile, polystyrene centrifuge tube. This was then centrifuged at 400 g for 30 minutes at 18°C. The mononuclear cell (MNC) layer which appeared as a thin white band at the plasma-Ficoll interface was harvested, washed three times in CMFSS and once in the RPMI 1640 (+ 10% FCS) culture medium,, before being counted and adjusted to either 1×10^6/ml or 1.11×10^6/ml as required. All cell experiments were carried out in sterile, large well, flat bottom culture plates at a final concentration of 1×10^6/ml. The plates were incubated at

FIG 3 **Preparation of Facb fragment from rabbit anti-calf RBC IgG**

The elution profiles of the chromatographic stages used in Facb preparation show:- (1) purification of rabbit anti-cRBC IgG using protain A-Sepharose 4B - peak **(a)** contains unbound material, peak **(b)** the rabbit IgG. (2) separation of Facb (peak **(c)**) and pFc (peak **(d)**) on G100 Sephadex, following plasmin digestion. (3) removal of undigested IgG (peak **(f)**) from Facb (peak **(e)**) using a protein A-Sepharose 4B column.




LKB INSTRUMENTS LTD. 6522 A
(a)
(b)
(1)
LKB INSTRUMENTS LTD. 6522 A
(c)
(d)
(2)
0
10
20
30
40
50
60
70
80
90
100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37°C in a humidified atmosphere containing 5% carbon dioxide.

Concentrations of reagents and conditions of culture described below were determined in a series of preliminary experiments, results of which appear in Chapter 3.

b) Facb cultures

0.9 ml volumes of washed PBMNC adjusted to 1.11 x 10^6/ml were aliquoted into culture plates and sterile Facb, diluted in medium, was added in 100 µl volumes to give final concentrations of 0.2, 2 and 20 µg/ml. 100 µl of medium was added to cultures not containing Facb. Pokeweed mitogen (PWM) was used to stimulate antibody synthesis. This was diluted in CMFSS and added in 20 µl volumes to give final reciprocal dilutions of 50, 200 and 1000. CMFSS was added to control cultures. The cells were cultured for 14 days and then the culture supernatants harvested by centrifugation. These were stored at -20°C before being assayed for IgG and rheumatoid factor (RhF), and in some cases, IgM.

c) Hagg cultures

PBMNCs adjusted to 1 x 10^6/ml were set up in 1 ml volume cultures +/- 5 µg/ml Hagg and +/- 1/200 final dilution of PWM. Cells were cultured for 5 days, harvested, washed three times in medium to remove the Hagg (which interferes with the ELISA) and then readjusted to 1 x 10^6/ml before culturing for a further 5 days. The supernatants were harvested by centrifugation and stored at -20°C before assaying for IgG and RhF.

d) Gamma-interferon (γ-IFN) cultures

The effect of γ-IFN on Facb- and Hagg-mediated suppression of

antibody synthesis was investigated in 4 normal individuals. Facb and Hagg cultures were set up as usual and cultured +/- a final concentration of 10 units γ-IFN/ml. Supernatants were assayed for IgG by ELISA.

e) 2-mercaptoethanol (2-ME) treated cultures

The effect of preincubating PBMNCs with the thiol-reducing agent 2-ME was examined. PBMNCs were isolated from fresh blood, washed as usual and incubated with 5 x 10^{-5}M final concentration of 2-ME for 1 hour at 37°C. The cells were then washed three times to remove the 2-ME and Facb and Hagg cultures set up as usual. The percentages of Facb rosetting cells were also determined before and after 2-ME treatment.

5. Facb ROSETTE CELL ASSAY

The Facb rosette cell assay was carried out as described by Hall et al (1980). Essentially, the assay consists of three steps as follows:-

a) Sensitisation of calf RBC

Calf RBC were washed three times in CMFSS and a 2% v/v cell suspension prepared. This was mixed with an equal volume of optimally diluted Facb and incubated at 37°C for half an hour. The sensitised cells (s-cRBC) were centrifuged and washed three times in CMFSS and a 1% v/v cell suspension prepared. This was kept on ice until required.

b) Incubation of PBMNC with sensitised calf RBC

PBMNCs were isolated as described above, washed three times in CMFSS and adjusted to 2 x 10^6/ml. 200 µl of this cell suspension

were mixed with an equal volume of 1% s-cRBC in an LP3 tube (Luckham Ltd, Sussex) and centrifuged at 400 g for 3 minutes at 4°C. The pelleted cells were incubated on ice immediately for 1 hour. Unsensitised cRBC were used as controls for non-specific rosetting.

c) Enumeration of Facb rosettes

After incubation a small amount of crystal violet was introduced into each tube and the cells resuspended on a rotating turntable inclined at an angle of 45° and operated at 20 rpm for 1 minute. The cells were then counted in a Neubauer improved haemocytometer. Any lymphocyte surrounded by 3 or more RBC was scored as a rosette. The results were expressed as a percentage of the total number of lymphocytes counted. A minimum of 200 lymphocytes were counted each time.

6. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

a) IgG and IgM ELISA

Flat bottom, polystyrene (Nunc immunoplate 1) microtitre plates were coated with an optimal dilution of goat anti-human polyvalent immunoglobulin in carbonate buffer, pH 9.6 for 18 hours at 4°C. The plates were washed in PBST and any available sites on the plastic blocked using 100 µg/well of PBS-1% BSA and incubating at 37°C for 1 hour. The blocking solution was removed by washing the plates in PBST, and IgG (or IgM) standard and test supernatants suitably diluted in PBS-0.5% BSA were then added to the appropriate wells and incubated for 1 hour at 37°C. This was followed by another wash in PBST. A 1/1000 dilution of goat anti-human IgG (or IgM) alkaline phosphatase conjugate in PBST was added in 100 µl volumes/well and the plates then re-incubated at 37°C for 1 hour. Unbound conjugate

was removed by washing the plates in PBST. The substrate, d-nitrophenyl phosphate, was diluted in diethanolamine buffer, pH 9.8, to a concentration of 1 mg/ml and added in 100 µl volumes/well and the plate incubated at room temperature until a predetermined IgG/IgM standard gave an absorbance value of approximately 1 at a wavelength of 405 nm. The plates were then read using the microELISA automatic plate reader. A standard curve of absorbance (E_{405}) against IgG/IgM concentration in ng/ml was constructed and immunoglobulin content of test samples determined using only the linear part of the curve. These values were then suitably corrected for dilution.

IgG and IgM standards

A normal serum pool (negative for RhF activity) was used as a source of IgG and IgM standards. The concentrations of these immunoglobulins were determined by nephelometry (by Mr K Case, Immunology Department, Royal United Hospital, Bath). The serum pool was aliquoted and stored at -90°C until required.

b) IgM rheumatoid factor ELISA

The conditions for the IgM RhF assay were essentially the same as those described above for the immunoglobulin ELISAs, with the following exceptions:-

i) Purified normal human IgG, at a concentration of 1 µg/ml in carbonate buffer, pH 9.6 was used to coat flexible, polyvinyl chloride microtitre plates.

ii) All test samples were tested neat and the RhF standard was diluted in medium.

iii) Anti-human IgM alkaline phosphatase conjugate was used to detect IgM RhF.

IgM RhF standard

IgM RhF had initially been purified from a strongly seropositive rheumatoid serum by affinity chromatography using purified human IgG linked to Sepharose 4B. This preparation was used to define some of the optimal conditions for the RhF ELISA. However, the activity of this material fell rapidly on storage and a second standard had to be selected.

A rheumatoid serum containing high titre RhF was used. This serum was repeatedly absorbed with Hagg and tested for RhF activity using the Rheuma-Wellcotest (a latex agglutination test) until no agglutination was observed. A normal serum was simultaneously absorbed with Hagg to determine the amount of IgM which was non-specifically removed during the absorption procedure.

The pre- and post-absorption sera were tested for RhF and IgM concentration by ELISA. The decrease in IgM concentration after absorption represented the amount of IgM RhF present in the serum. This figure was corrected for non-specific loss of IgM during absorption.

7. IMMUNOELECTROPHORESIS

10 ml of 1% w/v agarose (Type 1, low EEO) in a barbitone buffer, pH 8.6, were heated in a water bath until molten and then carefully poured onto a 8 x 8 cm glass plate resting on a levelling table. Once the gel was set, a pattern of wells and troughs was cut and only the gel from the wells was removed. 5 μl of test samples were placed into the appropriate wells. Normal human serum to which a small amount of bromophenol blue (BPB) had been added was always run as a control. The electrophoresis tank was filled with barbitone buffer and the gel connected to the buffer by means of paper wicks placed on

either side of the plate. The samples were electrophoresed under a constant voltage of 100V and the run stopped when the BPB had nearly reached the edge of the plate. Agarose was then removed from the troughs previously cut into the gel and the trough filled with 25µl of the appropriate antiserum. The plate was left overnight in a humid atmosphere at room temperature before being washed in three changes of 1M NaCl, once in distilled water and finally dried. The plate was then stained with Coomassie blue and differentiation obtained with destainer. Plates were finally dried in air and read.

8. MODIFIED LOWRY METHOD FOR PROTEIN ESTIMATION

Test samples and protein standard (200 µg/ml BSA solution) were appropriately diluted in 1 ml volumes and then 1 ml of copper tartrate added. The samples were mixed and allowed to stand for 10 minutes, after which 3 ml of diluted Folin and Ciocalteau's phenol reagent were added to each sample as quickly as possible. The samples were incubated at 50°C for 10 minutes, cooled and the absorbance measured at 650 nm. A standard curve of absorbance against protein concentrate was constructed and unknown values read off this and corrected for dilution.

9. PATIENT AND CONTROL GROUPS

Patients with early rheumatoid disease were drawn from the Early Synovitis Clinic at the Royal National Hospital for Rheumatic Diseases. These subjects were classified into early RA positive (mean age 55 years), early RA negative (mean age 54 years) and early palindromic RA (mean age 34 years) groups by Dr A D Woolf (Senior Registrar) and at each visit patients were assessed for disease activity.

All other patient groups were obtained from the clinics and wards at the hospital. The average ages of the established RA, OA and Felty's groups used in the Facb study were 56, 70 and 68 years respectively, and those of established RA, AS, OA and Felty's subjects used for the Hagg study were 62, 46, 68 and 74 years respectively.

At the time of investigation patients were either receiving no medication or only NSAIDs. Where second line therapy had been previously employed, only those patients with a wash-out period of not less than 8 weeks were used.

Normal healthy controls mean age 31 (for the Facb study) and 28 (for the Hagg study) were derived from laboratory and hospital personnel. Old normal subjects (mean age 69 years) were obtained from St Martin's Hospital, Odd Down, Bath, by courtesy of Dr R Jones.

10. STATISTICAL ANALYSIS

All data were analysed using the Mann Whitney U-test unless otherwise indicated. Correlations were conducted using Spearman's rank correlation test.

CHAPTER 3

RESULTS

The results presented in this chapter concern experiments carried out to determine optimal operating conditions for:-

1. The ELISA technique
2. Antibody synthesis *in vitro*.

SECTION 1 ELISA

The principles of enzyme-linked immunosorbent assays used to measure IgG and IgM and IgM RhF in culture supernatants are depicted in Figures 1 and 2. These assays had originally been established in Guilford microELISA cuvettes and then transferred to microtitre plates. Here optimal conditions were re-established using the IgM assay. Data from these experiments are presented below.

One of the first considerations was choice of solid support (ie type of plate) since although plastic nonspecifically adsorbs protein (Catt and Tredegar, 1967), different plastics do so to varying degrees. Four types of plates were examined (see Table 1) and of these the Dynatech 129B ELISA plates were found to be the best for both high protein adsorption and low inter- and intra- plate variation. However, these plates are sterile and therefore more expensive. Polyvinyl (PVC) plates were thus selected as the next best choice. Later IgG and IgM were carried out in Nunc immuno1 plates (Tissue Culture Services) but use of PVC plates was continued for RhF ELISAs as these plates exhibited a high adsorption of purified human IgG.

KEY TO FIG 1

= Goat antihuman polyvalent immunoglobulin

= IgG/IgM containing culture supernatants/standards

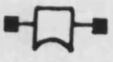
= Affinity purified antihuman IgG/IgM alkaline phosphatase conjugate

= d-nitrophenyl phosphate (native and degraded)

KEY TO FIG 2

= Pure human IgG

= IgM RhF containing culture supernatants/standards

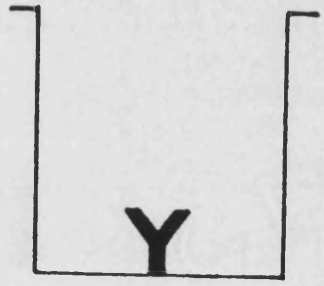


= Affinity purified antihuman IgM alkaline phosphatase conjugate

= d-nitrophenyl phosphate (native and degraded)

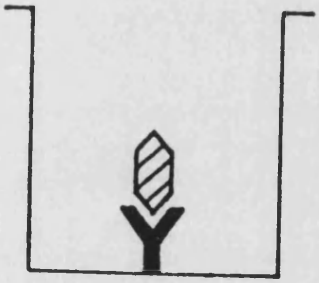
FIG 1 **Illustrating the double antibody ELISA used for quantifying IgG and IgM in culture supernatants**

*Excess binding sites on the solid phase were blocked using PBS-1% BSA before proceeding to step 2.



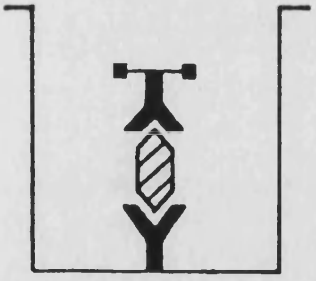
1. Antibody adsorbed to plate

*Wash



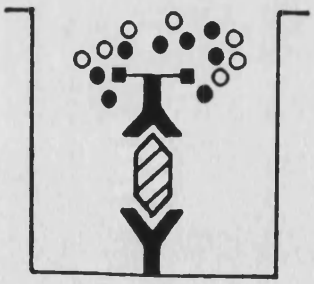
2. Test solution containing antigen added

Wash



3. Add enzyme labelled specific antibody

Wash

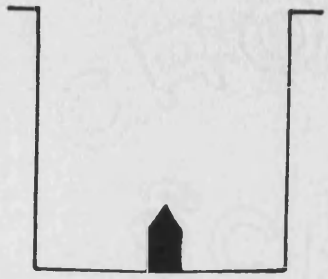


4. Add enzyme substrate

Amount hydrolysed = amount antigen present

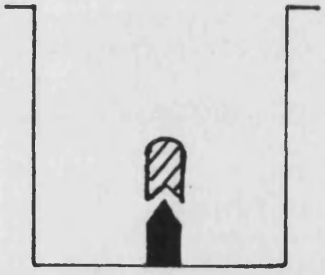
FIG 2 **Illustrating the indirect ELISA used for quantifying IgM RhF in culture supernatants**

*Excess binding sites on the solid phase were blocked using PBS-1% BSA before proceeding to step 2.



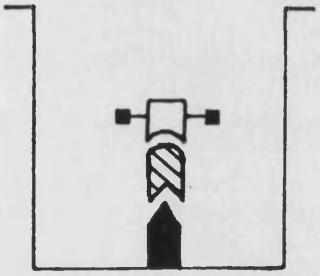
1. Antigen adsorbed to plate

*Wash



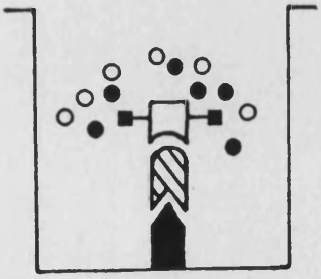
2. Add serum: any specific antibody attaches to antigen

Wash



3. Add enzyme labelled antiglobulin which attaches to antibody

Wash



4. Add substrate

Amount hydrolysed = amount antibody present

TABLE 1 **Showing the four types of plate tested for degree of protein adsorption and inter- and intra-plate variation when one concentration of IgM was incubated in all the wells of all the plates tested.**

Four microtitre plates of each of the types below were coated with goat antihuman polyvalent immunoglobulins antiserum and incubated with a given dilution of the IgM standard. The degree of adsorption (as indicated by absorbance values at 405 nm) and inter- and intra-plate variability of this were determined for each type of plate examined.

Plate tested	Average absorption	Average coefficient of variation within plates	Comparison of absorbancies between plates of the same type
Dynatech M129A	0.340	15.95%	$p > 0.05$
Dynatech M129B	1.147	7.30%	$p > 0.05$
Dynatech polyvinyl flexible	0.894	6.64%	$p > 0.05$
Nunc tissue culture plates	0.826	6.60%	$p > 0.05$

In the IgG and IgM assays goat antihuman polyvalent immunoglobulin antiserum was used to coat the solid phase. Dilutions ranging from 1/100 to 1/3200 were examined. The standard curves obtained are shown in Figure 3. No significant differences were found between these and a dilution of 1/400 was selected for future use.

In the IgM RhF assay ELISA plates were coated with 1-10 µg/ml pure human IgG. Tenfold dilutions of affinity purified RhF were used to construct standard curves. These are shown in Figure 4. A concentration of 1 µg/ml was selected for use in subsequent RhF assays.

Human IgG used in these assays was prepared from a pool of normal human serum by ion exchange chromatography on DEAE-Sephadex as described in Chapter 2.

For both assays, excess binding sites on plates were then blocked using PBS-1% BSA, shown to be optimal in preliminary experiments (data not shown).

Various solutions were tested for diluting the test samples/standards. Similar standard curves were obtained when PBS-Tween, medium and PBS-0.5% BSA were used as shown in Figure 5. With PBS the higher absorbances may be a result of some non-specific interaction, since the background was also found to be slightly higher with this diluent.

Since some supernatants had to be tested neat or at low dilutions, it was decided that a protein-containing solution such as PBS-0.5% BSA should be the diluent of choice, although PBS-Tween was equally as good and would have been cheaper. This would allow successive dilutions of the sample (culture supernatant containing 10%

FIG 3 **The effect of varying the dilutions of coating antibody on the IgM standard curve**

Microtitre plates were coated with 1/100 (○——○), 1/200 (□——□), 1/400 (●——●), 1/800 (△——△), 1/1600 (■——■) and 1/3200 (▲——▲) final dilutions of goat polyvalent antihuman immunoglobulin antiserum. An IgM standard curve was then constructed for each dilution used. Each point is a mean of two determinations.

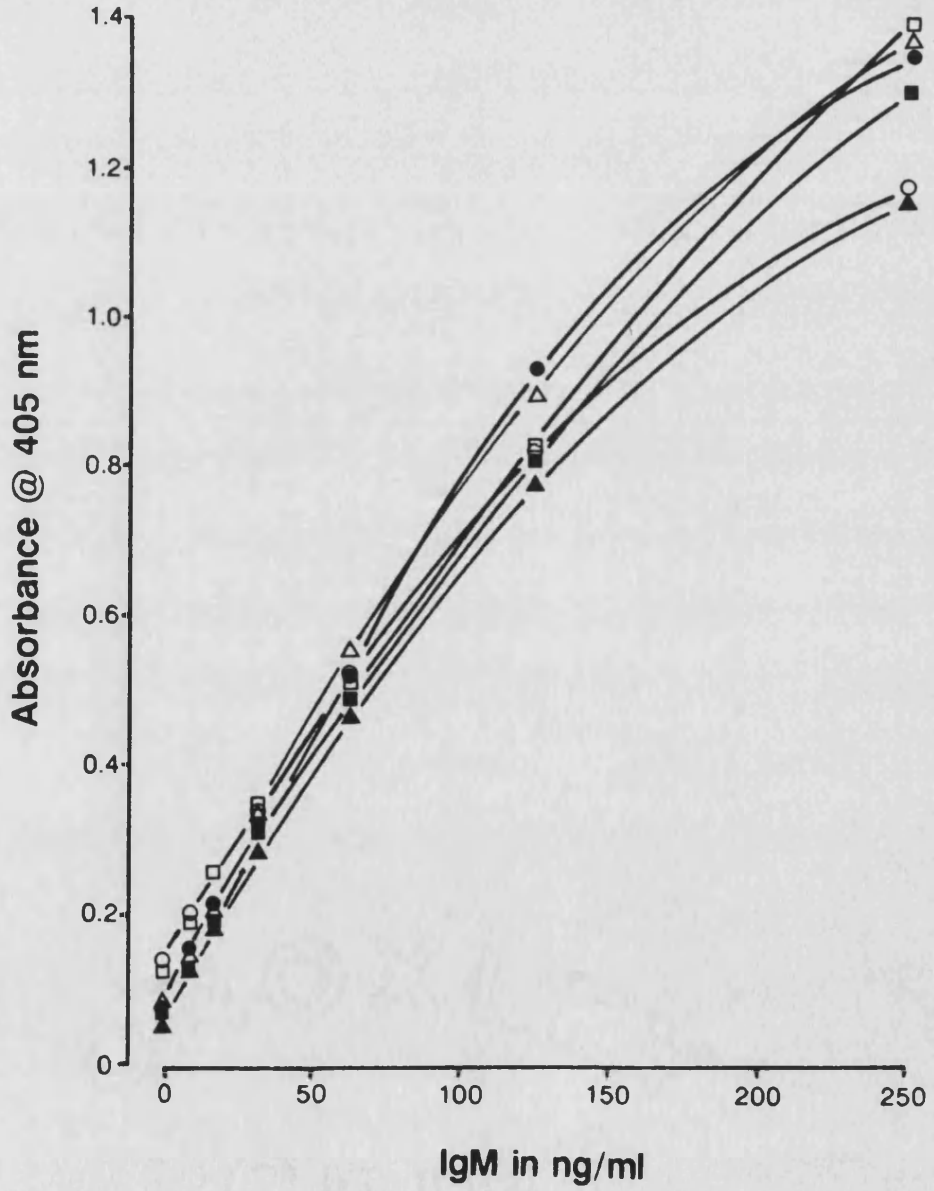


FIG 4 **The effect of varying coating concentrations of pure human IgG on the IgM RhF standard curve**

Microtitre plates were coated with 1 (●—●), 2.5 (□—□), 5 (○—○) and 10 (△—△) µg/ml IgG. Affinity purified IgM RhF was used as a standard. Each point represents a mean of two determinations.

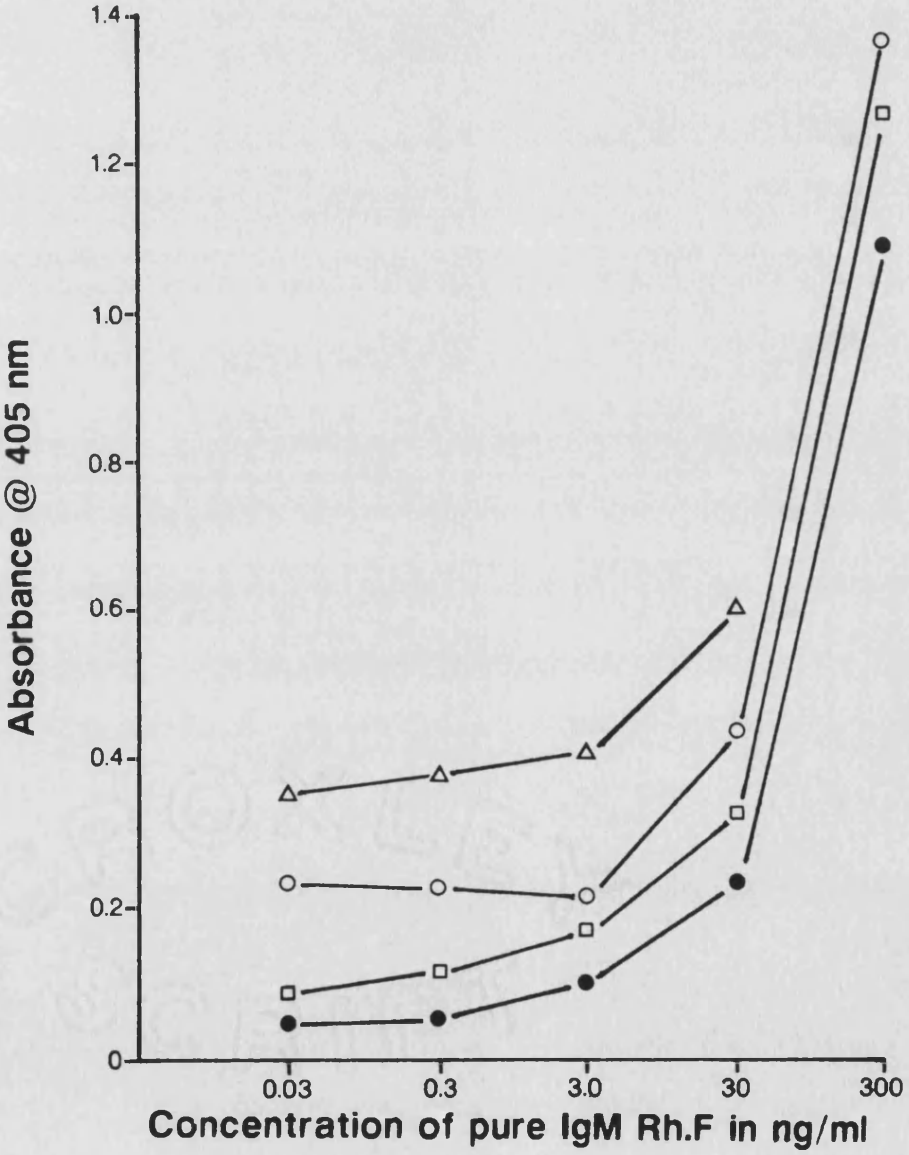
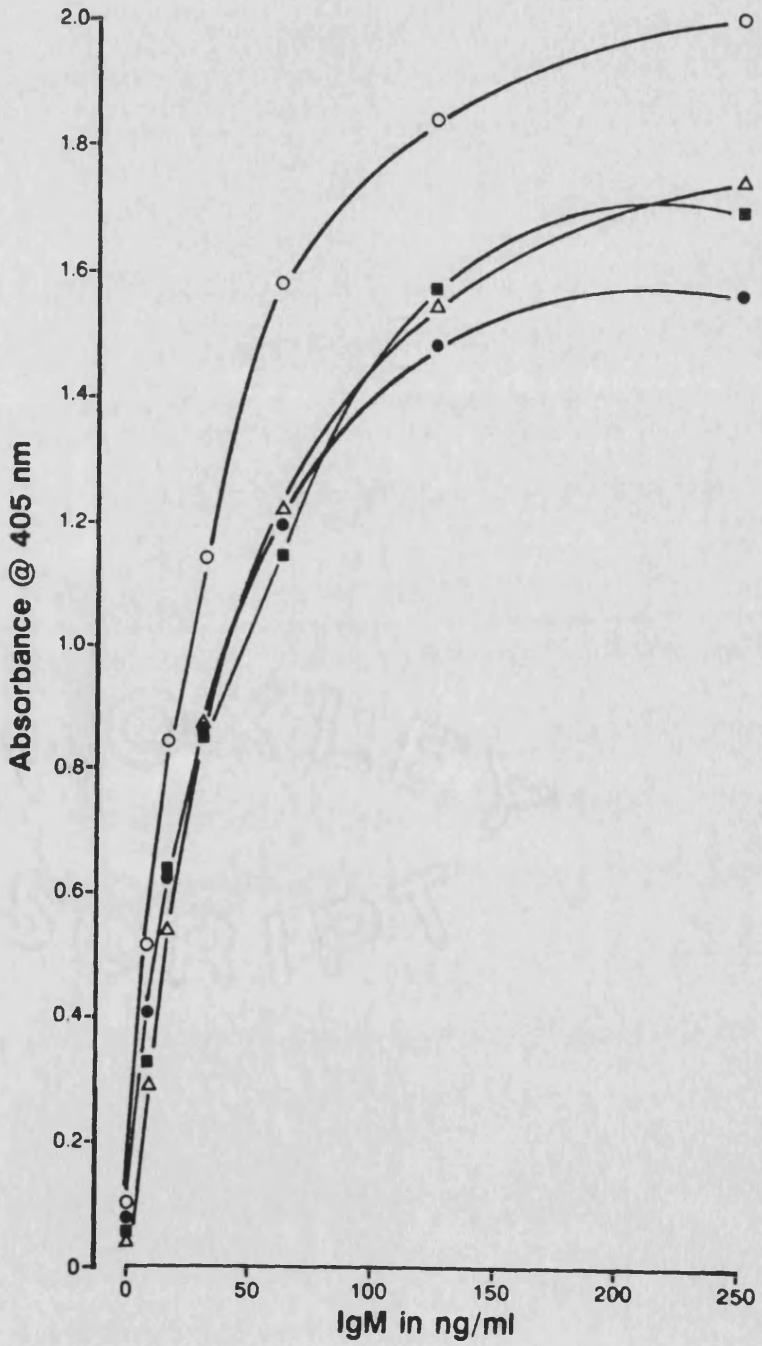


FIG 5 **The effect of different diluants on the IgM standard curve**

The serum standard was diluted in PBS (○——○), PBS-T (△——△), PBS-0.5% BSA (●——●) and medium (■——■). Each point is a mean of two determinations.



foetal calf serum) without producing an overall disturbance in the protein content of the system, which might otherwise affect the way the antigen interacted with the various immunological reagents used in the ELISA.

Antigen and conjugate incubating times and temperatures were also assessed. The results are shown in Figure 6. It was found that the background absorbance values were increased when antigen or conjugate incubation times were lengthened from 1 to 4 hours. These changes were more prominent with increases in time of conjugate incubation. Similarly, it was found that increasing the times of antigen incubation produced an increase in the slope of the standard curve. This was further elevated by prolonging incubation with conjugate. Although this enhanced slope meant a more sensitive assay, it also limited the range over which the linear curve was usable. It was thus decided that the assay should have a 1 hour antigen incubation at 37°C, followed by incubation for 1 hour with conjugate at the same temperature. This would result in a low background and a curve which was linear over a fair range of standard antigen concentrations.

Figure 7 shows the effect of using different dilutions of conjugate on the IgM standard curve. The manufacturers had recommended a final dilution of 1/3000 for use in ELISAs. However, it was found that a 1/1000 dilution produced a much better standard curve than the higher dilutions. Thus this dilution was used in subsequent assays. IgG conjugate was also used at a 1/1000 dilution, as recommended by the suppliers of this particular reagent.

Figure 8 shows degradation of the substrate (measured by the increase in absorbance at 405 nm) d-nitrophenyl phosphate, by anti-human IgG alkaline phosphatase conjugate. Plates had to be incubated

FIG 6 **The effect of varying antigen and conjugate incubation times and temperature on the IgM standard curve**

IgM standards were incubated for 1 hr (●——●), 2 hrs (▲——▲) or 4 hrs (■——■) at 37°C, followed by incubation with anti-IgM alkaline phosphatase conjugate for 1 hr, 2 hrs or 4 hrs at 37°C or for 24 hrs at 4°C. Absorbance was measured at a set time of substrate incubation. Mean values are shown.

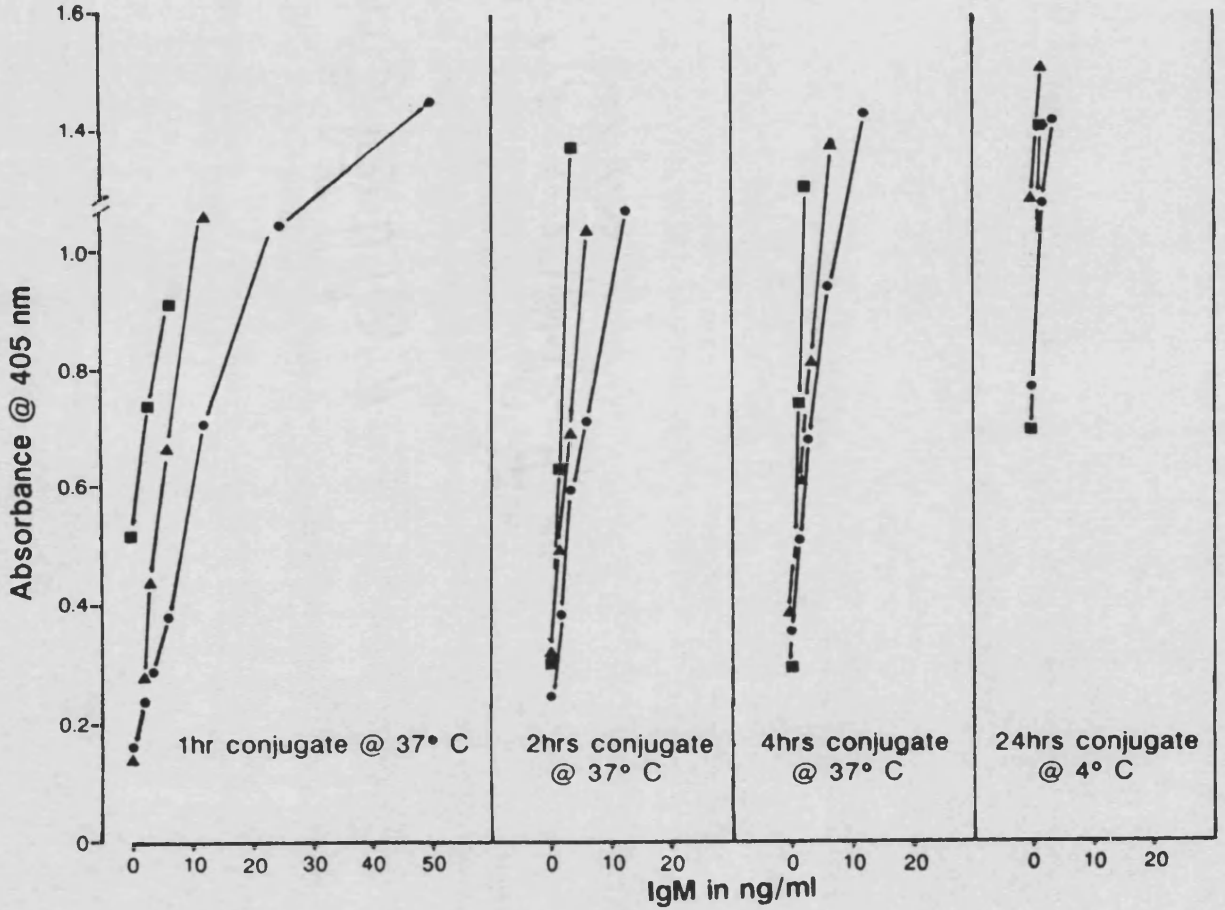


FIG 7 **The effect of different anti-IgM alkaline phosphatase conjugate dilutions on the IgM standard curve**

The conjugate was tested at final dilutions of 1/1000 (●——●), 1/2000 (▲——▲) and 1/3000 (■——■). Mean values of two determinations are shown.

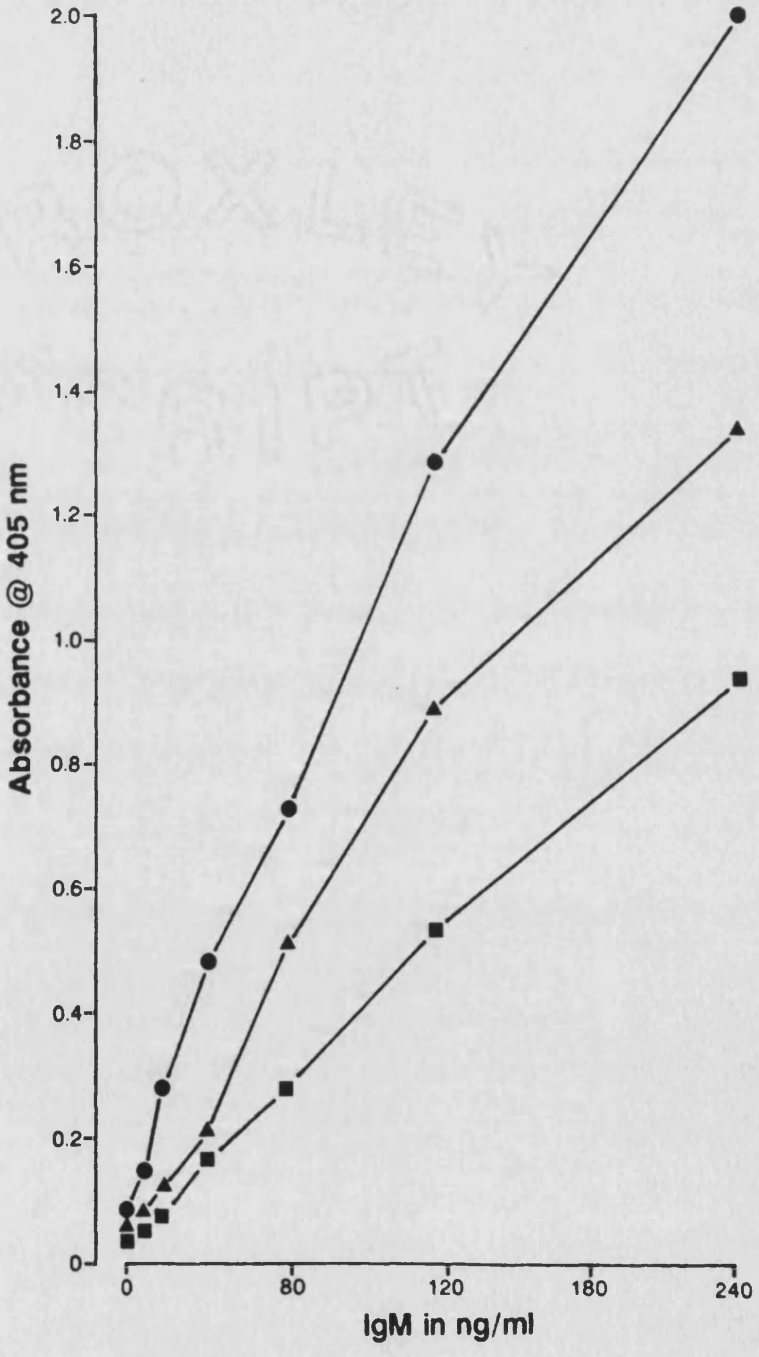
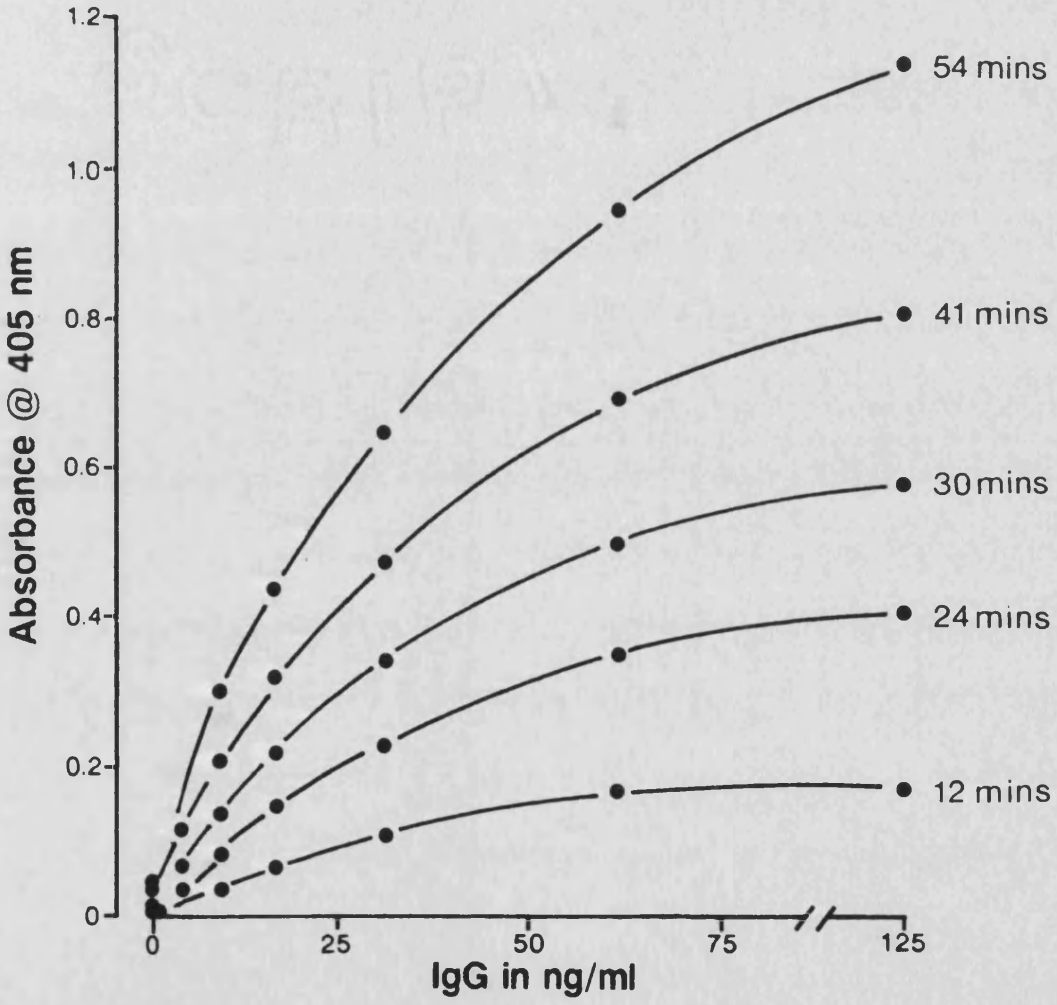


FIG 8 **Degradation of substrate with time as measured by an increase in absorbance at 405 nm**

An IgG ELISA was used to determine the linearity of the enzymic reaction.



usually for nearly an hour at 37°C to produce a usable standard curve. During this time the enzyme-substrate reaction was found to be linear. An hour's substrate incubation at 37°C had originally been selected as optimal. However, the realisation that different batches of conjugate had different potencies and therefore different rates of reaction and did not always need incubation at 37°C led to the decision that reaction should be terminated when a predetermined concentration of the standard reached an absorbance value of 1 at room temperature.

Summarising from the above:-

1. A 1/400 dilution of goat anti-human polyvalent immunoglobulins antiserum was used for coating the solid phase. For IgM RhF plates were to be coated with 1 µg/ml pure human IgG.
2. Plates were then blocked for an hour at 37°C with PBS-1% BSA.
3. Test culture supernatants and standards were diluted in PBS-0.5% BSA and the samples incubated for 1 hour at 37°C. Samples being examined for IgM RhF were tested neat and the standards diluted in medium.
4. Alkaline phosphatase conjugates were used at a final dilution of 1/1000 and the plates incubated for 1 hour at 37°C.
5. Enzyme-substrate reaction was incubated at room temperature until a given standard concentration reached an absorbance of 1. The plates were then read immediately using the ELISA microplate reader at 405 nm.

Reproducibility of IgM and IgG standard curves

Reproducibility of IgM and IgG standard curves was examined. This is shown in Figures 9 and 10. Each curve is a composite of five standard curves. The bar indicates one standard deviation.

FIG 9 **Reproducibility of the IgM standard curve**

Each point is a mean of 5 determinations and the bar represents one standard deviation.

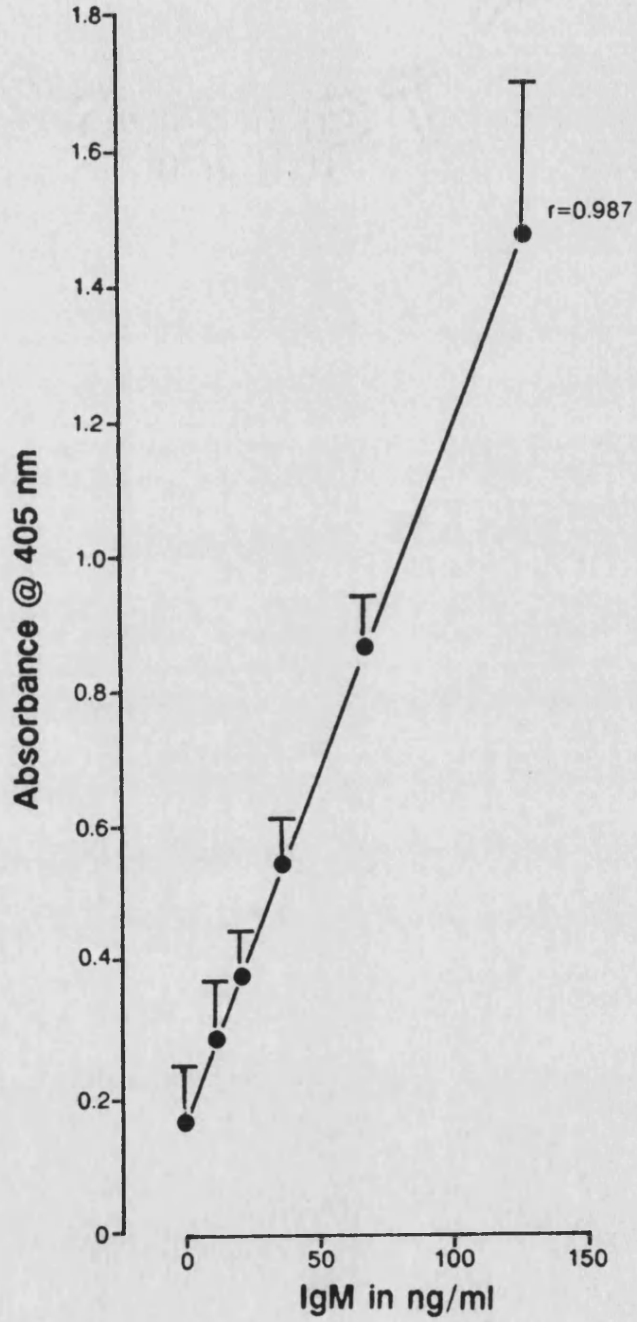
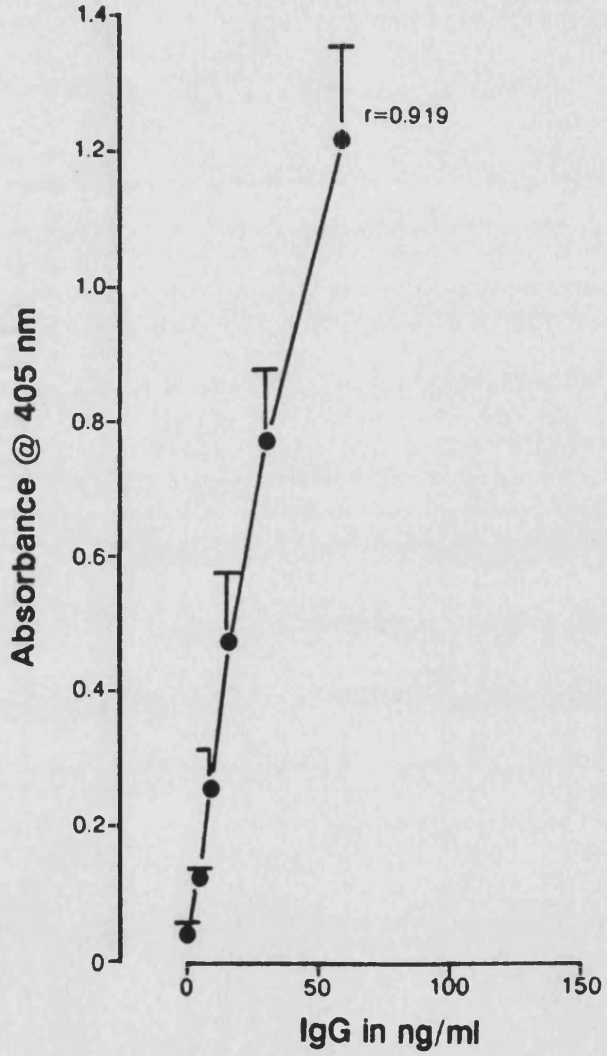


FIG 10 **Reproducibility of the IgG standard curve**

Each point is a mean of 5 determinations and the bar represents one standard deviation.



Sensitivity of IgM and IgG assays

Very low concentrations of IgM and IgG were set up in quadruplicate and the absorbances compared (one tail t-test) with the results obtained when no IgM or IgG was present. Sensitivity was defined as the lowest concentration of IgM or IgG which gave an absorbance value significantly different ($p < 0.05$) from the background. For IgG this was usually found to be 4 ng/ml and for IgM 8 ng/ml.

Specificity of IgM and IgG assays

This was ensured by using affinity purified anti-human IgM or IgG alkaline phosphatase conjugate. Figure 11 shows that anti IgM conjugate did not bind to pure human IgG coated plates but that a normal IgM standard curve was produced in goat polyvalent antihuman immunoglobulins coated plates. When standard curves were set up using pooled normal human serum and pure human IgG, parallelism was observed as shown in Figure 12, indicating that the same antigen was being detected.

IgM RhF standard

A very high titre rheumatoid serum was used as a standard in the IgM RhF ELISA. The percentage of IgM with RhF activity was determined in a manner similar to that described by Faith et al (1982). A 1/10 dilution of the RA serum was adsorbed with heat aggregated IgG and after each absorption, RhF activity was measured using the Rheuma-Wellcotest latex test. After 3 absorptions RhF activity was no longer detectable. The IgM concentrations of the starting material and the adsorbed samples were then measured by the ELISA system.

FIG 11 Specificity of the IgM ELISA

IgM standards were incubated in goat polyvalent antihuman immunoglobulin antiserum (●—●) and pure normal human IgG (○—○) coated microtitre plates. A normal IgM curve produced with the former but no reactivity was observed with the latter. Mean values of two measurements are shown.

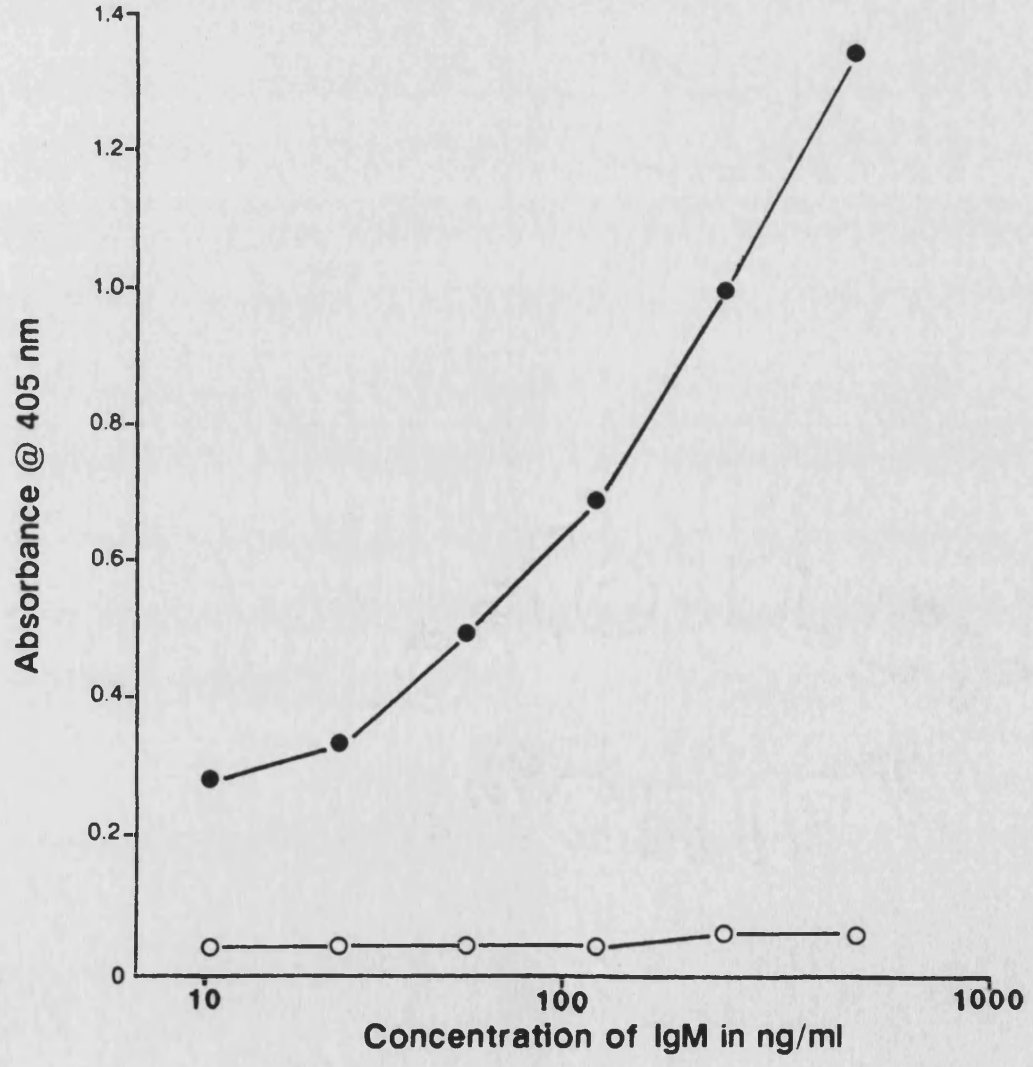
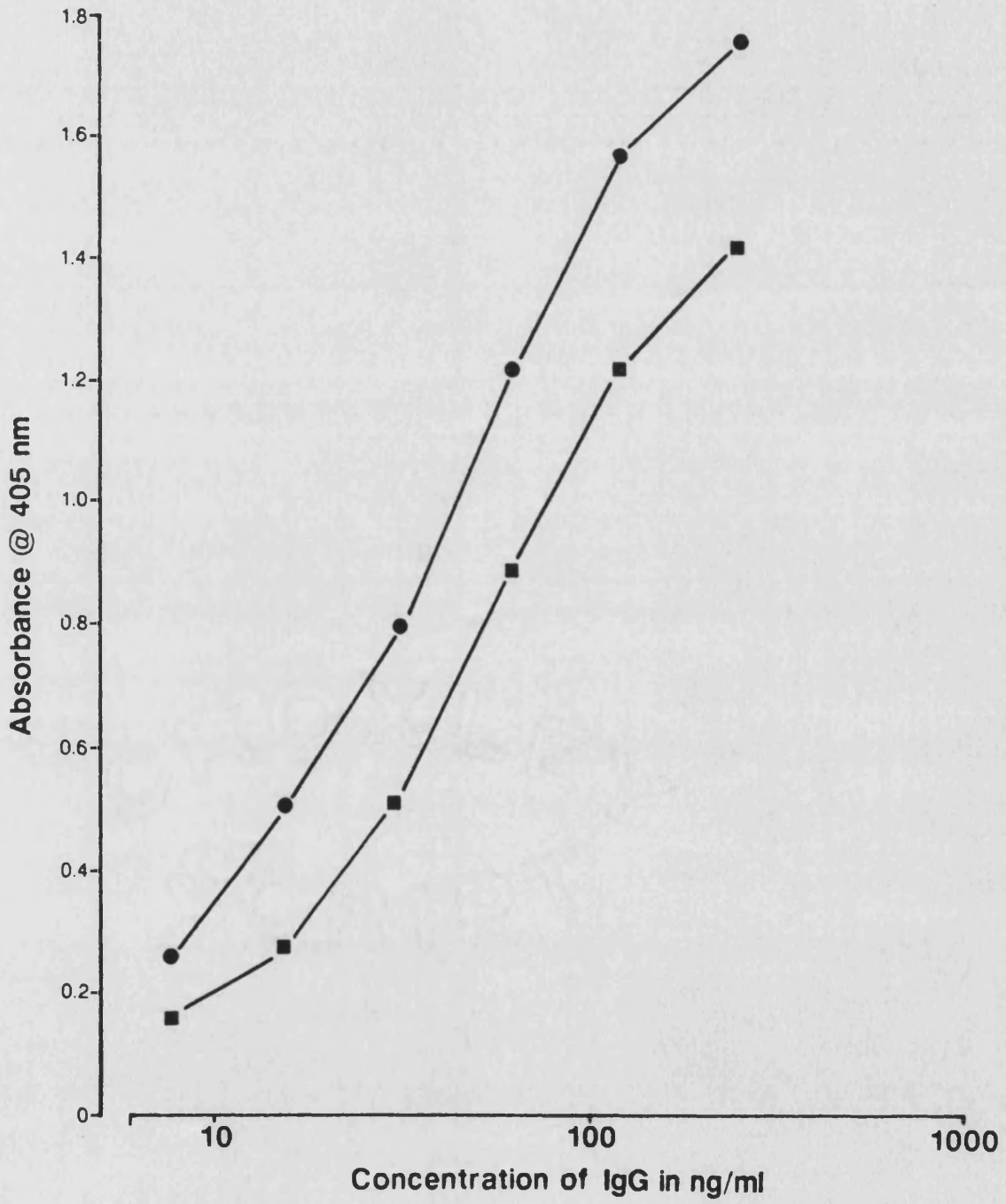


FIG 12 **Specificity of the IgG ELISA**

Parallelism between IgG curves obtained when purified human IgG (■—■) and pooled normal human serum (●—●) were used as standards.



A normal serum with the same IgM concentration (as measured by nephelometry) as the RA serum was also treated similarly as a control for nonspecific adsorption of IgM. This serum showed no RhF activity as determined by the latex test.

Table 2 shows the percentage of IgM removed at each adsorption of the 2 sera. Much of the RhF was removed during the first absorption. The second and third phases removed any remaining RhF. The results show that 73.3% of IgM in this particular serum had RhF activity. Similar values have been obtained by Panayi and co-workers (personal communication), especially in patients with systemic rheumatoid disease.

Thus high titre RA sera where percentage IgM with RhF activity had been quantified as above were used as standards in the RhF ELISA.

The effect of Facb on IgG, IgM and IgM RhF assays

Since culture supernatants would contain Facb, it was important to show that this ligand did not interfere with the immunoglobulin and RhF assays.

Figures 13, 14 and 15 show that Facb had no significant effect on IgG, IgM and IgM RhF curves respectively when it was added to the relevant standards at final concentrations of 2 and 20 µg/ml.

TABLE 2 Showing the percentage of IgM removed at each absorption of the RA and normal sera.

No of times absorbed	% of IgM absorbed by Hagg		% of IgM absorbed by Hagg in RA serum after correction for non-specific absorption of IgM
	RA serum	Normal serum	
1	71.1	8.3	62.8
2	77.7	9.1	68.6
3	86.0	12.7	73.3

FIG 13 **The effect of Facb on the IgG ELISA**

This was examined by diluting the IgG standard in PBS-0.5% BSA containing 0 (●——●), 2 (□——□) or 20 (△——△) µg/ml final concentration of Facb. Each point is a mean of two determinations.

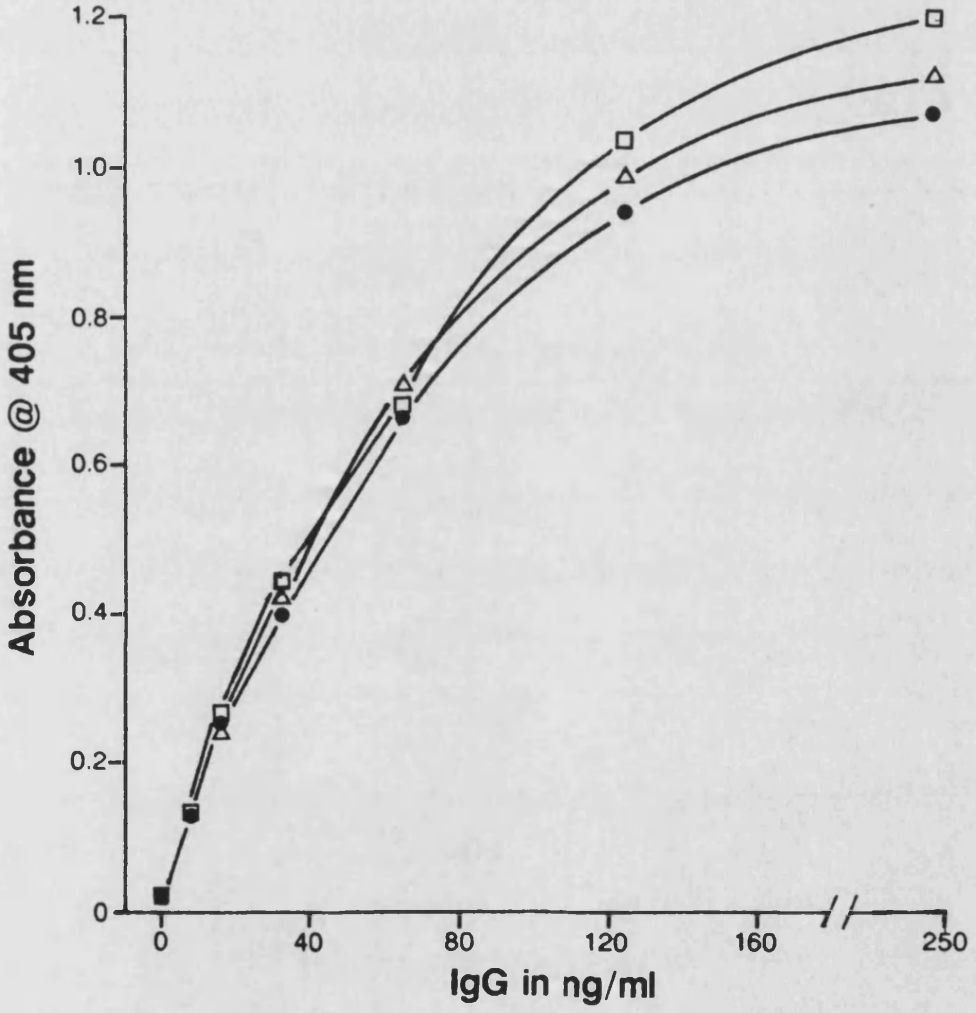


FIG 14 **The effect of Facb on the IgM ELISA**

This was examined by diluting the IgM standard in PBS-0.5% BSA containing 0 (●——●), 0.2 (△——△), 2 (□——□) or 20 (○——○) µg/ml final concentration of Facb. Each point is a mean of two determinations.

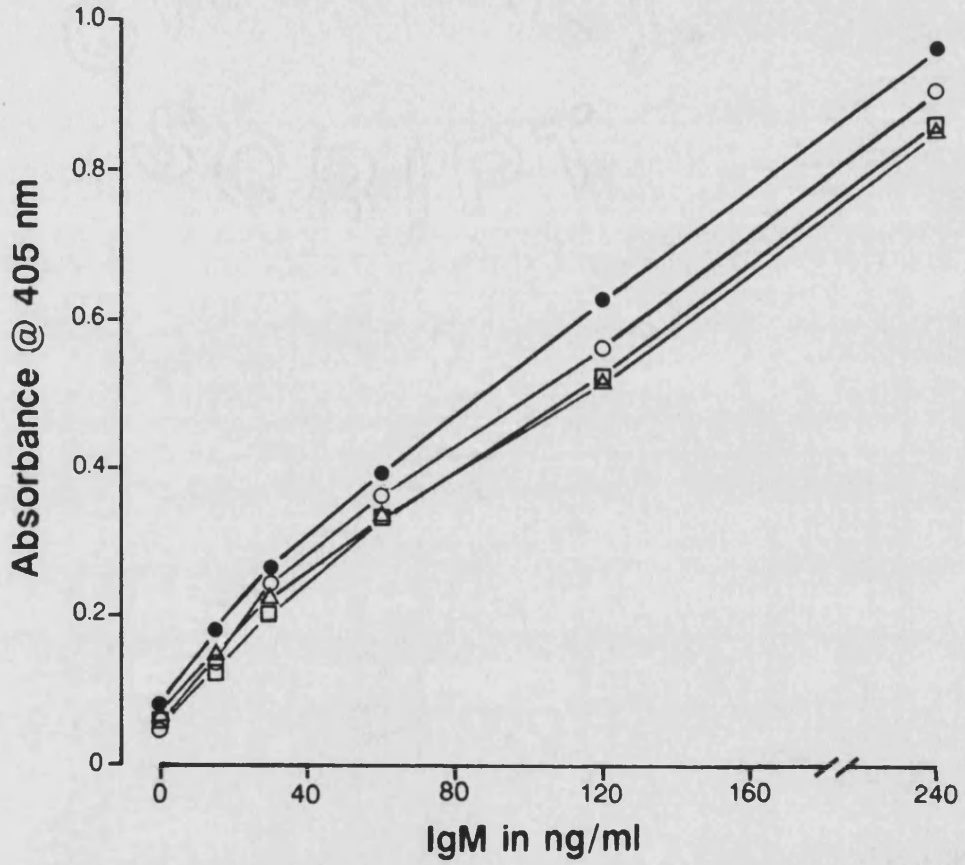
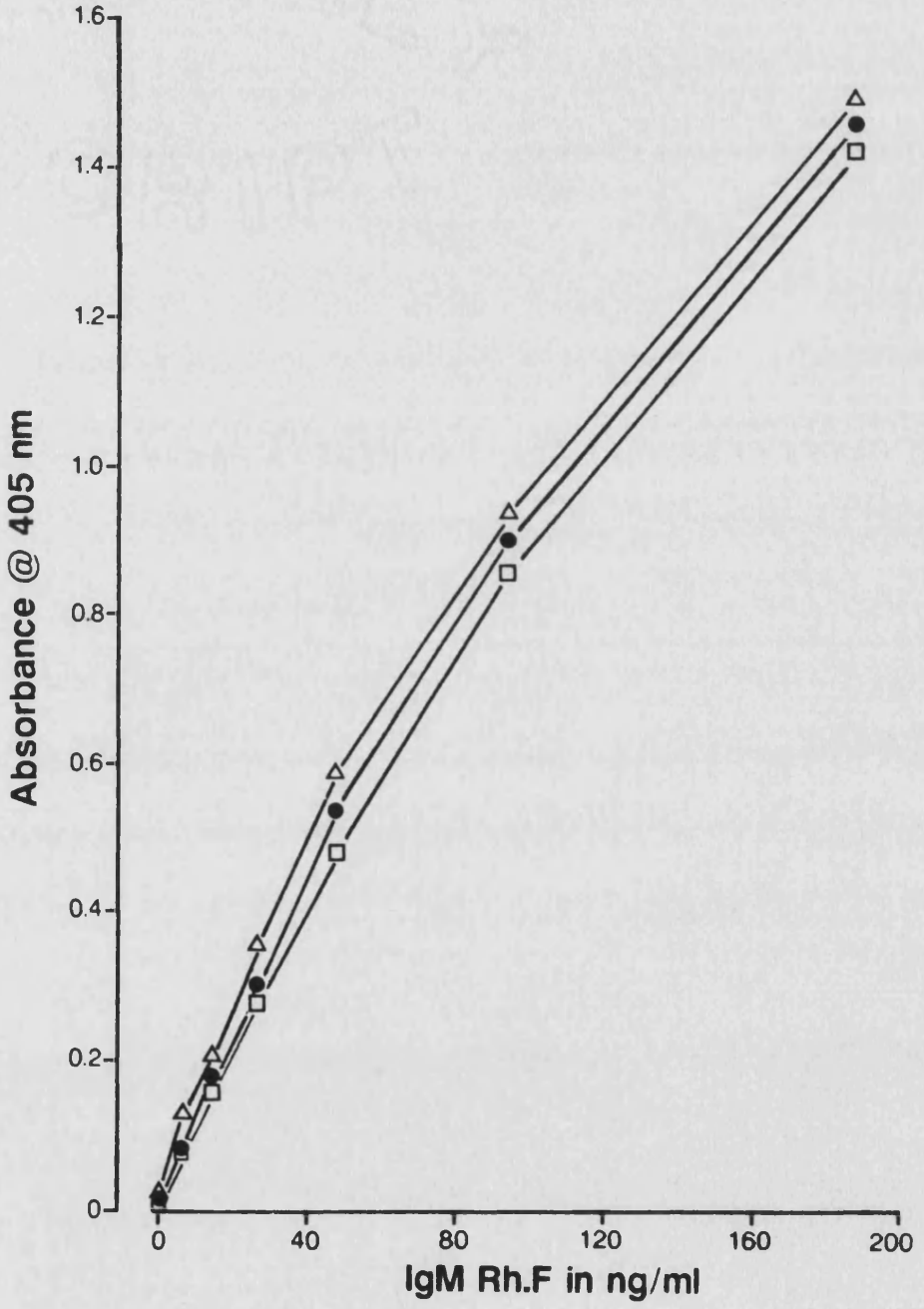


FIG 15 **The effect of Facb on the IgM RhF ELISA**

This was examined by diluting the IgM RhF standard in PBS-0.5% BSA containing 0 (●——●), 2 (□——□) or 20 (△——△) µg/ml final concentration of Facb. Each point is a mean of two determinations.



SECTION 2 ANTIBODY SYNTHESIS IN VITRO

Generation of immunoglobulin *in vitro* was achieved by stimulating isolated and washed PBMNC with pokeweed mitogen. This is a crude extract obtained from the *Phytolacca americana* root and is known to stimulate both T and B cells (Ling and Kay, 1975).

A. PWM dose response curves

Using a cell concentration of 1 x 10^6/ml shown by preliminary data to be optimal (Table 3), 1 ml cultures were set up with doubling dilutions of PWM. PBMNC from 7 healthy controls and 11 patients with RA were used. Culture supernatants were harvested on Day 7 and assessed for IgG and IgM content by ELISA. These results are shown in Figures 16 and 17. Peak responses were observed at final dilutions of 1/40 in normal controls and 1/80 - 1/160 in RA patients. PWM dilutions approaching 1/1000 and over returned immunoglobulin production to background levels.

IgG production in RA and normal subjects was not dissimilar, although the patients generated slightly higher levels of IgG compared to controls. But with spontaneous IgG production a marked difference ($p < 0.05$) was observed between RA patients and healthy controls with the former generating nearly three times as much IgG as the latter. With IgM a similarly striking difference was observed, but with PWM-stimulated cells. Here, rheumatoid patients produced much less IgM than healthy subjects with levels of significance reaching a p value of < 0.01 at peak response. This is in agreement with the findings reported by Olsen et al (1982). Spontaneous IgM production, however, was found to be similar in the two groups.

TABLE 3 Effect of increasing cell concentration on IgM synthesis (ng/ml) by normal PBMNC measured at Day 7.

PWM dose	Cell concentration x 10^6/ml			
	0.5	1.0	2.0	3.0
P0	17	61	87	122
P50	231	1385	1697	1165
P100	385	2315	1800	2178

n = 4

FIG 16 **PWM dose response curves in normal healthy controls**

PBMNCs from normal healthy controls were incubated with doubling dilutions of PWM for 7 days. Levels of IgG (●——●) and IgM (□——□) produced were measured using the ELISAs. Spontaneous IgG (- - - -) and IgM (— · — ·) generation was also assessed. Median values are shown and the corresponding interquartile ranges are omitted for purposes of clarity.

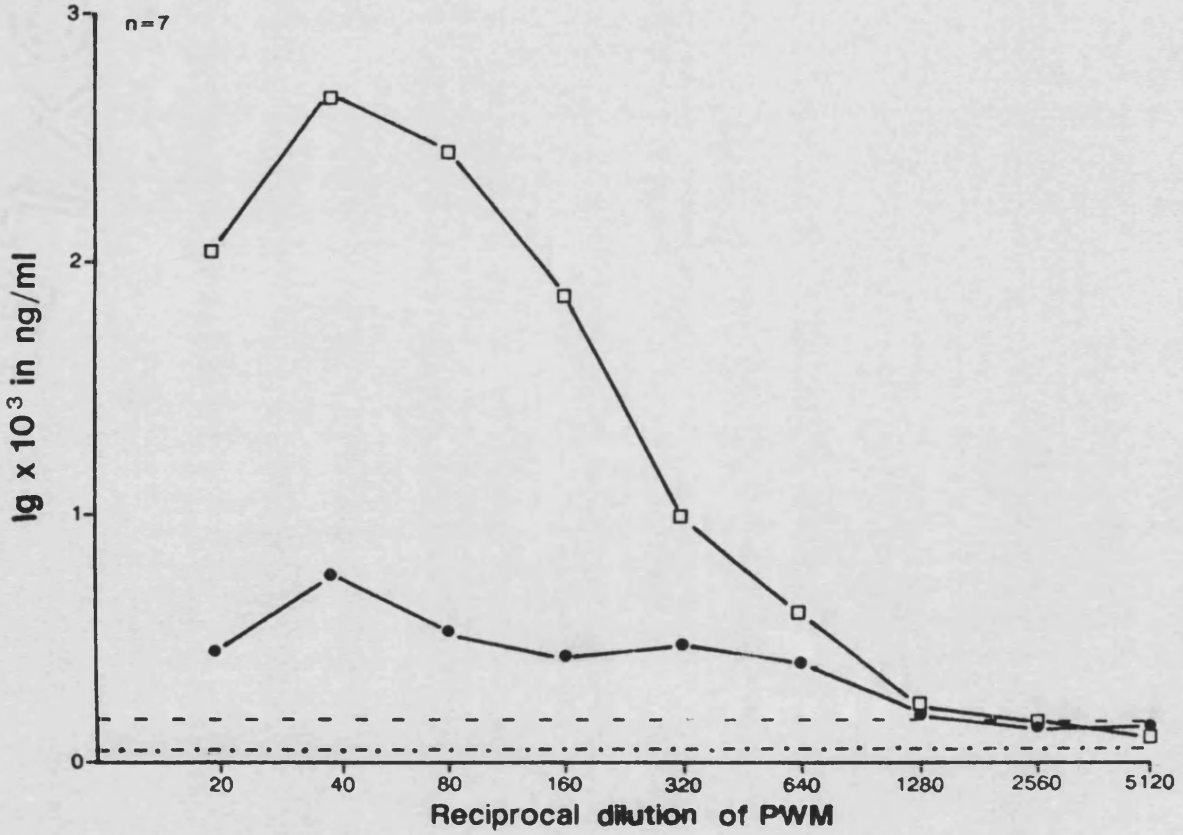
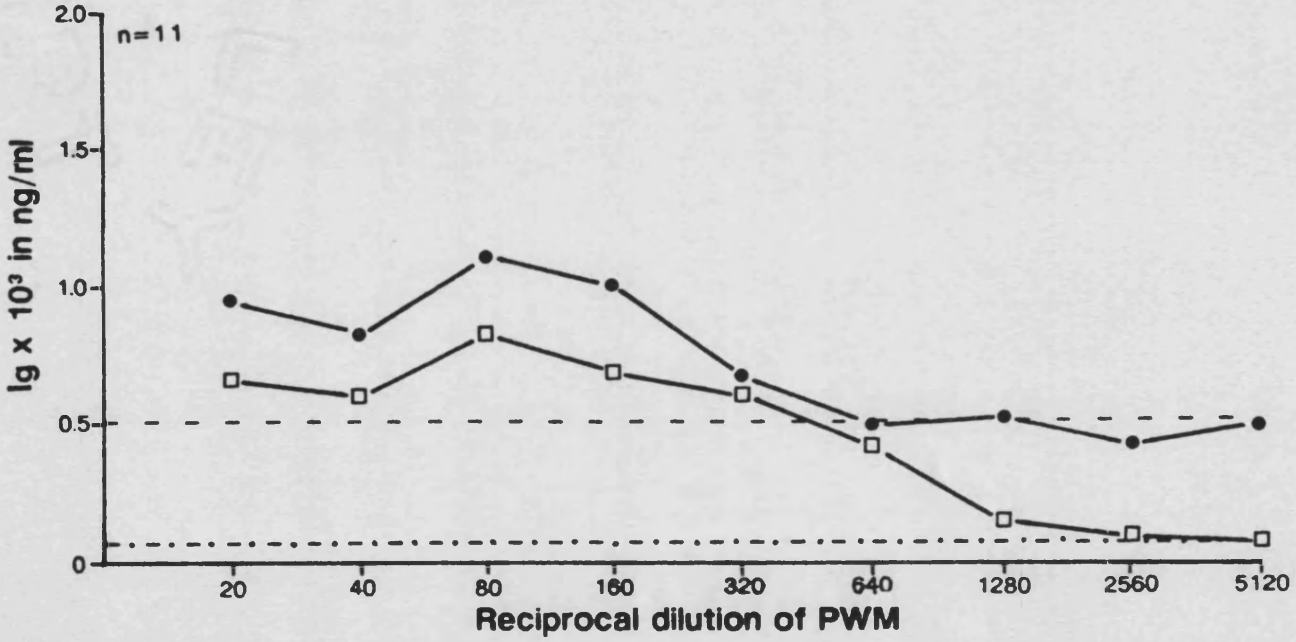


FIG 17 **PWM dose response curves in RA patients**

PBMNCs from RA subjects were stimulated with doubling dilutions of PWM. Levels of IgG (●—●) and IgM (□—□) were measured in day 7 cultures using the ELISAs. Spontaneous IgG (- - - -) and IgM (– · – · –) were also examined. Results are expressed as median values and the corresponding interquartile ranges are omitted for purposes of clarity.



In view of these data, final PWM dilutions of 1/50, 1/100 and 1/200 were selected for subsequent use in Facb experiments. However, batch-to-batch variation in the PWM preparations used led to peak responses occurring at dilutions as high as 1/1000. Thus a revised selection of PWM doses led to the use of the mitogen at final dilutions of 1/50, 1/200 and 1/1000 in the aforementioned experiments.

B. Antibody production over 21 days

Using a 1/50 dilution of PWM, antibody production of a 21 day period was examined. Figures 18 and 19 show Ig production in one index case. IgG and IgM were measured. For the first 5 days there was very little immunoglobulin production. However, between Day 5 and Day 10 there was an exponential increase in Ig concentration and this was followed by a much slower rise in Ig levels from Day 10 to Day 21.

A normal subject was used here and again levels of IgM were greater than IgG. This was also true of another normal individual examined at the same time (data not shown). Background Ig levels were found to increase but only very slowly, the increase becoming more marked after Day 4.

The data above thus indicated a period of at least 10 days to obtain a maximum level of Ig synthesis.

FIG 18 PWM-stimulated (●——●) and spontaneous (○——○) IgG production in one normal individual over a period of 21 days

Each point is a mean value of two determinations.

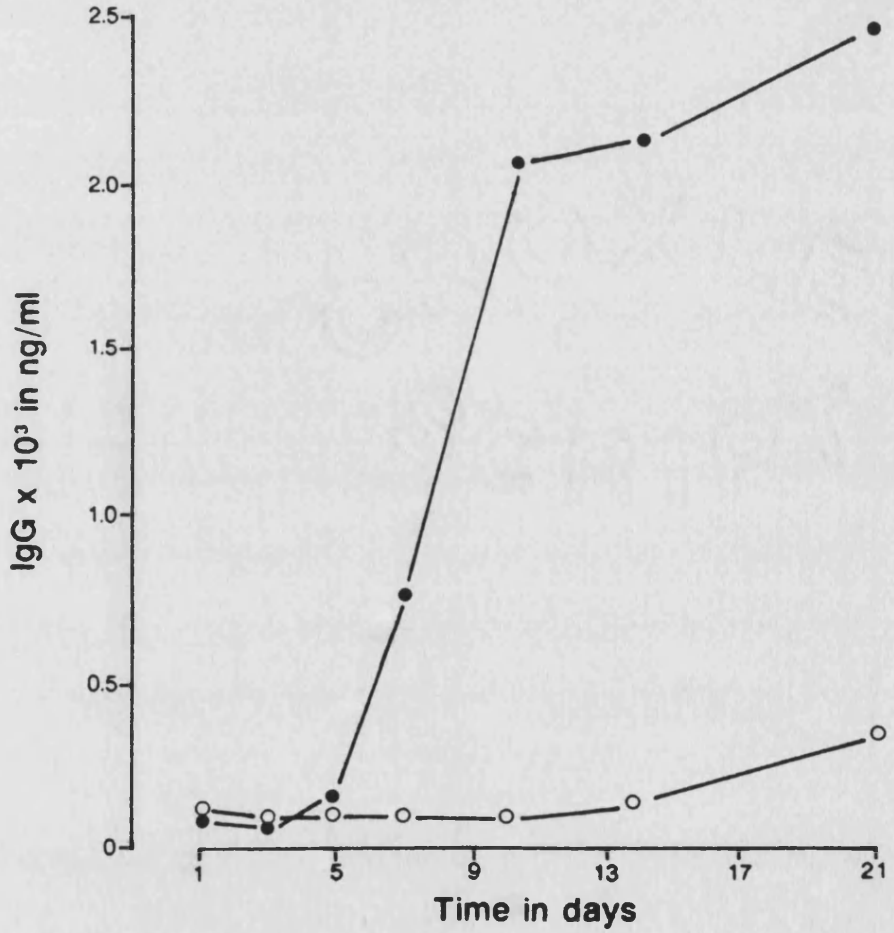


FIG 19 PWM-stimulated (●——●) and spontaneous (○——○) IgM production in one normal individual over a period of 21 days

Each point is a mean value of two determinations.

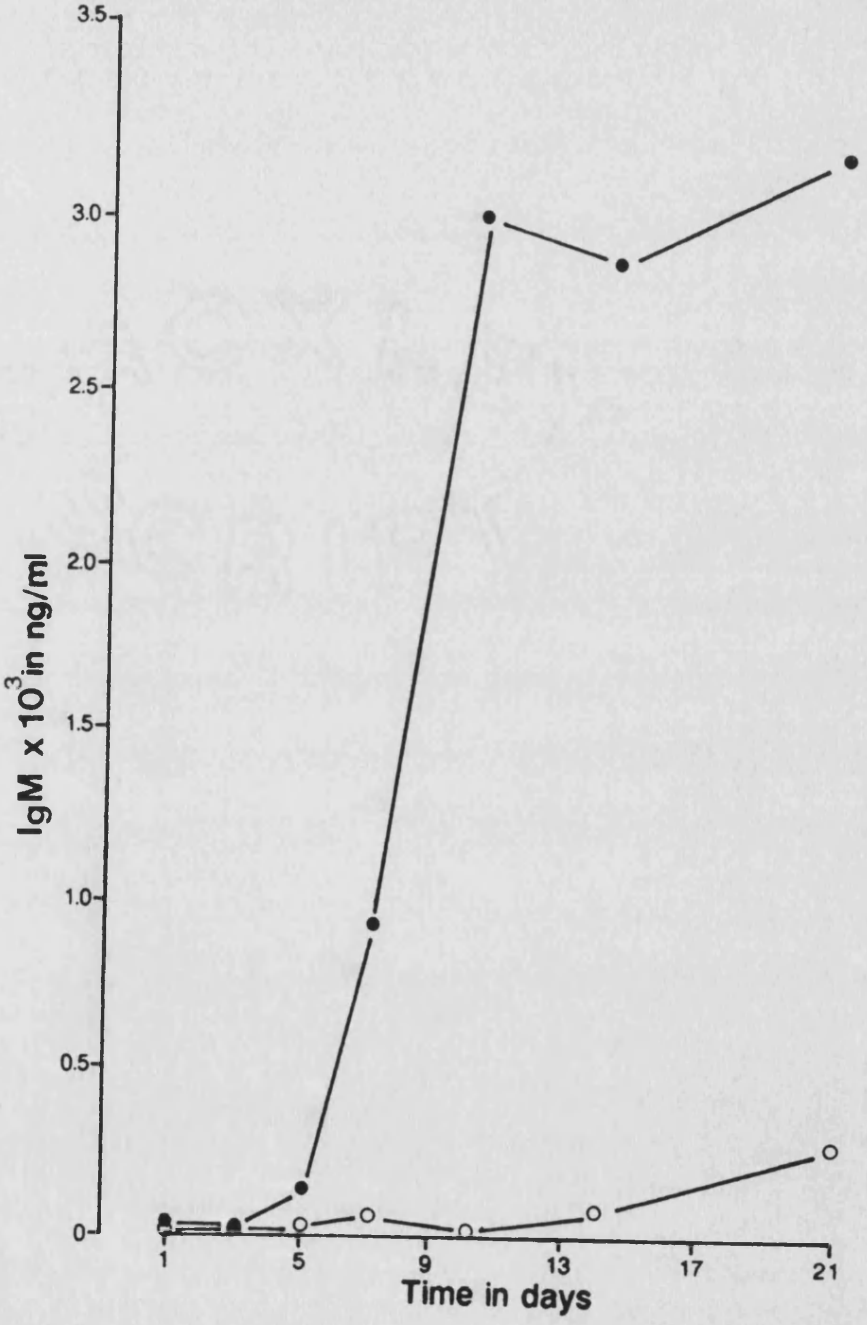
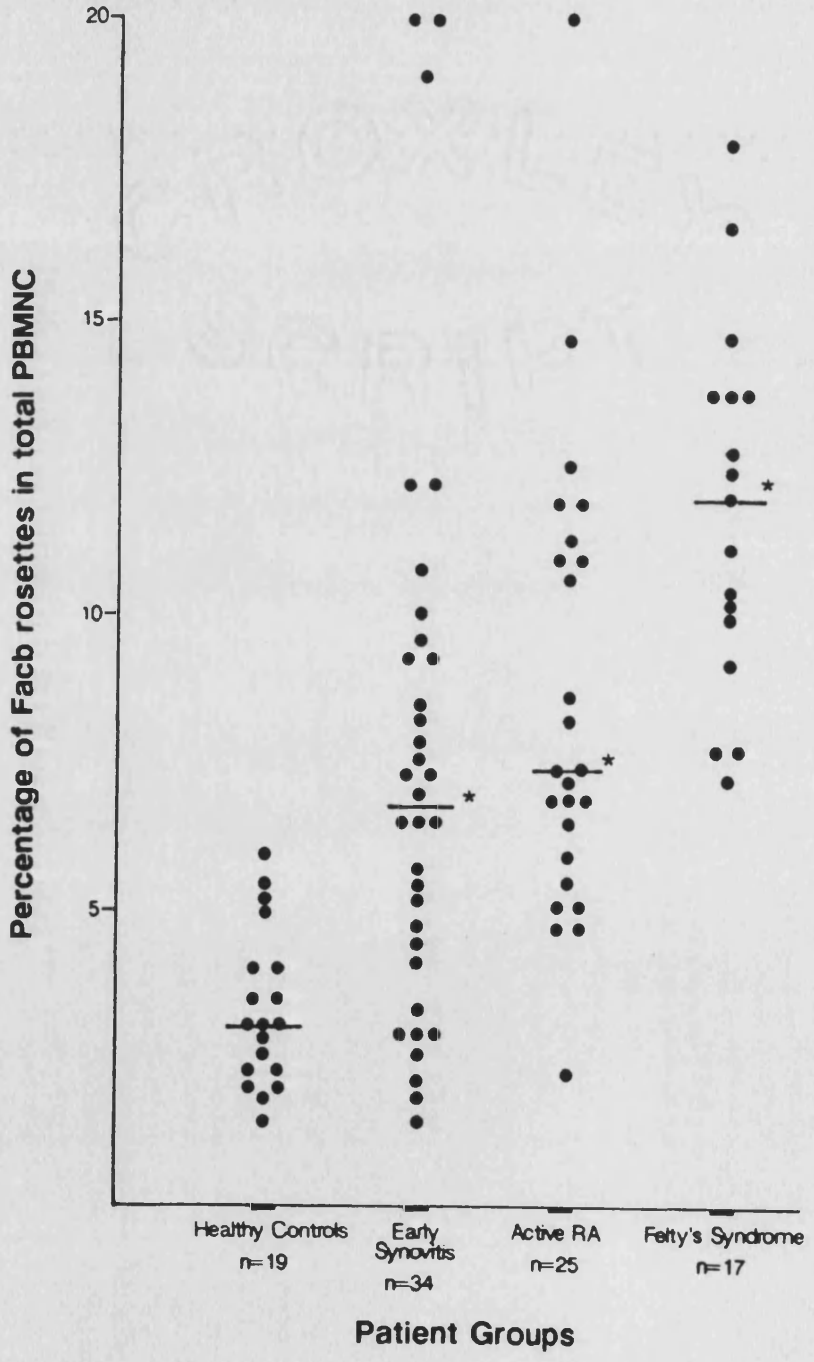


FIG 1 **Numbers of circulating FacbR$^+$ cells in normal, RA and Felty's patients**

Median values of percentage Facb rosette forming cells are indicated by the horizontal bars.

* $p < 0.01$ when compared to observations in normal healthy controls



CHAPTER 4

Facb RESULTS

The results presented in this chapter concern immunomodulation of antibody production by Facb. This is a fragment produced by enzymic digestion of the IgG molecule whereby the C_H3 domain is removed. Preliminary studies in this laboratory have shown that PBMNCs from patients with established RA are significantly less responsive to the suppressive effects of Facb compared to normal healthy controls, despite the observation that the percentage of $FacbR^+$ cells (Figure 1) in these patients is significantly higher than in controls. The greatest numbers are observed in subjects with Felty's syndrome, which can be regarded as representative of advanced disease.

The present study was undertaken to assess Facb-mediated feedback suppression in patients with early RA to determine whether a similar defect existed in these subjects and to evaluate whether this had any disease specificity.

SECTION 1

1. Initial experiments

It has already been established by previous workers that Facb is best used at final concentrations of 0.2, 2 and 20 μg/ml to produce suppression of the IgG response. However, it was not known what effects different incubation periods had on the emergence of Facb suppression. Nor was it clear whether Facb had to be present at the beginning of the culture period in order to be suppressive.

Therefore initial experiments were set up using normal PBMNCs stimulated with reciprocal dilutions of 50 (P50), 200 (P200) and 1000 (P1000) of PWM as determined in Chapter 3, and Facb concentrations of 0.2, 2 and 20 µg/ml. Results obtained here and in all subsequent experiments are expressed as a percentage of the PWM response where no Facb was present. The latter is sometimes referred to as the 'baseline' response in the text.

a) Time of incubation

Figure 2 shows Facb suppression of IgG synthesis in PBMNC cultures harvested on Days 7, 10 and 14. It was found that, although not significantly different, the best levels of suppression were observed on Day 14. It was therefore decided that subsequent Facb cultures should be incubated for a period of 14 days. This would have the added advantage that measurable quantities of RhF may accumulate in the cell culture supernatants.

b) Time of Facb addition

Using 14-day Facb cultures, the effects of adding the ligand on Day 0, 1, 4 and 7 were examined. Figure 3 shows that Facb was only suppressive if it was present at the beginning of the culture.

Patient studies were then commenced using 14-day PBMNC cultures where Facb was added at Day 0 in final concentrations of 0.2, 2 and 20 µg/ml to cultures stimulated with P50, P200 and P1000 dilutions of PWM.

FIG 2 **Facb-mediated suppression of the IgG response at P1000 on Days 7, 10 and 14**

PBMNCs from normal subjects were stimulated with PWM and Facb fragment added at final concentrations of 0.2, 2 and 20 μg/ml. Suppression of the IgG response was assessed and the results expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (T) are depicted.

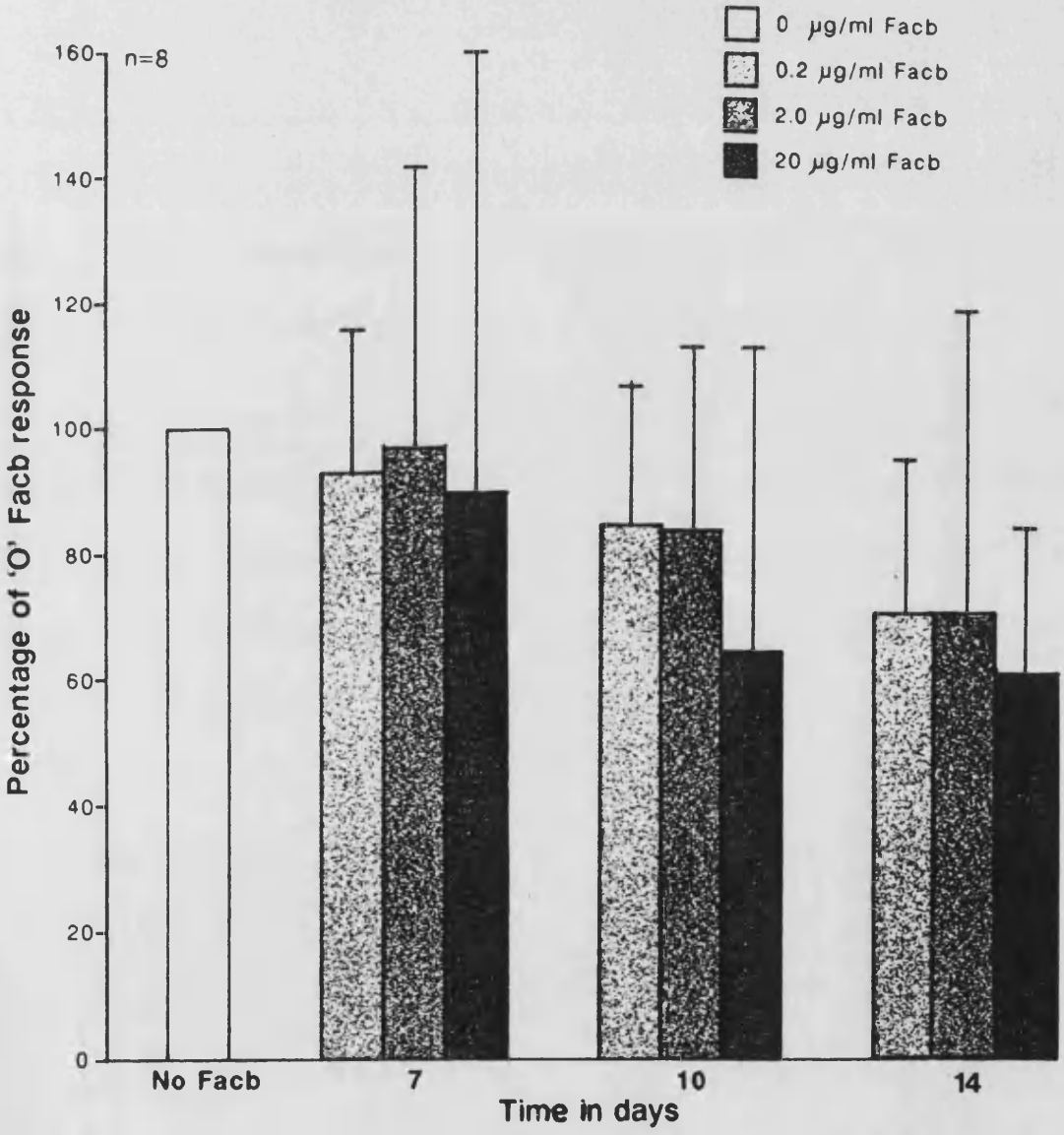
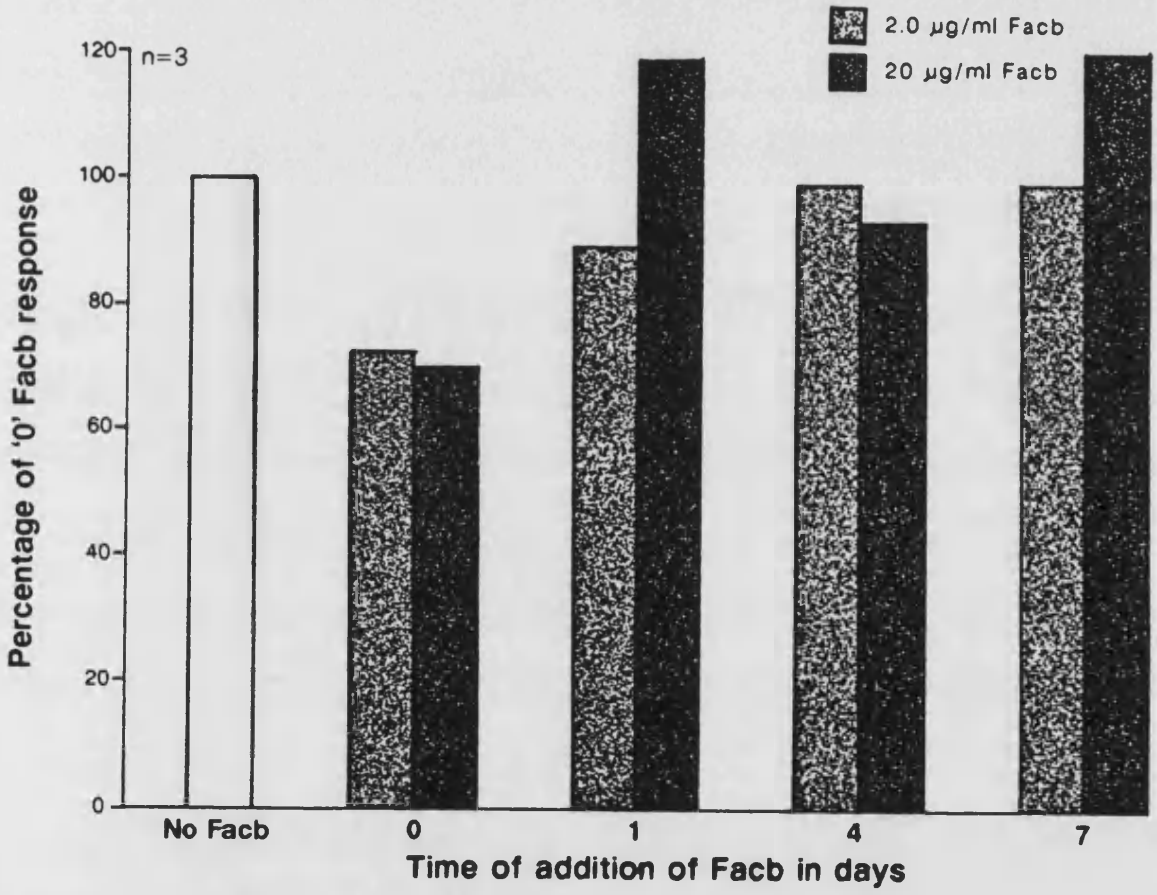


FIG 3 **The effect of adding Facb at different times during culture on feedback suppression of IgG production at P1000**

Normal PBMNCs were stimulated with PWM and Facb added at final concentrations of 2 and 20 μg/ml to cultures on days 0, 1, 4 and 7. Suppression of IgG response is expressed as a percentage of the Facb response where no ligand was present.



2. Facb suppression of PWM-driven IgG synthesis

The following series of graphs illustrates the effect of Facb fragment on PWM-induced IgG production in normal healthy controls, patients with early and established RA, subjects with OA and a small number of Felty's patients. Only those individuals producing >300 ng/ml IgG were considered. The absolute values of IgG levels generated in response to PWM in each group appear in Table 1 and later in Figures 12 - 15.

Figure 4 shows Facb suppression of IgG synthesis in normal healthy controls. It was found that as the concentration of mitogen was reduced the degree of suppression became greater. Thus at P50, the highest stimulating dose of PWM, the suppressive effects of Facb were negligible. At P200, suppression was observed at ligand concentrations of 2 and 20 ug/ml Facb and at P1000 all doses of the fragment were found to be suppressive. Here the IgG synthesis was reduced to 76%, 77% and 49% of the P1000 response. The values obtained at 0.2 and 20 ug/ml Facb were found to differ significantly ($p < 0.01$). Comparing the results obtained here with those seen at the two higher doses of PWM revealed significant differences at 2 and 20 ug/ml Facb at P50 ($p < 0.01$) and at 20 ug/ml Facb at P200 ($p < 0.01$). At all permutations of PWM dose and Facb concentrations, the responses were found to be highly variable.

Similar responses were obtained in patients with early RA positive and palindromic disease (see Figures 5 and 7). True suppression was only observed at P1000. In the former group these results were highly significantly different from those seen at the two higher doses of PWM. In the latter, levels of significance were reached at all concentrations of Facb tested when compared to the

TABLE 1 **The effects of Facb fragment on PWM-stimulated IgG production in test groups**

Median levels of IgG production (ng/ml +/- interquartile range) in response to three doses of PWM are shown, together with the effects of increasing concentrations of Facb on this production. Only those individuals generating > 300 ng/ml IgG are shown.

	PWM dose	P50				P200				P1000			
Patient groups and numbers	Facb in µg/ml	0	0.2	2	20	0	0.2	2	20	0	0.2	2	20
Normals		585	585	591	545	1800	1908	1474	1282	2520	1908	1933	1237
n = 11	+/-	1227	268	170	388	3220	634	329	369	2927	668	899	327
Early RA positive		623	662	644	669	1204	1111	1221	1163	900	692	577	506
n = 14	+/-	1009	232	195	318	3242	345	525	562	2204	161	141	273
Early RA negative		631	555	567	604	843	723	654	708	782	769	722	573
n = 8	+/-	647	151	380	620	455	20	166	370	547	195	225	163
Early palindromic RA		939	1048	957	868	1960	2183	1915	1909	3550	2840	2183	2102
n = 8	+/-	3470	356	124	432	7800	548	387	762	6700	633	159	395
Established RA		2180	2158	2280	2396	2680	2243	2417	2372	1146	974	1053	970
n = 20	+/-	4212	458	702	604	4000	603	834	1036	3143	232	453	644
OA		3400	3587	3483	4406	2270	2052	2259	2912	1324	1223	1484	1786
n = 8	+/-	3908	545	505	3032	9066	417	727	3938	4821	334	1023	3853
Felty's		622	614	601	465	1086	1144	1194	959	-	-	-	-
n = 6	+/-	1597	77	295	58	876	326	315	376	-	-	-	-

FIG 4 **Facb-mediated suppression of PWM-stimulated IgG response in normal healthy controls**

Facb was added at final concentrations of 0.2, 2 and 20 μg/ml to PBMNC cultures stimulated with 3 doses of PWM. Suppression of the IgG response was assessed and the results expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (I) are depicted.

* $p < 0.01$ when compared with equivalent Facb concentrations at P1000

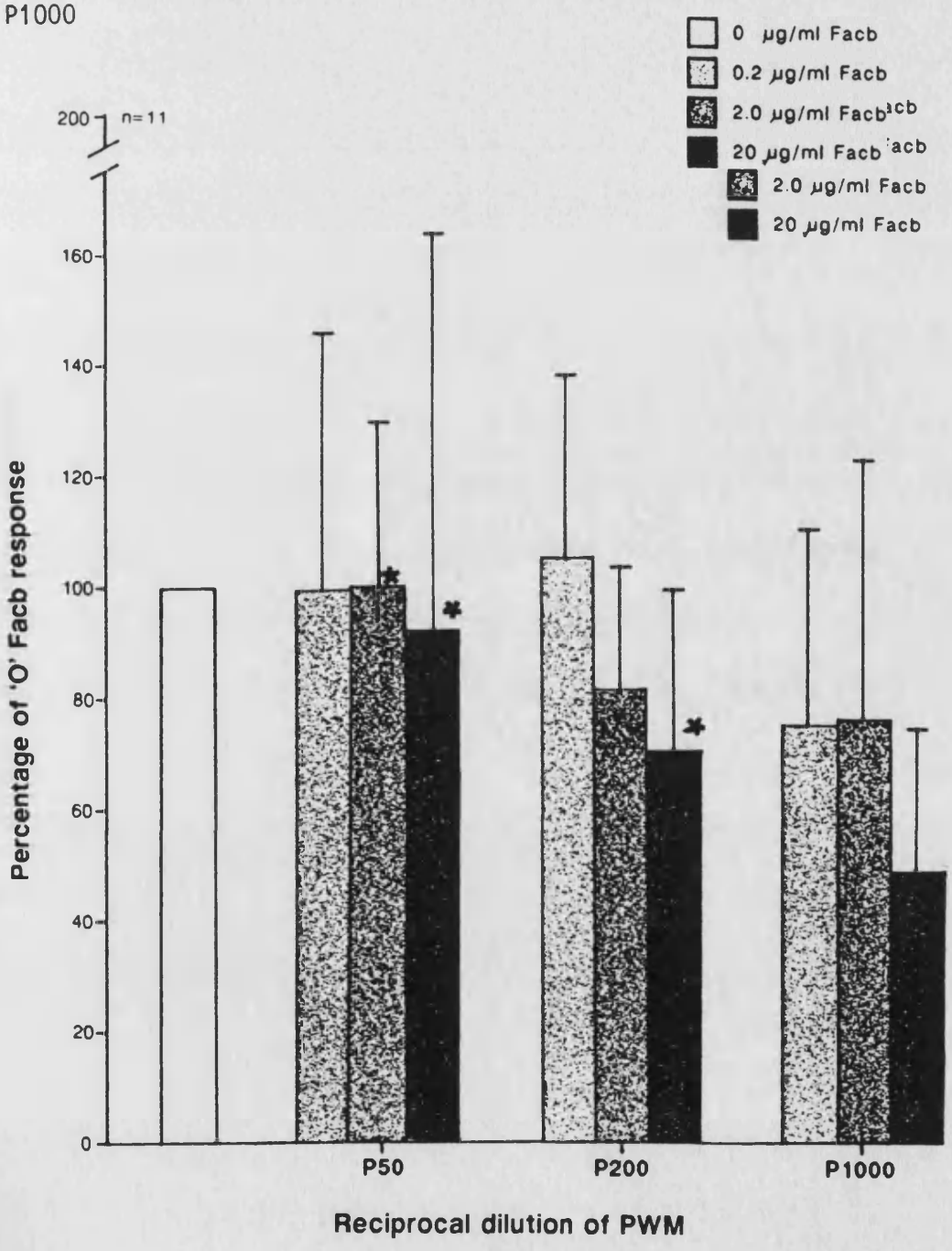


FIG 5 **Facb-mediated suppression of PWM-stimulated IgG response in early RA positive patients**

Facb was added at final concentrations of 0.2, 2 and 20 µg/ml to PBMNC cultures stimulated with 3 doses of PWM. Suppression of the IgG response was assessed and the results expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (T) are depicted.

* $p < 0.01$ when compared with equivalent concentrations at P1000

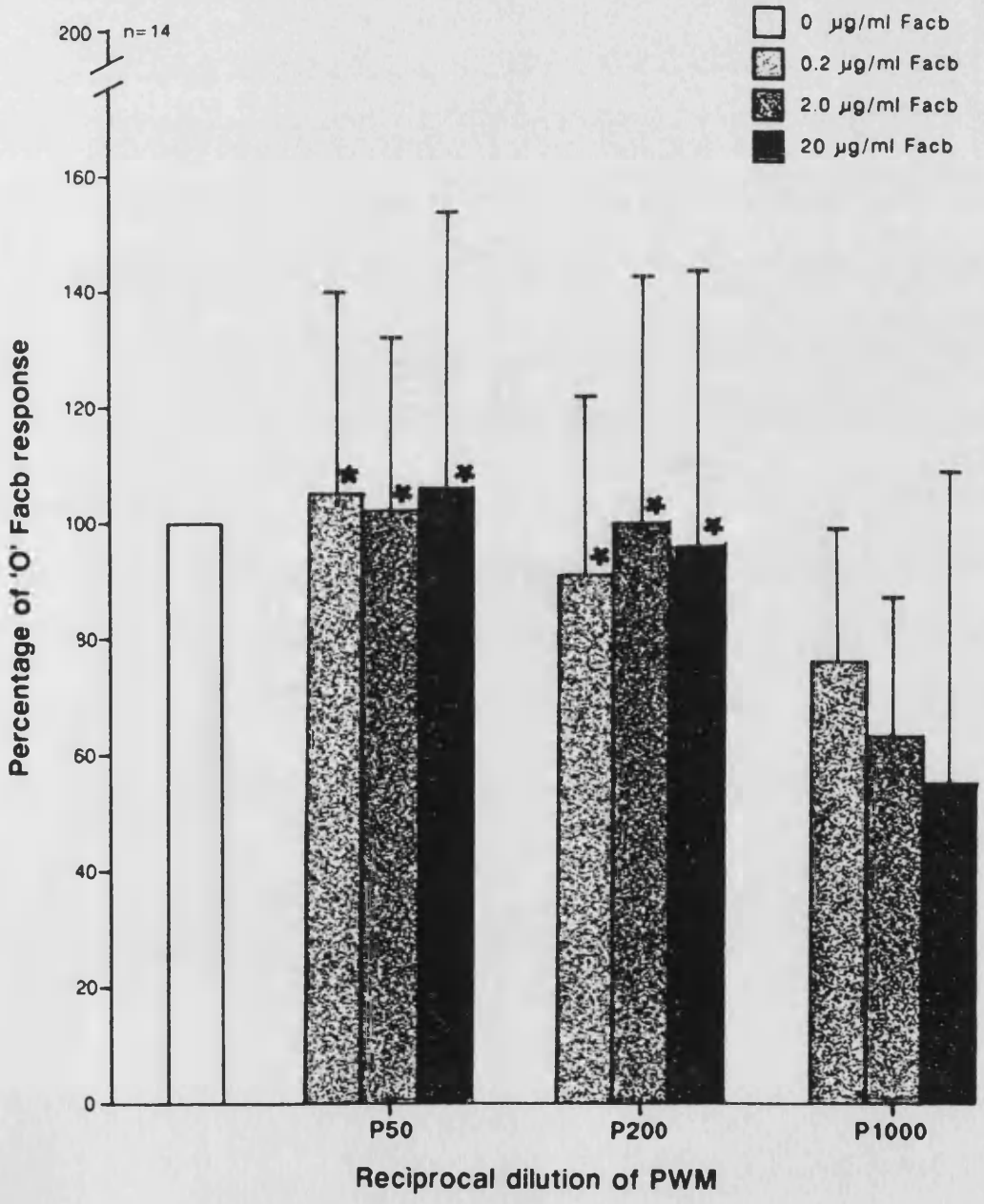
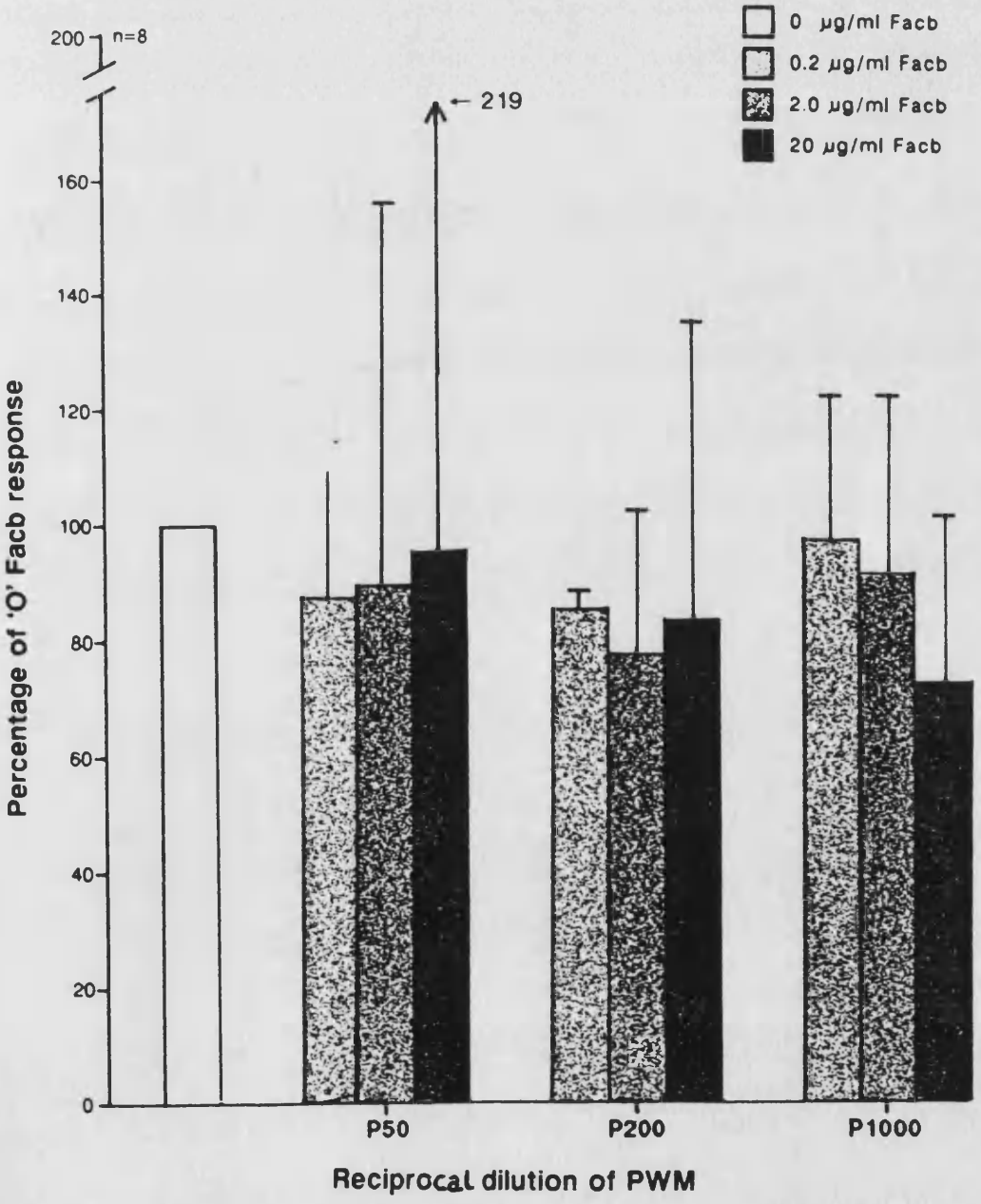


FIG 6 **Facb-mediated suppression of PWM-stimulated IgG response in early RA negative patients**

Facb was added at final concentrations of 0.2, 2 and 20 µg/ml to PBMNC cultures stimulated with 3 doses of PWM. Suppression of the IgG response was assessed and the results expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (T) are depicted.

No significant differences were observed between responses at the 3 PWM doses tested.



response at P200 and at 2 and 20 μg/ml for P50. The palindromic RA group contained both seropositive and seronegative patients. However, these were not treated as separate groups because of the small number of patients involved in each subset.

Patients with early RA negative disease were found to behave somewhat differently (Figure 6). Here a degree of suppression was observed at all doses of PWM but unlike the other two early RA groups, suppression did not become greater with increasing dilution of PWM. Indeed, statistical analysis showed that there were no significant differences either within a group of responses at a given PWM dose or between different PWM doses at a given concentration of Facb fragment. It is probable that any significant differences which might actually exist in this group were not evident because of (i) the small number of patients investigated and (ii) the variability of the response.

Figure 8 illustrates the effect of Facb on the IgG response in patients with established RA. There was no suppression at P50. Reducing the PWM dose to P200 produced a degree of suppression which differed significantly at 0.2 and 20 μg/ml from that observed at P50. Decreasing the PWM dose further to P1000, however, did not result in a greater degree of suppression. Thus like the early RA negative group suppression was not greatly affected by lowering the concentration of PWM. This group contained both seropositive and seronegative patients with a disease duration of 2 - 22 years. Preliminary data analysis had shown that in contrast to early disease, Facb suppression of IgG production did not differ significantly in patients with established seropositive and seronegative RA.

FIG 7 **Facb-mediated suppression of PWM-stimulated IgG response in patients with early palindromic RA**

Facb was added at final concentrations of 0.2, 2 and 20 µg/ml to PBMNC cultures stimulated with 3 doses of PWM. Suppression of the IgG response was assessed and the results expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (T) are depicted.

* $p < 0.05$ when compared with equivalent concentrations at P1000

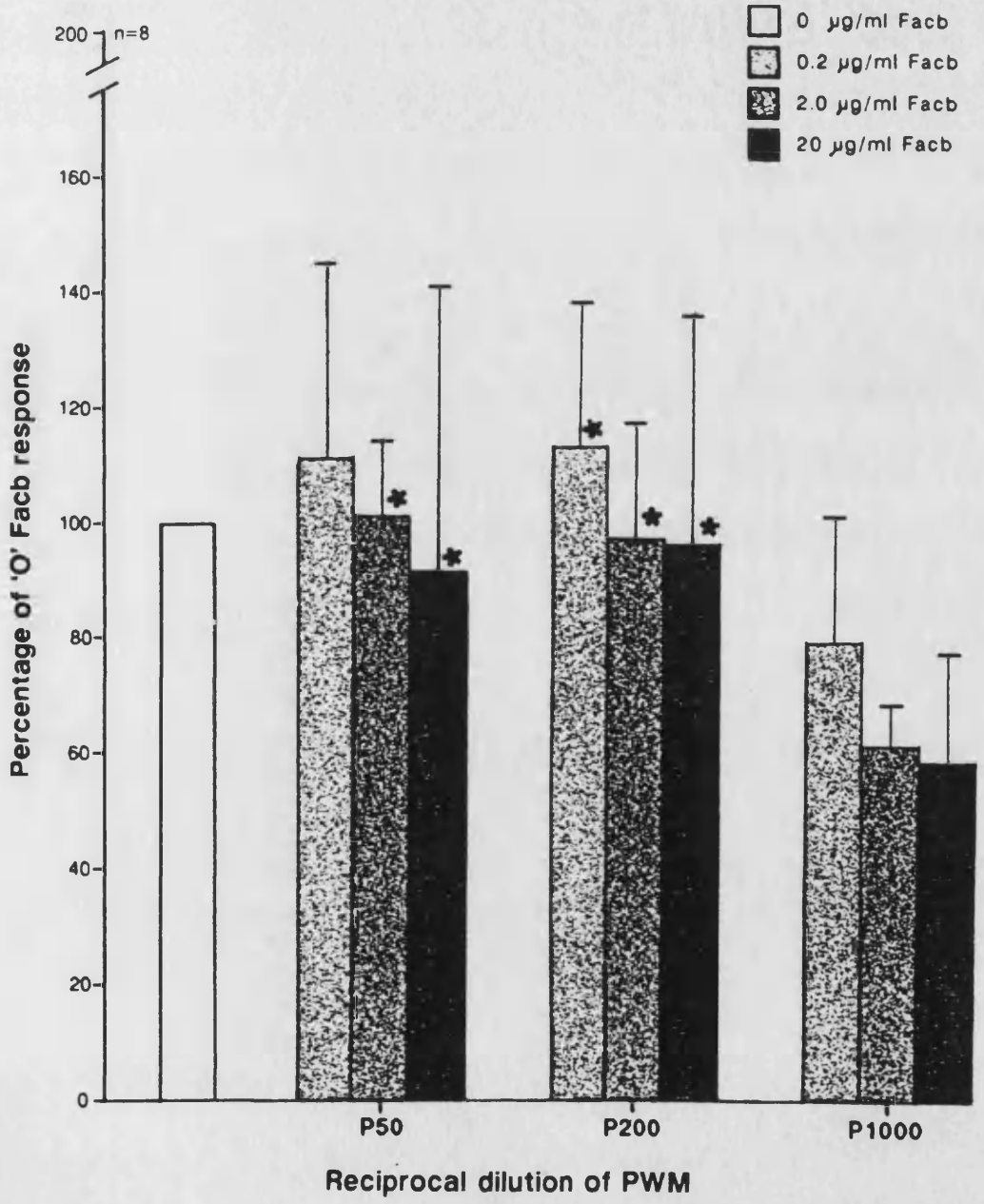
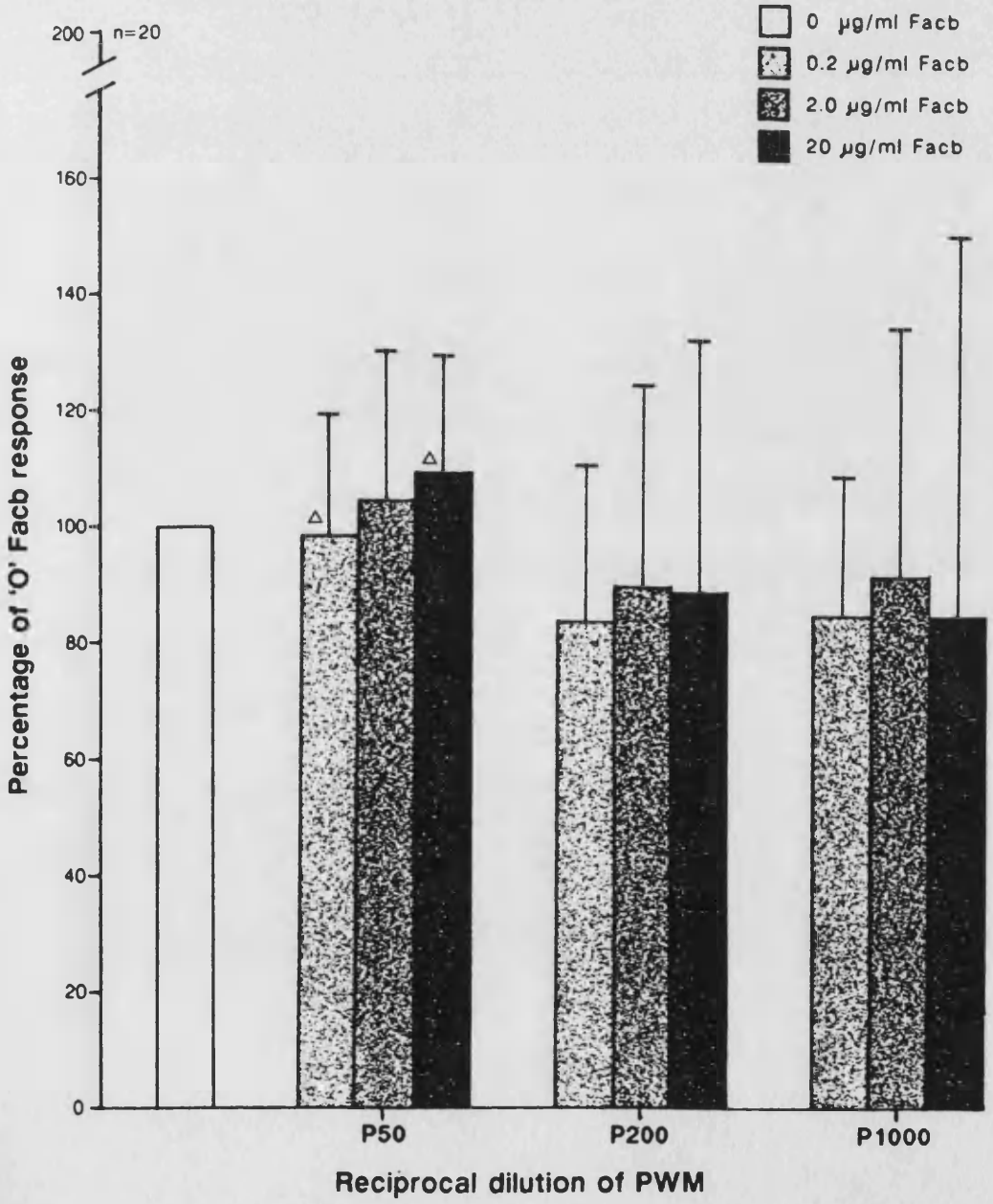


FIG 8 **Facb-mediated suppression of PWM-stimulated IgG response in patients with established RA**

Facb was added at final concentrations of 0.2, 2 and 20 μg/ml to PBMNC cultures stimulated with 3 doses of PWM. Suppression of the IgG response was assessed and the results expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (T) are depicted.

△ $p < 0.05$ when compared to equivalent concentrations at P200



Patients with OA (Figure 9) were also investigated. These patients were clearly different from any of those previously described. There was no suppression of antibody synthesis at any of the PWM doses examined. The overall picture instead was that of enhancement with increasing ligand concentration. However, no statistical differences existed between different Facb concentrations at a given PWM dose nor between a given Facb concentration at different PWM doses. This group also had the highest levels of variability of all the test groups examined, with the greatest variation occurring at an Facb concentration of 20 µg/ml.

In Figure 10 are shown the results obtained with a small number of Felty's patients. The graph shows an incomplete picture of suppression produced by Facb in this group, since data were available for only two patients at P1000 and therefore have not been shown. At P50 there was no suppression at 0.2 and 2 µg/ml Facb. However, at 20 µg/ml Facb the IgG response was reduced to 75% of the baseline level. This, however, was not significantly different from the values noted at the two lower concentrations of Facb. At P200 although the PWM concentration was reduced, very little suppression was produced. This group was found to have the lowest degrees of response variability, possibly because the patients constituted a well-defined group. However, the number of patients investigated was small and the number of individuals responding to PWM even smaller. Therefore the data presented above can at best only be used to draw tentative conclusions.

FIG 9 **The effects of Facb fragment on PWM-stimulated IgG response in OA patients**

Facb was added at final concentrations of 0.2, 2 and 20 μg/ml to PBMNC cultures stimulated with 3 doses of PWM. Suppression of the IgG response was assessed and the results expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (T) are depicted.

No significant differences were observed between responses obtained at the PWM doses tested.

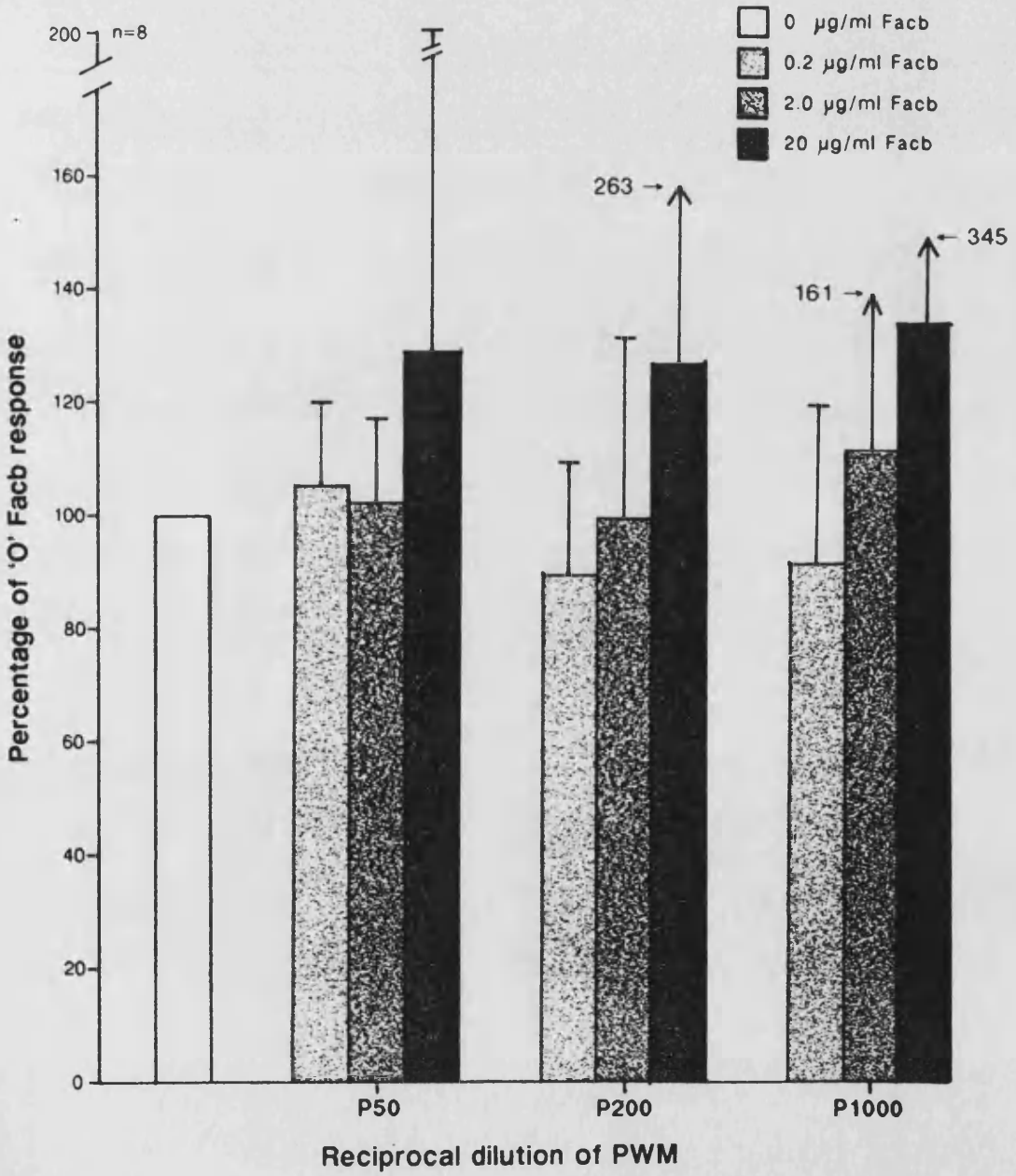
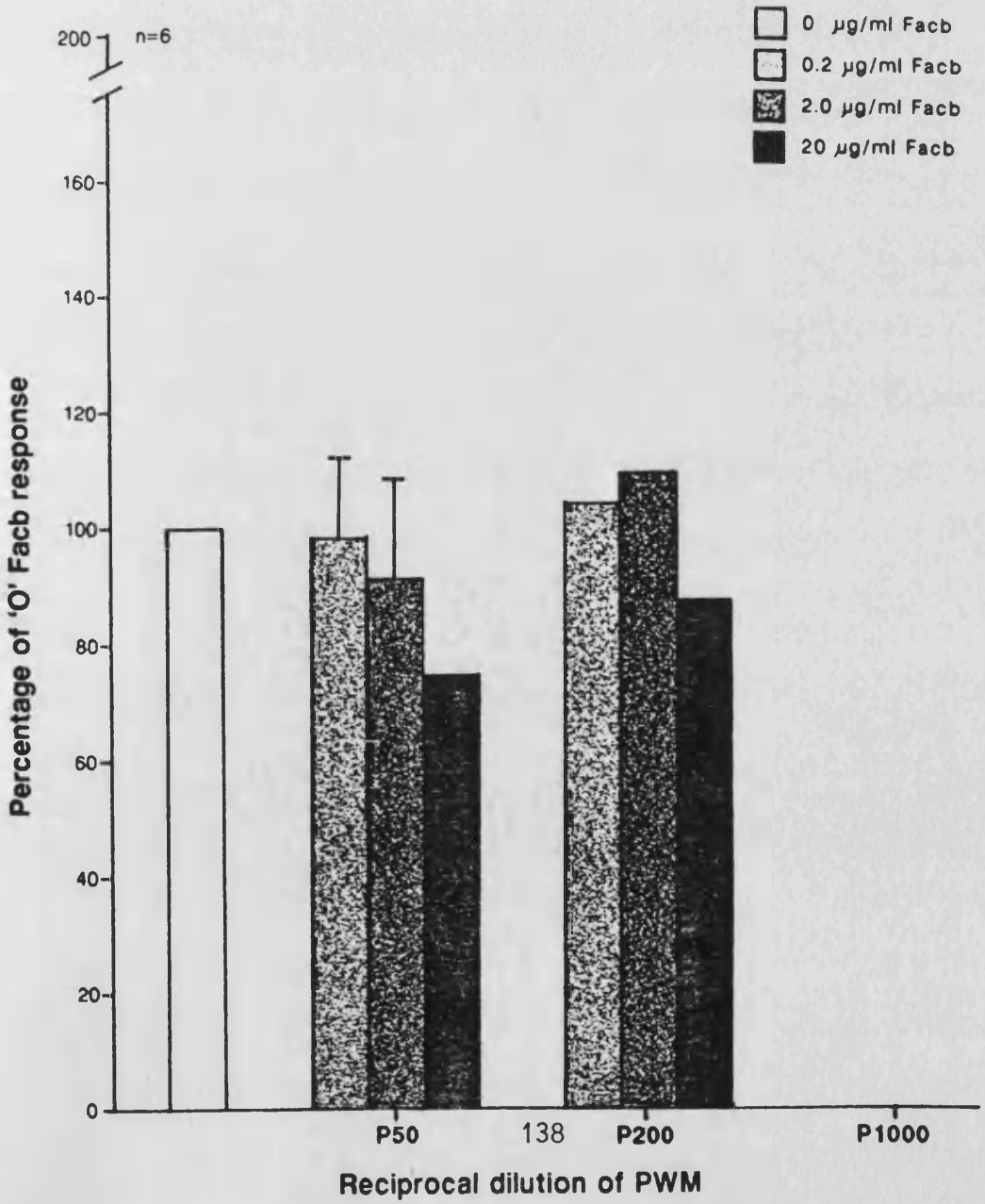


FIG 10 **Facb-mediated suppression of PWM-stimulated IgG response in Felty's patients**

Facb was added at final concentrations of 0.2, 2 and 20 µg/ml to PBMNC cultures stimulated with PWM. Suppression of the IgG response was assessed and results expressed as a percentage of the stimulated response where no Facb was present. Median values are shown and interquartile ranges (T) included where possible.

No significant differences were observed between responses obtained at the 2 PWM doses.



In summary:-

i) Facb suppression of IgG production was dependent on the dose of PWM used. Thus, there was virtually no suppression of antibody response at P50, but with decreasing PWM concentration, increasing Facb suppression was seen, with the greatest being seen at P1000. This is illustrated in Figure 11. Whilst this was true of normals, early RA positive and palindromic subjects, suppression in the early RA negative and RA groups was not found to be significant, even at the lowest PWM dose. In early seronegative disease similar levels of suppression were observed at all three concentrations of mitogen, whilst in established RA responses to Facb differed only at P50 and not between P200 and P1000. Alteration of PWM dose had no effect on Facb modulation of IgG response in OA patients. At all three concentrations of mitogen, antibody synthesis was found to be enhanced in the presence of the ligand.

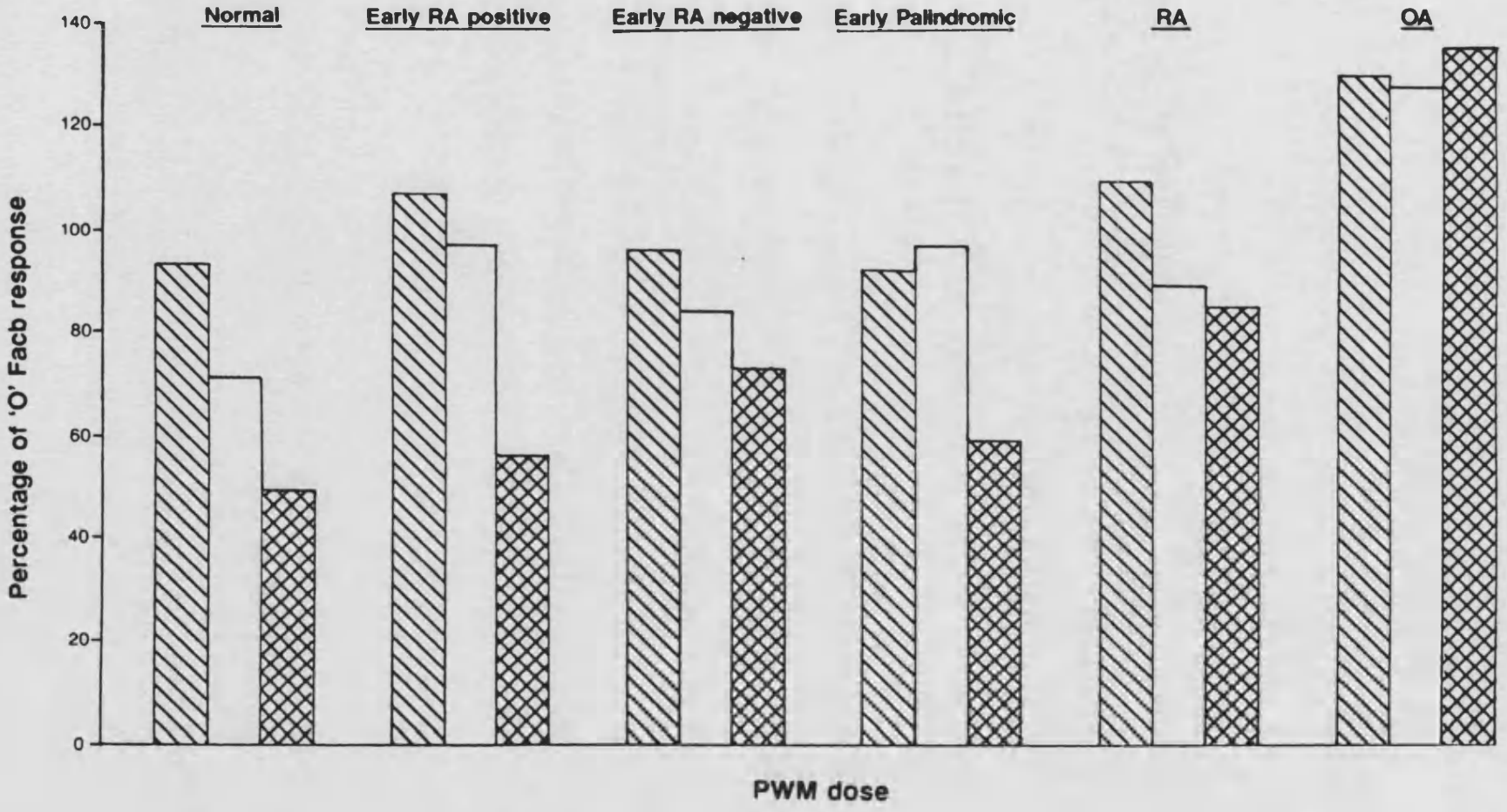
ii) At all doses of PWM and Facb the effect of the ligand on IgG production was highly variable. Greatest variation was most frequently seen at P50 with 20 μg/ml Facb and of all the test groups examined, patients with OA exhibited the greatest variability of response. This variability may also explain why, where suppression occurred it was not always in a truly dose-dependent manner.

3. Comparison of Facb suppression of IgG responses between test groups

The levels of suppression produced by Facb in the various groups were compared using the Mann-Whitney U-test. Although all doses of PWM were examined, only responses observed at P1000 were

FIG 11 **Variation in Facb-mediated suppression of the IgG response with PWM dose in control and patient groups**

Only results obtained with 20 µg/ml final concentration of Facb at P50 (▨), P200 (□) and P1000 (▩) are depicted. These are expressed as percentage of the stimulated response where no Facb was present. Median values are shown for each group. Interquartile ranges are omitted for purposes of clarity. In most groups suppression was greatest at P1000.



thought to be of any relevance since suppression was found to occur best at this dose. The results of the comparisons at P1000 are shown in Table 1A.

Patients with early RA positive and palindromic disease were not found to differ from normal healthy controls. However, responses seen in subjects with early RA negative and established disease were observed to reach levels of significance at 20 μg/ml Facb and those in OA patients were significantly different from controls at all the Facb concentrations examined. No differences of any biological significance were found when comparing the degrees of suppression between the early RA groups. However, when these patients were compared with subjects with established RA, significant differences were seen between the latter and patients with early RA positive and palindromic disease at 2 and 20 μg/ml Facb. In contrast, early RA negative subjects were not found to be different from patients with established RA at any Facb concentrations, nor were they different from OA patients. RA and OA patients were not found to differ from each other.

Felty's patients only exhibited some degree of suppression and this was at P50 with 20 μg/ml Facb. Comparing this with responses observed in other groups it was found that these patients were highly significantly different from early RA positive, OA and RA patients, but not from normal controls, palindromics and early RA negative patients.

In summary, at P1000 where the best suppression occurred, early seronegative patients behaved like RA and OA patients with regard to Facb suppression of IgG response, whilst those with early seropositive and palindromic RA were akin to normal healthy controls. However, early seropositive and seronegative patients themselves did

TABLE 1A **Comparison of Facb suppression of IgG response at P1000 in health and disease**

Clinical group		Levels of significance and Facb dose in μg/ml
Normal controls	: Early RA positive	NS
	: Early palindromic	NS
	: Early RA negative	20 **
	: RA	20 ***
	: OA	0.2 *
		2 *
		20 **
Early RA positive : seronegative : palindromic		NS
Early RA negative	: RA	NS
Early palindromic RA	: RA	2 *
Early RA positive	: RA	20 *
RA : OA		NS

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

not differ from each other. RA and OA patients were indistinguishable, in spite of the slightly different effects produced in each by the ligand. No conclusion could be drawn on Felty's patients because of insufficient data.

4. Facb suppression and PWM response

From Figures 4 - 10 it is clear that where there is suppression this occurs best at P1000. One explanation for this is that at P50 the IgG response is too powerful to be overcome and therefore suppression by Facb is minimal. However, when the stimulating dose is reduced to P1000, the IgG response is perhaps not so great and thus more easily suppressed. Figures 12 - 15 show the PWM dose response curves obtained with the test groups examined. It can be seen that in normal controls and in palindromics, far from the response falling at P1000, it was much higher than at P50. With the early seropositive and seronegative patients, although the overall IgG response was lower than in the former two groups, levels at P1000 in the early patients were not lower than those observed at P50. It should, however, be noted that in the seronegative group IgG synthesis at the three doses of PWM was similar. Higher levels of antibody production were obtained with patients with established RA. Although IgG production was lower at P1000 than at P200, the levels of Facb suppression seen at these two doses were similar. Data obtained with OA patients also serve to emphasise the point that lower levels of IgG production do not necessarily mean greater Facb suppression. Here Facb was found to have no effect even at P1000, where immunoglobulin production was observed to be the lowest.

In the light of the above data another explanation was

FIG 12 The IgG response at 3 doses of PWM in normal healthy controls

Median values are shown. (T) represent interquartile ranges.

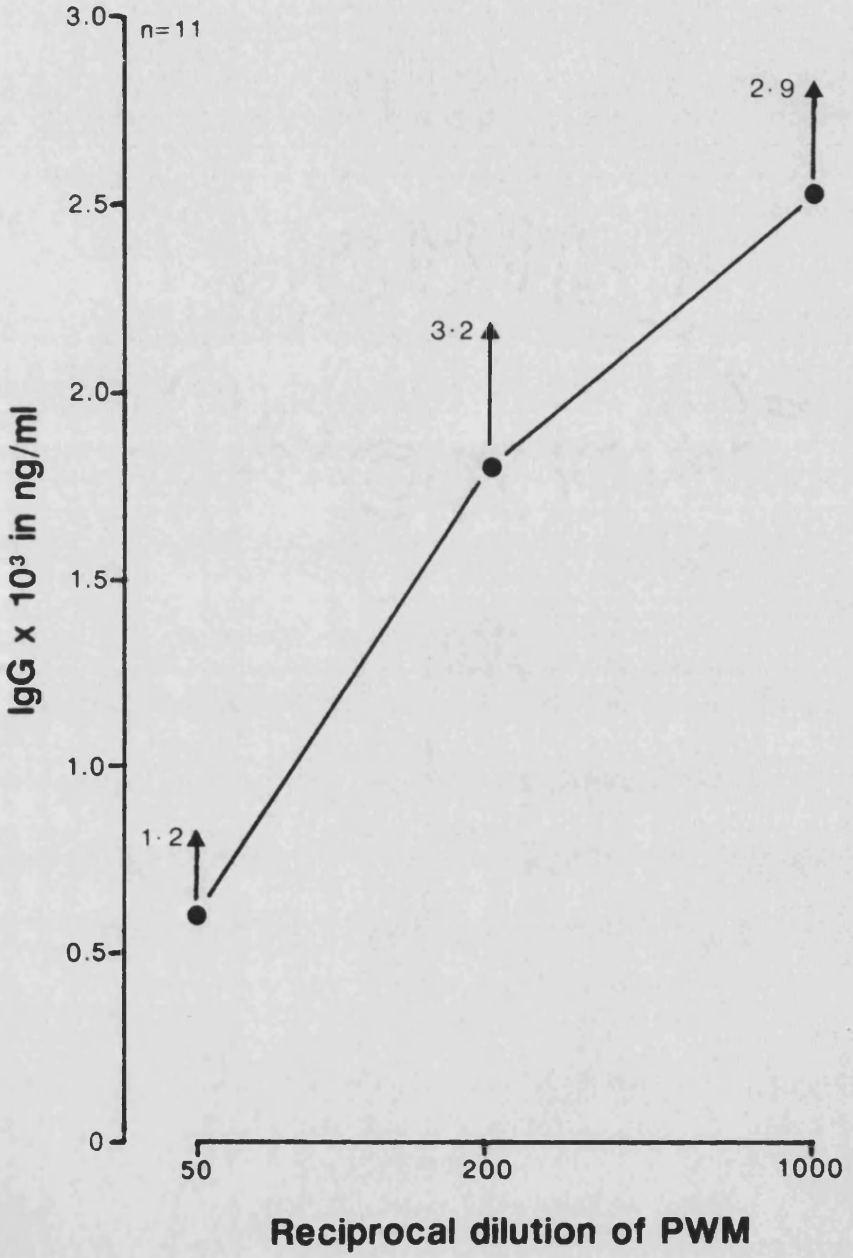


FIG 13 **The IgG response at 3 doses of PWM in early RA**

Early RA positive, negative and palindromic patients are represented by (▲——▲), (O——O) and (■——■) respectively. Median values are shown. Interquartile ranges are omitted for clarity.

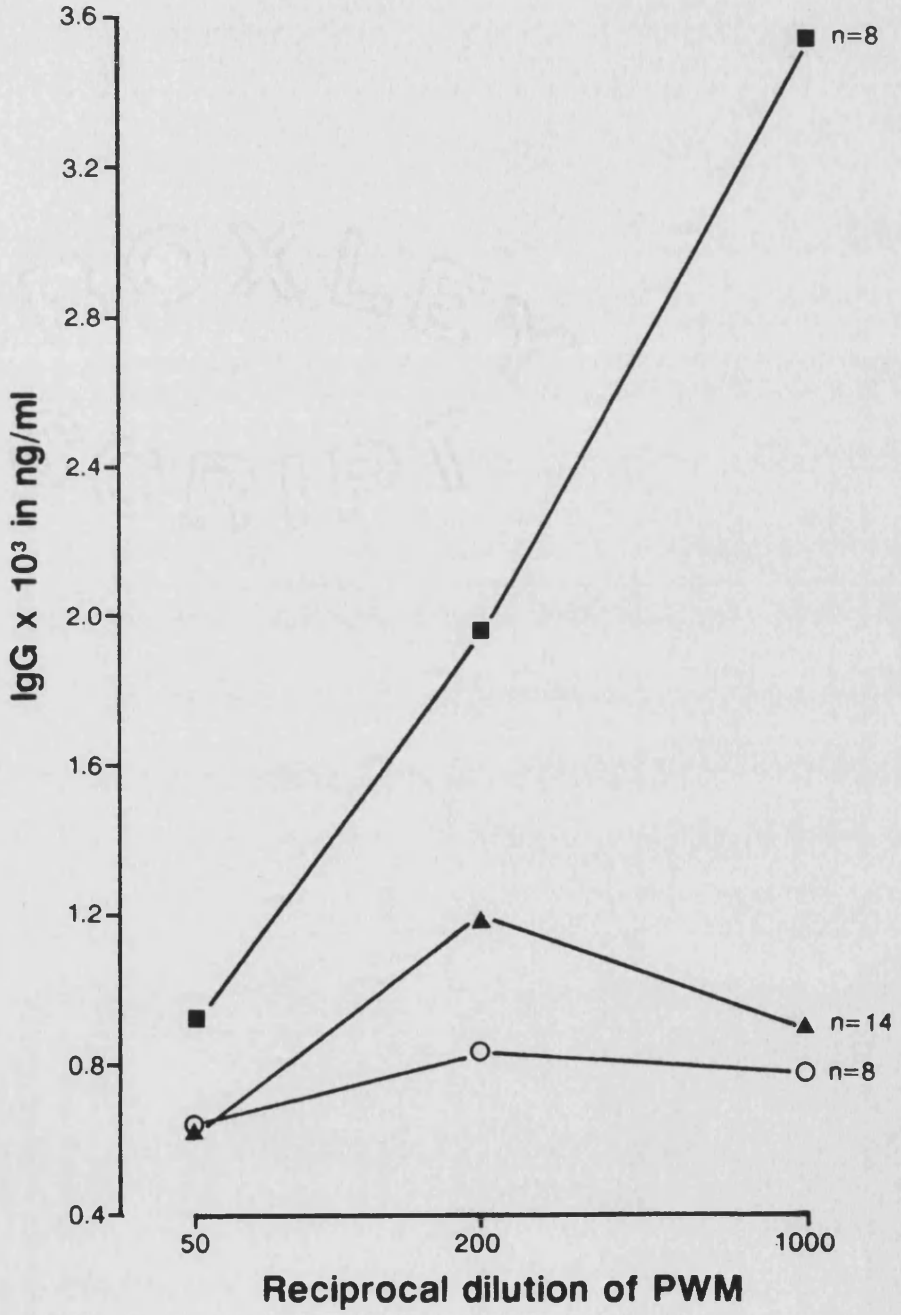


FIG 14 The IgG response at 3 doses of PWM in established RA patients

Median values are shown. ↑ represent interquartile ranges.

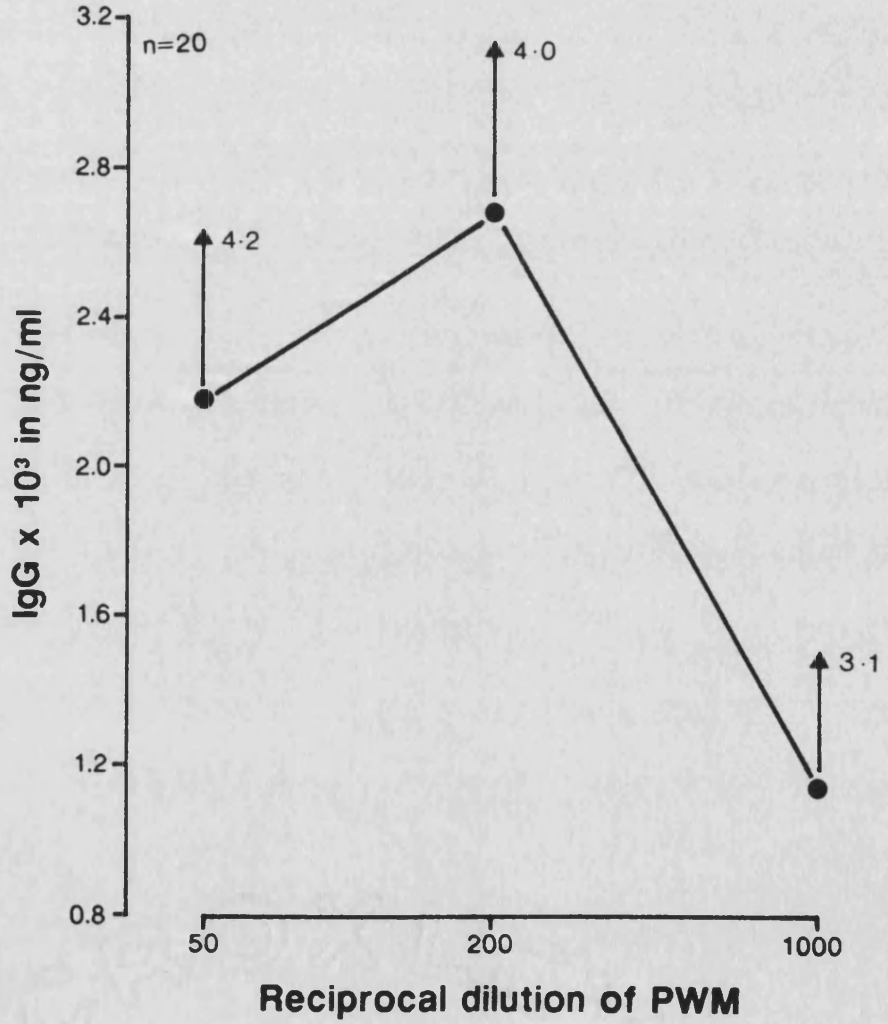
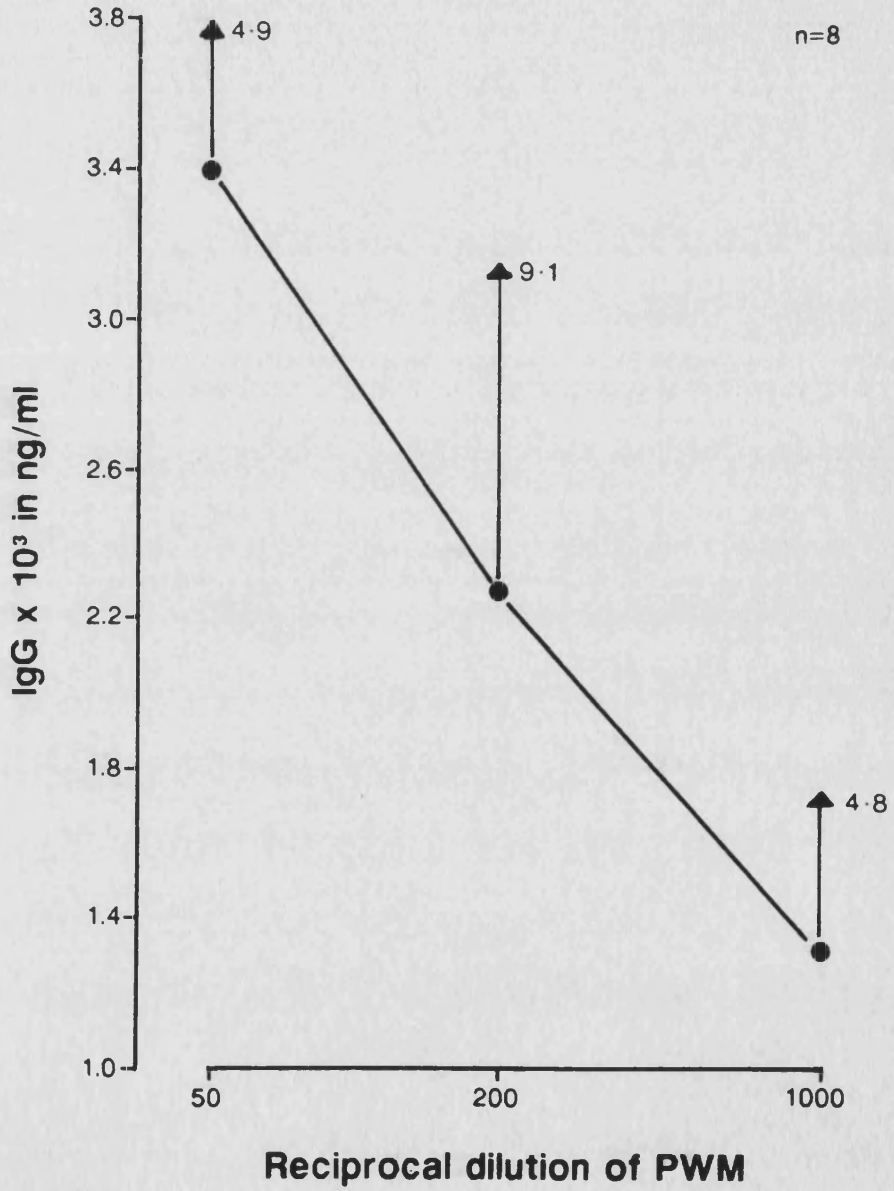


FIG 15 **The IgG response at 3 doses of PWM in OA patients**

Median values are shown. ↕ represent interquartile ranges.



considered - that with maximum stimulation, as for example in normal controls at P1000, the FacbR$^+$ population is expanded and more FacbR$^+$ cells are available for recruitment into negative feedback suppression. However, other factors must be involved since patients with established RA were found to possess higher levels of FacbR$^+$ cells compared to normal subjects but Facb-mediated suppression was impaired.

Another explanation of the better Facb suppression of IgG seen at P1000 is that PWM at higher concentrations might block the FacbRs on the PBMNC. This was investigated and the results are presented in the next section.

5. Effect of PWM on Facb rosettes

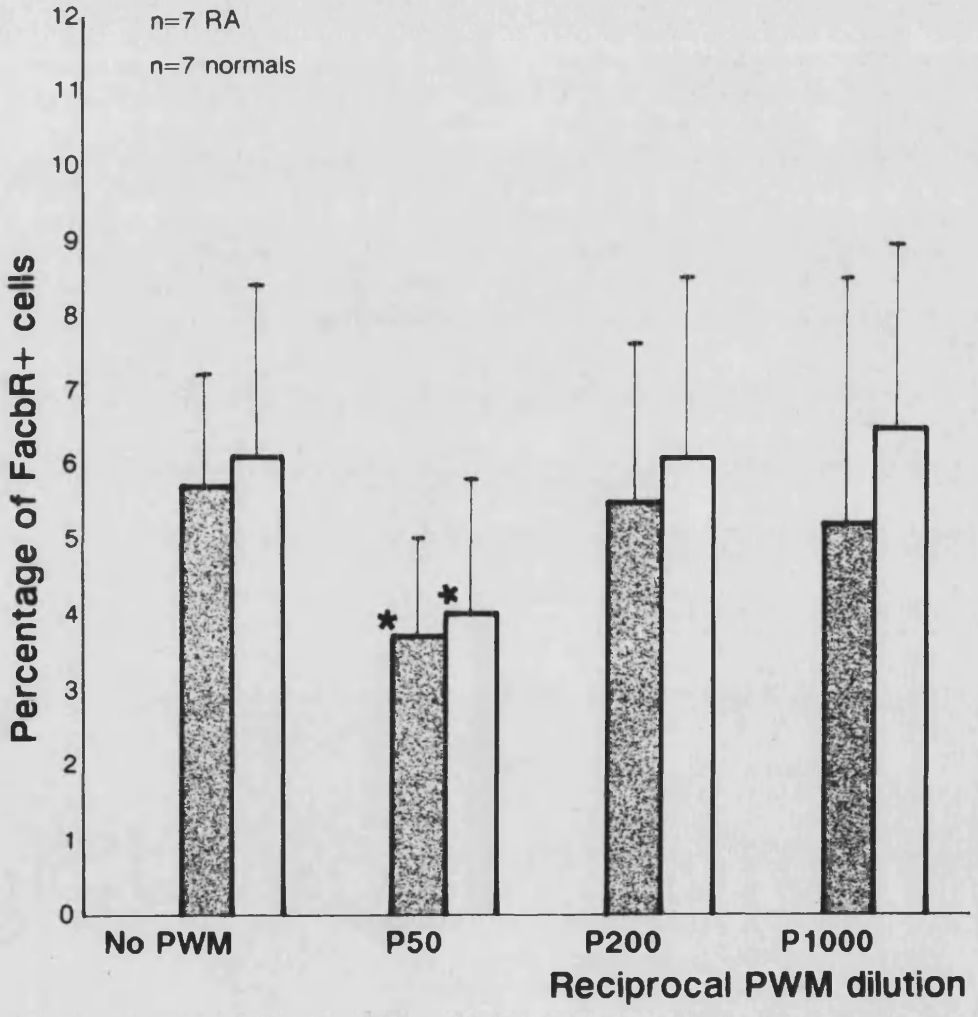
Facb rosette assays were performed in the presence of PWM at final dilutions of 50, 200 and 1000. Controls assays contained an appropriate amount of CMFSS. Both normal healthy controls and RA patients were examined. Results of these experiments appear in Figure 16. It can be seen that the addition of PWM at a 1/50 dilution markedly reduced the number of Facb rosettes; a p value of < 0.05 compared to control assays was obtained for both RA and normal subjects. When the PWM was added at reciprocal dilutions of 200 and 1000, percentages of Facb rosettes were found to return to near normal levels in both groups. Interestingly, in one normal healthy subject Facb rosettes were not found to be suppressed by PWM. This individual was also found not to respond to PWM *in vitro*. This, together with the data above, seems to suggest that Facb receptors and those for mitogen may be closely associated on the cell surface.

Since the presence of PWM or the response to it can alter the suppressive effects of Facb, it is possible that even at low doses,

FIG 16 **The effect of PWM on Facb rosettes**

PWM at final reciprocal dilutions of 50, 200 and 1000 was added to PBMNCs from RA (▩) and normal subjects (□), which were then assessed for percentage of $FacbR^+$ cells in the rosette assay. Median values and interquartile ranges (T) are shown.

* $p < 0.05$ when compared to rosette numbers observed in the absence of PWM



PWM might change the kinetics of this suppression. Therefore, suppression of spontaneous IgG synthesis by Facb was examined. Results of this are presented in the next section. Only those individuals producing more than 100 ng/ml of IgG in the resting state were examined.

6. Facb suppression of spontaneous IgG production

Figures 17 - 23 show the effects of Facb fragment on spontaneous IgG production in the various test groups. The absolute values of IgG generated spontaneously in each group appear in Table 2.

In normal healthy controls Facb was found to be suppressive at all concentrations, reducing the spontaneous IgG level to 86%, 72% and 65% of the original response with 0.2, 2 and 20 ug/ml of ligand respectively. Although these values were not significantly different from each other, it can be seen from Figure 17 that the suppression was dose-related. As with the PWM-driven response, the effects of the ligand on spontaneous antibody generation were variable. In all the other groups examined, levels of suppression were either lower, as for example with early RA patients, or practically nil, as with patients with established RA, OA and Felty's syndrome. Again in all groups variability of response was noted but not to the extent seen with PWM-stimulated cultures. Furthermore, as before, OA patients exhibited the highest levels of variation.

The degrees to which Facb suppressed spontaneous production of IgG in the various test groups were compared. These results appear in Table 2A. The data here differ from those found at P1000 (see Table 1A) in the following respects. Firstly, patients with early RA negative disease were found not to differ from normal healthy controls but did so significantly ($p < 0.05$) from subjects with established RA. However, no differences were seen when compared with

TABLE 2 **The effect of Facb fragment on spontaneous IgG production in test groups**

Median levels of spontaneous IgG production (ng/ml +/- interquartile range) observed in the various test groups are depicted. The effects of increasing Facb concentrations on this synthesis are also shown. Only those individuals generating ⩾ 100 ng/ml of IgG spontaneously were selected for analysis.

	Facb in µg/ml			
Test groups and numbers	0	0.2	2	20
Normals	163	140	118	105
n = 11	+/- 86	+/- 76	+/- 57	+/- 26
Early RA positive	211	205	192	186
n = 16	+/- 179	+/- 25	+/- 44	+/- 45
Early RA negative	143	125	123	121
n = 7	+/- 58	+/- 33	+/- 18	+/- 7
Early palindromic RA	137	121	127	122
n = 7	+/- 125	+/- 28	+/- 8	+/- 29
Established RA	183	179	187	202
n = 16	+/- 162	+/- 33	+/- 40	+/- 78
OA	310	297	291	307
n = 9	+/- 480	+/- 63	+/- 175	+/- 193
Felty's	272	277	254	283
n = 9	+/- 234	+/- 30	+/- 98	+/- 92

FIG 17 **The effect of Facb on spontaneous IgG production in normal healthy controls**

Results are expressed as a percentage of the unstimulated IgG response where no Facb was present. Median values and interquartile ranges (T) are shown.

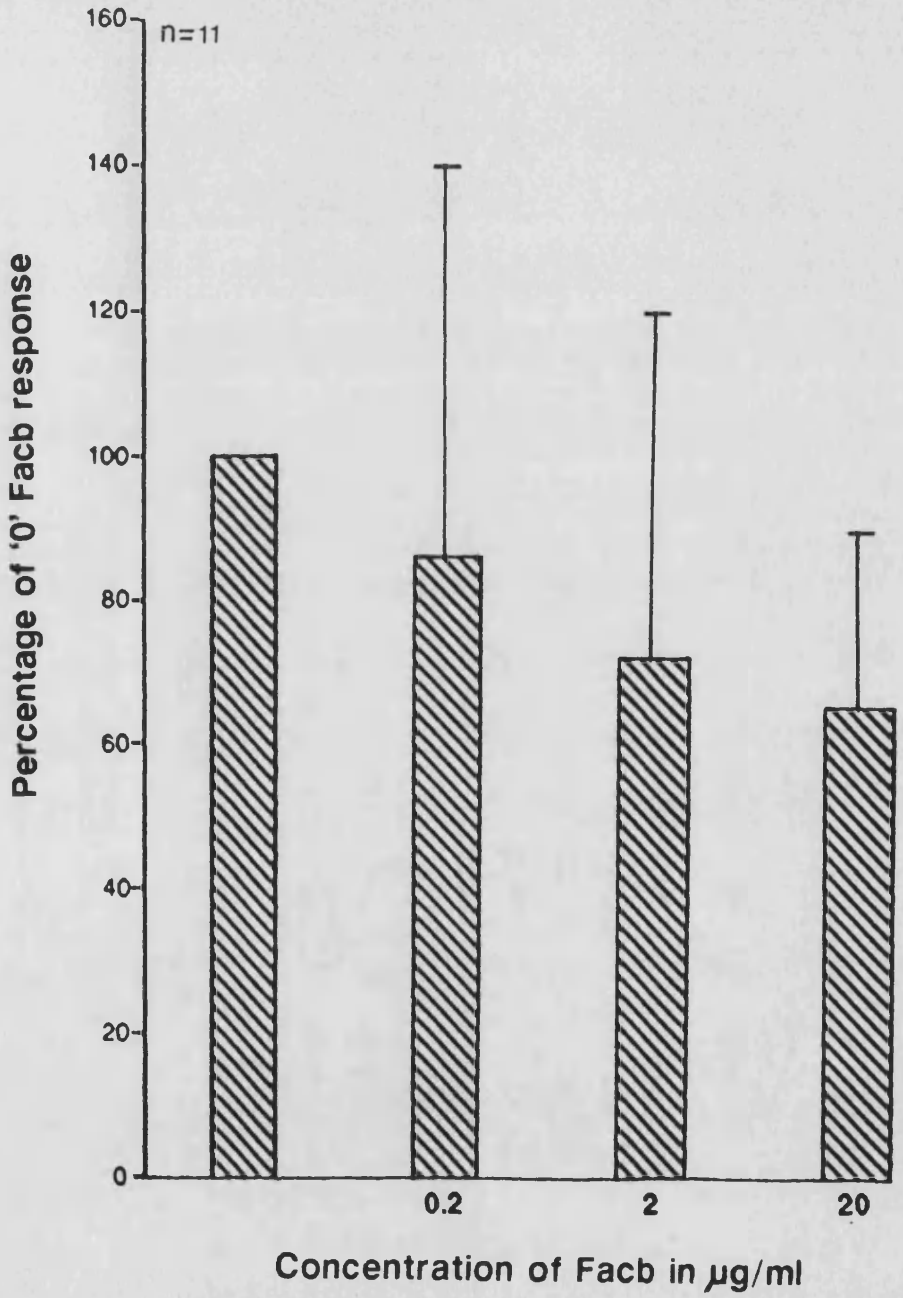


FIG 18 **The effect of Facb on spontaneous IgG production in early RA positive patients**

Results are expressed as a percentage of the unstimulated IgG response where no Facb was present. Median values and interquartile ranges (T) are shown.

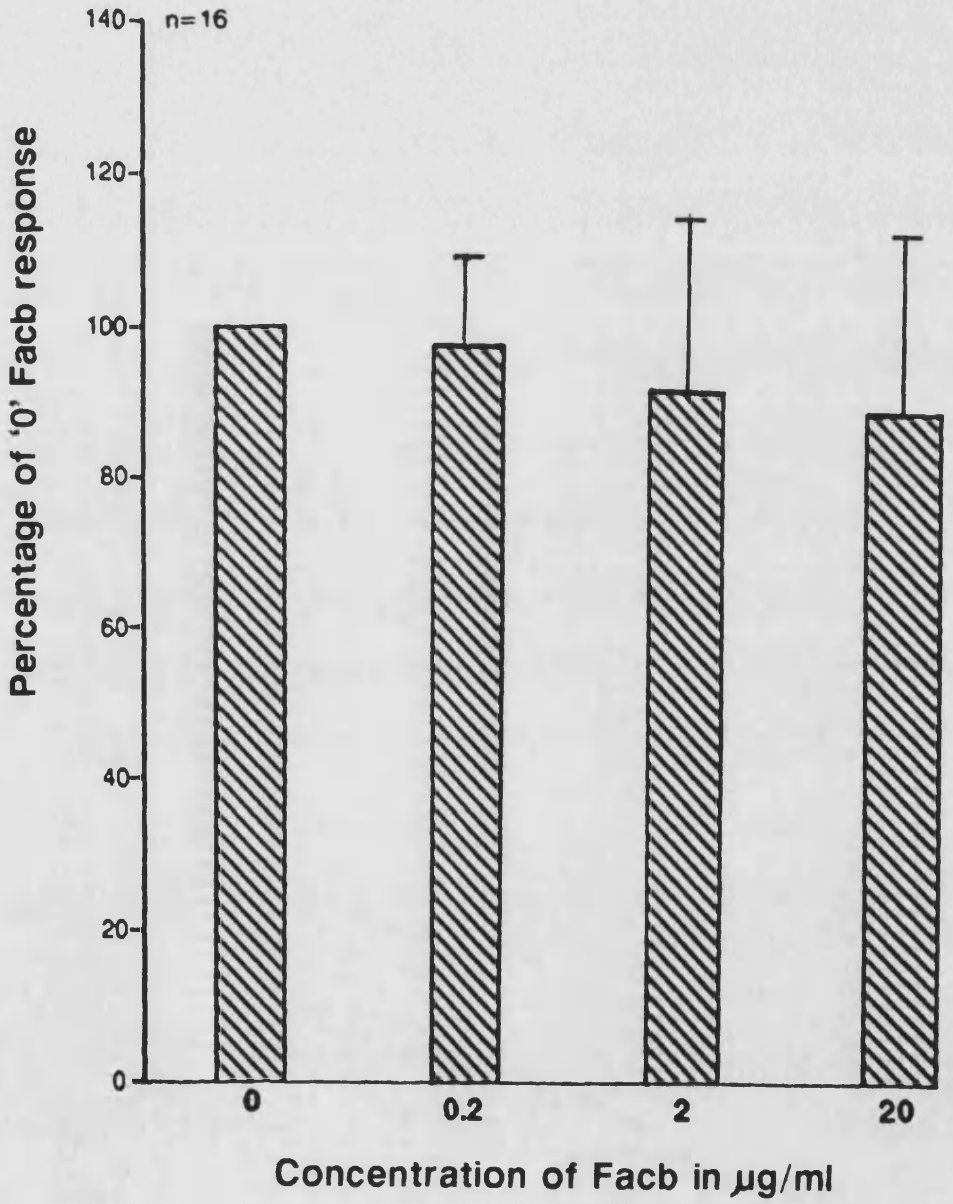


FIG 19 **The effect of Facb on spontaneous IgG production in early RA negative patients**

Results are expressed as a percentage of the unstimulated IgG response where no Facb was present. Median values and interquartile ranges (T) are shown.

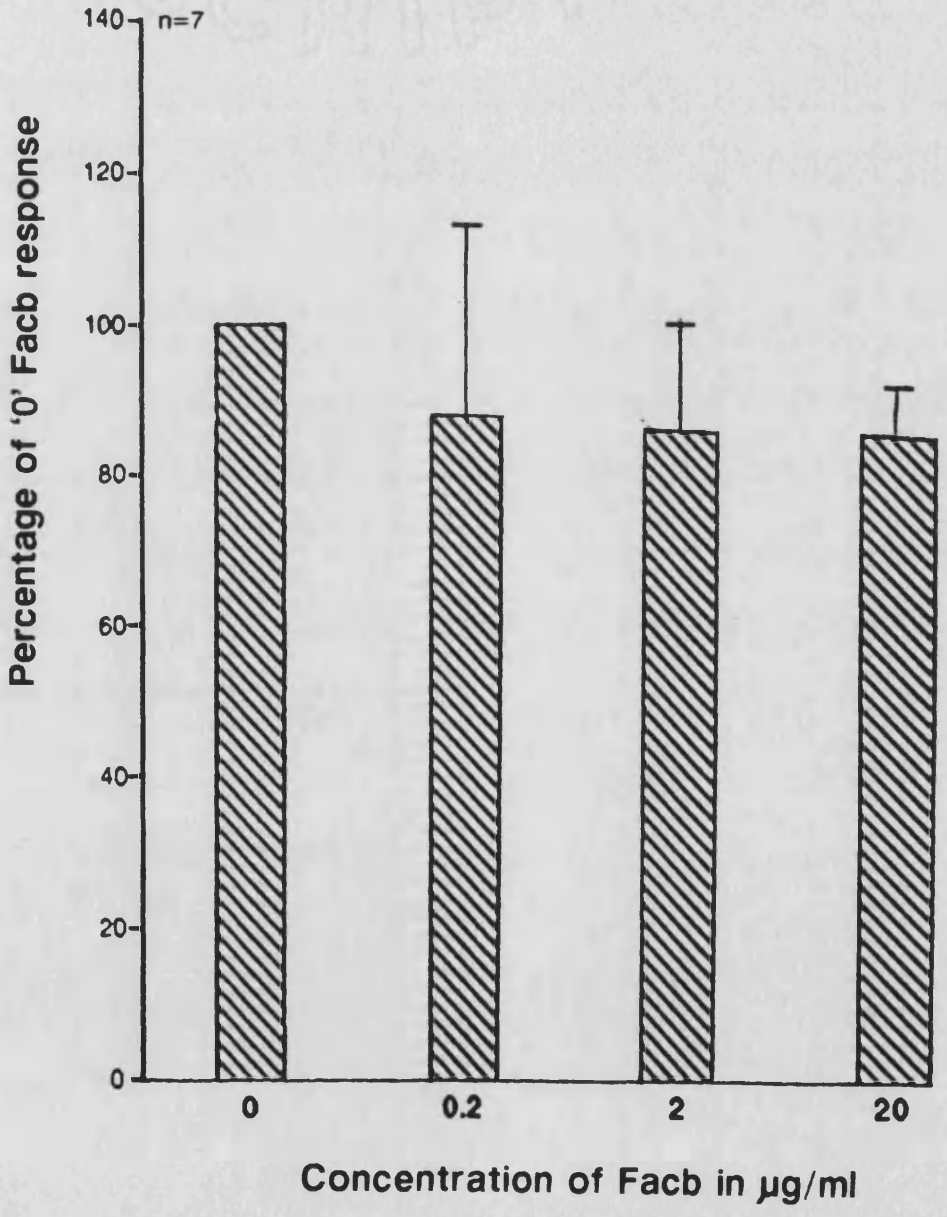


FIG 20 **The effect of Facb on spontaneous IgG production in early palindromic RA patients**

Results are expressed as a percentage of the unstimulated IgG response where no Facb was present. Median values and interquartile ranges (T) are shown.

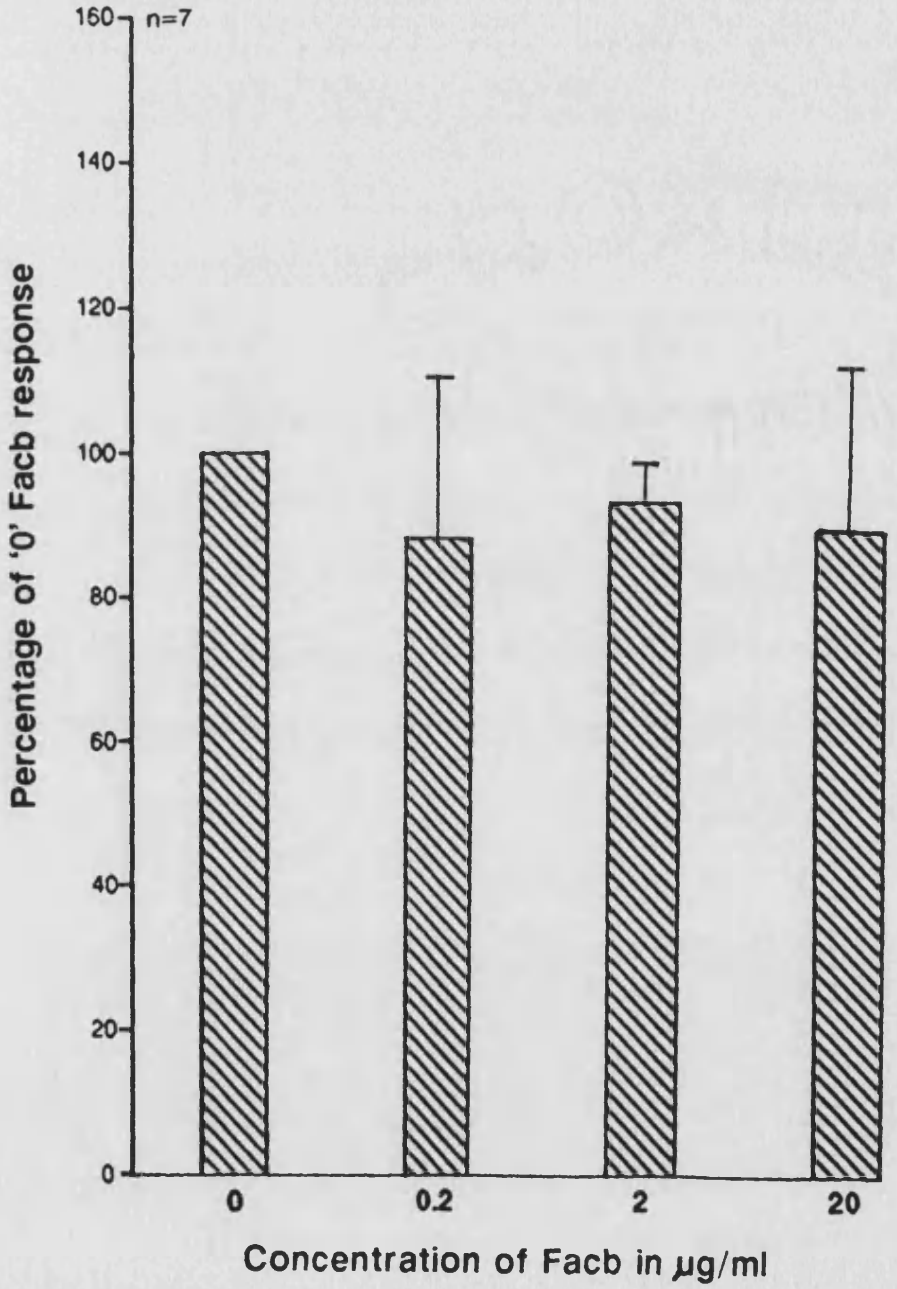


FIG 21 **The effect of Facb on spontaneous IgG production in established RA patients**

Results are expressed as a percentage of the unstimulated IgG response where no Facb was present. Median values and interquartile ranges (T) are shown.

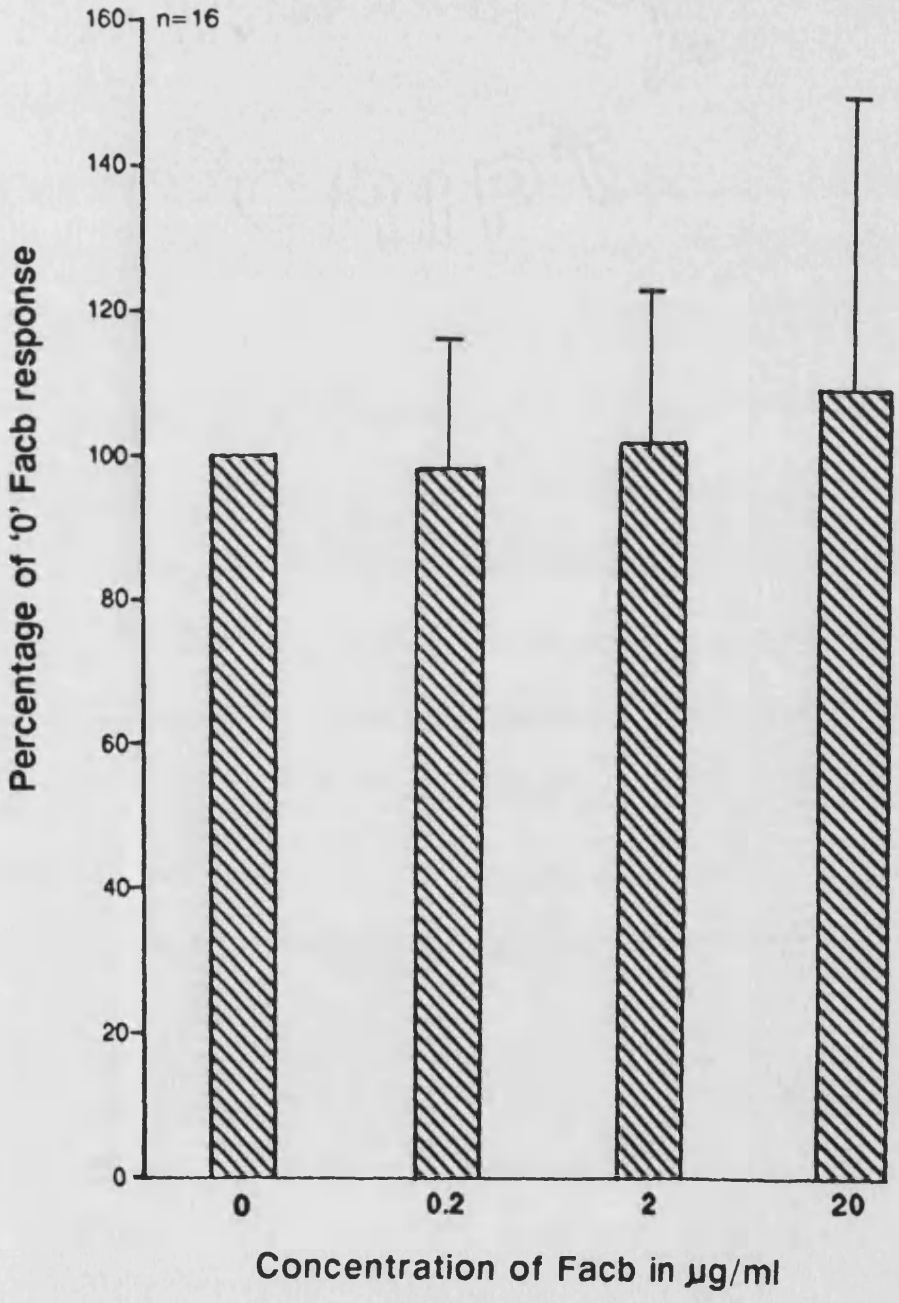


FIG 22 The effect of Facb on spontaneous IgG production in OA patients

Results are expressed as a percentage of the unstimulated IgG response where no Facb was present. Median values and interquartile ranges (T) are shown.

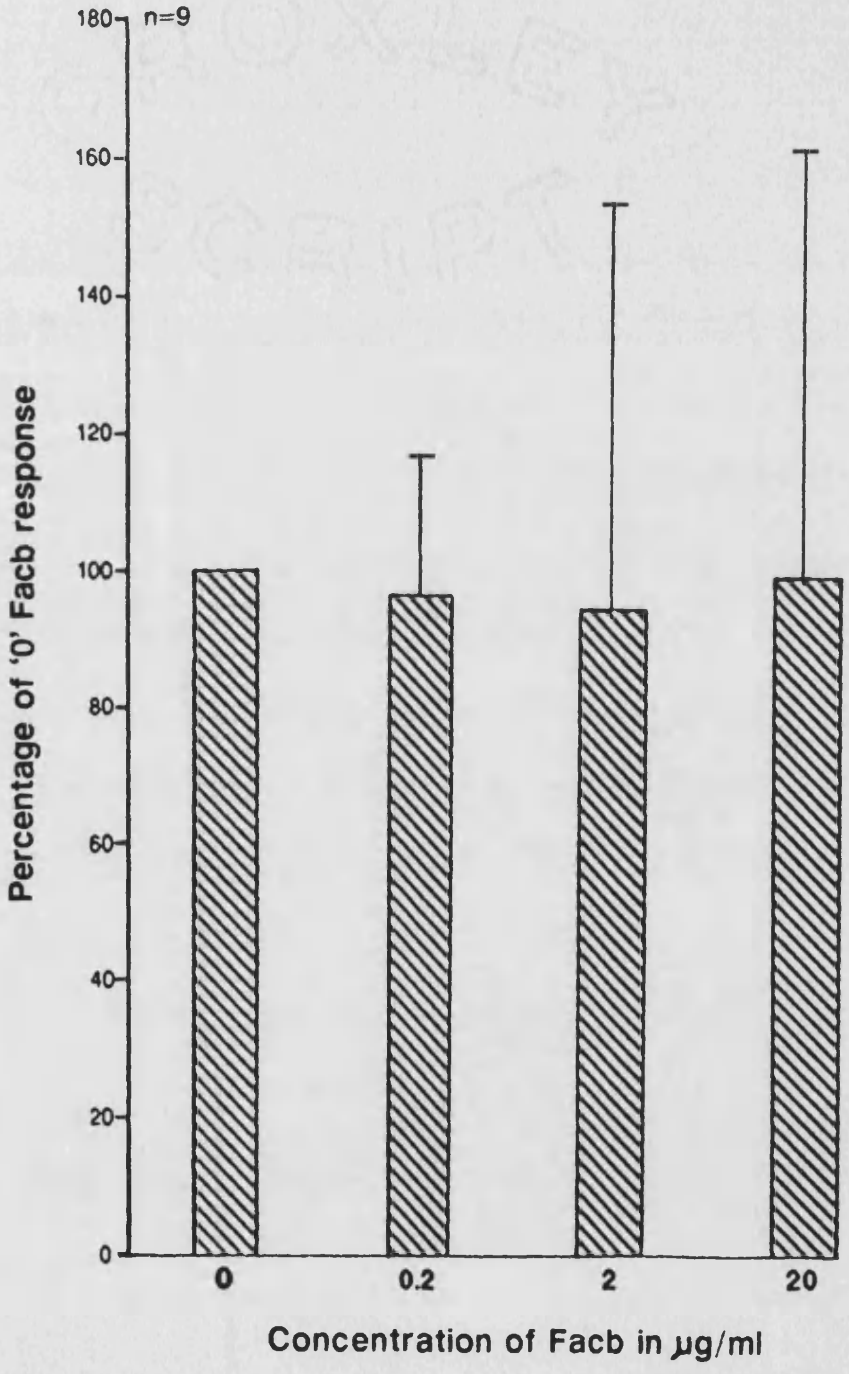


FIG 23 **The effect of Facb on spontaneous IgG production in Felty's patients**

Results are expressed as a percentage of the unstimulated IgG response where no Facb was present. Median values and interquartile ranges (T) are shown.

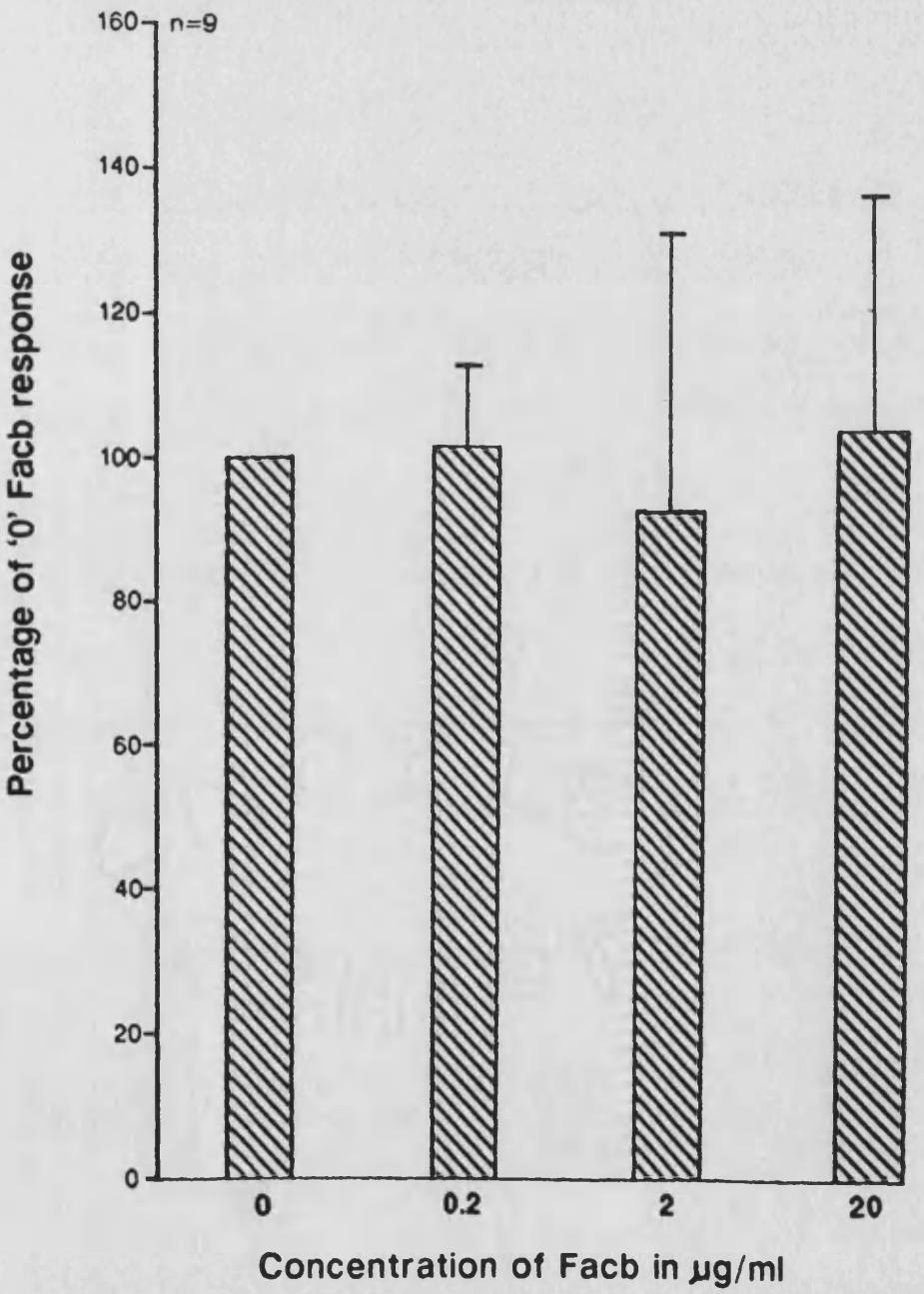


TABLE 2A Comparison of Facb suppression of spontaneous IgG production in health and disease

Clinical group		Levels of significance and Facb dose in µg/ml
Normal controls	: Early RA negative	NS
	: Early palindromic	NS
	: Early RA positive	20 **
	: RA	2 **
		20 **
	: Felty's	2 *
		20 **
	: OA	2 *
		20 **
Early RA positive : palindromic : negative		NS
Early RA negative	: RA	2 *
		20 *
Early RA positive Early palindromic	: RA	NS
Early RA positive negative palindromic	: Felty's	NS
RA : Felty's :OA		NS

* $p < 0.05$
** $p < 0.01$

Felty's patients. Secondly, early RA positive patients differed from normal subjects, although this was only at the highest concentration of Facb.

Comparing the data shown in Figures 17 - 23 with those obtained at P1000 in these groups produced very few results of statistical significance. This is surprising in view of the obviously greater suppression seen in stimulated cultures, especially in early RA. However, this can be explained by the large variability of response, particularly in the PWM-driven system. Comparison of these two sets of data suggests the following:-

i) In normal subjects PBMNC are able to respond to the suppressive effects of Facb in both the stimulated and resting state.

ii) PBMNC from early RA patients in unstimulated cultures are less responsive to FcR-mediated feedback suppression than controls. However, as seen in Section 1(2), cells from these patients possess the ability to be triggered by mitogen to suppressive activity, as for example in early RA positive and palindromic disease. This capacity for some reason appears to be impaired in early RA negative patients.

iii) Unstimulated PBMNC from RA subjects are not suppressed by Facb fragment. Furthermore, the ability to be induced to suppressor function is diminished or absent. Similarly, patients with OA are not suppressed in the resting state. Modulation of FcR cells by ligand in the presence of PWM in these patients serves to enhance the immunoglobulin response.

Felty's patients could not be compared in this manner because of a lack of data at P1000.

SECTION 2 FACB SUPPRESSION OF IgG RESPONSE AND DISEASE PARAMETERS

Associations between various disease parameters and Facb suppression of IgG synthesis were examined in early RA patients. All comparisons were made at the P1000 response. These are discussed below.

1. Facb suppression and disease activity

Patients with early RA were assessed for disease activity and the results expressed as a figure ranging from 0 (little or no activity) to 5 (severe synovitis and very active disease). Figure 24 shows the various parameters used to arrive at a grading of the disease. This is based on the Ritchie Articular Index.

The relationship between Facb suppression of IgG production at P1000 and disease activity was examined using only early RA positive patients. Early RA negative and palindromic subjects were not examined because in the former very little Facb suppression was observed (see Section 1(2)). In the latter, disease activity ranged only from 0 - 1.

Figure 25 shows how Facb suppression in early RA positive subjects varied with increasing disease activity at 0.2, 2 and 20 µg/ml Facb. As disease activity increased suppression became more apparent at all Facb doses. The best correlation is seen at 0.2 µg/ml with an r_s value of 0.56 ($p < 0.05$). At 20 µg/ml Facb levels of significance are approached with an r_s value of 0.455. At 2 µg/ml Facb r_s has a value of only -0.179, which is not significant.

The greater degree of Facb suppression with increasing disease activity seems puzzling at first sight. However, analysis of the number of $FacbR^+$ cells at different disease activities provides one

FIG 24 Clinical parameters used to assess disease activity in early RA patients

Disease activity was scored on a scale from 0 - 5 depending on active joints (painful and/or swollen), morning stiffness and plasma viscosity. Details of assessment are given below. Joints counted as used in Ritchie Index.

Score	Criteria
0	Plasma viscosity normal; no active joints
1	⩽ 2 active joints
2	⩽ 4 active joints
3	⩽ 8 active joints
4	⩽ 12 active joints
5	> 16 active joints

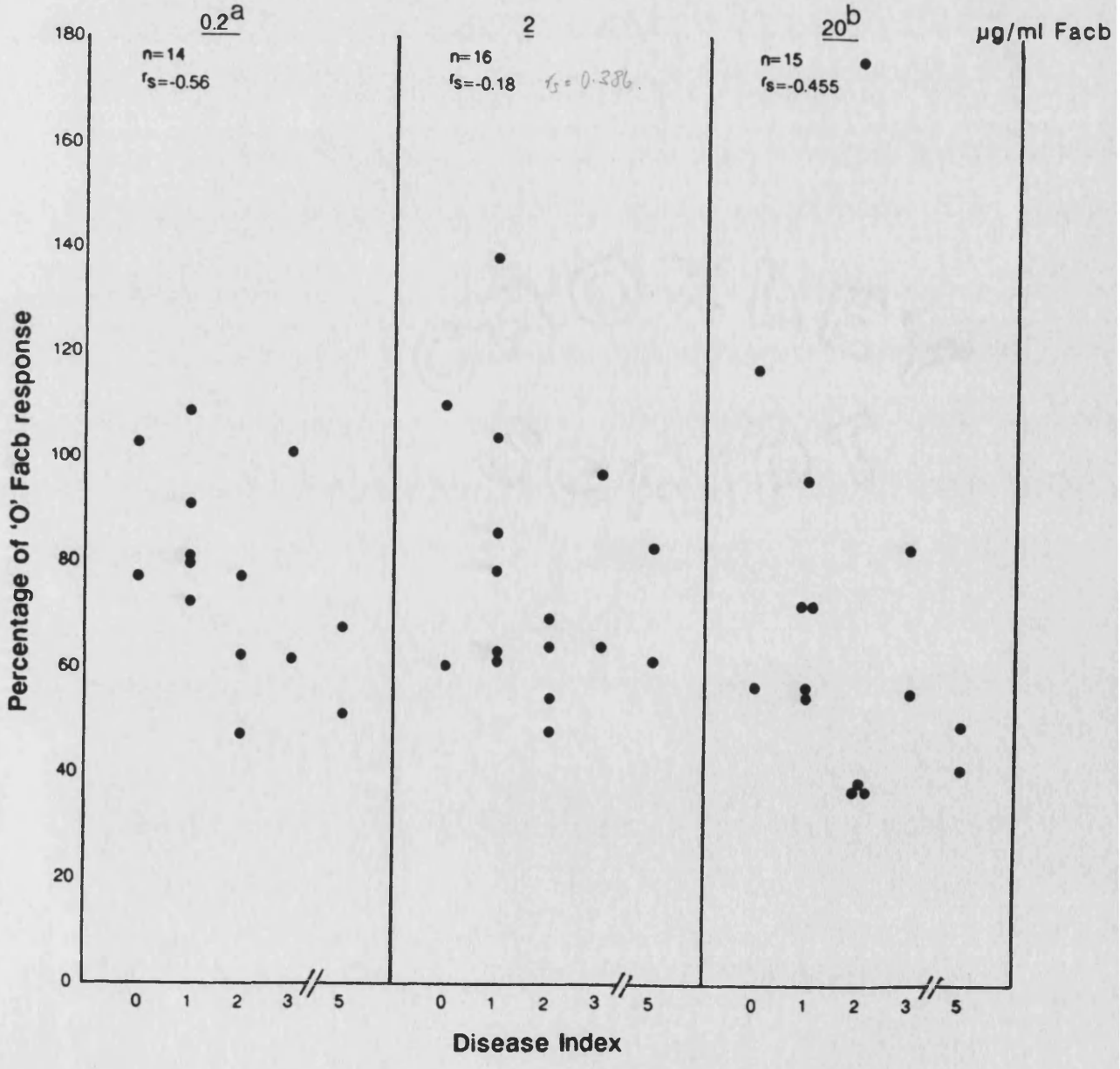
Early morning stiffness > 2 hours or plasma viscosity > 1.90 increased the score by 1

FIG 25 **Correlation of Facb-mediated suppression at P1000 in early RA positive patients and disease activity**

Disease activity was scored as ranging from 0 (inactive disease) to 5 (severe aggressive synovitis). Suppression of the IgG response is expressed as a percentage of the P1000 response where no Facb was present.

a $p < 0.05$

b p approaching significance at the 5% level



possible explanation. Figure 26 shows the increase in the percentage of FacbR$^+$ cells as disease becomes more active in early RA patients. It is possible that the increase in the number of FacbR$^+$ cells at high disease index might allow better suppression of the immunoglobulin response in these patients.

2. Facb suppression and disease duration

Association between Facb suppression of IgG synthesis and increasing disease duration was investigated. Responses noted in early seropositive RA patients at P1000 were compared with those seen in subjects with established disease of (i) 2 - 5 years and (ii) 5 - 22 years duration.

These data are illustrated in Figure 27. Although the degrees of suppression seen in patients with established disease are lower than those obtained with early RA positive subjects, no statistical differences were found. This is probably due to the large variability of response. Clearly, the findings here differ statistically from those noted when the established RA patient group is not subdivided according to the period of disease duration (see Section 1(2)).

3. Facb suppression and laboratory measurements of serum and blood components

Measurements of haemoglobin, platelets, CRP and plasma viscosity are frequently used by clinicians as indicators of disease activity. Relationships between these clinical parameters and Facb suppression of IgG response at P1000 were investigated. Other parameters examined included serum immunoglobulins (G, A and M), IgM RhF, alkaline phosphatase and serum thiols. All doses of Facb were examined using Spearman's rank correlation test.

FIG 26 Correlation of the FacbR$^+$ cell numbers with disease activity in early RA patients

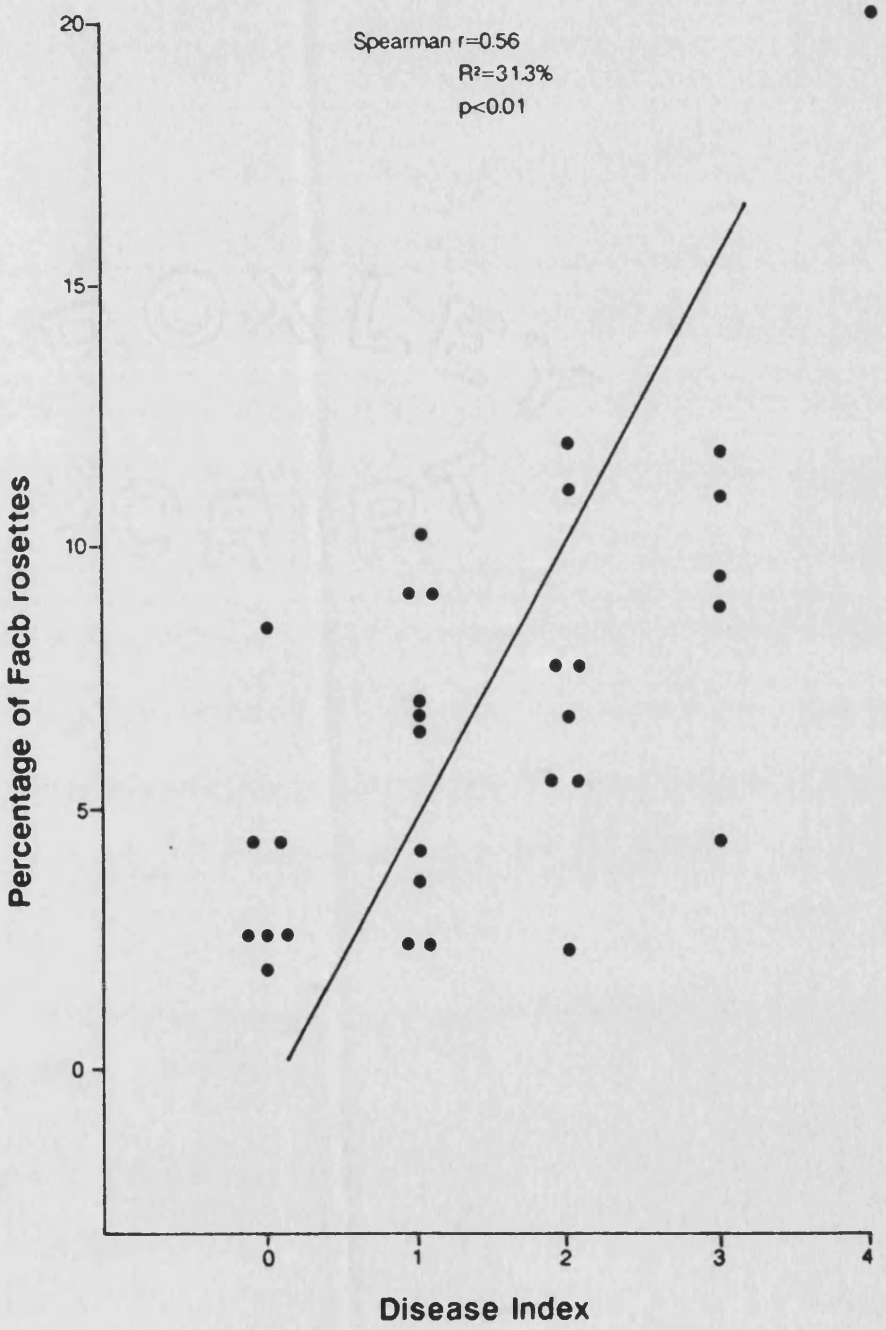
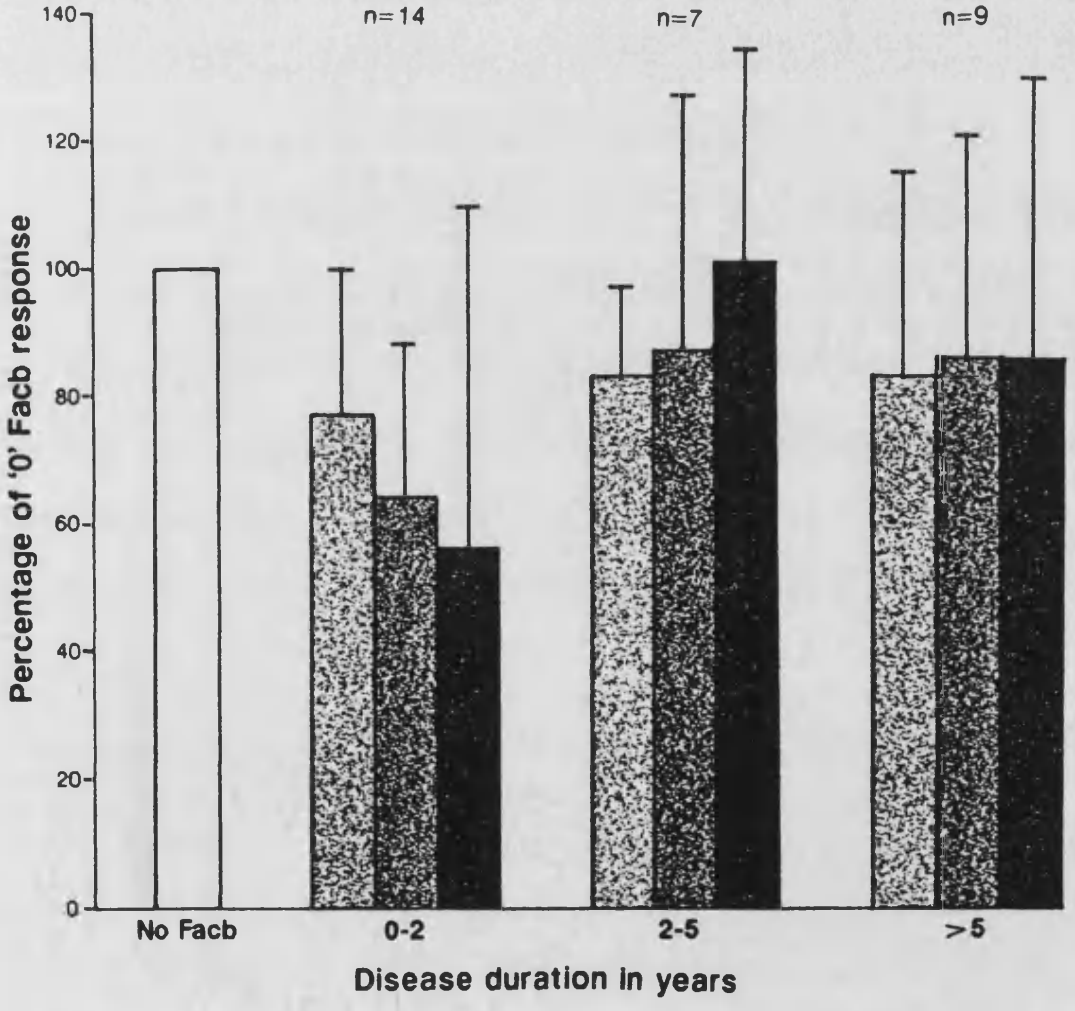


FIG 27 **The variation in Facb-mediated feedback suppression of the IgG response with disease duration**

Suppression of the P1000 response by 0.2 (▨), 2 (▩) and 20 (■) µg/ml of Facb was assessed. Results are expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (T) are shown.



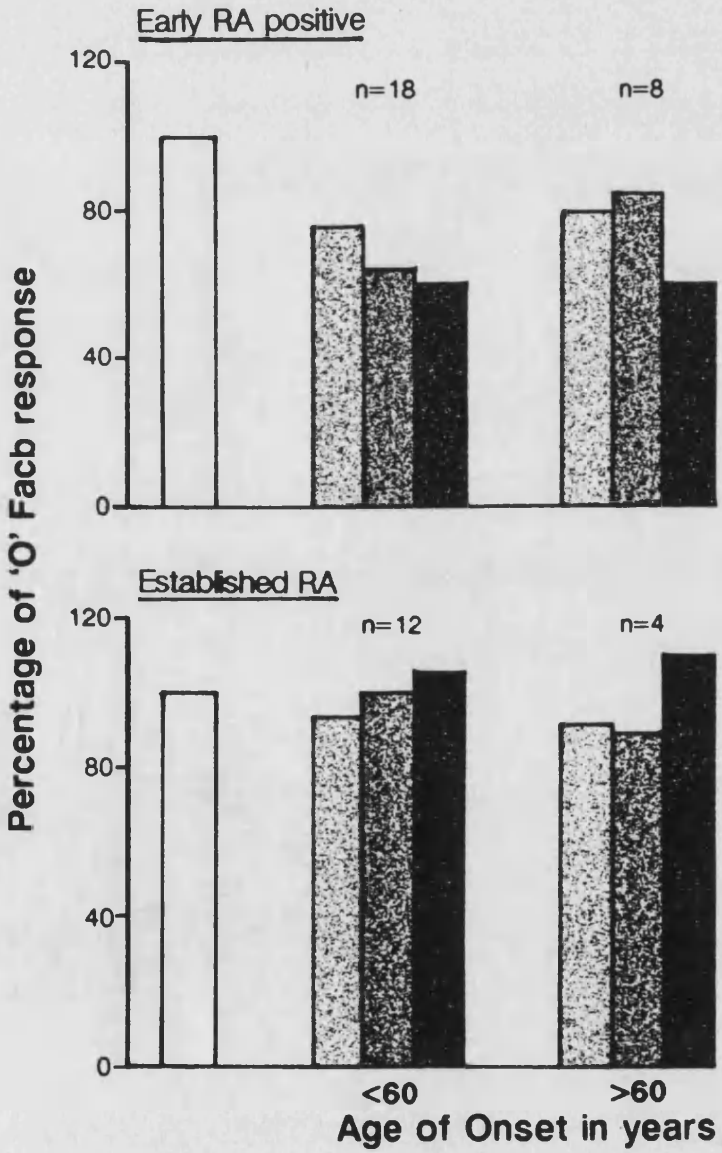
None of the above variables was found to be related to the degree of Facb suppression of antibody production in patients with early RA (data not shown).

4. Facb suppression and age of onset

Facb suppression of IgG response was examined in relation to the age of onset of disease. No significant differences were found between levels of Facb suppression seen in individuals with an early age of onset and those whose disease began in their sixties. These results are shown in Figure 28.

FIG 28 **The variation of Facb-mediated feedback suppression of IgG synthesis with age of onset**

P1000-stimulated PBMNC from early and established RA patients were incubated with 0.2 (▒), 2 (▒) and 20 (■) μg/ml final concentrations of Facb. Suppression of the IgG response was assessed and expressed as a percentage of the stimulated response where no Facb was present. Median values are depicted. Interquartile ranges are omitted for reasons of clarity.



SECTION 3 FACB SUPPRESSION OF RHEUMATOID FACTOR

The above results have all concerned Facb suppression of the IgG response. The question now arose as to whether these effects were limited to only this immunoglobulin or whether suppression extended to other types of antibody, in particular RhF. Clearly, this was important since if the addition of the ligand were found to produce suppression of RhF in normals but not in patients with rheumatoid disease then this would provide at least one explanation for the presence of this autoantibody in RA. Thus the production of RhF and its suppression by Facb was investigated.

1. Rheumatoid factor synthesis in controls and disease groups

Figures 29 to 36 show spontaneous and PWM-induced RhF production in the various test groups. It was found that both young and old normal, healthy controls produced no RhF spontaneously. The latter were individuals over 60 years of age who had been examined separately to determine whether RhF production was related in part to the process of aging, since the average age of the RA patients was higher than that of normal healthy controls obtained from the laboratory personnel. Clearly, age does not appear to affect spontaneous RhF production. OA patients were also not observed to generate RhF in the resting state. In patients with early seronegative or palindromic RA, very small numbers of subjects generated RhF spontaneously. This, however, was at low levels and since these were below the sensitivity of the assay, the values had to be considered as background. In early RA positive, established RA and Felty's patients numbers of individuals producing autoantibody at PO were greater, although the overall median values of RhF

FIG 29 Spontaneous and PWM-stimulated IgM RhF production in normal healthy controls

Horizontal bars represent median values

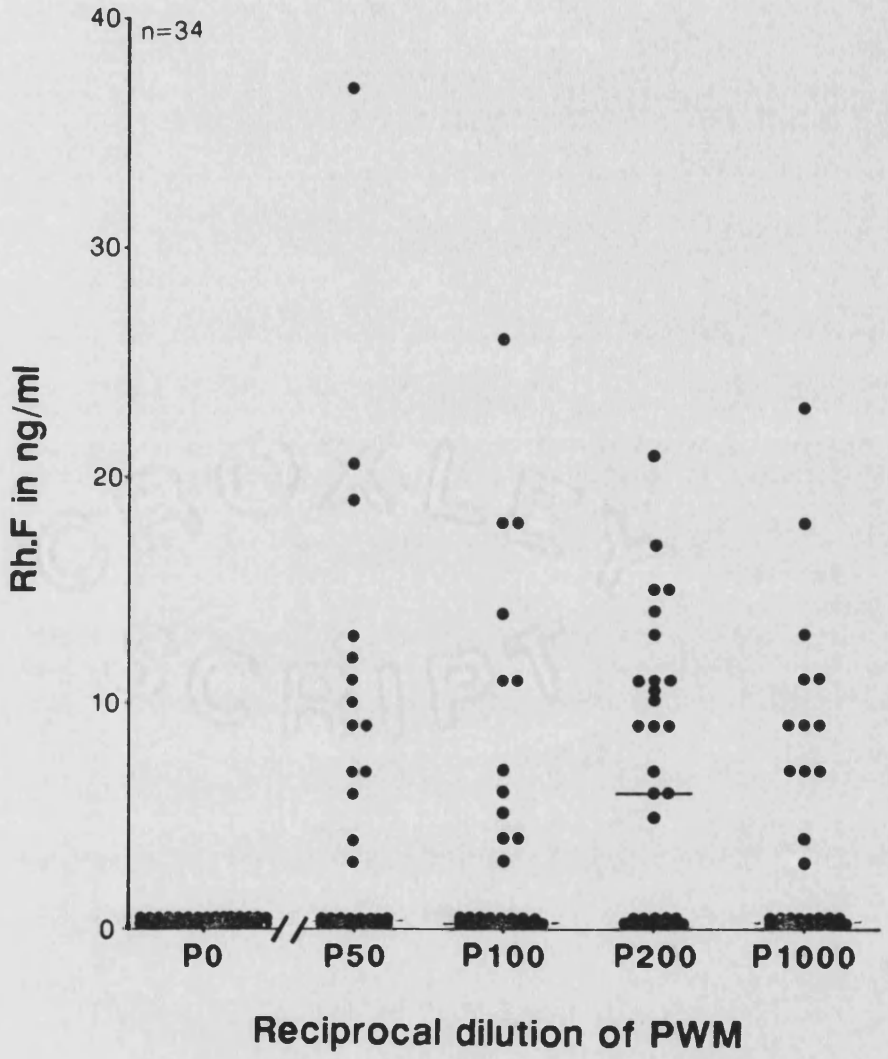


FIG 30 Spontaneous and PWM-stimulated IgM RhF production in early RA positive patients

Horizontal bars represent median values

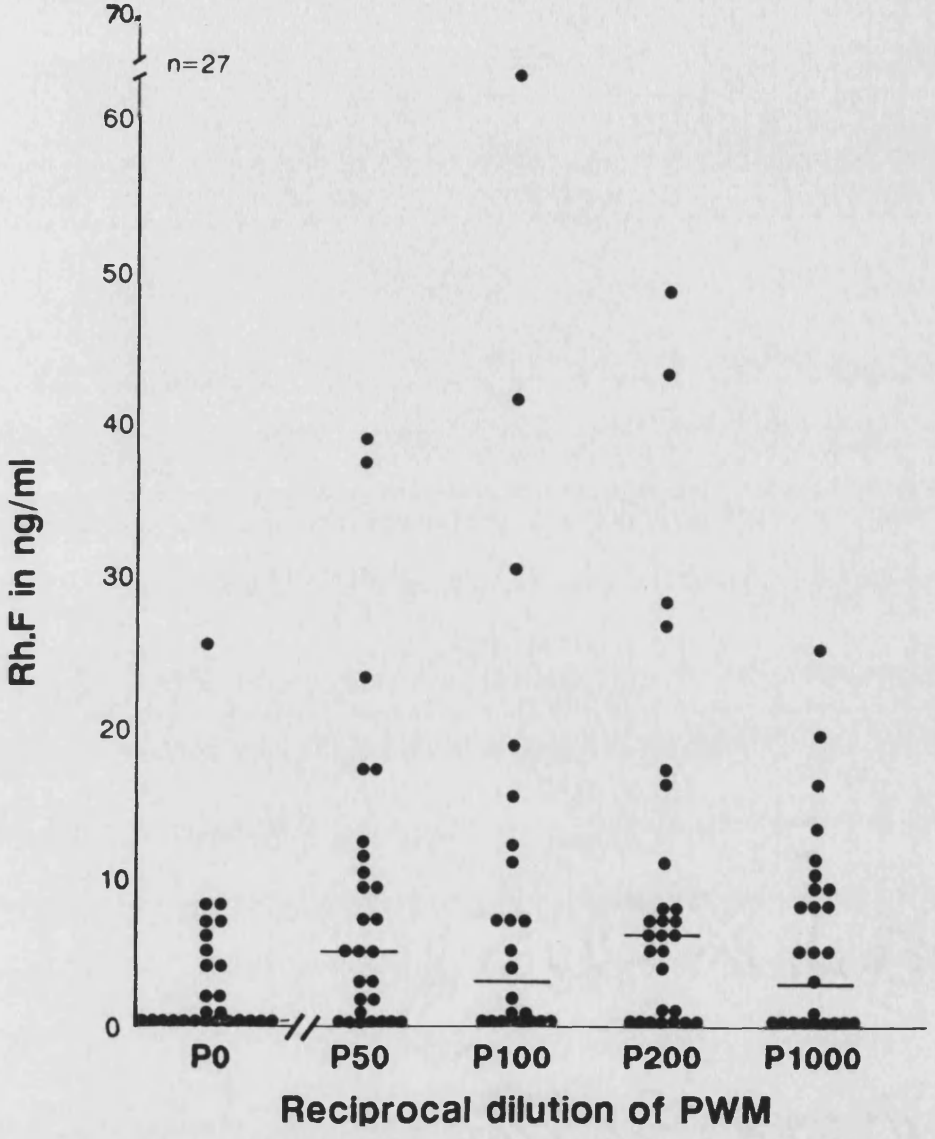


FIG 31 Spontaneous and PWM-stimulated IgM RhF production in early RA negative patients

Horizontal bars represent median values

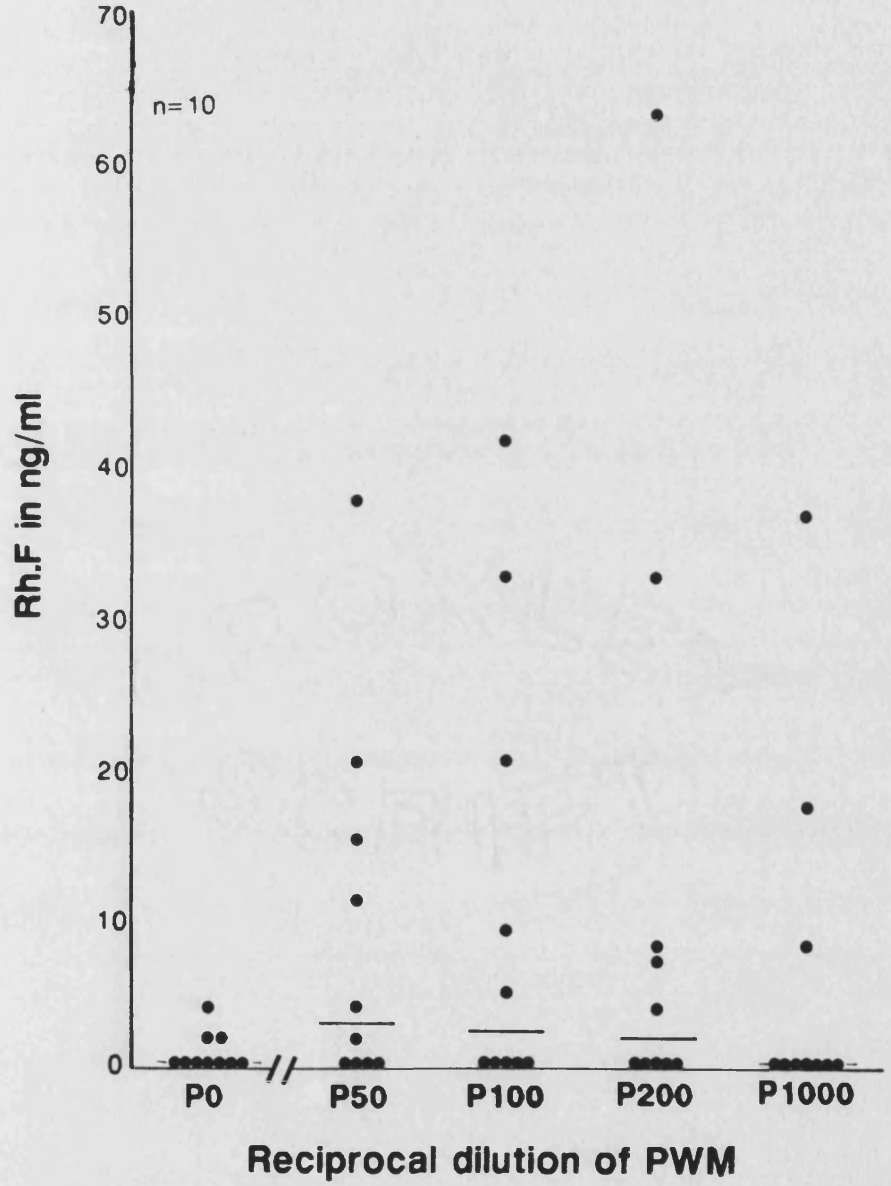


FIG 32 Spontaneous and PWM-stimulated IgM RhF production in early palindromic RA patients

Horizontal bars represent median values

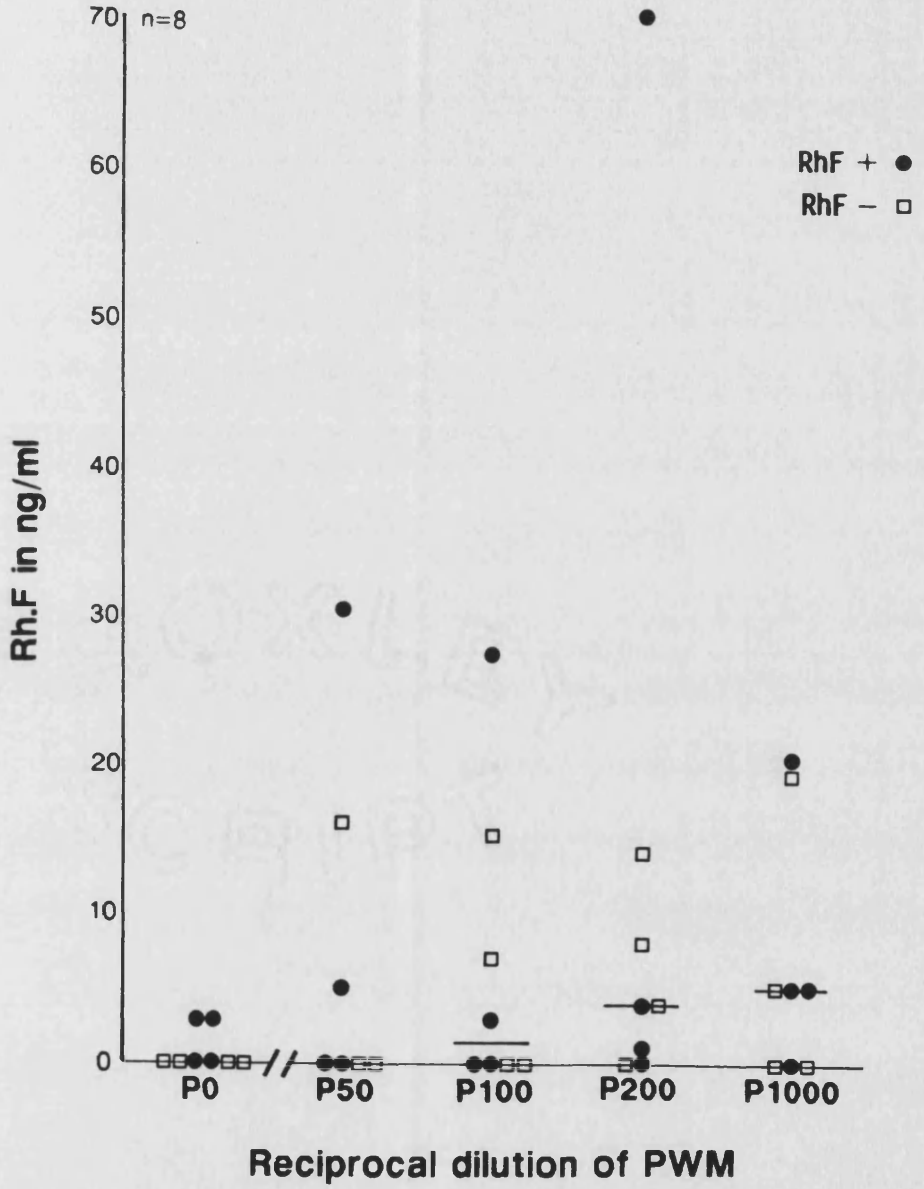


FIG 33 Spontaneous and PWM-stimulated IgM RhF production in established RA patients

Horizontal bars represent median values

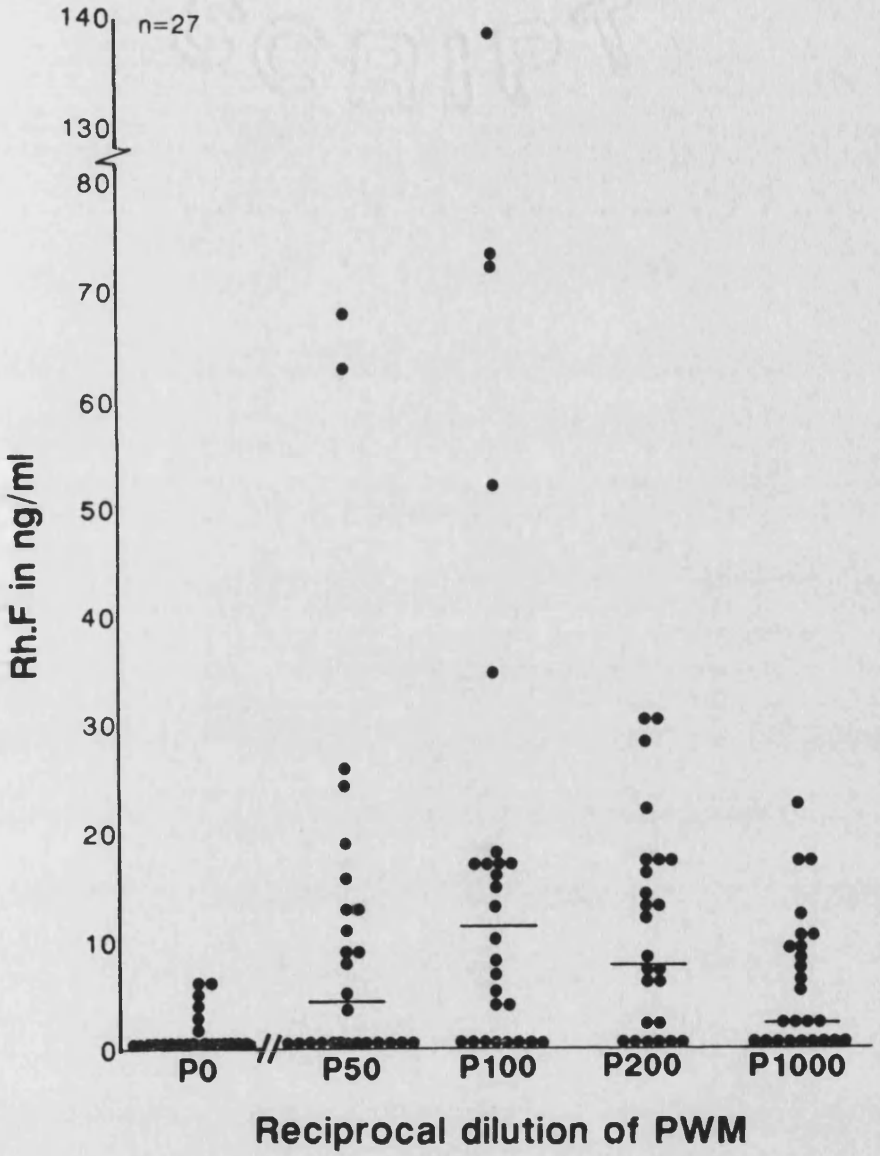


FIG 34 Spontaneous and PWM-stimulated IgM RhF production in OA patients

Horizontal bars represent median values

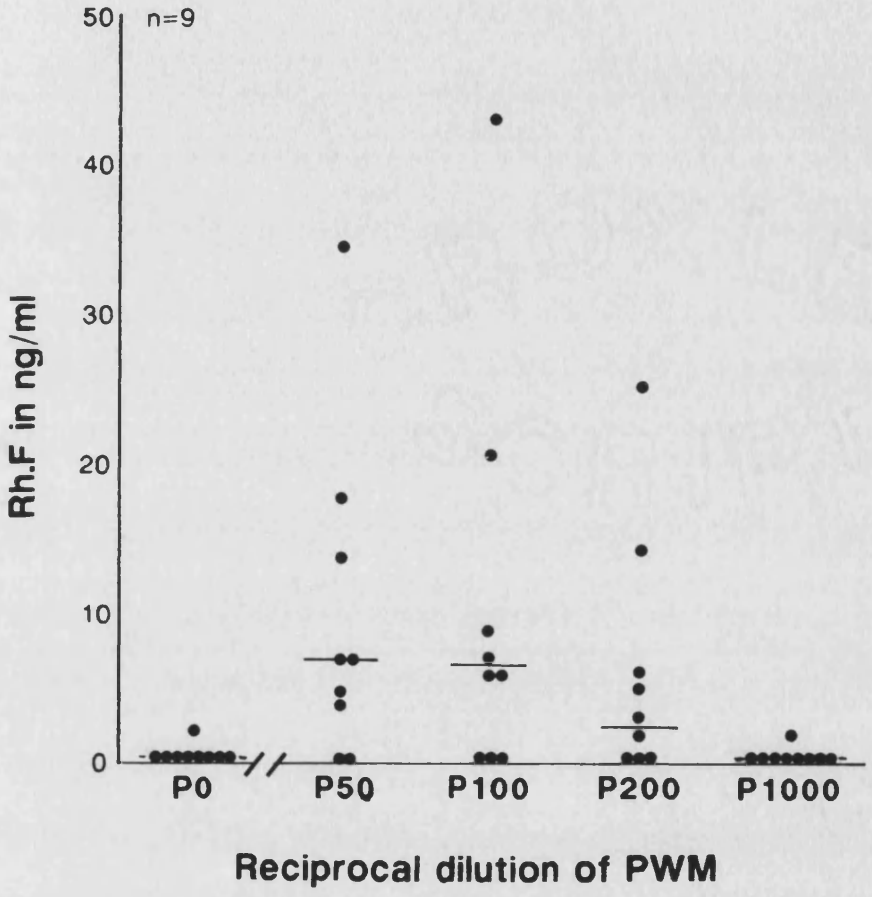


FIG 35 Spontaneous and PWM-stimulated IgM RhF production in Felty's patients

Horizontal bars represent median values

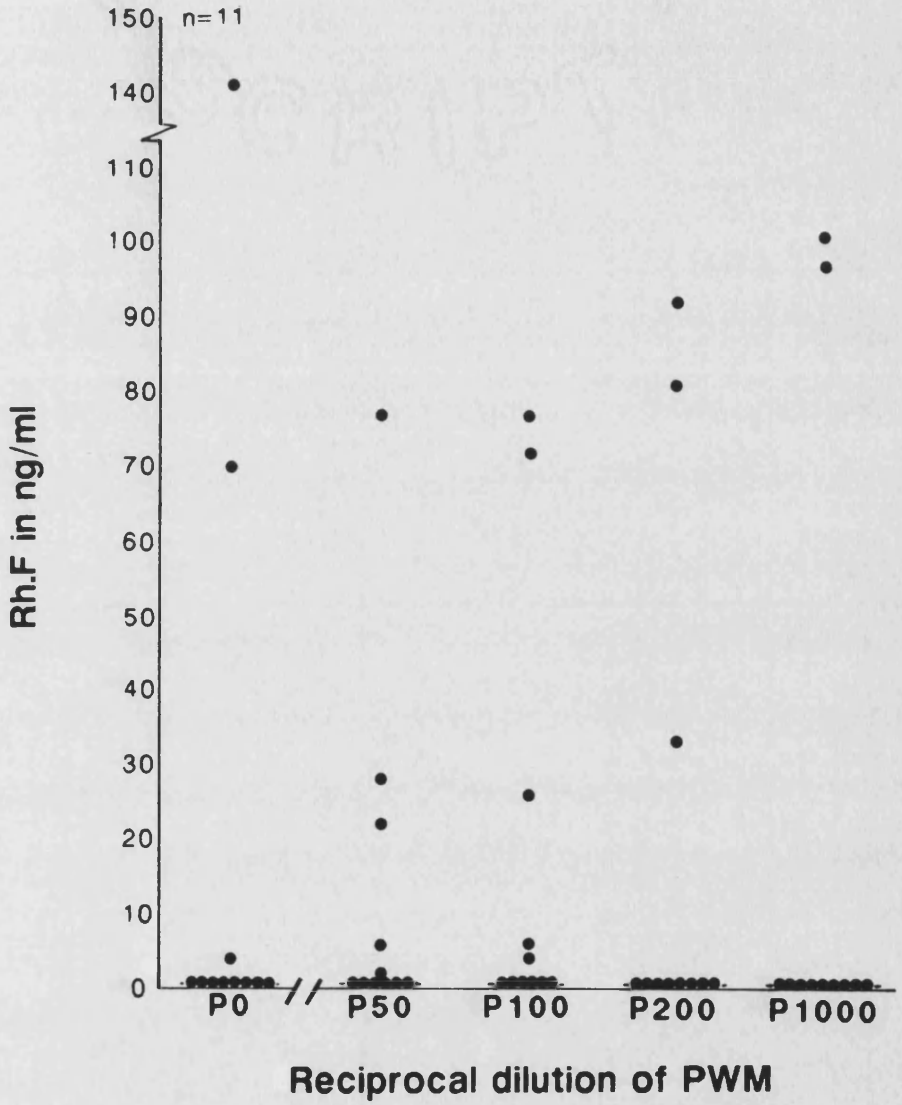
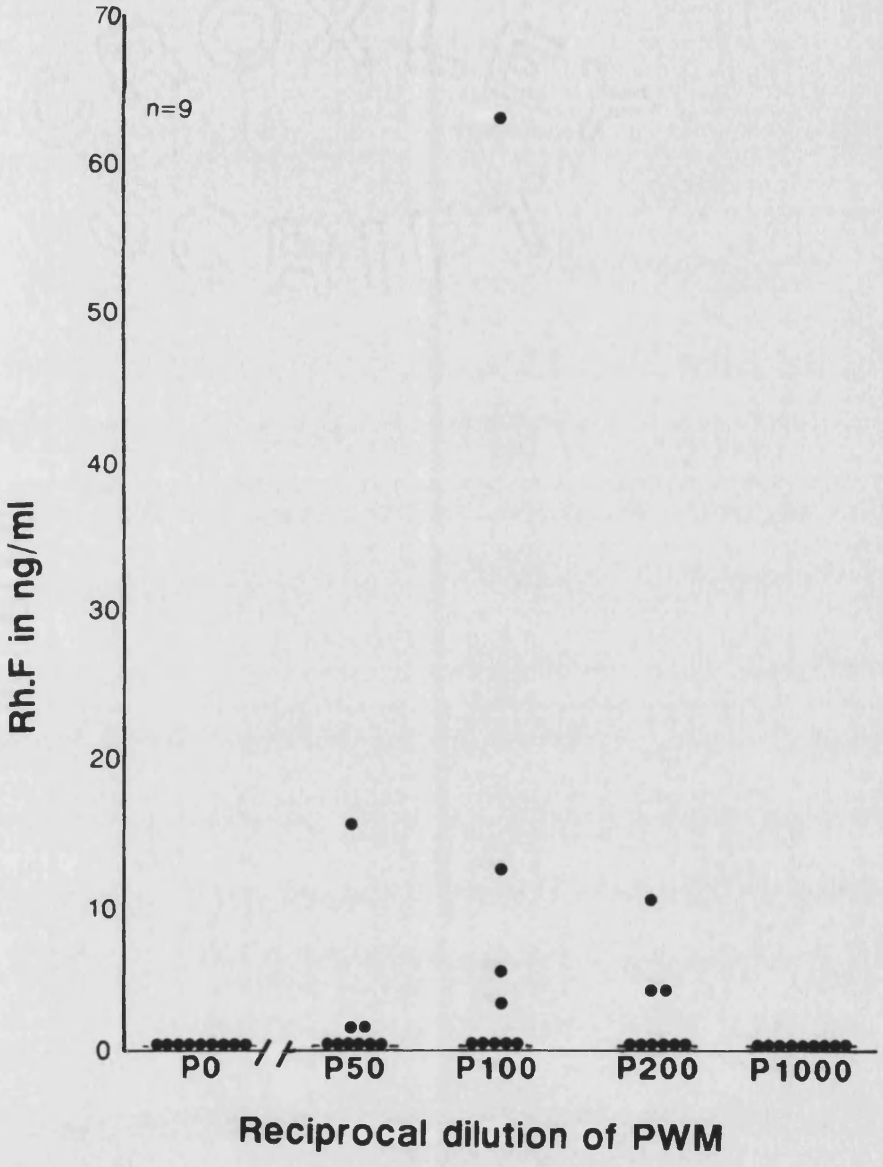


FIG 36 Spontaneous and PWM-stimulated IgM RhF production in old normal controls

Horizontal bars represent median values



production of each of these groups were zero. Using 5 ng/ml as the lowest detectable level of RhF in the ELISA, it was found that 22.2%, 7.4% and 18% of all the patients tested in early RA positive, RA and Felty's groups respectively, generated RhF spontaneously. Considering only these patients, median levels of RhF production in the absence of mitogen were 8 ng/ml in early RA positive, 6 ng/ml in RA and 106 ng/ml in Felty's patients. Thus, although in the former two groups similar levels of RhF were generated, the proportion of patients who were spontaneous generators of RhF was higher in the early RA group. In Felty's syndrome, although most patients did not produce the autoantibody spontaneously, the subjects who did had much higher levels of generation than in early RA positive and established RA groups.

With PWM the numbers of individuals synthesising RhF, as well as the levels of autoantibody production, were increased in most groups, except in Felty's patients where individuals with high levels of spontaneous RhF production were found to be a little suppressed. Overall median values of the autoantibody in the test groups were quite low. At peak responses these values were 6 ng/ml in both normal healthy controls and patients with early seropositive disease. In early RA negative and palindromic patients these values were < 5 ng/ml and 5 ng/ml respectively. A median peak value of 11.5 ng/ml was noted in RA subjects and of 7 ng/ml in OA patients. In both old normal controls and Felty's patients median values for all doses of PWM were zero. This further emphasises that old age is not a contributory factor for RhF production. Using only the responses obtained at P200, the relative proportions of subjects producing RhF in each of the test groups was determined. Values of > 5 ng/ml were regarded as a positive response. These data appear in Table 3. The

TABLE 3 IgM RhF production at P200

Clinical group	n	Range in ng/ml	Overall median RhF in ng/ml	% of subjects producing RhF
Young normal	34	0-21	6	50
Old normal	9	0-10	0	11.1
Early RA positive	27	0-48	6	56
Early RA negative	10	0-62	2	40
Palindromic	8	0-69	4	37.5
RA	27	0-30	7.5	63
OA	9	0-25	2.5	33
Felty's	11	0-94	0	33

percentages of individuals generating autoantibody were much improved in the presence of PWM, with the highest value (63%) occurring in patients with established RA. Similar values were obtained in normal (50%) and early RA positive (56%) subjects, whilst those in early RA negative (40%) and palindromic (37.5%) patients were slightly lower. In OA, Felty's and old normal groups these values were 33%, 33% and 11.1% respectively.

Using data from only early RA positive and established RA patients, it was found that levels of RhF produced in vitro did not correlate with concentrations of IgM and IgM RhF observed in the corresponding sera.

2. Facb suppression of RhF synthesis

Subjects who were found to generate RhF in vitro were further investigated for effects of Facb on this synthesis. Not all of the subjects originally found to synthesise RhF generated it on subsequent testing, so the patients numbers were smaller than anticipated. Also, not all doses of PWM gave rise to RhF synthesis, leading to unmatched numbers of patients at each dose.

The following graphs (Figures 37 - 42) show the effects of Facb on PWM-induced RhF synthesis. It was originally decided to use only those individuals generating > 10 ng/ml of RhF. However, it was discovered that some subjects who produced < 10 ng/ml without Facb produced more RhF with the ligand present.

Thus, in all the groups tested, though the response was variable, increasing concentrations of Facb fragment tended to enhance the production of RhF. However, suppression was also observed and

FIG 37 **The effect of Facb on PWM-stimulated IgM RhF production in normal healthy controls**

Median values are shown and interquartile ranges (T) are indicated where appropriate.

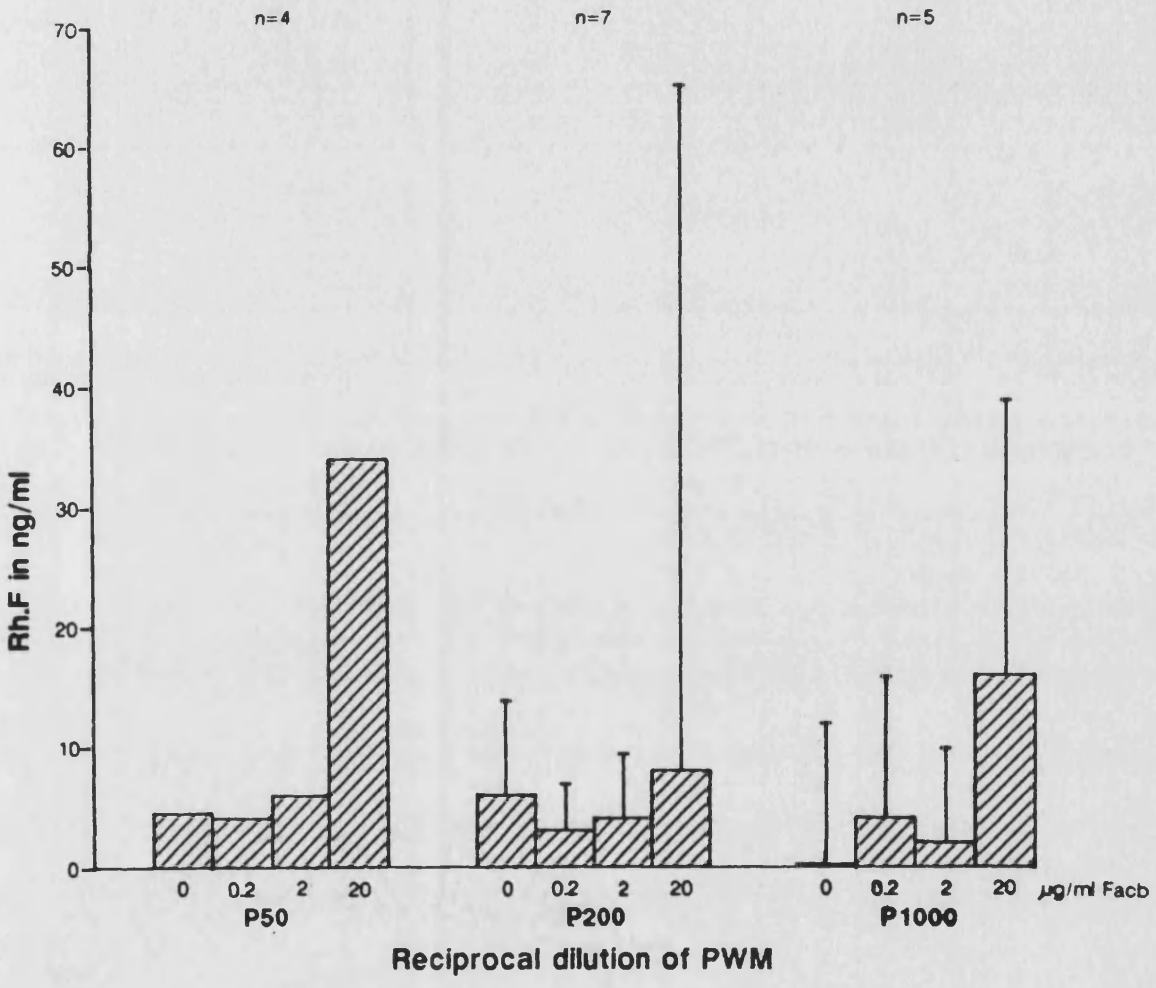


FIG 38 **The effect of Facb on PWM-stimulated IgM RhF production in early RA positive patients**

Median values of RhF and interquartile ranges (T) are shown.

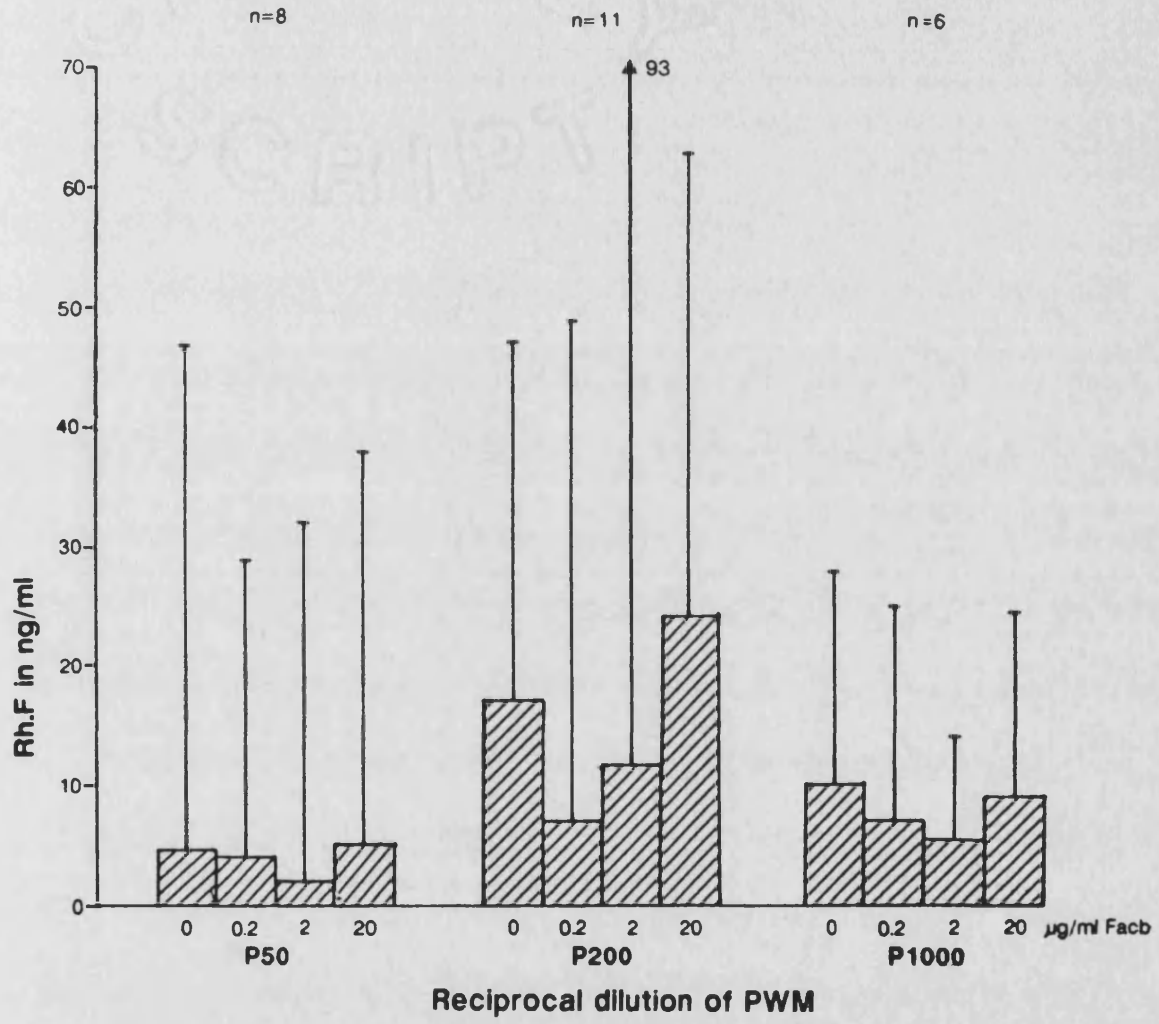


FIG 39 **The effect of Facb on PWM-stimulated IgM RhF production in early RA negative patients**

Median values are shown and interquartile ranges (T) are indicated where appropriate.

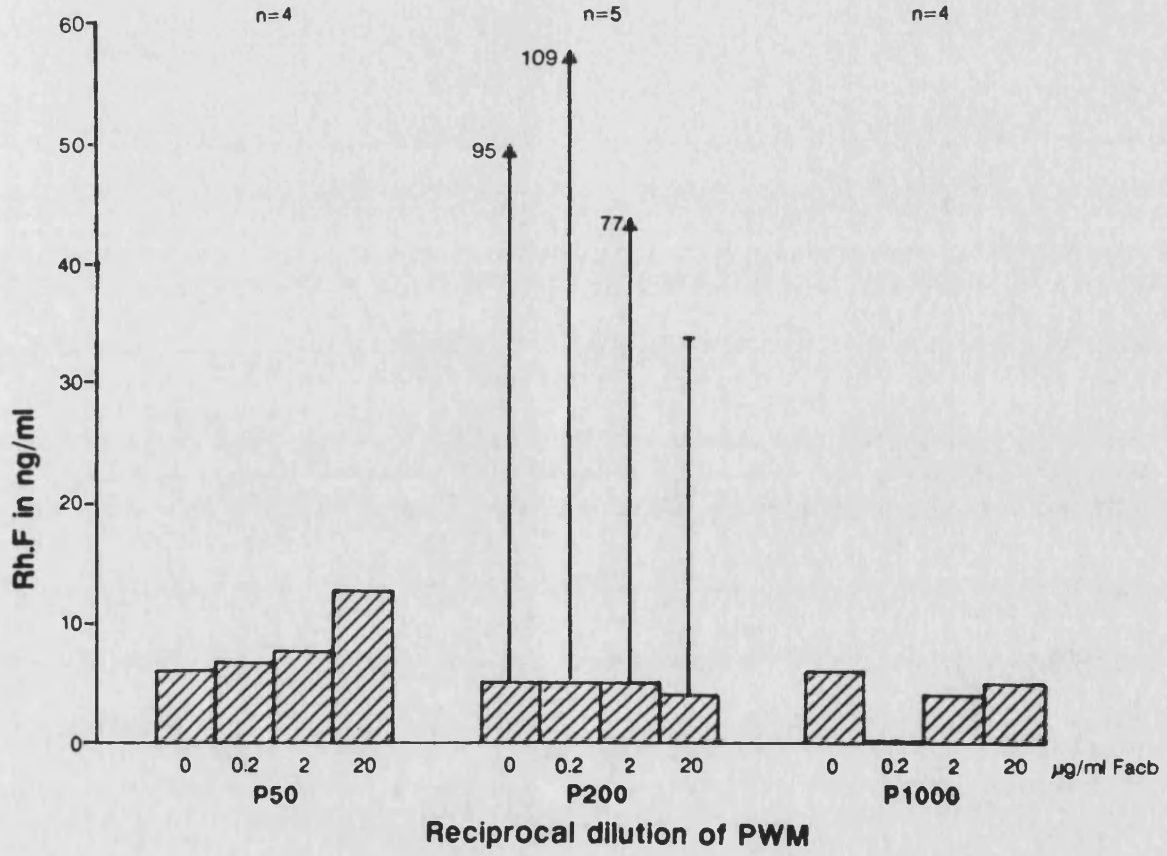


FIG 40 The effect of Facb on PWM-stimulated IgM RhF production in early palindromic RA patients

Median values of RhF and interquartile ranges (T) are shown.

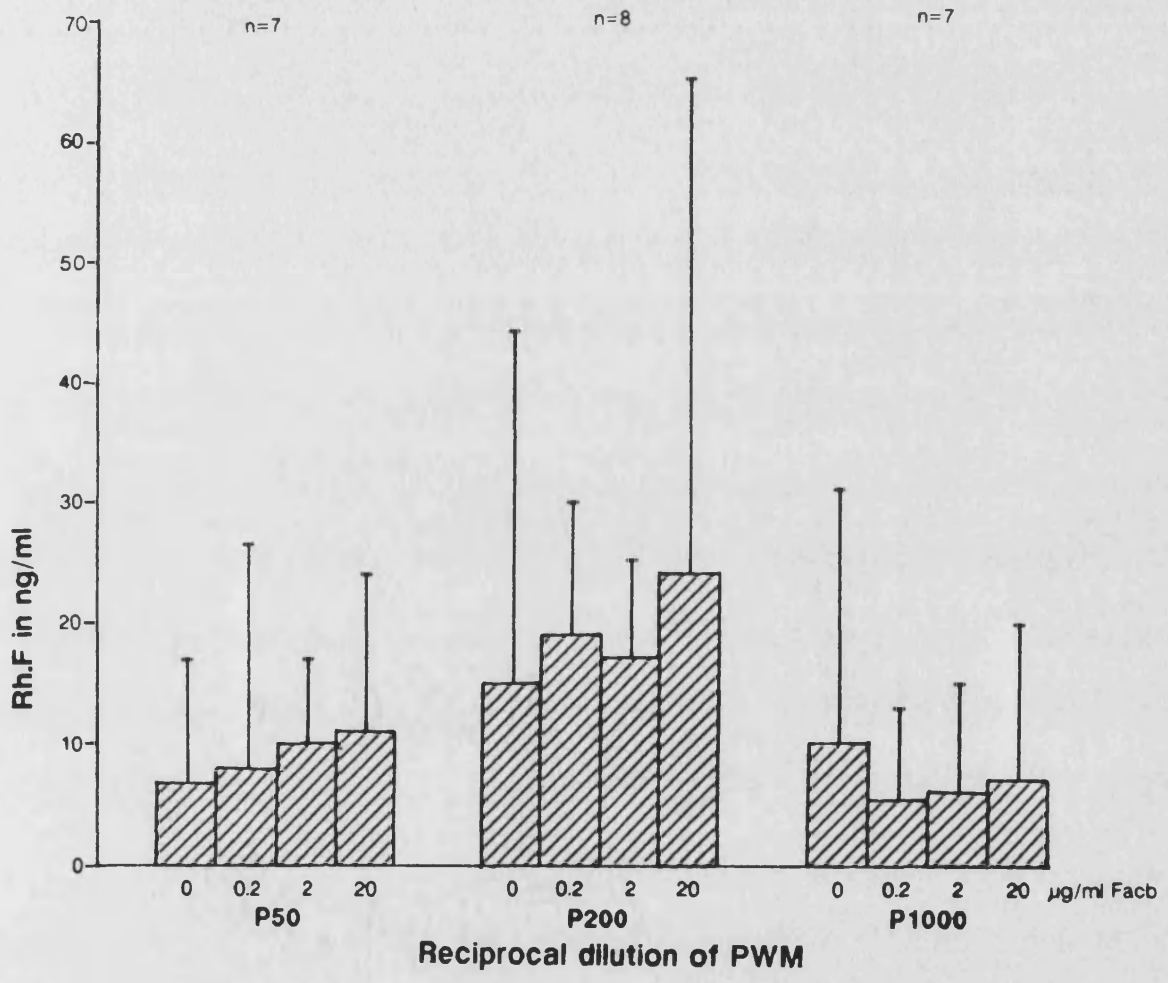


FIG 41 **The effect of Facb on PWM-stimulated IgM RhF production in established RA patients**

Median values of RhF and interquartile ranges (T) are shown.

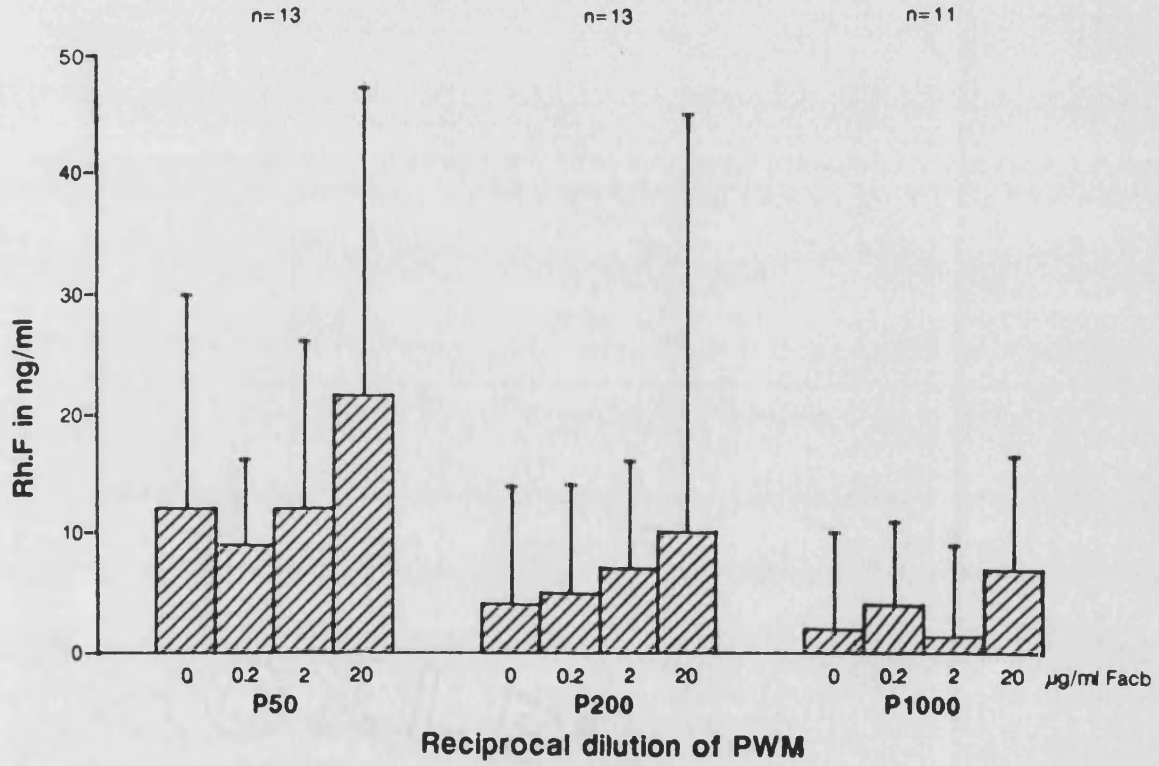
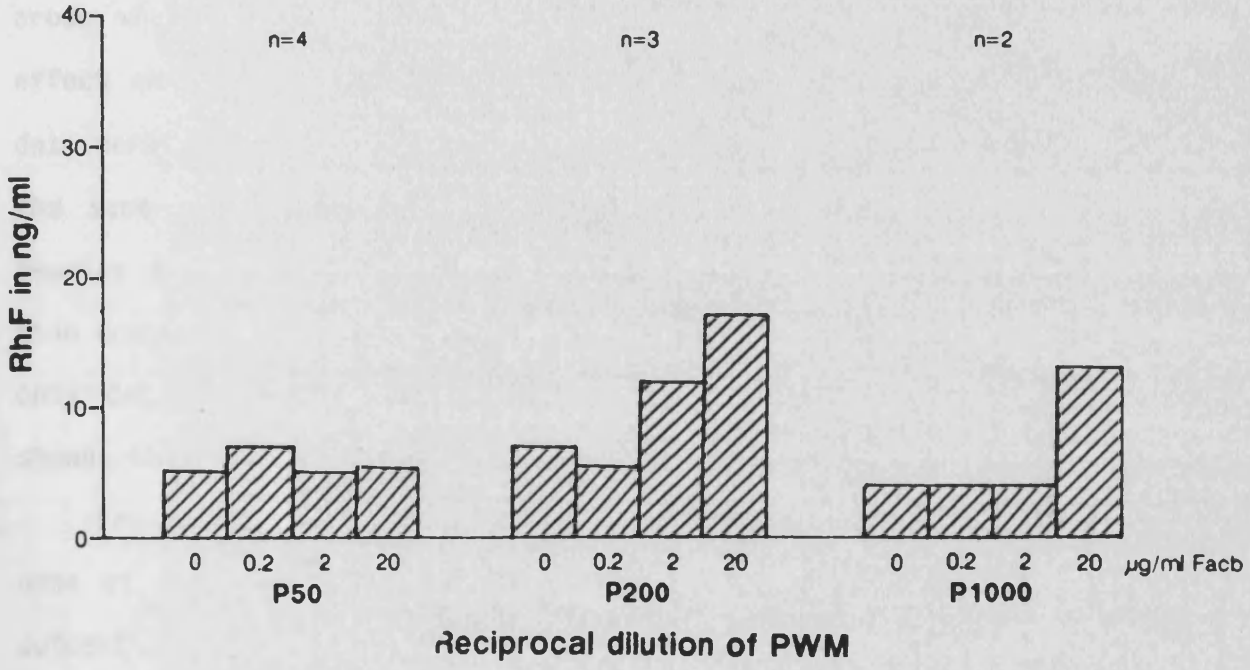


FIG 42 The effect of Facb on PWM-stimulated IgM RhF production in OA patients

Median values of RhF are shown.



here the lower concentrations of the ligand were more effective than 20 µg/ml. At the latter concentration RhF production was found to be unaffected or enhanced but rarely suppressed.

The data were further analysed by selecting out those individuals with > 10 ng/ml RhF and converting responses seen at 0.2, 2 and 20 µg/ml Facb to a percentage of the response where no Facb was present. This is presented in Figures 43 - 45. Also illustrated in these graphs are the corresponding IgG responses, since the question arose whether effects of Facb on RhF were part of a generalised effect on immunoglobulin production. It should be noted that these data were available for only a very small number of patients and that the same individuals were not present at all PWM doses. Thus, results of each PWM dose should be examined within that dose rather than comparing responses between the other doses examined. Although only data on early RA positive, palindromic and RA subjects are shown, the other groups were also examined.

From these graphs it can be seen that unlike IgG response, the dose of PWM used did not appear to affect Facb modulation of the autoantibody production. The response was highly variable and as seen earlier suppression was often better at 0.2 and 2 µg/ml Facb than at 20 µg/ml of the ligand, as illustrated by patients with palindromic RA (see Figure 44). The highest concentration of Facb was found in some cases to markedly enhance the autoantibody production. However, the most interesting observation here was that RhF production was not always suppressed in parallel or to the same extent as the IgG response; sometimes quite the reverse was observed. For example, at P200 in early RA positive patients (Figure 43) the IgG production was reduced whilst that of RhF was enhanced by increasing the concentration of Facb. Clearly, this indicates that

Facb modulates IgG and IgM RhF responses differently, either because it operates at different levels for the two immunoglobulins or because different kinetics are operational in each case.

Figures 43 - 45 should be regarded as purely illustrative. The responses were highly variable and the numbers of patients so small as not to allow accurate assessment of the extent to which RhF is suppressed by Facb.

In some of the above patients Facb suppression of the total IgM response was also examined. Figure 46 shows the effects of Facb on IgM RhF, IgM and IgG responses in early RA positive, negative, palindromic and established RA patients. It is clear that whilst the effects of Facb on RhF do not shadow those observed with IgM, there is nonetheless a greater similarity between these responses than between those of IgG and RhF. The graphs also illustrate the difference in the effect of Facb on IgG and IgM responses: the former was suppressed whilst the latter tended to be enhanced.

The extent to which IgG is seen to be suppressed in these data does not mirror that which has been presented in the earlier part of this chapter. However, it should be remembered that only a very small number of individuals are depicted and the response to Facb is highly variable.

FIG 43 **The effect of Facb on IgM RhF and corresponding IgG production at 3 doses of PWM in patients with early RA positive disease**

Results are expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (T) are shown where appropriate.

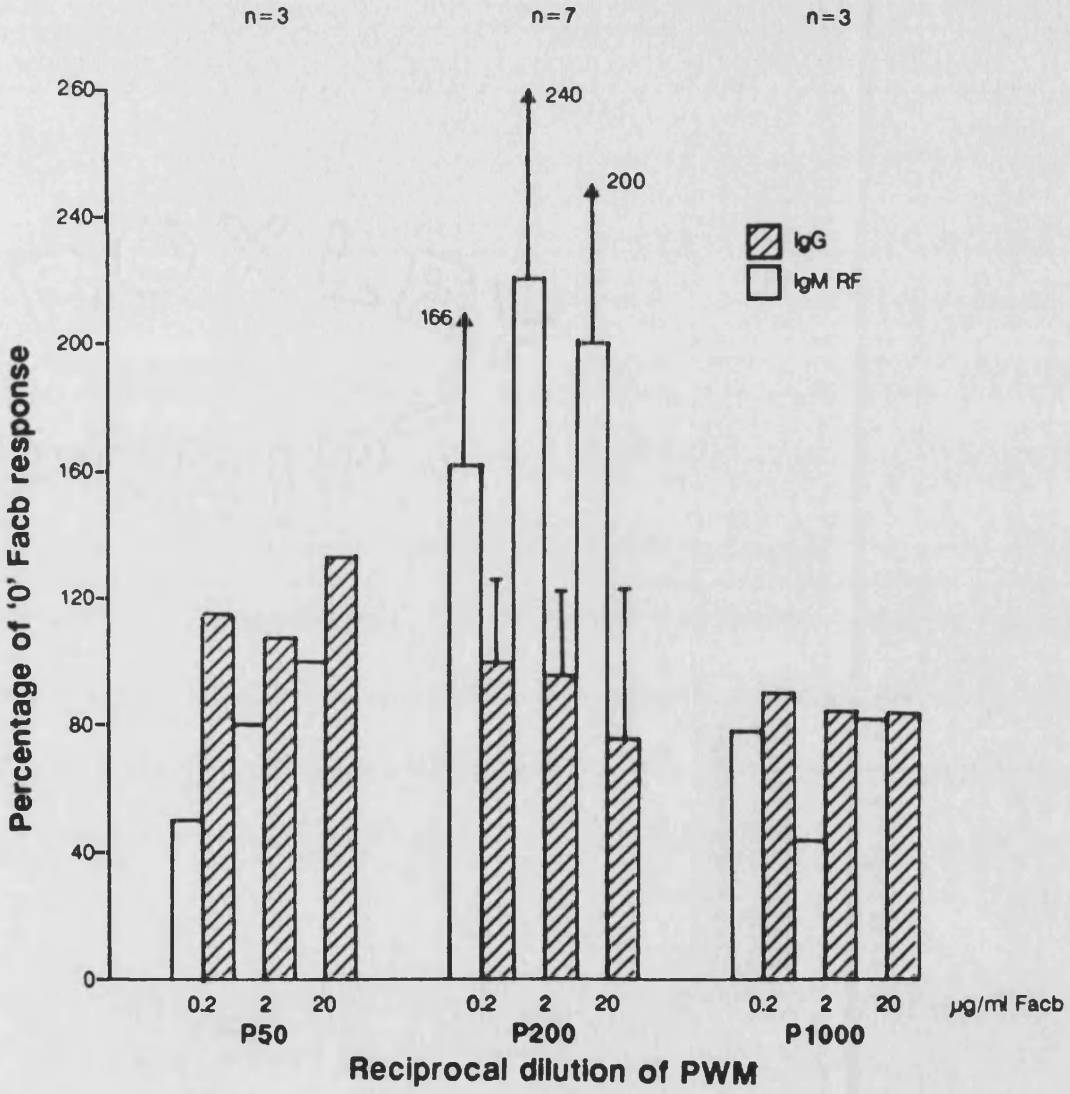


FIG 44 **The effect of Facb on IgM RhF and corresponding IgG production at 3 doses of PWM in patients with early palindromic RA**

Results are expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (T) are shown where appropriate.

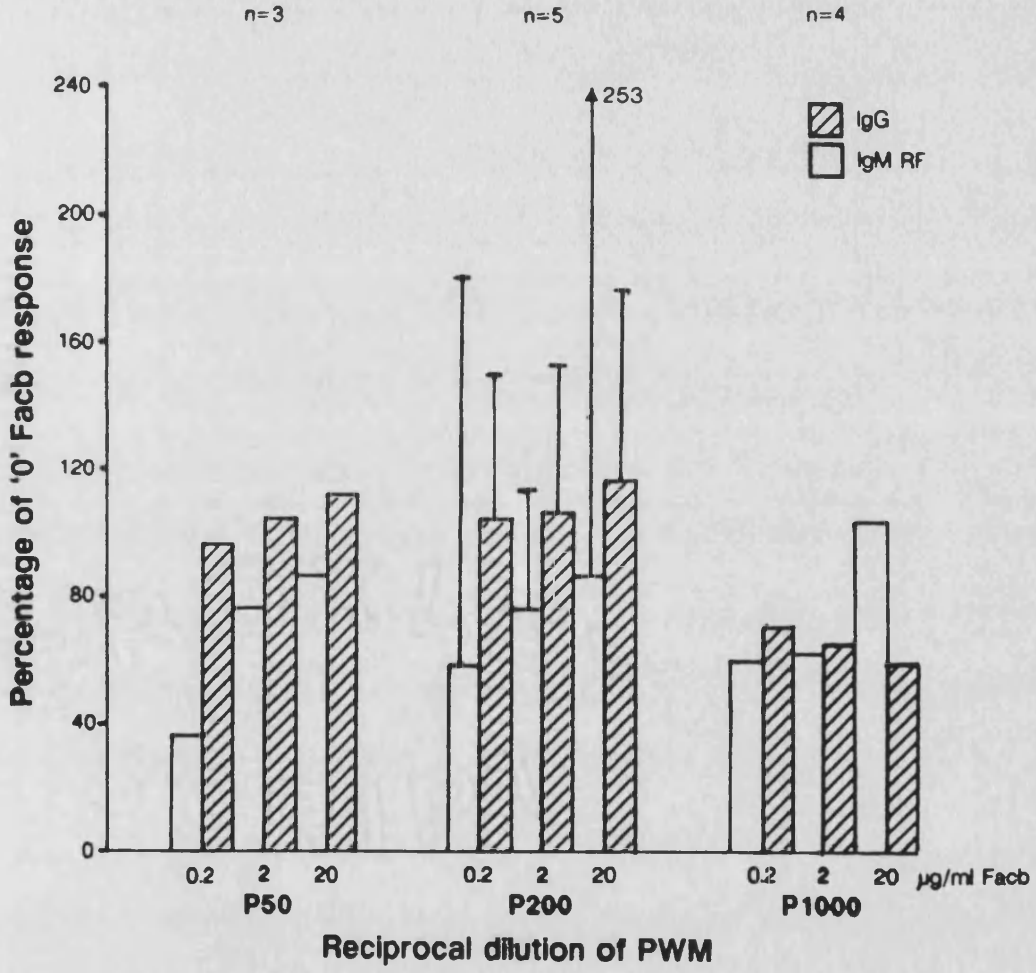


FIG 45 The effect of Facb on IgM RhF and corresponding IgG production at 3 doses of PWM in patients with established RA

Results are expressed as a percentage of the stimulated response where no Facb was present. Median values and interquartile ranges (T) are shown where appropriate.

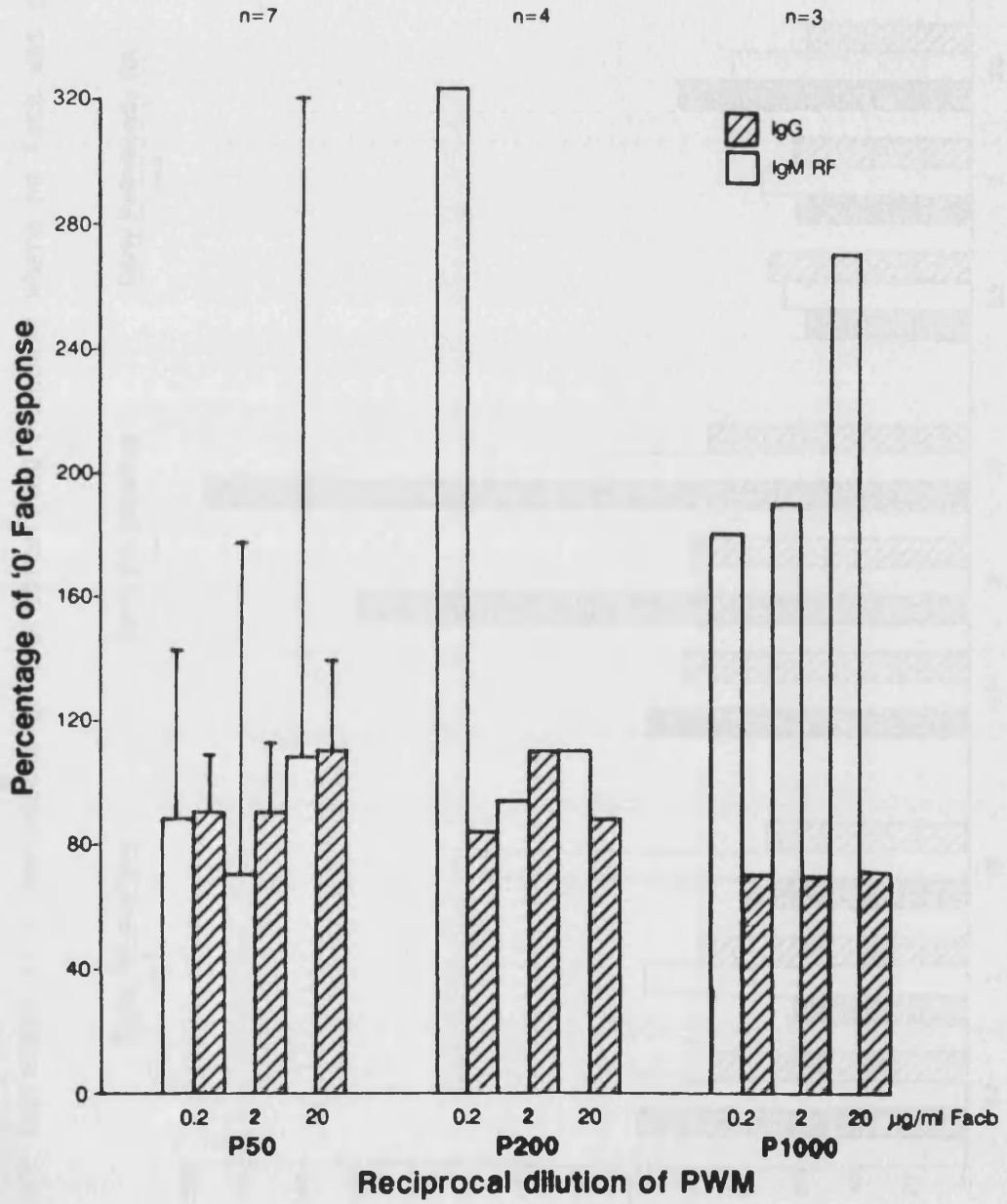
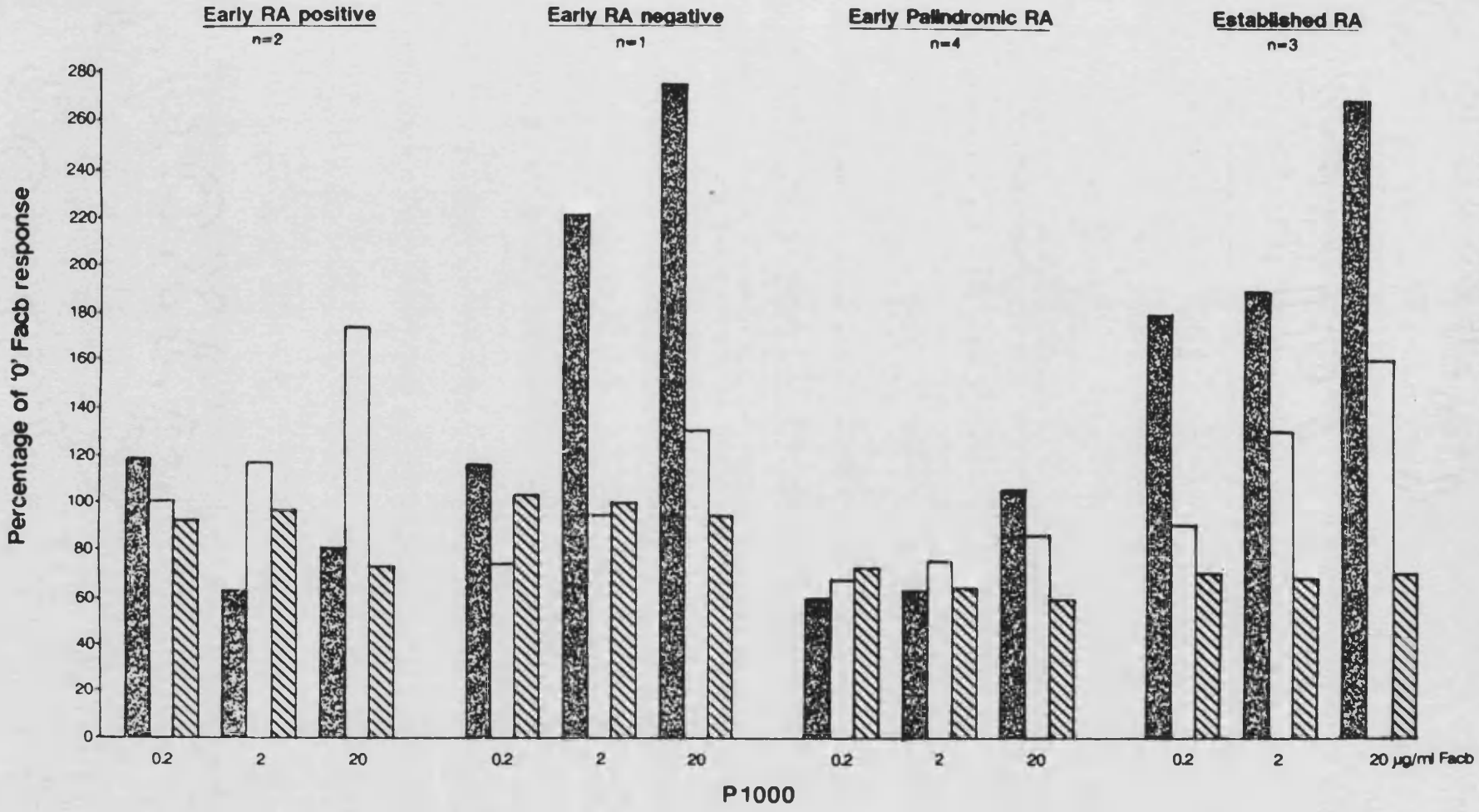


FIG 46 The effect of Facb on IgG (▨), IgM (□) and IgM RhF (▩) production at P1000 in early and established RA patients

Results are expressed as a percentage of the stimulated response where no Facb was present. Median values are shown.



SECTION 4 MECHANISMS OF Facb SUPPRESSION

Impaired Fc-mediated suppression of in vitro IgG production by RA PBMNC compared to normal healthy controls has been observed with Facb fragment. It is possible that this might result from ineffective binding of the ligand either because of a low number of receptors or because of low association constants of Fc binding. The former, however, is unlikely because, as Figure 1 shows, RA patients have significantly higher numbers of Facb rosetting cells compared to healthy controls. Furthermore, enhanced binding of labelled aggregated IgG by RA PBMNC has been reported by other workers (Wooley and Panayi, 1978). At present no information is available on association constants in health and rheumatoid disease regarding Facb receptors.

Another explanation for impaired feedback suppression comes from the observation that free SH groups are important in many immune responses (Hall, 1986). In view of the decreased levels of SH expression in RA (Lorber and Chang, 1968) and the observation that FcR function is dependent on SH groups (Abrahamson et al, 1971), the possibility existed that Facb-mediated suppression of IgG response may be modulated by thiol reagents. Thus the effect of pretreating PBMNCs with 2ME prior to incubation with Facb fragment was investigated in RA and normal subjects. Results were available for only a very small number of individuals in each group. It was found that although 2ME did not have a marked effect on Facb suppression of antibody synthesis, levels of suppression were slightly greater in PBMNC cultures pretreated with the thiol compared to those which had not been incubated with 2ME (data not shown). These experiments need to be repeated using a larger number of subjects.

The effect of 2ME on Facb rosettes was also examined. It was found that there was an increase in the number of rosettes following 2ME treatment, in agreement with previous findings in this laboratory (Eales, PhD thesis, 1982). These data are shown in Figure 47.

The effects of γ-IFN on Facb-mediated suppression of the IgG response were also examined. This molecule, amongst its various actions, is able to induce the expression of FcRs (Taylor-Papadimitriou and Rozengurt, 1985). Thus the effects of increased numbers of FcR^+ cells on negative feedback suppression were investigated using PBMNC from both normal and RA subjects. Cultures were set up as usual in the absence or presence of 10 units of γ-IFN, a concentration found by preliminary experiments to significantly enhance Facb expression in normal, healthy controls. Similar data (see Figure 48) were obtained here but levels of significance were not reached, probably because of the small number of individuals tested. Figures 49(a) and (b) show the effect γ-IFN had on Facb suppression of antibody response in a very small number of normal subjects. The data presented here differ from those described earlier because greater suppression was observed at the two higher doses of PWM than at P1000. However, in the presence of γ-IFN there was practically no suppression of IgG at P50 and P200 and at P1000 slightly greater suppression was observed at 0.2 and 2 μg/ml Facb. Similarly, in RA patients (Figures 50(a) and (b)) any suppression observed in Facb cultures (eg at P200) alone was negated in the presence of γ-IFN.

Looking at the corresponding PWM-induced IgG synthesis in normals in Figure 51, it can be seen that although the effects of γ-IFN were variable on IgG response, this was not significantly altered by adding γ-IFN to the cultures. RA patients were similarly

FIG 47 **The effect of 2ME on Facb rosettes**

PBMNC from normal and RA subjects were incubated with 2ME at a final concentration of 5×10^{-5}M for 1 hour at 37°C. Cells were washed and then assayed for FacbR expression in the rosette assay. Results are expressed as median values of percentage Facb rosetting cells.

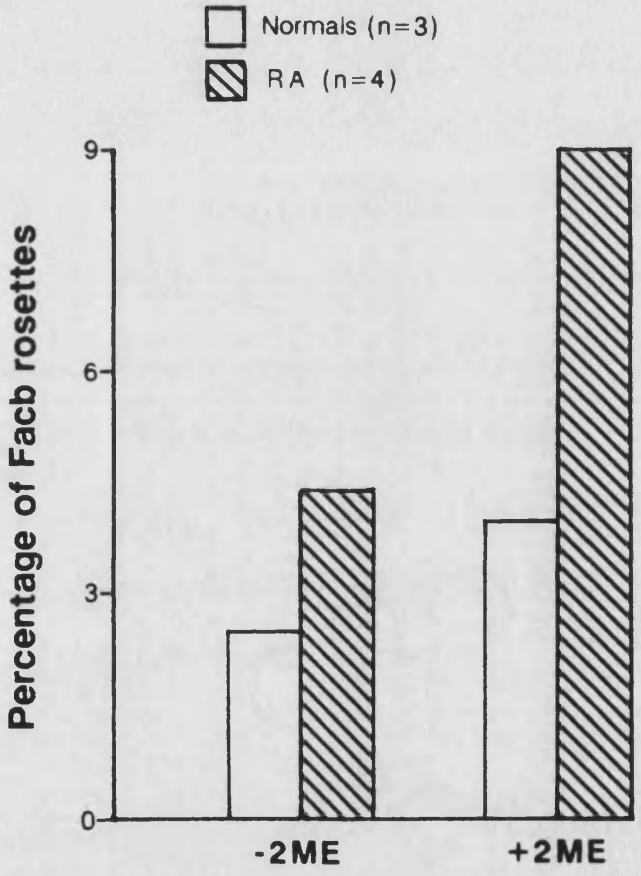


FIG 48 **The effect of γ-IFN on Facb rosettes**

PBMNC from normal subjects were incubated in the presence (▨) or absence (▧) of 10 units/ml γ-IFN for 18 hrs at 37°C. Percentage of FacbR$^+$ cells were then determined. Median values and interquartile ranges (T) are shown.

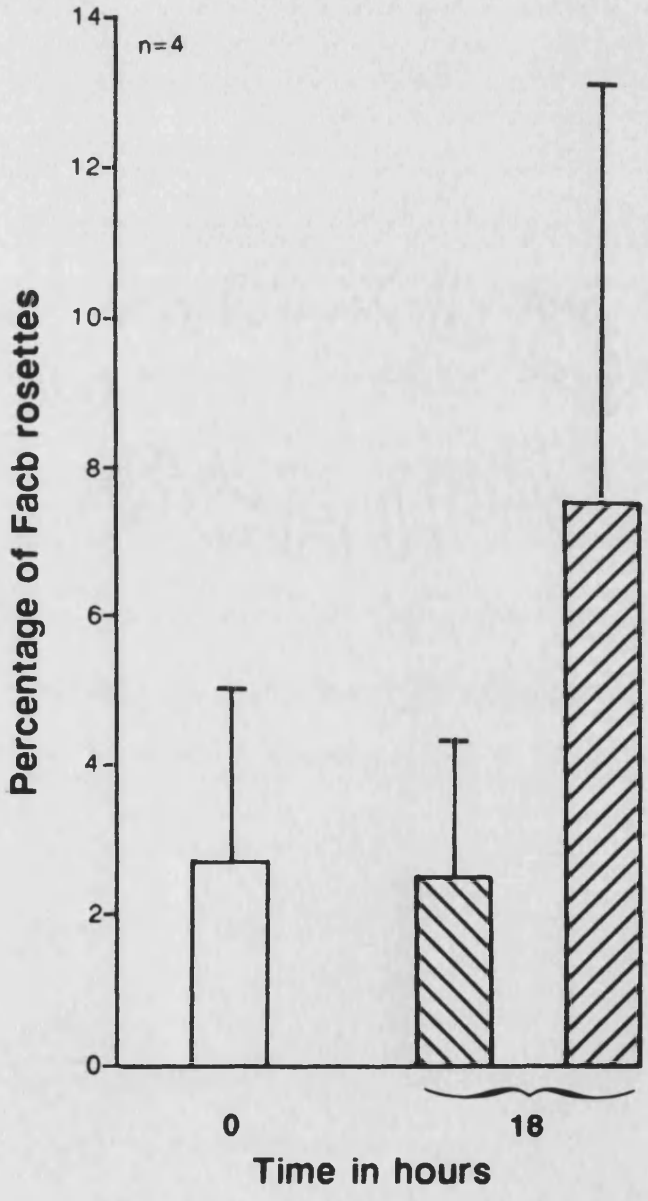


FIG 49 **The effect of γ-IFN on Facb-mediated feedback suppression of PWM-stimulated IgG response in normal healthy controls**

PBMNC were stimulated with 3 doses of PWM and Facb added at final concentrations of 0.2 (▨), 2 (▩) and 20 (■) µg/ml. The cultures were incubated in the presence or absence of 10 units of γ-IFN. Results are expressed as a percentage of the stimulated response where no Facb was present.

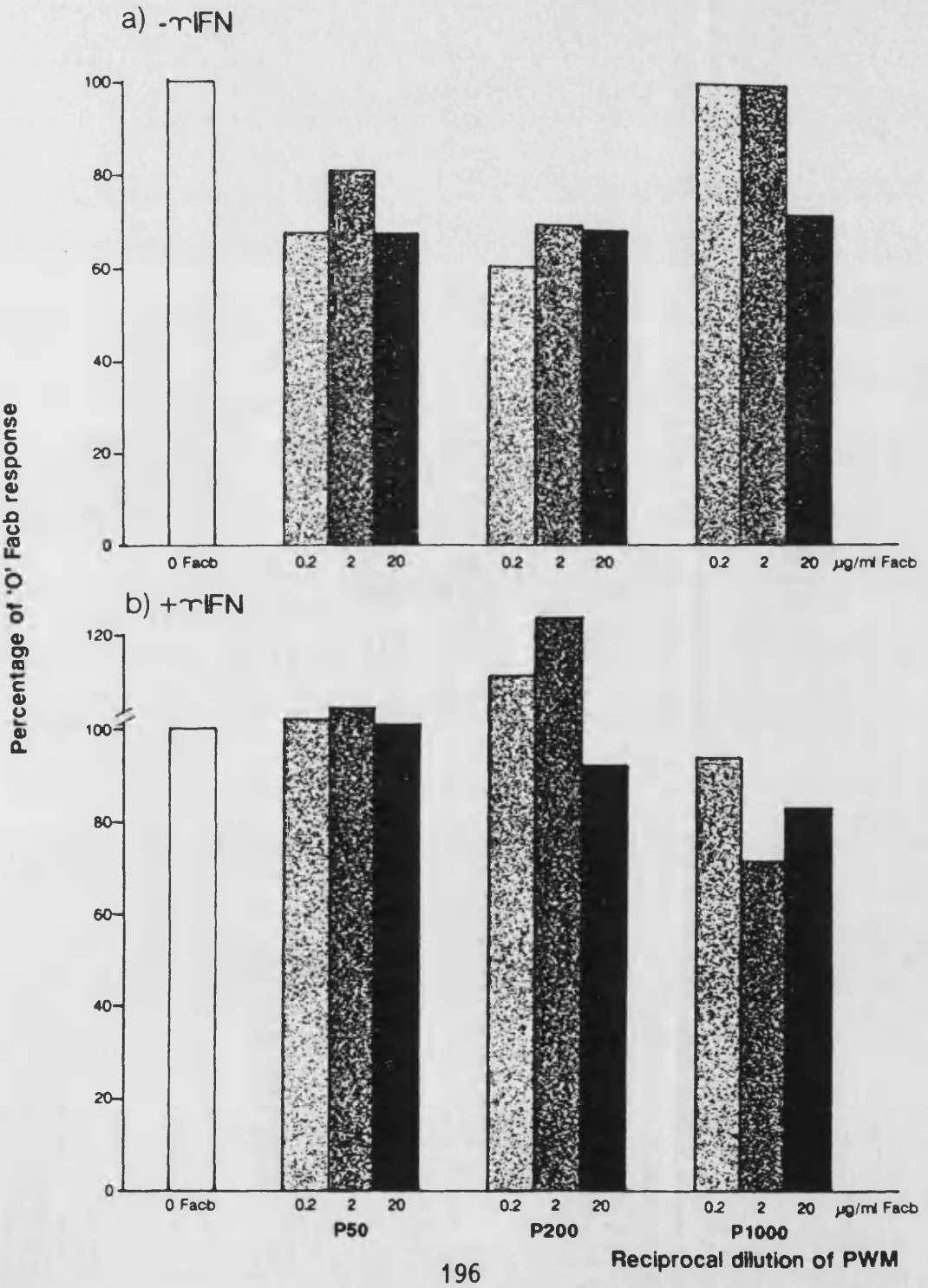
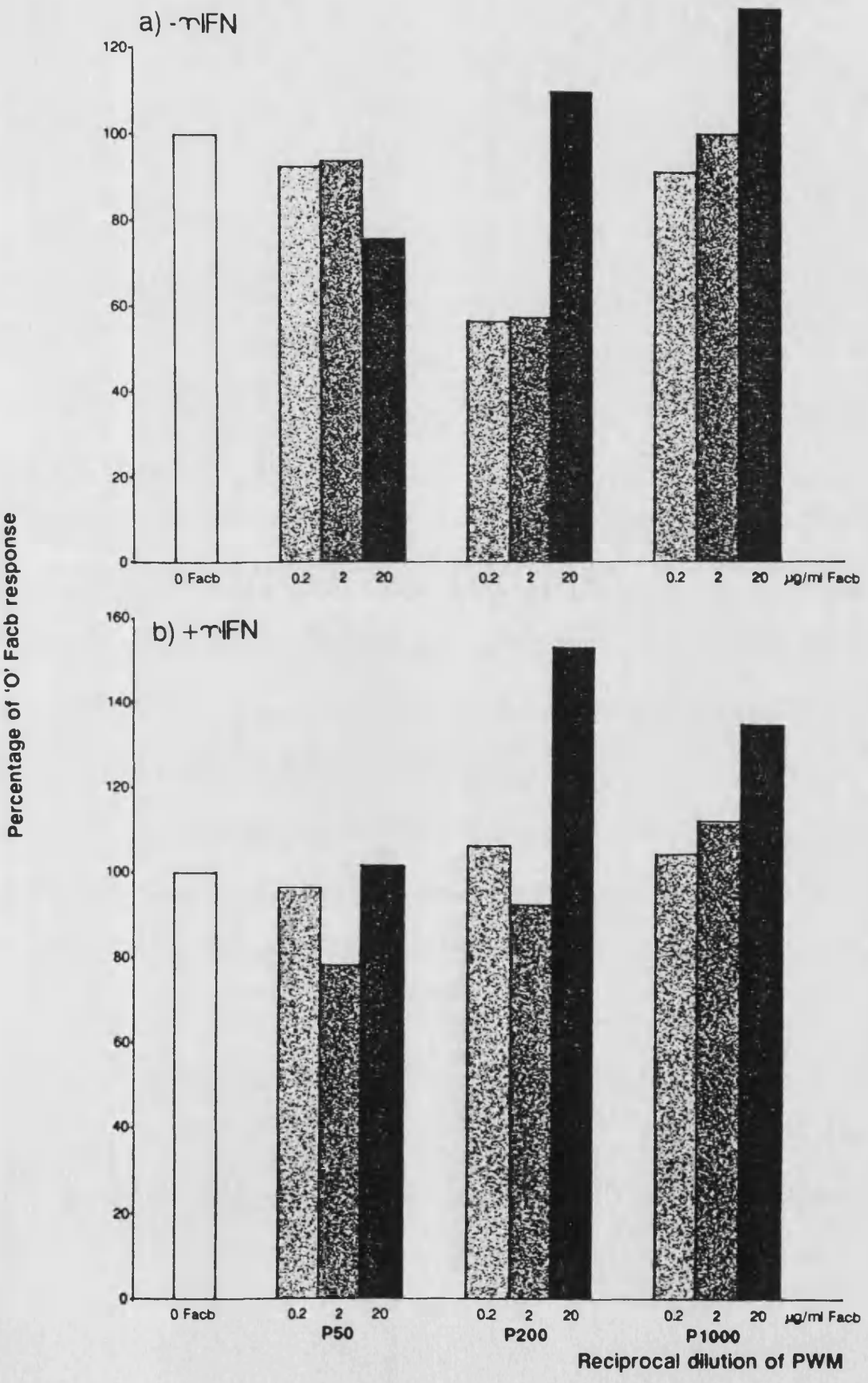


FIG 50 **The effect of γ-IFN on Facb-mediated feedback suppression of PWM-stimulated IgG response in patients with established RA**

PBMNC were stimulated with 3 doses of PWM and Facb added at final concentrations of 0.2 (), 2 () and 20 () μg/ml. The cultures were incubated in the presence or absence of 10 units of γ-IFN. Results are expressed as a percentage of the stimulated response where no Facb was present.


a) -γIFN
b) +γIFN
Percentage of 'O' Facb response
0 Facb
0.2
2
20
µg/ml Facb
P50
P200
P1000
Reciprocal dilution of PWM

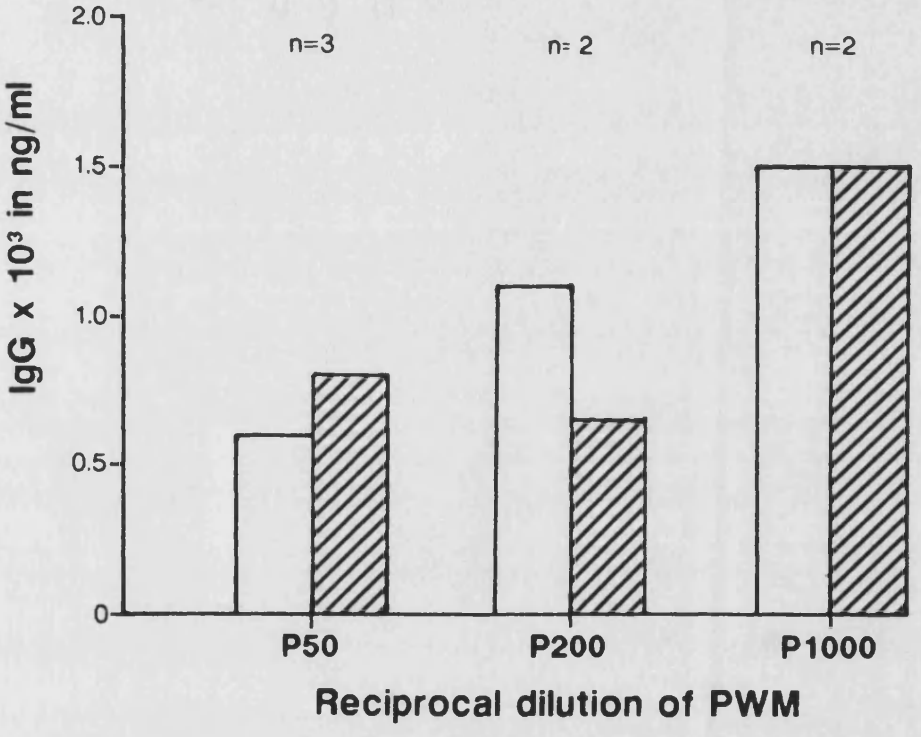
investigated (data not shown) but of the five patients examined only two individuals responded to PWM and then not at all doses. However, there was no evidence of an effect on IgG production.

Thus from the above it would appear that γ-IFN does not act directly on the immunoglobulin producing cells themselves but is able to reduce or negate the suppression produced by Facb. This could be either by rendering the Ig synthesising cells refractory to the suppressive effects of $FacbR^+$ cells or by acting on the latter and preventing suppressor activity.

Like the 2ME experiments, the numbers of individuals shown are very small. The above experiments need to be repeated to confirm the preliminary findings reported here.

FIG 51 **The effect of γ-IFN on IgG synthesis in normal subjects**

PBMNC were incubated for 14 days in the presence or absence of 10 units of γ-IFN. Levels of IgG were measured by ELISA. Median values of IgG are depicted in the figure.



CHAPTER 5

HAGG RESULTS

The results presented in this chapter concern modulation of antibody synthesis by heat aggregated IgG. A purified myeloma protein of the IgG_1 subclass was employed to produce Hagg. This was isolated from human serum using ion-exchange chromatography as described in Chapter 2, and was always used freshly aggregated at 63°C for 30 minutes for each experiment.

This ligand had not previously been used in this laboratory to investigate negative feedback suppression of immunoglobulin synthesis. Thus initial experiments involved setting up an assay for just this purpose. Since Hagg would interfere with IgG and RhF ELISAs, a two-step culture system had to be employed. PBMNC were first reacted with Hagg and PWM for a period of 5 days and then washed in fresh medium (to remove ligand) and recultured to generate supernatants which were then assessed for antibody production by the ELISA system. Results of the preliminary experiments which determined the operating conditions of this assay appear below in Section 1.

SECTION 1

1. Suppression of IgG synthesis by Hagg

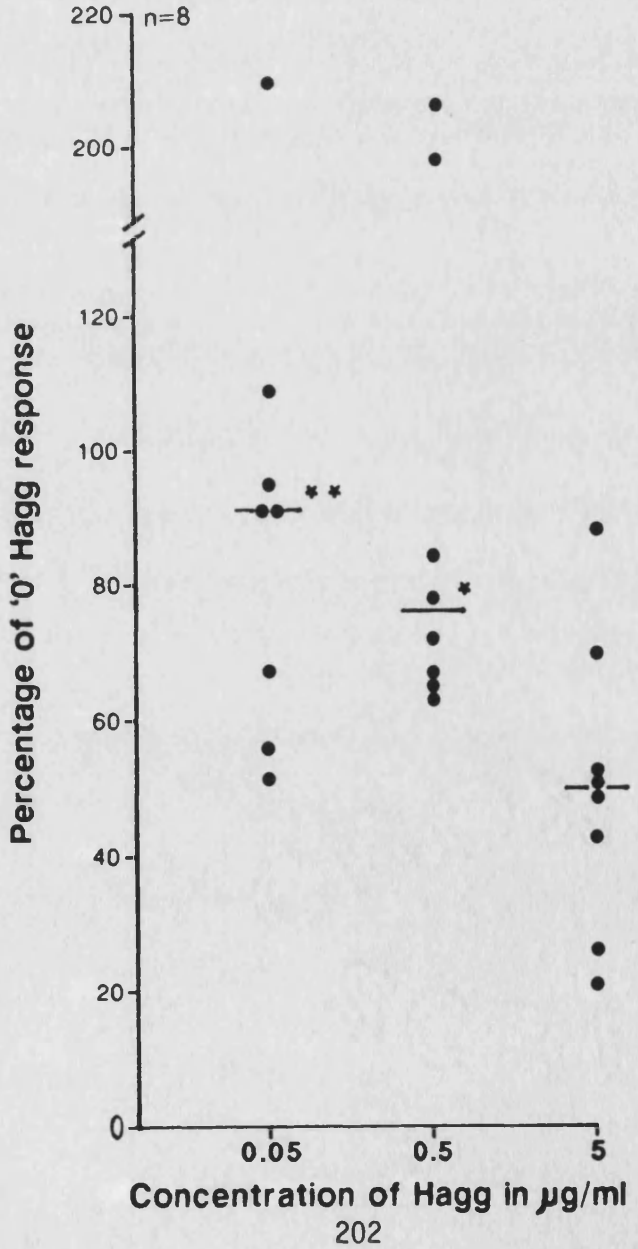
Heat aggregated IgG_1 was added to PWM-stimulated (P200) cultures of normal PBMNC at final concentrations of 0.05, 0.5 and 5 µg/ml. Figure 1 shows that suppression was found to occur best when Hagg was present at a concentration of 5 µg/ml. The IgG response was reduced by 50% at this concentration, giving p values of < 0.05 and < 0.01 when compared with suppression

FIG 1 **Dose-dependent suppression of PWM stimulated IgG response by Hagg**

Normal PBMNC were stimulated with P200 and incubated with 3 doses of Hagg for 5 days. Cells were washed and then re-incubated in fresh medium for a further 5 days. Suppression of the IgG response was assessed and results expressed as a percentage of the stimulated IgG response where no Hagg was present. Horizontal bars represent median values.

* $p < 0.05$ when compared with suppression at 5 µg/ml Hagg

** $p < 0.01$ when compared with suppression at 5 µg/ml Hagg



observed at 0.5 and 0.05 μg/ml Hagg respectively. Responses seen at these 2 latter concentrations did not differ from each other ($p > 0.05$). In 2 subjects it was found that the 2 lower doses of Hagg produced enhancement instead of suppression. These subjects were also found to exhibit less suppression at 5 μg/ml Hagg.

Figure 2 shows data obtained when equivalent concentrations of aggregated $F(ab')_2$ and BSA were added to normal PBMNC and cultured as for Hagg. $F(ab')_2$ was found not to suppress the antibody response, whilst BSA exhibited some activity. This almost certainly was due to the presence of small amounts of IgG in BSA preparation. Casein was then tested as an alternative protein (data not shown). This was found not to suppress the IgG response at the two higher concentrations, 5 and 0.5 μg/ml.

These experiments thus show that Hagg suppression of the IgG response seen in Figure 1 was due to the presence of Fc and not just to an excess of protein present in an aggregated form.

2. **Time of incubation o. Hagg cultures**

PWM-stimulated PBMNC from normal subjects were set up with 0.05, 0.5 and 5 μg/ml Hagg and the cultures incubated for a total of 10 or 14 days. Figure 3 shows that in one index case Hagg suppression of the IgG response was better at Day 10 than at Day 14. In another individual where only Day 14 cultures were set up, no suppression was observed (data not shown).

3. **Time of addition of Hagg**

Using a final concentration of 5 μg/ml Hagg in P200-stimulated normal PBMNC cultures, it was found that suppression of IgG

FIG 2 **The effect of heat aggregated $F(ab')_2$, BSA and IgG_1 on PWM stimulated IgG response in one index case**

Purified $F(ab')_2$ fragment (▢) and BSA (▩) were heat aggregated at 63°C for half an hour and added to P200 stimulated normal PBMNC cultures at a final concentration of 5 µg/ml. The effects of Hagg (▨) were simultaneously investigated. Suppression of the IgG response is expressed as a percentage of the PWM stimulated response where no Hagg was present.

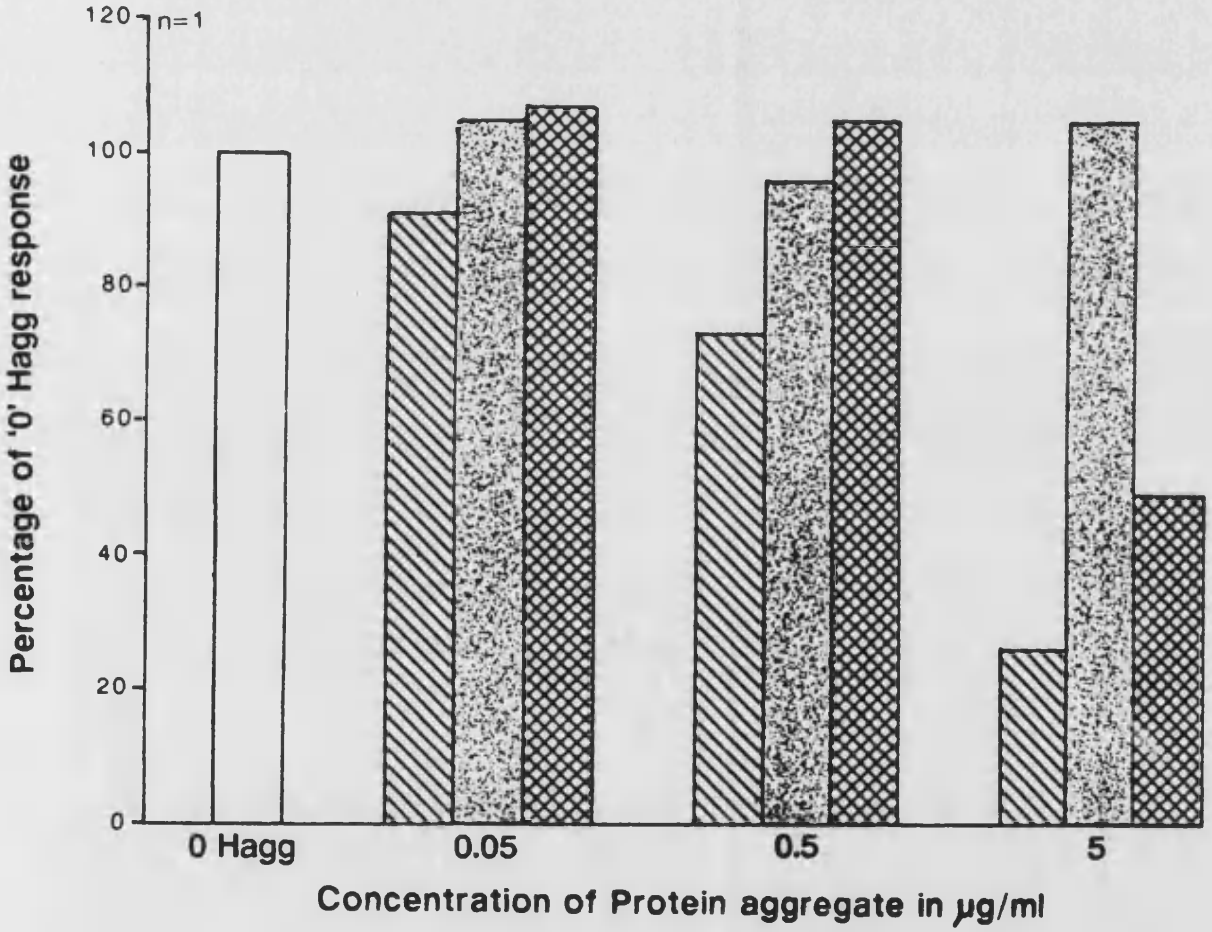
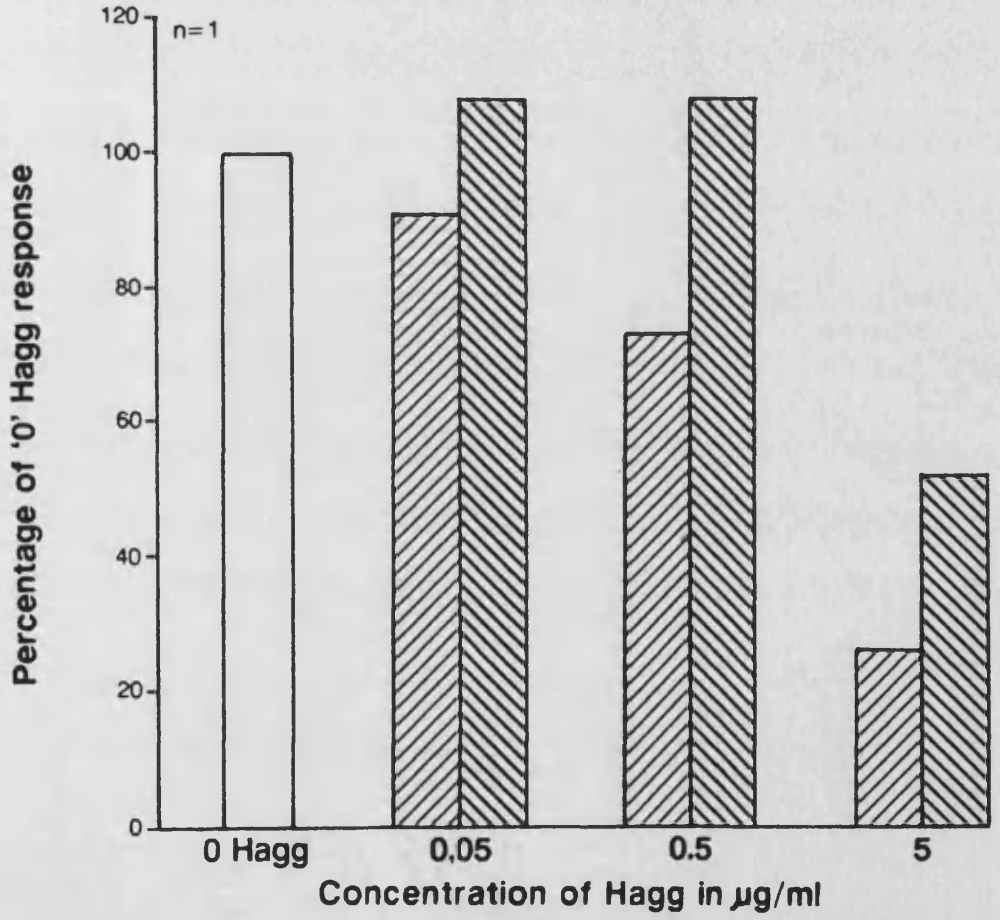


FIG 3 **Hagg suppression of PWM-stimulated IgG response on days 10 and 14 in one index case**

P200 stimulated normal PBMNCs were incubated with 3 doses of Hagg for 5 days, then washed and recultured in fresh medium for a total of 10 (▨) or 14 (▧) days. Suppression of the IgG response was assessed and expressed as a percentage of the stimulated IgG response where no Hagg was present.



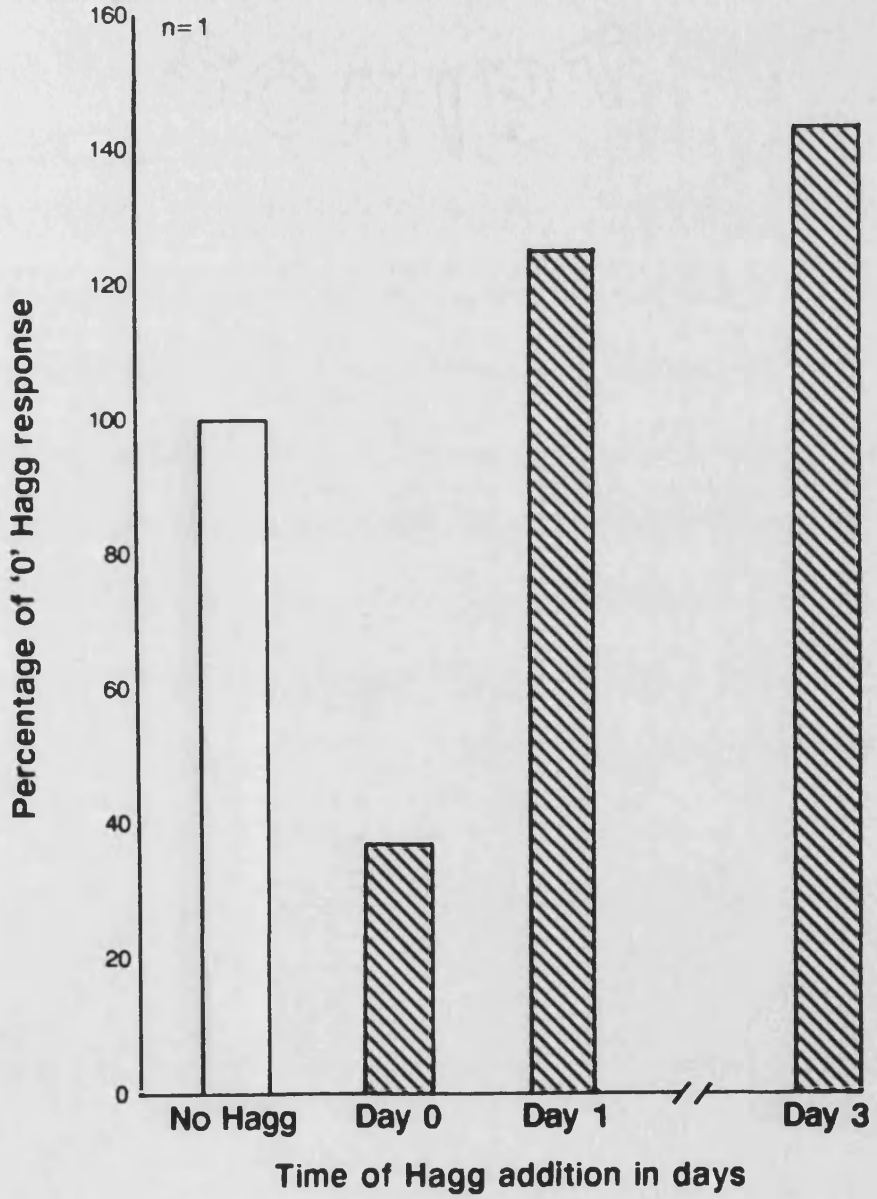
production only occurred when the ligand was present at the beginning of the culture. Adding Hagg on Day 1 and Day 3 was ineffective. These data are shown in Figure 4.

From the above it was decided that subsequent Hagg experiments should be conducted using PWM at a dilution of 1/200 and that Hagg should be added at a final concentration of 5 µg/ml at the beginning of the culture period, which was to last a total of 10 days.

Using these conditions Hagg suppression of IgG synthesis in various test groups was examined. These results appear in Section 2.

FIG 4 **The effect of varying time of Hagg addition on feedback suppression of PWM-stimulated IgG response**

Normal PBMNC were stimulated with P200 and a final concentration of 5 μg/ml of Hagg added on days 0, 1 and 3. Cells were washed on day 5 and recultured in fresh medium for a further 5 days. The degree of suppression was assessed and results expressed as a percentage of the stimulated IgG response where no Hagg was present.



SECTION 2

1. Suppression of IgG production by Hagg in health and disease

The effect of Hagg on both spontaneous and PWM-stimulated IgG responses was examined in young and old normal healthy controls and in patients with RA, AS, OA and Felty's syndrome.

Hagg was found to have no significant effect on spontaneous IgG synthesis. Table 1 shows that the median values of IgG production were either totally unaffected or slightly reduced. Only with RA patients were levels of IgG slightly raised compared to cultures where no Hagg was present. It should be noted, however, that within each group the individual responses were variable. This explains why in groups such as old normals (ON) and Felty's patients the response range increased, although with Hagg the median values were found to be reduced.

The effects of Hagg on PWM-induced IgG production in various test groups are illustrated in Figures 5 - 10. Only individuals producing > 300 ng/ml IgG have been considered.

Figure 5 shows Hagg suppression of IgG synthesis in normal healthy controls. The PWM response was quite variable but both high and low responders, with the exception of one individual, exhibited a suppressed antibody response upon addition of Hagg. A median value of 1568 ng/ml fell significantly to 780 ng/ml ($p < 0.05$).

A degree of suppression was also observed in most of the subjects in the various test groups, with the exception of one or two individuals who exhibited an enhanced or unaltered IgG response. With RA patients the median value fell from 2844 ng/ml to 1747 ng/ml IgG. With AS IgG was reduced from 1813 ng/ml to 955 ng/ml and in OA from 1073 to 598 ng/ml. In old controls the median values appear to indicate a lack of suppression. This, however, is misleading, since

TABLE 1 Showing the range and median values of spontaneous IgG production in the absence and presence of Hagg in normal healthy controls and patients with RA, AS, OA and Felty's syndrome

Test group	n	- Hagg		+ Hagg	
		Range (ng/ml)	Median (ng/ml)	Range (ng/ml)	Median (ng/ml)
Normals	20	0 - 120	15	0 - 55	15
RA	15	0 - 54	14	0 - 61	20
AS	10	0 - 82	32.5	5 - 75	29
OA	14	0 - 62	23	0 - 53	18.5
ON	10	3 - 73	16	0 - 103	13.5
Felty's	4	5 - 117	27	9 - 155	23

FIG 5 **Hagg suppression of PWM-stimulated IgG synthesis in normal healthy controls**

P200 stimulated PBMNC were incubated in the presence or absence of 5 µg/ml final concentration of Hagg for 5 days. Cells were then washed and recultured in fresh medium for a further 5 days. IgG content was assessed by ELISA. Median levels are indicated by the horizontal bars.

* $p < 0.05$ when compared to the P200 response in the absence of Hagg.

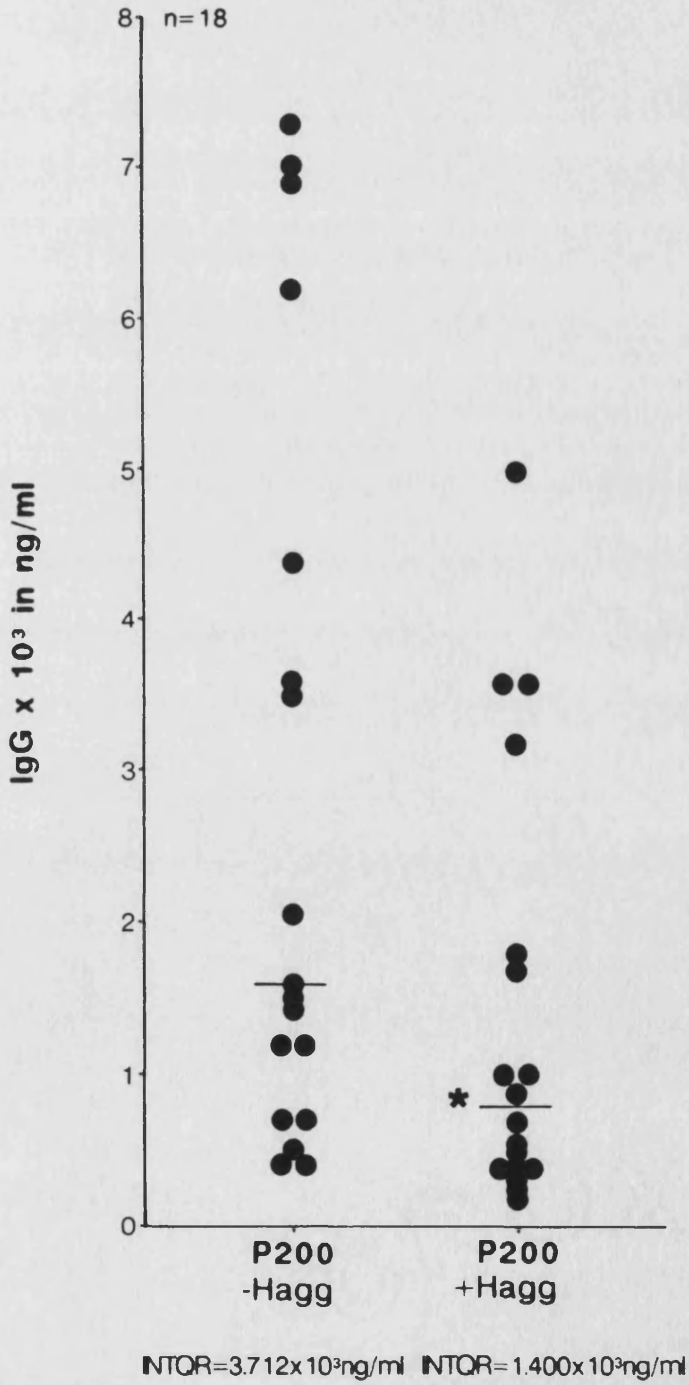


FIG 6 **Hagg suppression of PWM-stimulated IgG synthesis in patients with established RA**

P200 stimulated PBMNC were incubated in the presence or absence of 5 μg/ml final concentration of Hagg for 5 days. Cells were then washed and recultured in fresh medium for a further 5 days. IgG content was assessed by ELISA. Median levels are indicated by the horizontal bars.

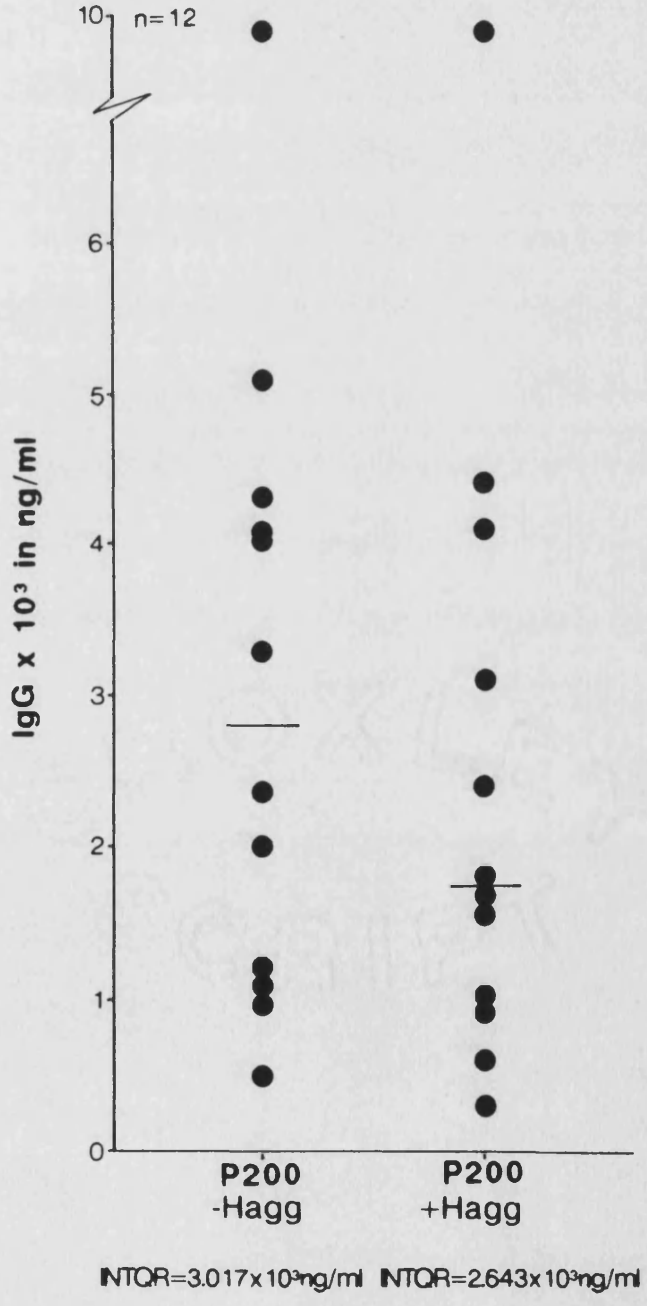


FIG 7 **Hagg suppression of PWM-stimulated IgG synthesis in AS patients**

P200 stimulated PBMNC were incubated in the presence or absence of 5 µg/ml final concentration of Hagg for 5 days. Cells were then washed and recultured in fresh medium for a further 5 days. IgG content was assessed by ELISA. Median levels are indicated by the horizontal bars.

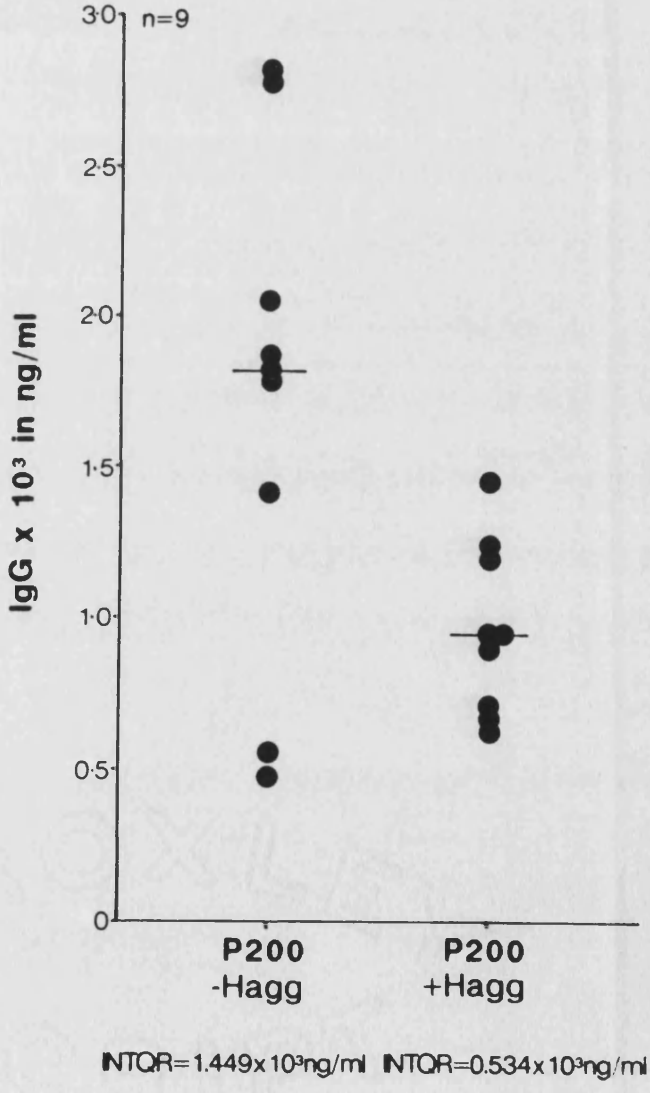


FIG 8 **Hagg suppression of PWM-stimulated IgG synthesis in OA patients**

P200 stimulated PBMNC were incubated in the presence or absence of 5 µg/ml final concentration of Hagg for 5 days. Cells were then washed and recultured in fresh medium for a further 5 days. IgG content was assessed by ELISA. Median levels are indicated by the horizontal bars.

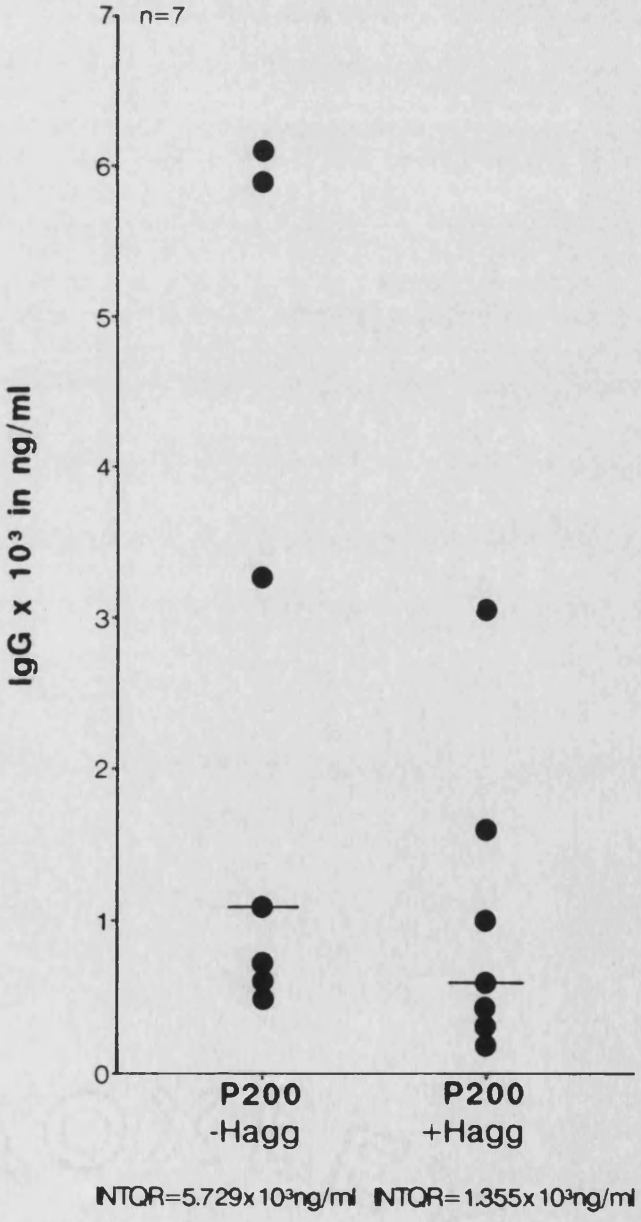


FIG 9 **Hagg suppression of PWM-stimulated IgG synthesis in old normal subjects**

P200 stimulated PBMNC were incubated in the presence or absence of 5 μg/ml final concentration of Hagg for 5 days. Cells were then washed and recultured in fresh medium for a further 5 days. IgG content was assessed by ELISA. Median levels are indicated by the horizontal bars.

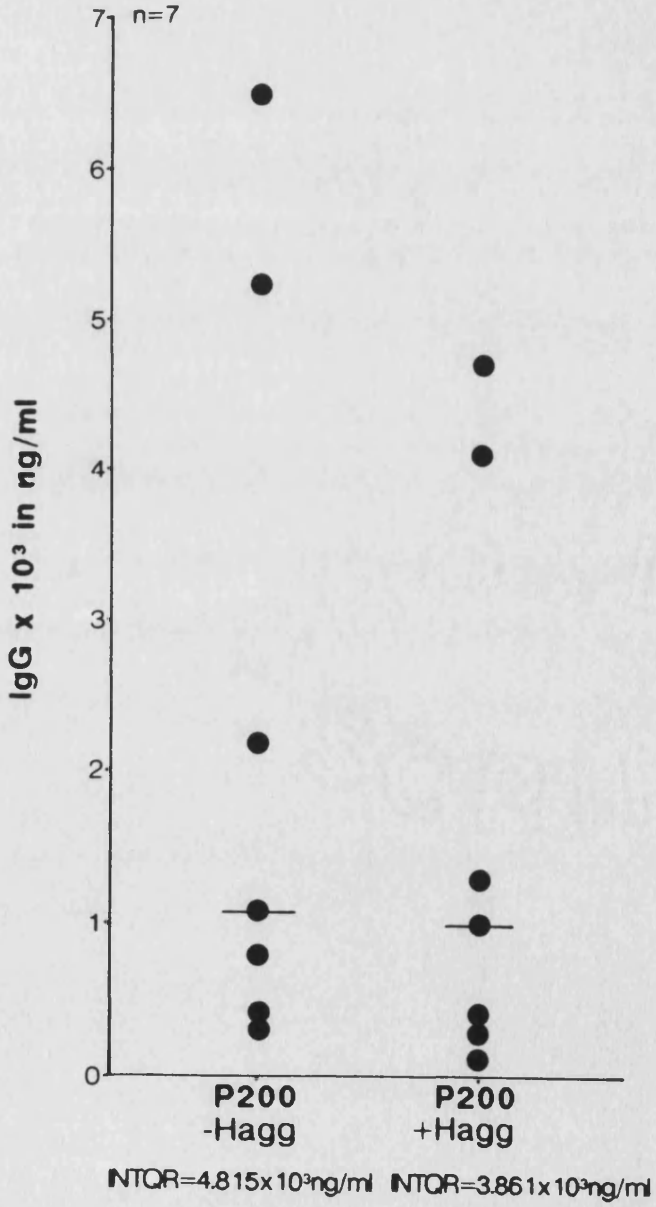
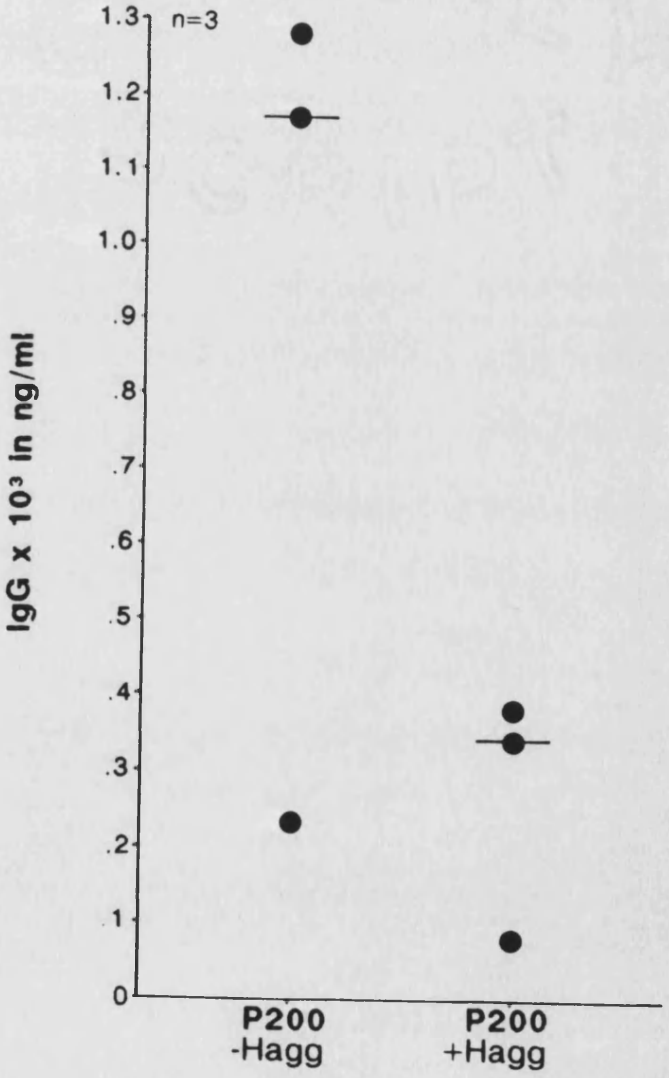


FIG 10 **Hagg suppression of PWM-stimulated IgG synthesis in Felty's patients**

P200 stimulated PBMNC were incubated in the presence or absence of 5 µg/ml final concentration of Hagg for 5 days. Cells were then washed and recultured in fresh medium for a further 5 days. IgG content was assessed by ELISA. Median levels are indicated by the horizontal bars.



most subjects were found to be well suppressed when individual responses were examined. None of the above data was found to reach levels of statistical significance when P200 +/- Hagg results were compared within each group using the Mann-Whitney U-test. Felty's patients were not included in this analysis because of the very low number of patients available. In this group only 2 subjects responded to PWM adequately, whilst the third was a low responder and the fourth did not respond at all. However, all three responding subjects exhibited marked suppression with the median value falling from 1170 ng/ml to 340 ng/ml. A larger number of patients would need to be tested in order to evaluate the effects of Hagg on IgG synthesis in this subgroup of RA.

It can be seen from the above that RA patients generated much more IgG than normal subjects. This, however, did not reach statistical significance. Immunoglobulin levels in patients with AS were only slightly raised above normals, whilst those in OA, old normals and Felty's subjects were slightly lower. The OA group was found to contain a large number of non-responders. Only 7 of the 14 individuals tested responded to PWM. Possibly another dose of mitogen might have yielded a better result.

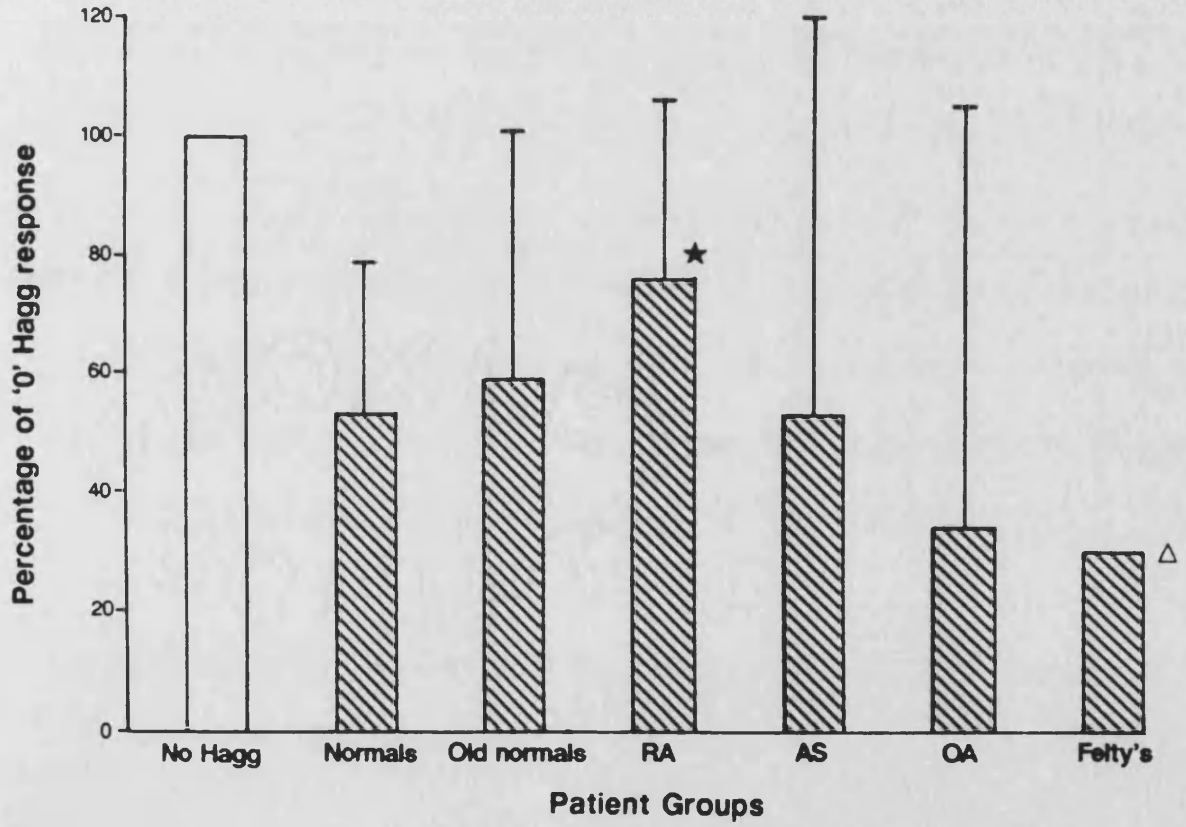
Because of the different levels of IgG production, it was not possible to directly compare the extent to which Hagg suppressed the antibody response in each of the above groups. The P200 + 5 µg/ml Hagg results were thus converted to percentages of the antibody response where no ligand was present. These data are shown in Figure 11. Responses in normal subjects fell to a median of 53% of the P200 response when Hagg was added to the cultures. This value was 76% in patients with RA, 53% in AS subjects, 34% in the OA group, 59% in old

FIG 11 **Comparison of Hagg-mediated suppression of the PWM-stimulated IgG response in normal and patient groups**

Suppression is expressed as a percentage of the P200 response where no Hagg was present. Median values and interquartile ranges (T) are shown.

★ $p < 0.02$ when compared to the suppression observed in normal healthy controls

△ not statistically analysed because of the small number of patients available.



normals and 30% in Felty's patients. Comparing these results using the Mann-Whitney U-test, it was found that only the RA group showed significantly less suppression compared to normal healthy controls ($p = 0.016$). No differences were found between control subjects and any of the other groups examined, nor were these groups, including old normals, different from RA patients. This is probably explained by the greater variability seen in response to Hagg in these groups. This, at least in the OA group, may be attributed partly to age, since the interquartile range in old normals was found to be larger than that observed in the young controls. (It should be noted that the term 'normal controls' in the above text refers to the young healthy subjects and not to the group comprising the old normals.)

Felty's patients were not included in the above statistical analysis because of the very small numbers of patients involved. However, these patients were well suppressed, although they form a subgroup of RA. A larger number of these patients needs to be investigated in order to confirm these data.

2. The effect of Hagg on IgM RhF production

The effect of Hagg on spontaneous and PWM-induced IgM RhF production was also examined. These data appear in Figures 12 - 17.

Addition of Hagg completely abolished low levels of spontaneous RhF synthesis. In one RA patient and one Felty's subject, who produced 35 and 14 ng/ml of RhF respectively, Hagg was found to markedly suppress the autoantibody response. This dropped to 30% of the original level in the RA patient and to 21% in the Felty's subject. Only in one person, an old normal, was Hagg found to elicit RhF production and then only to very low levels (< 5 ng/ml).

FIG 12 **The effect of Hagg on spontaneous and PWM-stimulated IgM RhF production in normal healthy controls**

Hagg was added at a final concentration of 5 µg/ml to PBMNC cultures in the absence or presence of PWM (P200). Median values are represented by the horizontal bars.

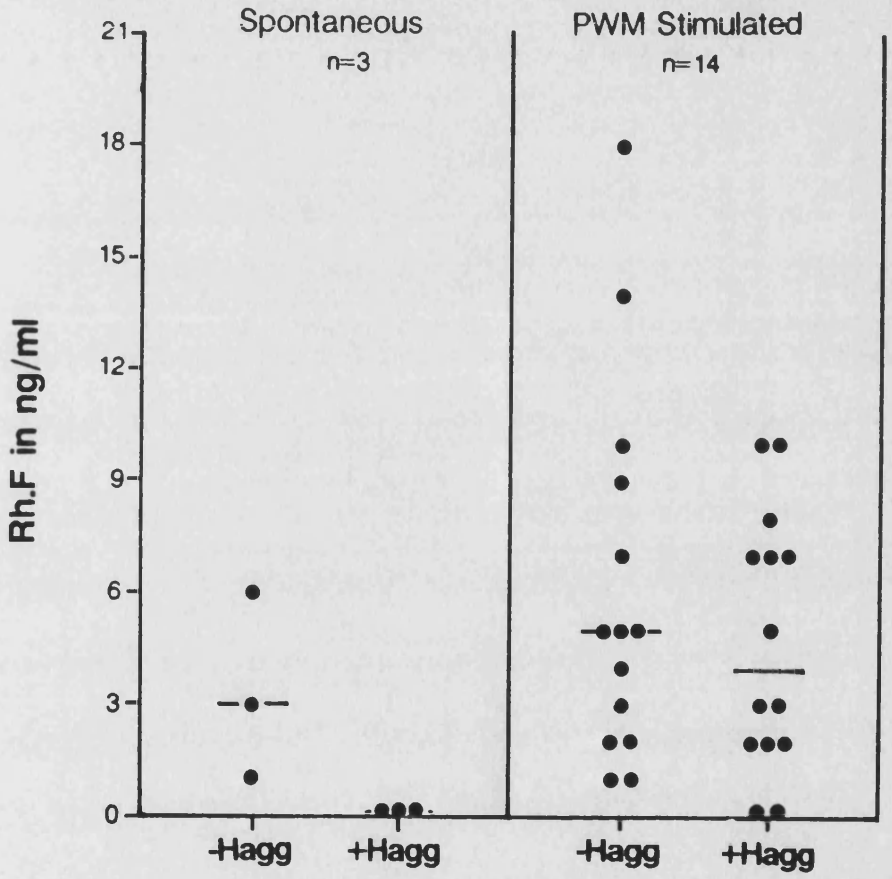


FIG 13 The effect of Hagg on spontaneous and PWM-stimulated IgM RhF production in established RA patients

Hagg was added at a final concentration of 5 µg/ml to PBMNC cultures in the absence or presence of PWM (P200). Median values are represented by the horizontal bars.

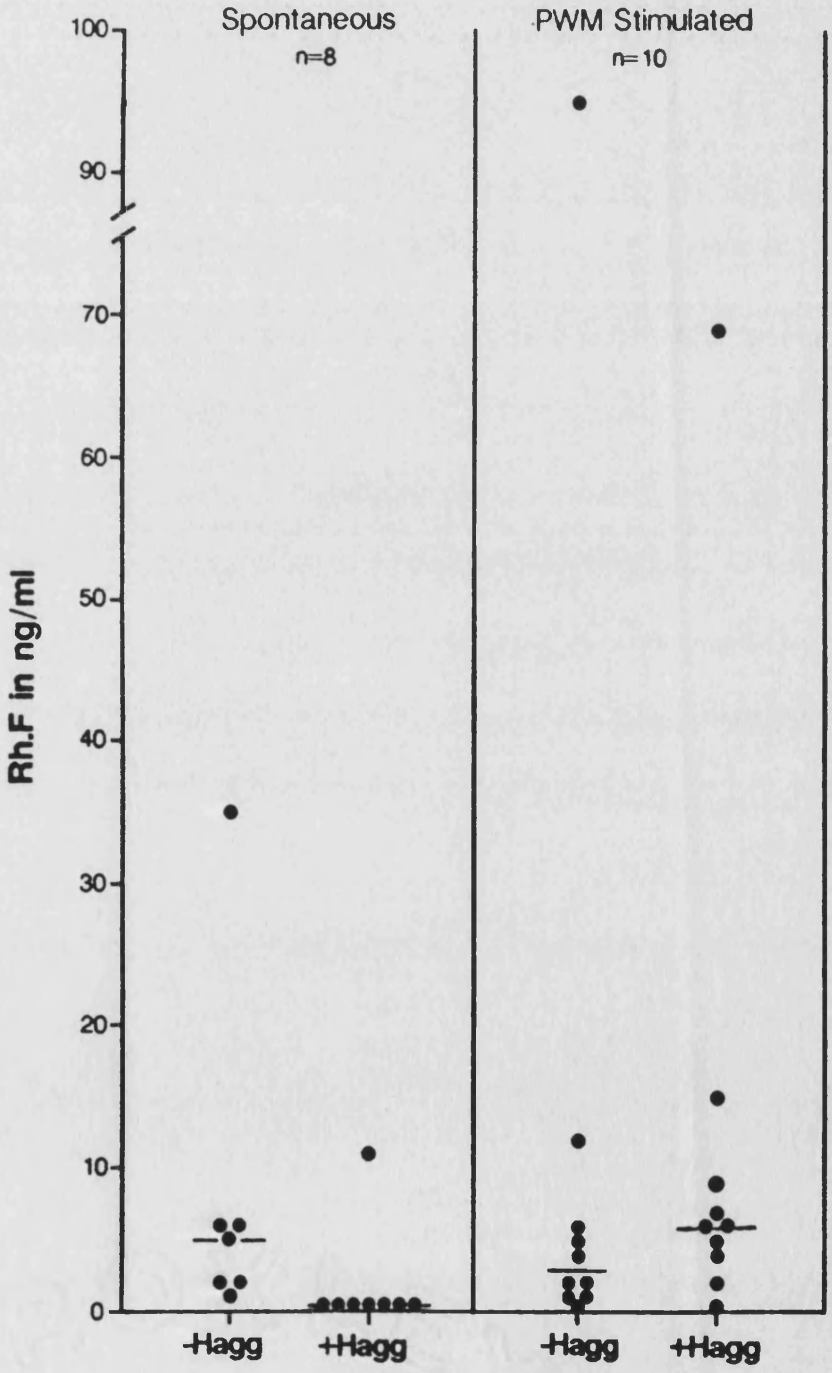


FIG 14 **The effect of Hagg on spontaneous and PWM-stimulated IgM RhF production in OA patients**

Hagg was added at a final concentration of 5 µg/ml to PBMNC cultures in the absence or presence of PWM (P200). Median values are represented by the horizontal bars.

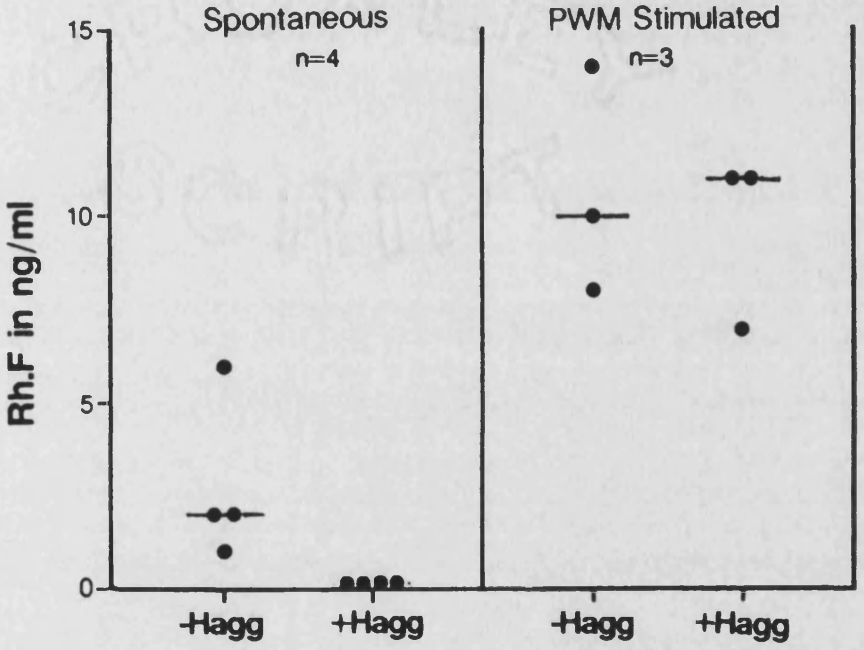


FIG 15 **The effect of Hagg on spontaneous and PWM-stimulated IgM RhF production in AS patients**

Hagg was added at a final concentration of 5 µg/ml to PBMNC cultures in the absence or presence of PWM (P200). Median values are represented by the horizontal bars.

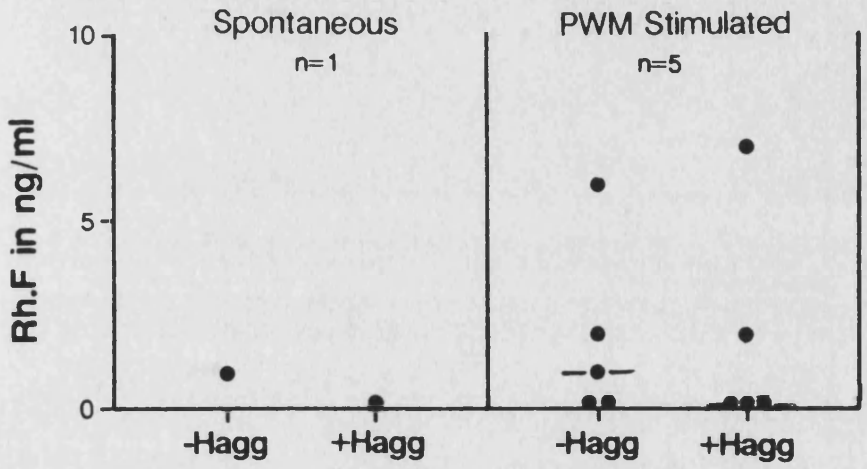


FIG 16 **The effect of Hagg on spontaneous and PWM-stimulated IgM RhF production in Felty's patients**

Hagg was added at a final concentration of 5 µg/ml to PBMNC cultures in the absence or presence of PWM (P200). Median values are represented by the horizontal bars.

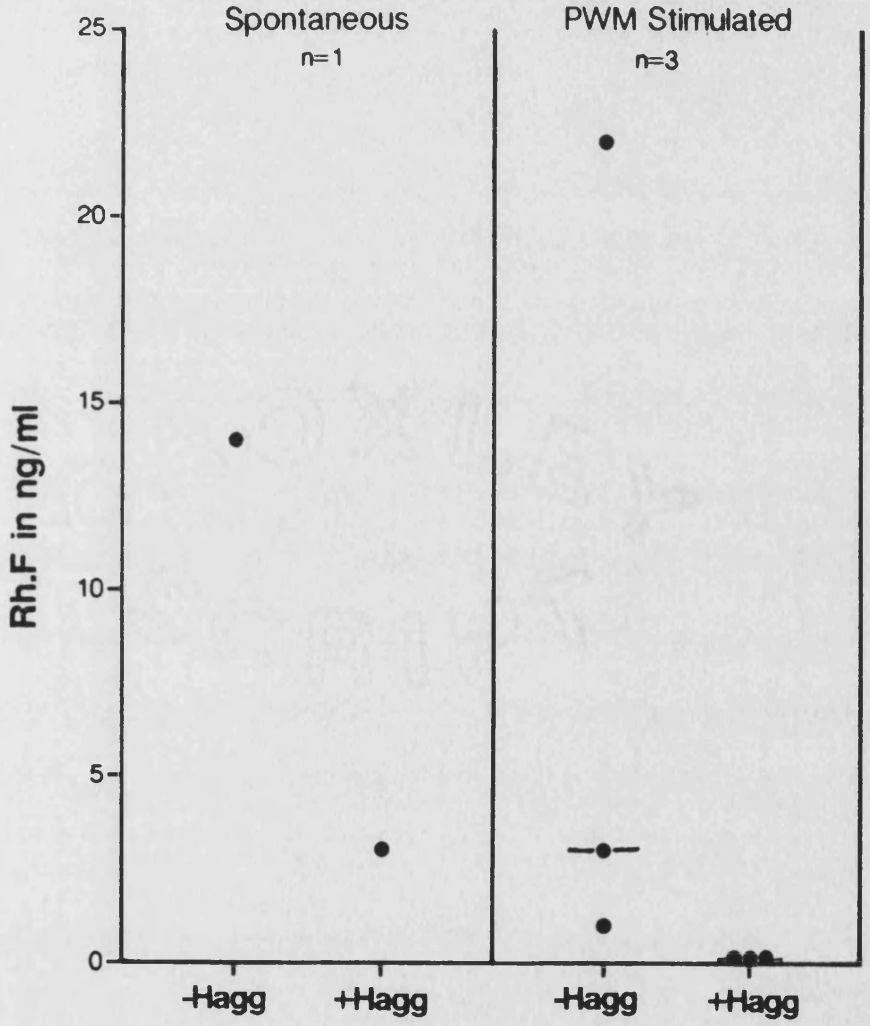
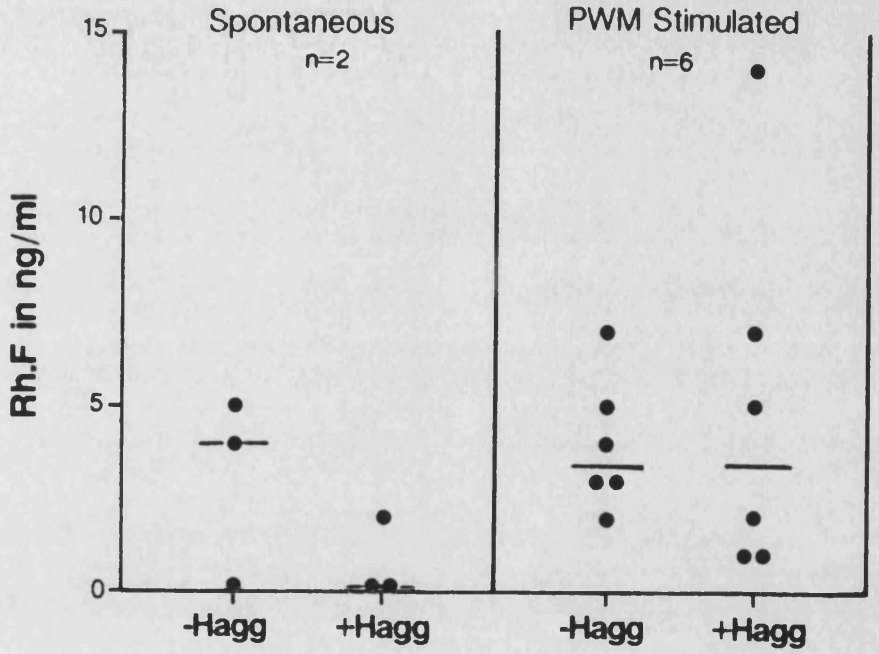


FIG 17 **The effect of Hagg on spontaneous and PWM-stimulated IgM RhF production in old normal subjects**

Hagg was added at a final concentration of 5 μg/ml to PBMNC cultures in the absence or presence of PWM (P200). Median values are represented by the horizontal bars.



Stimulation of PBMNCs led to an increase in the number of individuals generating autoantibody in most groups. However, the median levels of RhF were not always enhanced above those observed with spontaneous production. The addition of Hagg produced variable responses. In normal and AS subjects the median values were slightly reduced; in old normals there was no change and in OA and RA subjects there was enhancement. This, however, was only of note in the latter group where a two-fold increase (3.5 to 6 ng/ml) in RhF synthesis was observed. In Felty's patients, on the other hand, PWM-induced RhF production was completely suppressed in both low and high generators of autoantibody.

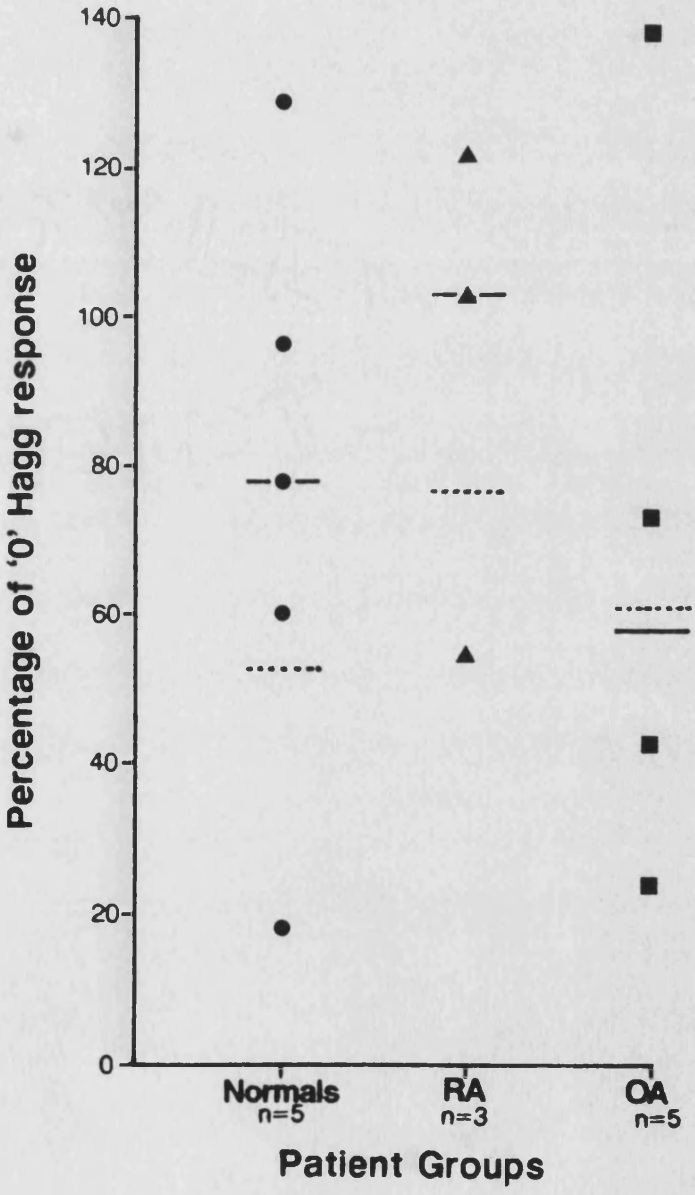
Thus is would appear from the above data that Hagg is able to suppress spontaneous RhF production, but when PBMNC are polyclonally stimulated with mitogen, a variable effect is produced, which in RA subjects tends towards enhancement.

Part of this variability might result from effects on total IgM synthesis in normals. Supernatants from 6 normal, 6 RA and 6 OA PBMNC cell cultures were examined for the effect of Hagg on IgM production. Figure 18 shows that IgM is not as well suppressed as the corresponding IgG response (at least in normals and RA) and that the effect of the ligand is highly variable but even with IgM the RA subjects are less suppressed compared to controls. Clearly, more subjects needed to be examined, since of the 18 people tested, 6 were non-responders for IgM. Certainly for RA subjects, IgM production is reported to be reduced compared to controls (Olsen et al, 1982). This was also indicated in Chapter 3.

Percentage suppression of RhF by Hagg could not be suitably compared between patient groups shown in Figures 12 - 17 because of the low levels of RhF generated. However, using only those individuals

FIG 18 **The effect of Hagg on PWM-stimulated IgM and IgG responses in normal, RA and OA subjects**

Suppression of the IgM and IgG synthesis by 5 µg/ml Hagg is expressed as a percentage of the P200 stimulated response where no ligand was present. Median values for IgM (———) and for IgG (--------) responses are shown.



producing > 10 ng/ml RhF, percentage Hagg suppression of the autoantibody was compared with the corresponding effects of Hagg on IgG production at P200.

Figure 19a shows that in some cases IgG was suppressed much more than RhF whilst in others (Figure 19b) the situation was reversed. Furthermore, it was found that Hagg may have opposing effects on IgG and RhF generation (Figure 19c). Thus, like Facb in Chapter 4, Hagg may modulate polyclonal IgG and specific RhF responses differently in the same person and between individuals.

3. Comparison of Hagg and Facb suppression of IgG response

Data presented in Chapter 4 and here suggest that Hagg is a better suppressor of IgG synthesis than Facb. This is illustrated in Figure 20 which depicts suppression of antibody response in normal PBMNC by Facb at concentrations of 0.2, 2 and 20 μg/ml and by Hagg at 0.05, 0.5 and 5 μg/ml. Statistically significant difference between the two ligands is reached only when Hagg is used at 5 μg/ml.

One obvious explanation for differences observed in Facb- and Hagg-mediated suppression .s that the former is not able to bind to the relevant Fc receptors as strongly as the latter. Thus inhibition experiments were carried out using PBMNC from RA patients, which gave a higher level of $FacbR^+$ cells then normal subjects. PBMNC were incubated with different concentrations of Hagg and Facb (the ranges included those concentrations normally employed in culture) for half an hour on ice before being set up in an Facb rosette assay as usual. The results of these experiments appear in Figures 21 and 22. Preincubating PBMNCs with increasing concentrations of Facb, whilst giving rise to variations in the percentage of rosettes, did not affect the level of Facb rosettes observed ($p > 0.05$), even when the

FIG 19 **Modulation of PWM-stimulated IgG and IgM RhF responses by Hagg**

The effects of Hagg on IgG (▨) and corresponding IgM RhF (□) production are depicted. Results are expressed as a percentage of the P200 response where no Hagg was present. Median values are shown. In **(a)** IgG is better suppressed than RhF. In **(b)** IgM RhF is suppressed better than the corresponding IgG response. In **(c)** IgM RhF is markedly enhanced whilst IgG is suppressed.

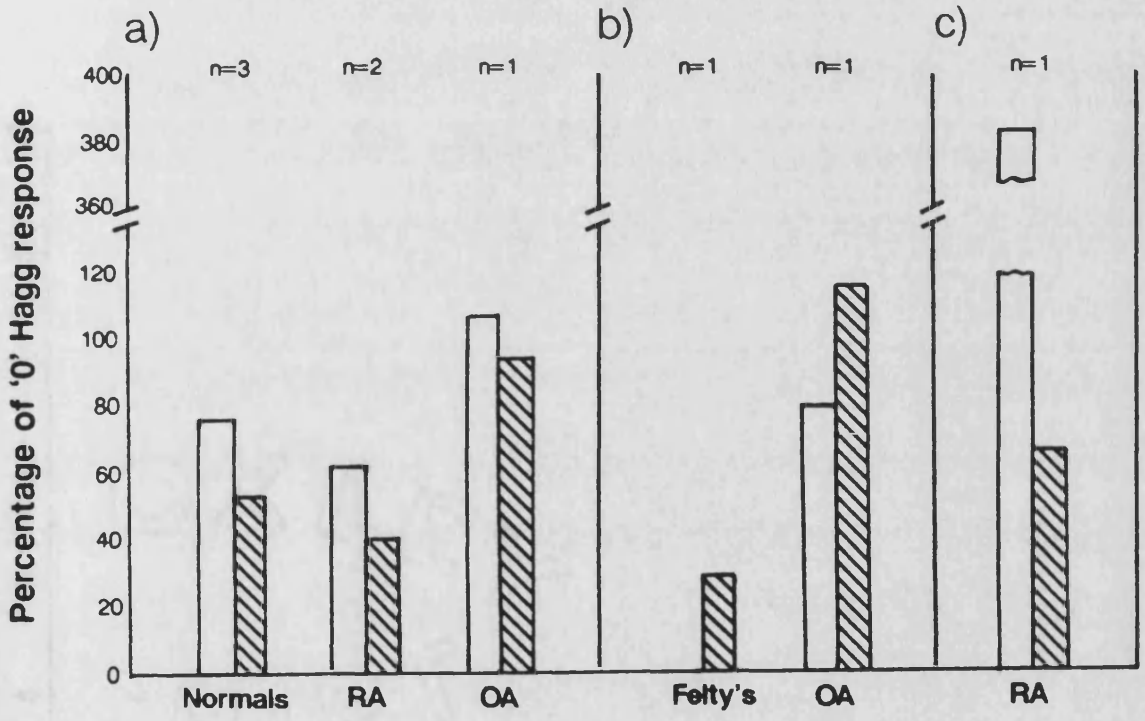


FIG 20 **Comparison of Hagg and Facb suppression of IgG response at P200 in normal healthy controls**

Suppression is expressed as a percentage of the stimulated response where no ligand was present. Facb and Hagg data are represented by (■—■) and (●—●) respectively, where each point in the former is a median value of 16 observations and in the latter of 19 determinations.

** $p < 0.01$ when the degree of suppression is compared with that

* $p = 0.02$ observed at 5 µg/ml Hagg

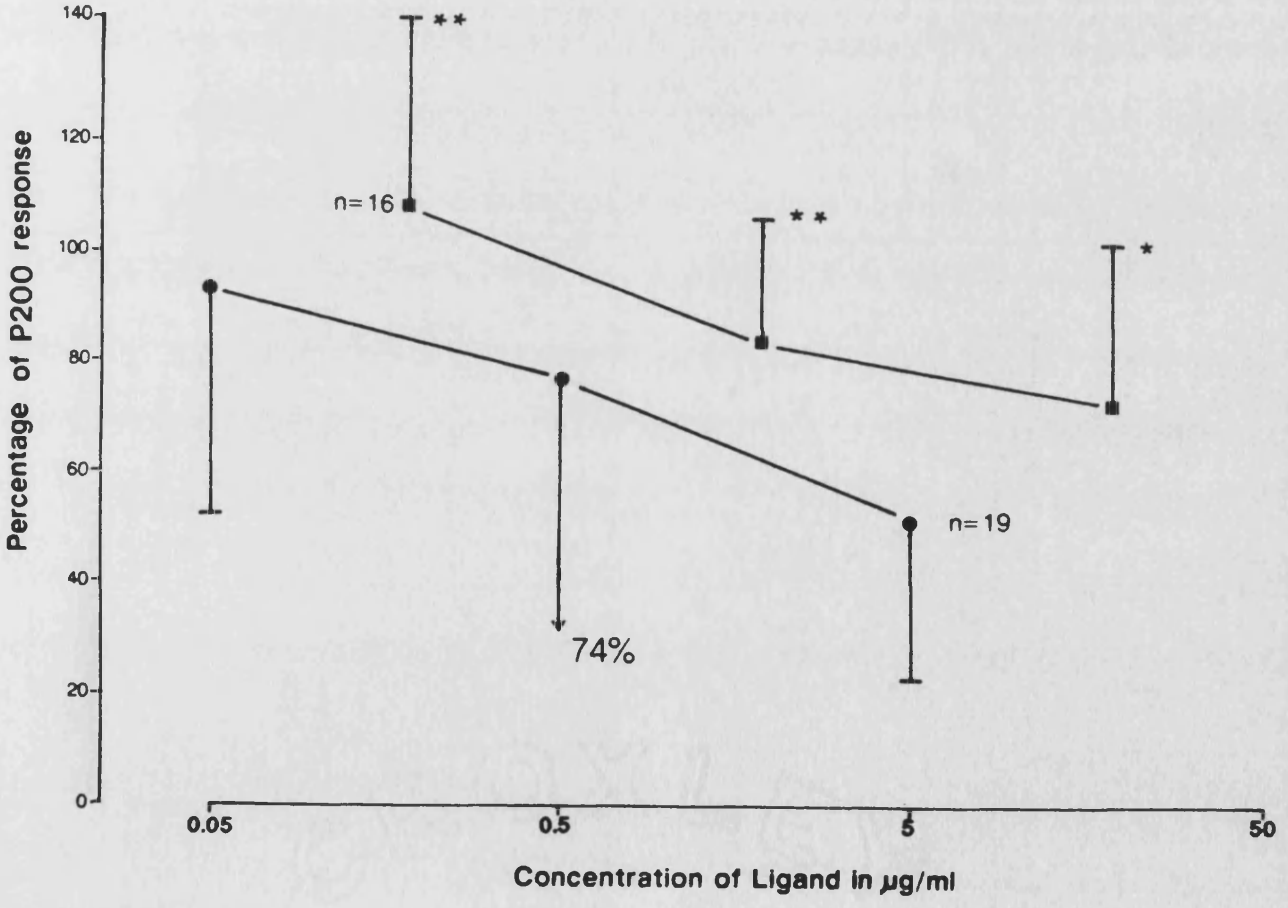
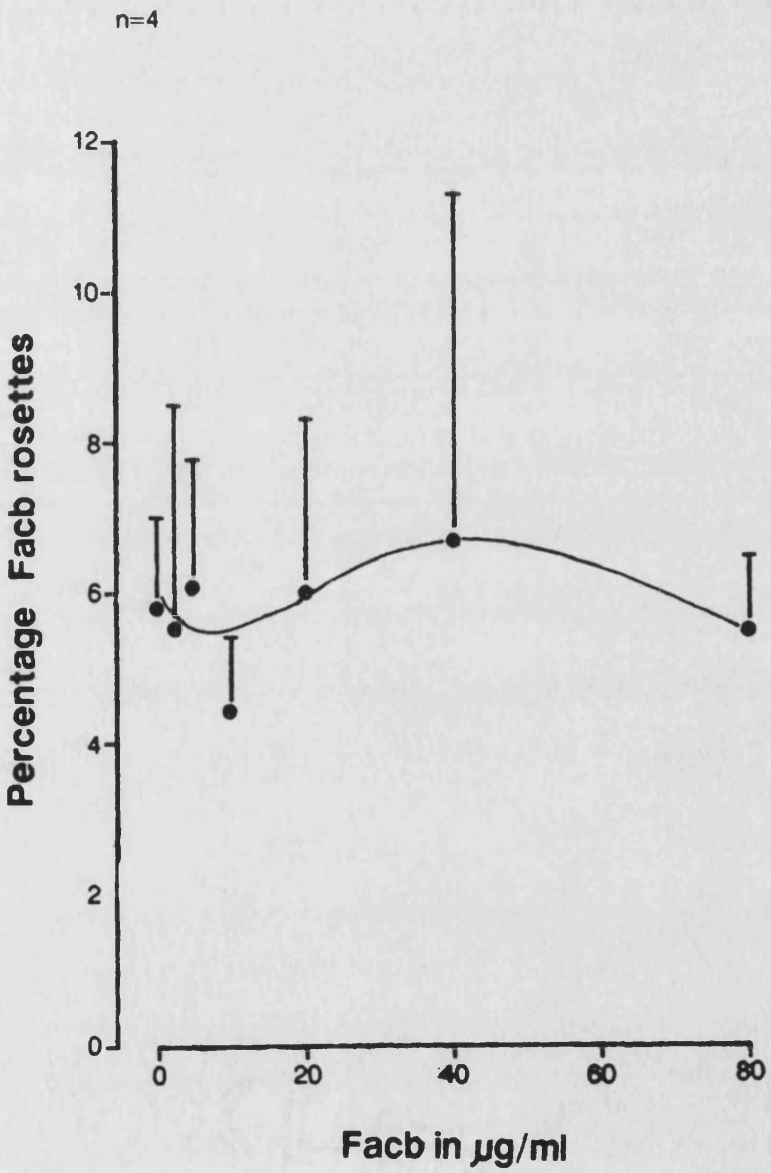


FIG 21 **The effect of Facb on percentage of Facb rosetting cells**

RA PBMNC were pre-incubated with increasing concentrations of Facb for 30 mins on ice and examined by the rosette assay for FacbR expression. Each point is a mean of 4 determinations and the (T) represents one standard deviation.



concentration increased to 80 μg/ml. With Hagg the percentage of Facb rosettes fell dramatically ($p < 0.05$) from 6.5% to 1.4% and 2.7% at concentrations of 22 and 11 μg/ml respectively, indicating that Hagg is clearly better than Facb for inhibiting rosette formation. This perhaps is not surprising since it should by the very nature of its multivalency be able to do so. Although Hagg would appear to be a much more effective ligand than Facb, it should be remembered that Hagg is also able to recruit other FcR^+ cells apart from those bearing the Facb receptor into the process of negative feedback suppression.

Table 2 shows the suppression of IgG production in normal healthy controls and RA patients at P200 with Facb and Hagg. It can be seen that even with a superior ligand, FcR-mediated feedback suppression is not improved in RA subjects. Comparing results obtained with Facb with those observed in Hagg cultures reveals no statistical differences. In normal individuals, however, suppression is significantly improved upon using Hagg. The Table also includes median values of IgG which shows that within a test group Facb and Hagg cultures at P200 had comparable levels of IgG production. So differences in suppression or the lack of them were not due to differences in lymphocyte stimulation.

Comparison of Facb and Hagg effects on RhF production is not really possible because of the very small number of patients producing sufficient autoantibody to be analysed in this way.

FIG 22 **The effect of Hagg on percentage Facb rosetting cells**

RA PBMNC were pre-incubated with increasing concentrations of Hagg for 30 mins on ice and examined by the rosette assay for FacbR expression. Each point is a mean of 4 determinations and the (T) represents one standard deviation.

* $p < 0.05$ when compared to the percentage of $FacbR^+$ cells observed in the absence of Hagg

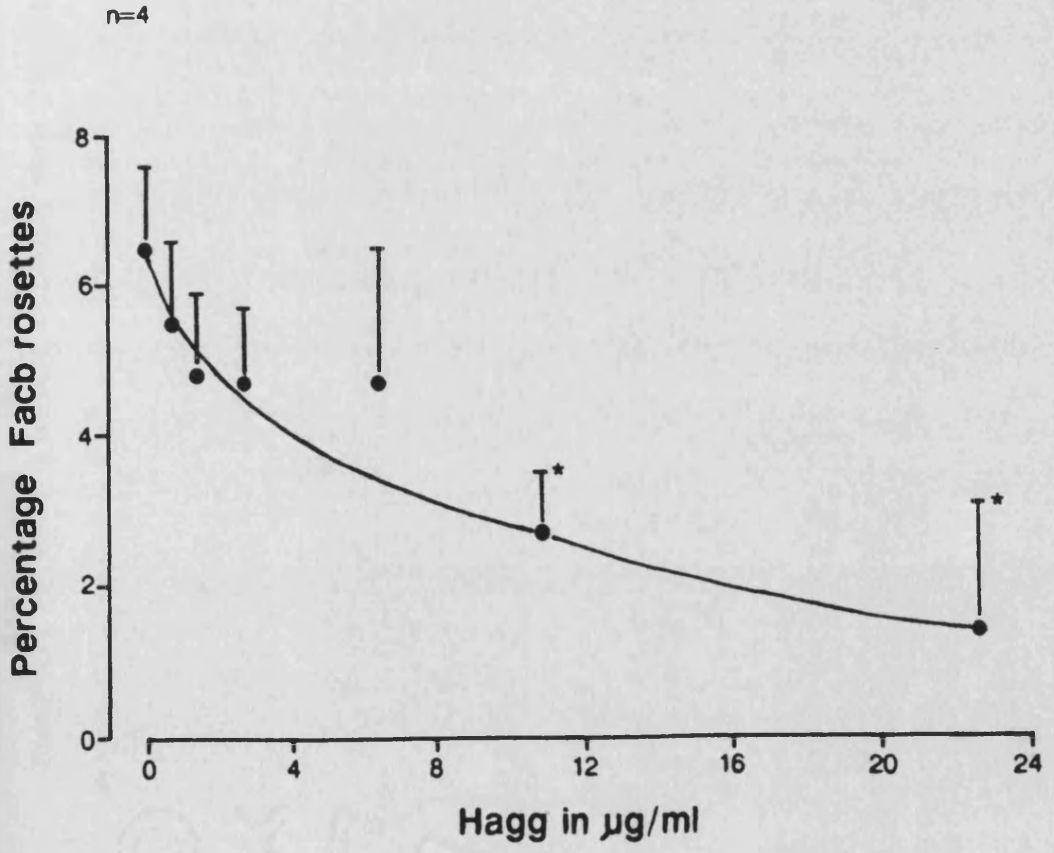


TABLE 2 Comparing suppression produced by Facb and Hagg in normal healthy controls and RA patients at P200.

Asterixes refer to p values obtained when results of Facb and Hagg were compared using the Mann-Whitney U-test. ** $p < 0.01$; * $p = 0.02$

Test groups	F A C B			H A G G		
	n	IgG in ng/ml	Results expressed as % median value of the P200 response alone	n	IgG in ng/ml	Results expressed as % median value of the P200 response alone
Normals	16	1800	P200.2 = 106% ** P2002 = 82% ** P200.20 = 71% *	19	1600	P200 + Hagg = 53%
RA	22	2680	P200.2 = 84% P2002 = 90% P20020 = 89%	12	2800	P200 + 5 ng/ml Hagg = 76%

CHAPTER 6

DISCUSSION

Mechanisms controlling antibody synthesis in RA are defective. This is clear not only from the observations that hypergammaglobulinaemia and autoantibody formation are common findings in RA, but also from the joint pathology where numerous plasma cells are actively synthesising immunoglobulin (Munthe and Natvig, 1972). Such observations have led to intensive investigations of the pathways which regulate antibody synthesis in this disease. Many of the *in vitro* studies have centred on T cell function and these have revealed that suppressor cell activity in RA is impaired, especially in the joint (Chattopadhyay et al, 1979 a,b; Abdou et al, 1981; Romain et al, 1982; Sakane et al, 1982). Currently, this is believed to be due to a lack of suppressor inducer cells in the $CD4^+$ population (Emery et al, 1987; Salmon et al, 1987). However, another mechanism exists which is also important in controlling the antibody response and this is Fc-mediated negative feedback suppression. Here the immunoglobulin is able to suppress its own synthesis by interaction with the Fc receptor on the responding cell. Exactly how this suppression occurs is not clear, but two schools of thought exist; first, that antibody interferes with T-B cell co-operation (Hoffman et al, 1980) and second, that it switches off B cells from immunoglobulin synthesis and diverts them to memory cell production (Kölsch et al, 1980), a process which can be reversed by T cell factors. Recently, a subpopulation of FcR^+ cells has been described which bears receptors for the C_H2 region of rabbit IgG (Hall et al, 1980). These cells are referred to as $FacbR^+$ cells and have been found to

participate in negative feedback suppression (Eales et al, 1984). Earlier work in this laboratory has shown that in patients with established RA, Facb-mediated feedback suppression of IgG response is impaired in comparison to that observed in normal healthy controls (Eales et al, 1985).

On the basis of these data the question arose as to whether this defect was also present in early RA and furthermore, did it express any disease specificity? This would not only provide information concerning the early aetiopathogenetic events in RA, but more importantly may be of potential use to the clinician. Rheumatoid disease in its early stages is difficult to distinguish from other forms of polyarthritis seen in the Early Synovitis Clinic. Clearly, a parameter which could indicate with some degree of accuracy the existence of RA would be of enormous benefit in terms of early treatment and management of the patient.

The present study was thus undertaken to investigate Facb-mediated negative feedback suppression in patients with early RA.

The subjects used in the investigation were all drawn from the Early Synovitis Clinic, where they were classified into early RA positive, early RA negative or early palindromic RA subgroups. The last group had originally been subdivided into RhF positive and RhF negative sets, but in the final analysis no distinction was made between them because of the small number of patients in each subset. Control groups consisted of normal healthy individuals, patients with established RA as positive controls and OA subjects as controls for chronic arthritis. Felty's patients were also tested, being representative of advanced disease.

Antibody production was induced by PWM at doses selected as optimal during preliminary experiments, which were carried out using PBMNC from normal and established RA subjects. In these early experiments, it was found that whilst PWM-driven IgG response in the two groups was similar, both in terms of the levels of immunoglobulin produced and kinetics, nearly three times as much IgG was generated spontaneously by PBMNC from RA patients compared to controls. This is in agreement with data obtained by other authors who have reported high levels of spontaneous Ig secretion by B cells (Al-Balaghi et al, 1982). However, when Facb and Hagg cultures were examined, it was found that spontaneous IgG production by RA PBMNC was similar to that observed in normal cultures; furthermore, the kinetics of the IgG response to PWM were different. At P200 higher levels of immunoglobulin were generated by established RA subjects compared to normal healthy controls, whilst at P1000 the situation was reversed. It is thus possible to obtain different immunoglobulin responses on different occasions even though the operating conditions remain the same throughout. It is therefore not surprising that conflicting data exist in the literature regarding PBMNC responses to mitogens in vitro. Part of this variability may arise from the PWM preparation itself, which at best is just a crude extract of the parent root. Another explanation is that of patient selection. Most investigators treat RA patients as a whole and do not classify them into subsets of early and established disease - as has been done in the present study - or else group the patients according to treatment, eg NSAIDs or immunosuppressant therapy.

Although the results were not significant in the present report, it was found that established RA patients generally produced more IgG than controls, whilst in patients with early palindromic RA levels of

Ig production were similar to normal controls and in patients with early seropositive and seronegative disease, these were slightly less. Other authors have reported either significantly depressed (Segond et al, 1979; Poikonen et al, 1982; Pardo et al, 1984) or normal (Bell et al, 1981; Patel et al, 1983) in vitro antibody responses in RA. Thus, RA patients need to be much more clearly defined in investigative studies.

Examination of the effect of Facb on IgG in the various test groups revealed that suppression of antibody response was dependent on the dose of PWM used. In groups where suppression was observed, ie in normal healthy controls, early RA positive and palindromic patients, it was found that suppression became apparent as the PWM concentration was reduced. Thus suppression was best observed at the PWM dose P1000. This raised the question of whether IgG synthesis was lower at this dose compared to other doses tested and therefore was easier to suppress.

Examination of the immunoglobulin production at the three PWM doses showed, for instance,that in normal and palindromic subjects, IgG synthesis on the contrary was maximal at P1000. Therefore, maximum stimulation appeared to generate maximum suppression. These data are in agreement with those presented by Weigle and Berman (1979) who reported that the greatest degree of suppression produced by Fc fragment in Fab immunised mice was obtained in those individuals in whom the antibody response was good. With low responders on the other hand, poor suppression was observed. Analysis of disease activity in early RA positive patients also showed that as activity increased Facb suppression became greater. Furthermore, there was a direct correlation between the number of $FacbR^+$ cells and disease activity. These data suggest that when the

immune system is highly activated, the FacbR$^+$ population is expanded and more of these cells are recruited into the process of negative feedback suppression.

However, other factors must be involved. Firstly, in patients with established RA, numbers of FacbR$^+$ cells were significantly higher compared to normal healthy controls, but Facb-mediated feedback suppression in these subjects was impaired. Secondly, in early RA positive subjects the IgG responses at P200 and P1000 were not very different, yet no suppression was observed at the former dose. Experiments with both humans (Goulding et al, 1986) and animals (Eales et al, 1984) have shown that secondary responses or immunisation regimes lead to an increase in the number of FacbR$^+$ cells. Similarly, there have been numerous reports of increased FcR expression upon cell stimulation (Van Boxel and Rosenstreich, 1974; Kramer et al, 1975; Eales et al, 1984; Goulding et al, 1986). The discrepancy between high FacbR$^+$ expression in patients with established RA and the inability to suppress might therefore be explained if the former is seen just as an indicator of cell activation and not as indicative of the ability to suppress.

The lack of Facb suppression at higher concentrations of mitogen, as for example in normal, early RA positive and palindromic subjects, may be partly explained by PWM blockade of Facb receptors, though this was shown by Facb rosetting experiments to be significant only at P50. It is possible that at the lowest PWM dose cells which are not stimulated at higher concentrations of mitogen come into play and allow emergence of suppression. Alternatively, a critical concentration of some cell modulating mediator may be achieved. For example, it is known that TRF can bind to FcR (Schimpl et al, 1977). During the immune response this is believed to compete with Fc for

binding to FcR (Kölsch et al, 1980). It is possible that PWM stimulates TRF production and that this is greater at P50 than at P1000, which might explain the emergence of Facb suppression at the latter dose. Al-Balaghi et al (1984) have reported increased production of B cell differentiation factor in RA. This is synonymous with TRF and might explain why in established RA subjects FcR-mediated (both Facb and Hagg) feedback suppression is impaired. This, however, assumes that in early RA positive and palindromic disease TRF production is similar to healthy controls. The situation for some reason may be different in early RA negative subjects.

Although no statistical differences were observed between the three early RA groups, it was found that in contrast to the other groups, IgG production in patients with early RA negative disease was not suppressed by Facb fragment, even when the PWM dose was reduced to P1000. It has been suggested by some authors (Doabloug et al, 1980; Alarcon et al, 1982a; 1984) that seronegative RA is a different disease entity from seropositive disease. With reference to Facb suppression the data obtained here would tend to support such a suggestion. Preliminary data analysis of Facb suppression in patients with established seronegative and seropositive RA, on the other hand, revealed no differences between the two subgroups. However, this is perhaps not surprising since it has been found that in the established RA group as a whole very little suppression was produced by Facb fragment. Since the majority of the subjects in this group were seropositive, this suggests that as the disease progresses normal Facb suppression in early RA positive disease gradually becomes impaired. By this token it would therefore be expected that in Felty's subjects very little suppression of IgG synthesis would occur. Data at P1000 unfortunately are not available

for Facb suppression in this group, but as far as Hagg suppression is concerned, Felty's patients were found to be well suppressed. However, with $FacbR^+$ expression there is a gradation with developing disease, with percentages of $FacbR^+$ cells varying in the order: normals < early RA < established RA < Felty's patients. The data above also suggest that in early RA negative disease Facb suppression of IgG response does not differ at all from established seronegative RA, since in both instances Facb suppression appears to be impaired. This suggests that perhaps $FacbR^+$ cells are a more prominent feature of seropositive disease than seronegative RA. The overall lack of suppression in the OA group is hard to explain but may in part be due to the high variability of response in individual patients. The role of the immune system in OA is less marked than in RA. Perhaps the similarity in the results obtained in OA and RA reflects any chronic disease state.

What produces the gradual impairment in feedback suppression in RA is not clear. However, at least two possibilities exist and these include old age and some aspect of disease chronicity.

Old age was found not to be responsible for the differences observed between early RA positive and established RA patients, since the average ages of these test groups were remarkably similar - 56 and 55 years respectively. Furthermore, when the ages of onset were examined in relation to Facb suppression it was found that subjects with early RA positive disease behaved in a similar fashion, regardless of whether the disease started before or after 60 years of age and that these data differed from those observed in established RA patients who had been similarly analysed. The latter were found to be less well suppressed irrespective of the age of onset. Thus

these observations seem to suggest that changes in the degree of Facb suppression may be related to the developing disease process.

The association between disease duration and Facb suppression was therefore investigated. It was found that though no significant differences were obtained, patients with disease duration of < 2 years were better suppressed than those who had had the disease for > 2 years or for > 5 years.

The possibility that impaired Facb suppression might become more apparent with progressing disease is further indicated by data obtained when the effect of Facb on spontaneous IgG production was examined. Here it was found that whilst Facb was able to suppress antibody production in normals in a dose-dependent manner, very little suppression was observed in the test groups. Here, some degree of difference was observed with early RA positive patients but not with early RA negative and palindromics compared to normal controls, whilst OA, RA and Felty's subjects were found to differ to a slightly greater extent. The difference between the early RA positive patients and the early RA negative and palindromic groups at PO is probably because the former represent a more exacerbated picture of RA than the latter two groups, which exhibit a milder form of the disease. Comparing these data with observations at P1000, these findings seem to suggest that whilst early RA positive and palindromic patients cannot suppress well at PO, they can nonetheless be mitogenically stimulated to suppress. In early RA negative subjects this capacity is impaired for some reason. In patients with established disease no suppression occurs at PO and furthermore, cells cannot be triggered to suppression even by mitogen stimulation. So gradually as rheumatoid disease develops the ability to suppress becomes impaired.

In studies investigating T suppressor cell function (Sakane et al, 1982), it was found that in early RA T cells did not suppress, nor were B cells responsive to suppressor signals, but as disease progressed it was found that T cells could be induced to suppressor activity by Con A but the B cells still remained unresponsive. Clearly, Facb fragment affects suppression differently from the suppressor T cells since in early RA positive disease B cells are responsive to FcR mediated feedback suppression.

Exactly what aspect of feedback suppression alters with progressing disease is not clear. Is it that the capacity of $FacbR^+$ cells to suppress is impaired or do B cells in established RA become less responsive to FcR-mediated suppression? Mixing experiments should have been conducted where Facb rosetting cells and B lymphocytes from RA patients were separated and mixed with either normal B cells or normal $FacbR^+$ cells respectively to determine where this defect might lie. However, one would first have to take MLR interactions into consideration and ensure that these did not interfere with this pathway of suppression.

It is possible that <u>in vivo</u> activation may in fact be responsible for the impaired FcR-mediated feedback suppression seen in RA. There are numerous reports which show that in RA, cells are activated both in the joint (Yu et al, 1980; Burmester et al, 1981; Galili et al, 1981; Fox et al, 1982; Duke et al, 1983) and in the circulation (Yu et al, 1980; Burmester et al, 1981; Papadimitriou et al, 1982). This is indicated by spontaneous ^3H-thymidine uptake (Horwitz et al, 1970; Stratton and Peter, 1978; Froebel et al, 1984) and Ig synthesis (Al-Balaghi et al, 1982) as well as by enhanced expression of HLA-DR antigens (Yu et al, 1980; Burmester et al, 1981; Fox et al, 1982).

Another explanation comes from the observation that SH groups

are important in FcR function (Abramson et al, 1971). In RA patients levels of sulphydryl groups are reported to be reduced (Lorber and Chang, 1968), raising the possibility that these may be related to the degree of feedback suppression observed in these patients.

The effects of γ-IFN and 2ME on Facb-mediated feedback suppression were investigated in a small number of normal healthy controls and established RA patients. Only limited data are available from these experiments because of a lack of response to PWM stimulation in the individuals tested. However, from the information available, it was found that whilst both 2ME and γ-IFN enhanced $FacbR^+$ expression, these substances appeared to be having opposing effects. With the former the degree of suppression was slightly better and with the latter there seemed to be a negation of the effects of Facb fragment on antibody synthesis. It is reported that γ-IFN may augment ongoing antibody responses (Taylor-Papadimitriou and Rozengurt, 1985). So it would appear that in the presence of γ-IFN and Facb antibody producing cells are subject to opposing effects and in the preliminary experiments reported here, the effects of γ-IFN appear to be much more potent than those of Facb. In RA it is possible that in areas of γ-IFN production, FcR mediated feedback suppression may be overcome by the stimulating influences of γ-IFN, thus leading to enhanced antibody production. The above experiments should be repeated using a much larger sample group.

The relationships between various clinical parameters and the degree of Facb suppression were also examined and these were found to be in no way related to each other. Hall et al (1980) also reported a lack of correlation with $FacbR^+$ expression and laboratory assessment of clinical activity. The mechanisms of Facb-mediated feedback

suppression were not investigated in the present study. However, it is known this occurs through interference with accessory cell function (Eales PhD thesis, 1982). Other studies in this laboratory (Goulding et al, 1986) have indicated that FacbR$^+$ cells are probably involved in the early stages of the immune response. Data presented here support this since it was found that Facb fragment had to be present at Day 0 for effective suppression to occur.

During the course of this study several preparations of Facb were used. Whilst most batches were found to suppress, it was discovered that with one or two preparations, although the Facb fragment could be used successfully in the rosette assay to detect FacbR$^+$ cells, it was not suppressive. Again, this emphasises the dichotomy between FacbR$^+$ expression and the ability to suppress. Thus the experiments conducted using these particular batches of Facb were not valid. It is not clear exactly why this variation in Facb preparations should occur. One possibility is that as the serum source aged, the immunoglobulin became much more fragile and, under the acidic conditions used in the preparation of Facb, was prone to cleavage at amino acid positions other than those normally reactive with plasmin. This may result in the loss of amino acids which, whilst not affecting the actual binding to the receptors (since rosetting still occurs) may prevent triggering of the cell for suppression, possibly by affecting cell receptor interaction with the G proteins in the plasma membrane. Examining the 3D structure of the IgG molecule it can be seen that the C_H3 domains are in close association and this is thought to stabilise the C terminal of the Fc region. The C_H2 regions differ by not being similarly paired, which imparts to the region a relatively open structure. This region is

thus referred to as 'soft' and is believed to possess a degree of inherent instability (Burton, 1985). Therefore, it is likely that during preparation damage can occur more readily to this area. This also suggests that the structural nature of the Facb fragment may in addition result in a looser or less avid binding to the receptor and this would make it easier to dislodge it. This was found to be true when Facb fragment and Hagg were compared for their respective abilities to inhibit Facb rosette formation; Hagg was found to be a much more effective inhibitor.

During the immune response numerous mediators are released and some of these (eg TRF), as mentioned earlier, are able to compete with the FcR binding ligand for the receptor site. Since there are likely to be individual variations in the amounts of these competing factors generated, different degrees of competition must exist in each individual culture system between the Facb and the competing factors, thus producing a wide range of responses. This might explain why in Facb cultures with a ligand that may bind loosely to its receptor and therefore be easily replaced, there is such a wide variation in the degree of suppression produced in immunoglobulin synthesis. Clearly, an investigation of association constants would be most useful.

Thus in view of the variability of the response observed in Facb cultures and the possibility that this may in part be due to the nature of the ligand, another FcR binding ligand was examined. This was heat aggregated IgG, which was prepared from myeloma IgG_1 ; a defined subclass of IgG was employed rather than a heterogeneous IgG preparation in view of the varying effects different subclasses of immunoglobulin have on antibody-mediated feedback suppression (Henry

and Jerne, 1968; Collisson et al, 1983). Again it was found that in patients with established RA feedback suppression of immunoglobulin synthesis was impaired compared to young normal healthy controls. However, in comparison to normal subjects over the age of 60 years, significant differences were not obtained. This seems to suggest that age may be partly responsible for the depressed levels of Hagg-mediated suppression in RA. However, this does not hold true since firstly, no significant differences existed between young and old normal healthy controls and secondly, in OA patients with an average age of nearly 70 years, the median level of suppression was greater than that even in young normals. However, no significant differences were found between these two groups or between OA and RA subjects. This is probably because of the high variability of response observed in this group. These data and those obtained with Facb cultures seem to suggest that OA subjects possess an innate heterogeneity to FcR modulation of the immune response. The patients in this study were not divided into primary or secondary OA; perhaps this has some bearing on the outcome of FcR-mediated feedback suppression. Similarly with ankylosing spondylitis patients, though the median level of Hagg suppression was the same as the young normals, the wide variability in response did not allow any significant differences to emerge when compared to established RA patients. It is felt that Hagg suppression of antibody synthesis in these groups should be reassessed using much larger sample groups.

Although no significant differences were obtained between the OA and AS patients and subjects with established RA, the data do indicate that the latter are less receptive to negative feedback suppression and that this is not made greater by the use of a much more potent FcR ligand. However, interestingly, the very small

number of Felty's patients examined were found to be well suppressed by Hagg. This poses the question as to whether FcR-mediated suppression differs between systemic disease and that where active synovitis exists. Papadimitriou et al (1982) reported that increased levels of circulating low density lymphocytes were only found in patients with synovitis but not in subjects with active EA disease, suggesting further separation in immunological activity between these two phases of the disease.

Exactly how Hagg-mediated suppression occurs was not investigated in the present study. However, it was found that like Facb, Hagg had to be present on Day 0 for suppression to occur, but the kinetics differed in that Hagg suppression emerged earlier, on Day 10, compared to Facb where suppression was most obvious on Day 14. This may be because the former is a much stronger FcR ligand or may operate via different pathways to suppress the immune response compared to Facb, since Hagg can bind to numerous cell populations whilst the Facb fragment binds only to a selected cell type. It has been suggested that Hagg may produce suppression of Ig synthesis by suppressing T helper cell activity (Moretta et al, 1979) or triggering phospholipase A_2 activity in macrophages, resulting in PGE_2 generation, which then serves to stimulate T suppressor cells (Saito-Taki and Nakano, 1983). Le Thi Bich-Thuy and Revillard (1984) showed that one or the other of these mechanisms could be activated depending on the experimental protocol. Thus in peripheral blood samples where cells had been obtained by defibrination, only IgG suppression occurred and this was thought to happen via T cell activation. Where heparinised blood was used non-isotype suppression was found to occur and this was thought to be due to PGE_2 generation. Although all major classes of Ig producing cells were suppressed, the

degree of suppression was much greater with IgG synthesising cells. Preliminary data presented in Chapters 4 and 5 showed that IgM production was not as well suppressed as IgG synthesis by Facb amd Hagg.

B cells are also believed to participate in FcR-mediated feedback suppression (Masuda et al, 1978; Miyama-Inabi et al, 1982). Pisko et al (1986) showed that Hagg stimulated the production of a suppressive B cell factor (SBF) from normal PBMNC. In RA subjects there is a decrease in the amount of SBF produced in response to Hagg (White et al, 1986) compared to levels generated by PBMNC from young and old normal healthy controls. In the test system used by White et al (1986) the old normals differed from young controls, suggesting the age was an important factor in the reduction of SBF production. The data obtained by these authors and those presented in the present study are not directly comparable because of the differences in experimental conditions.

It is felt that the Hagg experiments described here should be repeated using larger numbers of test individuals and extended to include early RA subjects.

The effect of Facb and Hagg on RhF production was also examined since the question arose as to whether the immunoregulatory effects of these ligands affected only IgG production or whether they were generalised and extended to other types of antibody. Furthermore, was autoantibody production modulated differently from normal antibody responses?

Thus, RhF production in the various test groups was examined. Overall median levels of RhF production were low in both Facb and Hagg cultures, probably because of the large number of non-responders in each group.

It was found that whilst young and old normal healthy controls, OA, early RA negative, early palindromic and AS patients generated very little or no RhF spontaneously, both early RA positive and established RA subjects were found to produce RhF at PO. These data agree with the findings of other workers (Koopman and Schrohenloher, 1980a; Tsoukas et al, 1980; Patel et al, 1983; Alarcon et al; 1984). One explanation for unstimulated autoantibody production in these patients comes from the work of Patel and Panayi (1984) who reported that in RA there exists increased T helper cell activity for the production of RhF. Examination of the relative proportions of patients generating spontaneous RhF showed them to be 22% and 7.4% in the early RA positive and established RA subjects respectively. Higher values (40 - 50%) have been reported in RA by other workers (Koopman and Schrohenloher, 1980a; Olsen et al, 1982) and the differences between their data and those presented in this thesis are most probably due to patient selection.

With PWM stimulation, RhF production was stimulated in all groups. No significant differences were found in the percentage of subjects responding to PWM in the various test groups. However, in agreement with data reported in the literature (Koopman and Schrohenloher, 1980b; Alarcon et al, 1984) 50% of young normal healthy controls (in the Facb study) were stimulated to autoantibody production at P200. The percentage for early RA positive was slightly higher compared to controls and those for early RA negative, palindromics, OA and Felty's subjects were lower. Whilst some reports (Alarcon et al, 1982b) show that seronegative patients are similar to controls in their ability to produce RhF <u>in vitro</u> following cell stimulation, others have reported that these patients have significantly decreased levels of RhF production compared to

controls (Pasquali et al, 1981). Pasquali et al (1984) relate this to the presence of anti-idiotypic antibodies in seronegative subjects. However, it is possible that this difference may be related to the choice of stimulating agent, ie EBV versus PWM, since each of these polyclonal activators has been found to activate different, non-overlapping populations of B cells (reviewed Olsen and Jasin, 1985).

The percentages of responding subjects in the OA, Felty's, palindromic and early RA negative groups reported in the current work are probably artificially high because of the small number of patients examined compared to the main groups of young normals, early RA positive and established RA.

Rodriguez et al (1982) reported that the ability to generate RhF in vitro became greater with age. However, this was not found to be true in the current investigation: levels of RhF production in young and old normals were comparable.

Suppression of PWM-driven RhF production by Facb was examined and found not to vary with the dose of PWM as reported for IgG synthesis. Although the effects of this fragment on autoantibody production were variable, it was found that the lower doses of Facb were suppressive whilst with increasing concentration of the ligand the RhF levels either returned to the response observed where no ligand was present or were enhanced. This is in contrast to the data obtained for IgG synthesis where higher concentration of ligand produced greater suppression. In the small number of individuals examined both Facb and Hagg were found not to significantly suppress IgM production and whilst the effects produced by Facb on IgM RhF were more akin to those seen with IgM production, they were not

always observed to be similar, indicating that RhF production may be differently modulated from the generalised IgM response. This is further emphasised by the observation that in some subjects whose cells did not produce RhF with PWM alone, the presence of Facb led to autoantibody production. Thus it would appear that Facb can induce RhF synthesis. In the joint where numerous enzymes are present, partially degraded IgG molecules may enhance autoantibody production. Morgan and Weigle (1983) reported that a peptide derived by macrophages from Fc fragment could act as an adjuvant in antibody response. It is possible that a similar activity may be generated from Facb fragment here and also in vivo. Oxygen free radical damaged IgG may also stimulate RhF production in the rheumatoid joint.

Whilst similar effects were produced by Facb in all test groups and in PWM-stimulated and unstimulated cultures, those seen with Hagg were found to be more definitive.

In all patients spontaneous RhF production was completely eliminated or strongly suppressed by Hagg. However, with PWM-driven cultures results were variable. In most groups RhF levels of production were found to be suppressed or unchanged. Only in RA subjects was a two-fold enhancement seen in the level of RhF synthesis in the presence of Hagg.

It would therefore appear that whilst Facb fragment does not vary in the effects it produces in different groups, Hagg has a different effect on RA PBMNC compared to those from other test groups.

It is possible that RA cells may be sensitised to Hagg already and therefore see it not as an FcR immunomodulator but as an antigen. TaO et al (1984) have recently reported that at concentrations as low

as 0.1 ug/ml, Hagg behaves as a stimulant for RhF production by RA cells. This is not found in normal controls and this effect is different from that observed by Pisko et al (1982) who employed much higher concentrations of Hagg (100 ug/ml). Here Hagg was found to behave as a polyclonal stimulator of immunoglobulin production, during the course of which RhF was also generated. Clearly, the data and the conditions presented in this thesis are similar to those of TaO and co-workers (1984). However, whatever the concentrations of Hagg, in view of the large numbers of immune complexes present in the joint (Winchester et al, 1970; Zvaifler, 1973; McDuffie, 1978), the potential for antibody production exists in the synovial compartment.

Mechanisms controlling RhF production are not clearly understood. However, it is known that RhF generation in vitro is susceptible to T_H and T_S influences (Tsoukas et al, 1980). What role feedback suppression plays is not clear,but the very nature of the anti-IgG antibody may not allow participation in this control mechanism. It is well established that IgM antibodies have a potentiating effect on the immune response, thus it is possible that IgM RhF may serve to enhance its own production in vivo rather than suppress it. This production is probably increased by two factors. Firstly, there is a lack of T cell suppressor influences in the synovial joint and secondly, much of the IgG produced in the synovial compartment has RhF activity. IgG RhF is known to self-associate and this would remove much of the Fc necessary for FcR-mediated suppression. IgM RhF will furthermore prevent other non RhF IgG from mediating this process by complexing with their Fc regions. Clearly, for the in vitro assay system described in the present investigation, this is not a consideration because of the very low amounts of IgM RhF generated in the cultures. The lack of suppressor influences in

the RA joint would thus allow expansion of a precommitted RhF producing cell population.

It has recently been reported that RA subjects possess higher numbers of $CD5^+$ B cells compared to normal controls (Plater-Zyberk et al, 1985). These are thought to be committed to autoantibody formation (Hardy et al, 1987). It is possible also that cells such as these may be modulated slightly differently from the PWM-stimulated cells examined in the current investigation, since $CD5^+$ cells are thought to represent a relatively immature subpopulation of the B cell repertoire, whilst cells responsive to PWM are mature lymphocytes which are committed to high rates of immunoglobulin production (Ault and Towle, 1981). Similarly, the data presented in this thesis should be regarded in the light of the observation by Ault and Towle (1981) that only a small percentage of the total B cell population is stimulated by PWM and the possibility exists that normal Ig production by other classes of B cells may vary in kinetics of or susceptibility to FcR-mediated feedback suppression. Thus other B cell activators should be also be used to more fully assess this mechanism in RA.

In view of the questions asked at the initiation of this investigation, the following may be concluded:-

1. FcR-mediated feedback suppression in early RA positive and palindromic RA patients is not impaired and that defect of this control mechanism develops with progressive disease.
2. Data suggest that Facb feedback mediated suppression may be more pertinent to seropositive disease than to seronegative disease, since the results obtained in the present study indicate that

IgG synthesis by PBMNC of seronegative patients is not susceptible to suppression by Facb.

3. Defects in FcR feedback suppression cannot be used to separate RA from different disease entities or from some subsets of early disease because of the wide variability observed in response to FcR binding ligands.

4. Modulation of RhF production by FcR ligands is complex. Certainly in RA the possibility that these ligands may behave as antigens cannot be ruled out. Furthermore, it is likely that other immunoregulatory pathways, eg those involving anti-idiotypic antibodies or T suppressor cells, are much more relevant to control of IgM RhF synthesis rather than the non-specific suppression mediated by Facb fragment and Hagg.

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