The production of human monoclonal antibodies from autoimmune patients

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THE PRODUCTION OF HUMAN MONOCLONAL ANTIBODIES FROM AUTOIMMUNE PATIENTS

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Summary.

Three HAT-sensitve cell lines were examined for their suitability in producing human monoclonal antibodies (MCA) by fusion with peripheral blood lymphocytes (PBL). The human cell line LICR-LON-HMy2 (HMy2) was found to generate hybrids with too low an efficiency to be useful. Two mouse myeloma cell lines, NS1/1.Ag4.1 (NS1) and X63-Ag8.653 (X63) both fused with high efficiencies. X63 was selected for intensive study on the basis of a higher fusion frequency, and lack of production of mouse immunoglobulin. This line proved to be an excellent fusion partner. Hybrids were formed, from both normal and autoimmune patients, which supported the secretion of useful amounts of human immunoglobulins M, G and A of both kappa and lambda isotypes.The hybrids were robust in culture, cloned efficiently and were easily recovered from storage in liquid nitrogen. Hybrid instability was not a problem. Of thirteen hybrids selected for intensive study, twelve were successfully cloned and continued immunoglobulin secretion through a period of observation of six to eight months in continuous culture. Two fusions were performed with X63 and PBL transformed by Epstein-Barr virus (EBV) and one fusion with tonsil cells transformed by EBV. Both the fusion frequency and proportion of hybrids secreting human immunoglobulins were increased in fusions with EBV transformed cells. It is concluded that EBV

transformation followed by fusion with X63 represents a highly efficient method of producing human MCA.

The possibility of producing human monoclonal autoantibodies was investigated by fusing PBL from patients with myasthenia gravis (MG) or systemic lupus erythematosus (SLE). All MG patients had demonstrable serum autoantibodies to the acetylcholine receptor (AChR). The SLE patients were selected for high serum autoantibody titres to Ro, La or nRNP. Ten fusions with MG PBL yielded 459 human immunoglobulin-secreting hybrids. No antibody activity to the AChR was detected by RIA in any of the supernatants. Twelve fusions with SLE PBL yielded 220 human immunoglobulin-secreting hybrids. No MCA were detected by ELISA which reacted specifically with either Ro, La, nRNP or DNA. When these MCA were screened by indirect immunofluorescence against fixed human cells, two gave even cytoplasmic staining patterns, and twelve stained cytoplasmic filaments. The filaments recognised were characterised as being intermediate filaments. In addition to binding intermediate filaments, one MCA was found to react with microfilaments. When MCA derived from other sources were screened against fixed cells, similar high proportions were found with anti-intermediate filament activity. MCA to intermediate filaments were found in hybrids derived from EBV-transformed tonsil cells (3/48), normal PBL (3/54), EBV transformed PBL (5/80) and MG PBL (3/65). In addition to binding intermediate filaments, one MCA from a

myasthenic patient also reacted with DNA. MCA to intermediate filaments are thus not related to the autoimmune status of the donors, and may not represent conventional antibody-antigen interactions. The high proportion of MCA to intermediate filaments may be related to the frequent occurrence of serum antibodies with this reactivity in a number of different autoimmune and infectious diseases.

Contents.

Abbreviations.

Ab : Antibody(ies)

AChR : Acetylcholine receptor

AET : 2-aminoethylisothiouronium bromide hydrobromide

Ag : Antigen

Alpha-BTX : Alpha bungarotoxin

ALD : Alcoholic liver disease

ANA : Anti-nuclear antibody(ies)

APC : Antigen presenting cell

BSA : Bovine serum albumin

CAH : Chronic active hepatitis

CCL : Chronic lymphocytic leukaemia

CELIA : Competitive enzyme linked immunosorbent assay

DMSO : Dimethyl sulphoxide

DNA : Deoxyribonucleic acid

DNAse : Deoxyribonuclease

EAMG : Experimentally acquired myasthenia gravis

EBV : Epstein-Barr virus

EDTA : Ethylenediaminetetra-acetic acid

EGTA : Ethyleneglycol-bis-(Beta-amino-ehyl ether)
N,N'-tetra-acetic acid

ELISA : Enzyme linked immunosorbent assay

FCS : Foetal calf serum

HAT : Hypoxanthine, aminopterine and thymidine

HT : Hypoxanthine and thymidine

Ig : Immunoglobulin(s)

LPS : Lipopolysaccharide

MCA : Monoclonal antibody(ies)

MCTD : Mixed connective tissue disease

MHC : Major histocompatibility complex

Mr : Relative molecular mass

NCS : Newborn calf serum

NP40 : Nonidet P-40

PAGE : Polyacrylamide gel electrophoresis

PEG : Polyethylene glycol

PBC : Primary biliary cirrhosis

PBL : Peripheral blood lymphocytes

PBS : Phosphate buffered saline

PMSF : Phenylmethane sulphonyl fluoride

PWM : Pokeweed mitogen

RA : Rheumatoid arthritis

RF : Rheumatoid factor

RIA : Radioimmunoassay

RNA : Ribonucleic acid

SD : Standard deviation

SDS : Sodium dodecyl sulphate

SLE : Systemic lupus erythematosus

SRBC : Sheep red blood cells

SS : Sjogren's syndrome

UV : Ultra-violet

Chapter One. General Introduction.

It has been widely held for many years that the function of the immune system is primarily, if not entirely concerned with the protection of the individual against infectious agents. Ehrlich's famous phrase horror autotoxicus, expressing the idea that self-reactivity is an abnormal and pathological condition, has long been a pervasive influence in immunology. Hand in hand with this notion, the question of why an animal does not normally make deleterious responses to its own antigens, has been a central question. The first serious attempt to explain how self unresponsiveness might be acquired was put forward by Burnet as a corollary to his clonal selection theory. This hypothesis postulates that each clone of lymphocytes gives rise to cells making antibody of only one specificity. Lymphocytes of a particular clone respond to contact with the corresponding antigen by proliferation and differentiation into plasma cells. Burnet also suggested that antibody diversity is generated by rapid somatic mutation, especially in embryonic development. Since some of these randomly generated clones would be likely to react with self-antigens, he suggested that these would be eliminated if they came into contact with the corresponding autoantigen during the critical period of development. Thus self-reactive lymphocytes would be selectively deleted (Burnet and Fenner 1949). This would predict that if animals were exposed to foreign antigens during the critical period, they would be tolerant to

them in adult life. This indeed was confirmed experimentally by Medawar and his colleagues (Billingham et al. 1953,1956). Animals injected at birth with either allogenic tissue or heterologous protein antigens were unable to reject allogenic skin grafts, or make antibodies to the proteins later in life. Within a few years numerous reports of tolerance induction to a variety of antigens appeared (Hanan and Oyama 1954, Dixon and Maurer 1955, Simonsen 1955, Weigle and Dixon 1959, Smith and Bridges 1958). In general, large doses of the foreign protein were employed and it was found easier to induce tolerance in immature rather than mature animals. Lower doses of antigens were found to be required for tolerance induction in young animals, and tolerance was both more profound and of longer duration.

In its simplest form however, the clonal deletion hypothesis explaning natural self-tolerance is no longer considered tenable. With the weight of considerable experimental evidence, self-reactivity is now believed to be a normal feature of the immune system. It is convenient to consider this evidence in relation to Talal's (1977) suggested three stages of response to self; auto-recognition, autoimmunity, and autoimmune disease.

1. Auto-recognition.

This describes the role of self-recognition in the normal functioning of the immune system. It includes the fundamental principles facilitating regulatory interactions of the cells of the immune system. The binding of antigen to receptors on B cells does not usually result in the triggering of B cells unless a helper T cell also recognises the antigen. In addition to recognising the antigen, helper T cells appear to promote B cell differentiation only if certain antigenic determinants on the B cell surface are complementary to receptors on the T cell. These cell surface antigens and receptors are determined by the major histocompatibility complex (MHC). Within the MHC lies a locus of genes which influence the immune response (the immune response or Ir genes); this expresses itself on the surface of cells of the immune system as class II antigens (Ia antigens in mice, HLA-D antigens in humans). Recognition of class II antigens by helper T cells appears to be nearly always necessary in T cell antigen dependent differentiation of B cells.

Antibodies or T cell factors directed against the individual's own receptors are the essential ingredients in the network theory of immune regulation proposed by Jerne (1973,1974a,1974b) and extended by others (Kohler 1975, Richter 1975, Hoffman 1975). This theory attempts to explain the behaviour of the immune

system by the interactions of antigens, idiotypes and anti-idiotypes. The configuration of every antigen-binding receptor, or idiotype, allows interaction with either of two structurally complementary substances. One is the specific antigen, and the other is the receptor of another clone of lymphocytes, the anti-idiotype. The idiotypes and anti-idiotypes may reside on either T or B cells and their antigen binding products (antibodies or T factors). The anti-idiotypes themselves can also recognise structurally complementary antigens, and be recognised in turn by other receptors, anti-anti-idiotypes. The whole immunological web is thus seen as mutually interacting and self regulating. The network theory has received some experimental support. Spontaneous synthesis of anti-idiotype antibody has been detected during immunisation of an animal to a particular antigen (Kluskens and Kohler 1974).Injection of anti-idiotype antibodies has been shown to suppress the particular T and B cells bearing the idiotype (Nisonoff and Bangasser 1975). Under certain circumstances, anti-idiotype antibodies have been shown to trigger lymphocytes (Trenker and Riblet 1975).

The state of auto-recognition includes Grabar's (1983) interpretation of the immune system's physiological function, where immunoglobulins are seen as transporters of catabolic materials. This is one interpretation of numerous experiments which have provided ample evidence that autoantibodies and the B

cells which produce them are a ubiquitous feature of the immune system. Studies with radiolabelled autoantigens, for example thyroglobin, have shown that autoantigen binding B lymphocytes are present normally in the blood of healthy subjects (Roberts et al. 1973). Normal B cell populations can be triggered to produce autoantibodies against virtually all self-constituents with appropriate immunisation or stimulation (Elson et al. 1979). Polyclonal B cell mitogens such as LPS, when injected into normal mice have been shown to induce serum autoantibodies to IgG (Dresser 1976, 1978, Izui et al. 1979a), DNA (Fourney et al. 1974, Izui et al. 1979b), erythrocytes (Cunningham 1974), and thymocytes (Izui et al. 1979b). An extremely high proportion (75%) of lymphocytes stimulated to immunoglobulin secretion by LPS in vitro produce autoantibodies (Dziarski 1982a). The LPS-induced autoimmune state in vivo is self-limiting, and the autoantibodies disappear within a short time after the LPS injection (Dziarski 1982b).

In addition to evidence for the existence of B cells capable of producing autoantibodies, sensitive methods of detection indicate that autoantibodies against a large number of self-antigens are found naturally in the serum of normal, healthy individuals. Darr and Fabre (1981) developed a highly sensitive binding immunoassay, depending on exposing 25 µl of serum for 1 hr on ice to 25 µl of a suspension of homogenised brain, liver or heart. After washing, the homogenate was then incubated with

radiolabelled rabbit anti-human $F(ab)_2$ antibody. Every human serum examined was found to react with the 3 homogenates. The autoantibodies were found to be IgM in class, were largely human tissue specific and were found in all age groups from 2 to 84 years. Dighiero and Aureamas (1982a, 1982b) studied 9 common autoantigens; tubulin, actin, thyroglobulin, myoglobin, fetuin, transferrin, albumin, cytochrome and collagen. Immunoabsorbent columns were made by coupling each antigen to glutaraldehyde-activated, polyacrylamide gels. Pooled sera from 800 normal donors and three individual donors were passed through the columns and the retained protein eluted using acid. The recovered immunoglobulin was then tested by ELISA against the appropriate antigen. The results showed that both the pooled and individual sera contained antibody activity which was specific for each of the antigens tested, the three major isotypes were represented, that IgG was most abundant and that binding was mediated by the Fab fragment. The authors also examined 61 monoclonal paraproteins from human sera. Four had autoantibody activity against actin and 1 against tubulin. Autoantibodies in normal sera have been also found to spermine (Bartos et al. 1980), and myelin (Edington and Delessio 1970 Hooper et al. 1972, Hawkins et al. 1980).

Autoimmunity.

This state is characterised by enhanced autoreactions concomitant to non-self reactions such as in infectious disease and injury. The autoimmune state, like the disturbing influence, is only temporary and the immunological network is fundamentally intact. Several mechanisms may be operating to induce autoimmunity in infectious disease. During the course of infection, tissues may be damaged and release antigens. The autoimmune response may be merely a physiological mechanism for removing these products. Alternatively a self antigen may be modified e.g. by proteolytic enzymes, to such an extent that it is not recognised as self and initiates an immune response. Another possible mechanism may be termed bacterial mimicry. Several lines of evidence suggest that certain infectious agents may be capable of infecting a particular host because they share structures which are extremely similar antigenically and immunogenically to those possessed by the host (Drach 1973). Antibodies produced to the infectious agent may then be expected to cross-react with autoantigens. Finally, many bacterial agents and certain viruses (e.g. EBV) are capable of behaving as B cell mitogens. These are able to polyclonally activate B cells to produce a variety of antibody specificities including autoantibodies. From Dziarsky's work with the bacterial, polyclonal mitogen LPS (1982a, 1982b), it would seem that

either there is preferential stimulation of cells with autoantibody specificities, or there are an extremely large number of such cells in the normal individual. Whatever the explanation of the appearance of autoantibodies in infectious disease, they have been found to many different antigens in many diseases. Rheumatoid factors, smooth muscle antibodies and ANA have been reported in many diseases (table 1.1).

Table 1.1 RF,ANA and anti-smooth muscle (SMA) autoantibodies found in infectious diseases.

Antibody specificity	Infection
RF	Syphilis
RF,ANA	Leprosy
RF,ANA	Tuberculosis
RF	Malaria
RF	Schistisomiasis
RF,ANA,SMA	Infectious mononucleosis
RF,SMA	Infectious hepatitis
RF	Influenza A
RF	Rubella
RF,ANA,SMA	Cytomegalovirus

(After Williams, 1977).

Antibodies to cytoplasmic cytoskeletons have been frequently found in human sera, and have been the subject of a recent review (Kurki and Virtanen 1984). The cytoskeleton of vertebrate cells consists of detergent and salt insoluble, cytoplasmic and nuclear fibrillar structures. The cytoplasmic cytoskeleton contains three major elements distinguishable by size; the microtubules (20-11 nm), intermediate filaments (7-11 nm) and the microfilaments (5-6 nm). Identification of cytoplasmic cytoskeletal structures can be achieved by their typical morphology or better still by the effect of certain drugs which specifically alter their cytoplasmic distribution. Cytochalasin B specifically disrupts microfilaments. Colchicine has no effect on microfilaments, but disrupts microtubules and causes intermediate filaments of the vimentin type to collapse into a perinuclear bundle. Vinblastine again has no effect on microfilaments, causes intermediate filaments to collapse into a perinuclear bundle and also disrupts microtubules leaving characteristic tubulin paracrystals in the cytoplasm (Norberg et al. 1979, Pollard 1981, Nagayama and Dales 1970, Goldman and Knipe 1973, Kuznetsov et al. 1981, Luduena et al. 1981).

Serum antibodies to cytoskeletal structures have been reported to be elevated in a many autoimmune and infectious diseases (Tables 1.2, 1.3, 1.4 after Kurki and Virtanen 1984, Pedersen et al. 1982).

Table 1.2 Microfilament antibodies in human sera.

Diagnosis	% of population with antibodies
CAH	55-67
PBC	53
ALD	25
RA	8
SLE	13
SS	36
Normals	3

Table 1.3 Microtubule antibodies in human sera.

Diagnosis	% of population with antibodies
CAH	13
PBC	7
ALD	50
RA	15
SLE	8
SS	21
CREST	87
Viral infections	14
Hahimoto's	56
Graves disease	41
Normals	3-9

Table 1.4 Intermediate filament antibodies in human sera.

Diagnosis	% of population with antibodies
CAH	88
PBC	93
ALD	50
RA	54-80
SLE	30
SS	64
Degenerative joint disease	16
Viral infections	65
Normals	14-63

The specificity of microfilament antibodies is mainly anti-actin (Lindman et al. 1976, Kurki et al. 1980). It has been shown by affinity chromatography that sera from normal blood donors may contain trace amounts of antibodies to actin and tubulin (Guilbert et al. 1982). An increased incidence of antibodies against myosin has been demonstrated in sera from patients with polymyositis (Wada et al. 1983). Patients with CAH may also have circulating antibodies to erythrocyte spectrin (Gorbars et al. 1981).

Microtubule antibodies are rare in the normal population. Increased tubulin binding activity has been observed in patients with alcoholic liver disease (Kurki et al. 1983) and in patients with infectious mononucleosis or autoimmune disease of the endocrine organs as compared to healthy controls or systemic autoimmune patients. Most patients with CREST syndrome have antibodies to kinetochores (Moroi et al. 1980).

Intermediate filament antibodies have been frequently reported in human sera. Antibodies (predominantly of the IgM class) are found to be elevated during acute viral infections such as hepatitis, chicken pox, measles, mumps and infectious mononucleosis. Titres fall during convalescence (Toh et al. 1979). Mortazavi-Milani et al. (1984a), reported the appearance of elevated titres (>1:80 by immunofuorescence against fibroblasts) of anti-intermediate filament antibodies in 82% of sera from acutely infected malaria patients.

Similar levels were found in rabbits infected with Trypanosoma brucei (Mortazavi-Milani et al. 1984b). Serum antibodies to intermediate filaments and other cytoskeletal components have been found in infectious mononucleosis, and such antibodies can be elicited in vitro by infection of normal PBL with EBV (Whitehouse et al.1974, Mortazavi-Milani et al. 1982). 74 out of 113 sera from patients with infectious hepatitis, chickenpox, measles and mumps reacted with intermediate filaments of cultured fibroblasts (Toh et al.1979). The class of immunoglobulin was predominantly IgM, and elevated titres were seen in the acute phase of infection. Titres fell in convalescence.

In all these cases, autoantibodies are formed during an infection and recovery is accompanied by loss of autoantibody production. This contrasts to the situation found in autoimmune disease where autoantibody production is a chronic condition.

3. Autoimmune disease.

This describes severe derangements of the immune network that are essentially non-reversible from within the immune system and lead to pathological conditions. It would simplify matters if the term autoimmune disease was applied only to those cases where it can be shown that the autoimmune process contributes directly to the pathogenesis of the disease. In practice

however, it is usually the case that all chronic diseases clearly associated with autoantibody formation are regarded as autoimmune, unless it can be shown that the immunological phenomena are purely secondary. This reflects our state of ignorance of the role of autoimmunity in disorders involving immunological abnormalities, and means that the distinction between autoimmunity and autoimmune disease is necessarily blurred.

4. Classification of autoimmune diseases.

In the absence of a known underlying mechanism for autoimmune disease, these diseases are usually classified as organ-specific or systemic, or at some point on the spectrum between the two. In organ-specific autoimmune disease, the antibodies are usually specific for one or more antigens of a particular organ or tissue and the lesions are confined to these regions. Furthermore, the lesions can be reproduced experimentally in animals, to a certain degree, by injecting the antigen in Freund's complete adjuvant. One example of an organ specific autoimmune disease is Hashimoto's thyroiditis. In this case there is a specific lesion in the thyroid involving infiltration by mononuclear cells, destruction of follicular cells and germinal centre formation, accompanied by the production of circulating antibodies specific for thyroid constituents. Other examples of

organ-specific autoimmune diseases include encephalomyelitis, orchitis and pernicious anaemia.

At the other end of the spectrum are the non-organ specific or systemic diseases. These are characterised by the presence of antibodies to various tissue or organ antigens, and the lesions are widespread. The classical example of this type of disease is systemic lupus erythematosus (SLE). Pathological changes are widespread and mainly characterised by lesions to connective tissue affecting the skin, kidney glomeruli, joints, serous membranes and blood vessels. An extremely varied assortment of autoantibody specificities is seen, including antibodies to DNA and other nuclear components found in all cells of the body. Other examples include scleroderma, mixed connective tissue disease (MCTD) and rheumatoid arthritis (RA).

Many autoimmune diseases fall between these extremes. They usually involve antibodies capable of reacting with various tissues, even though the lesions may be restricted. An example is myasthenia gravis (MG), where the primary lesion is a loss of functional acetylcholine receptors (AChR) on skeletal muscle. Antibodies to AChR are found in the serum and there is much evidence to suggest that they are responsible for the muscular weakness observed in the disease. However, some myasthenics have antibodies to other antigens e.g. ANA and anti-striated muscle antibodies. Sjogren's syndrome, primary biliary cirrhosis and some forms of

haemolytic anaemia are also examples of 'intermediate' autoimmune diseases.

That this classification may be of some aetiological significance is suggested by the finding of overlap of autoimmune disorders. There is a tendency for more than one autoimmune disorder to occur in the same individual, and when this happens the association is often between diseases within the same region of the autoimmune spectrum. Thus patients with autoimmune thyroiditis have a much higher incidence of pernicious anaemia than would be expected. Serologically there is even greater overlap. Thyroid antibodies for example, are found in 50% of pernicious anaemia patients (Roitt 1980). At the non-organ specific end of the spectrum SLE is clinically associated with RA, haemolytic anaemia, dermatomyositis and Sjogren's Syndrome. ANA and RF are common features in all these disorders (Roitt 1980).

There is also an increased tendency to develop cancer in autoimmune disease. In patients with organ specific disease, cancer of the target organ is more likely, whereas non-organ specific disease patients are more susceptible to generalised lymphoreticular cancer.

5. Theories of tolerance to self antigens, and mechanisms of breakdown in autoimmune disease.

Because of anatomical barriers, certain body constituents do not normally come in contact with the

lymphoreticular system. Consequently immunological tolerance is not established. Any trauma which results in the release of these sequestered antigens would then provide an opportunity for autoantibody formation. In the case of sperm and lens, this seems to be the case, as experimental release of these components directly into the circulation can provoke autoantibody formation. However, the great majority of autoantigens involved in autoimmune diseases are not sequestered, and the injection of unmodified extracts of tissues does not elicit autoantibody formation. Thus one has to consider what the normal mechanisms of control of the autoimmune response are, and how these may be by-passed in autoimmune disease.

In 1975 Nossal extended and subtly altered Burnet's deletion theory. He proposed that at some stage in their maturation from stem cells to antibody producing cells, B lymphocytes go through a phase during which contact with antigen induces only tolerance and not immunity. This differs from Burnet's theory in that the stage of differentiation of the lymphocyte is important rather than the whole animal. Indeed it could be shown that primary B cells which start to express surface IgM are highly susceptible to induction of tolerance, and that tolerance is very rapidly induced in these cells (Nossal and Pike 1975, Metcalf and Klinman 1976, Nossal et al. 1979). The mechanisms of this tolerance induction are unknown, but it may be related to the observation

that immature B-cells are unable to regenerate receptors once removed by antigenic modulation (Bruynes et al.1976, Raff et al.1975). Since B-cells have a short life span, and as there is a continuous generation of new B-cells, functional inactivation has to be a continuous process whenever new lymphocytes mature that possess receptors for self determinants. Thus there may be circumstances under which some B-cells with anti-self activity escape. The concentration of autoantigen may be too low, or the affinity of the antibody may be too low for the antigen. Tolerance in immature B-cells is more easily induced with multivalent antigens (Metcalf and Klinman 1976, Elson 1977) and in clones with high affinity receptors rather than low affinity receptors (Nossal et al. 1979). Although elimination of particular clones of lymphocytes may thus play a role, there are probably other more important processes that produce self-tolerance.

It has been demonstrated that antibody can contribute to an unresponsive state by competing with lymphocyte receptors for available antigens (Dresser and Gowland 1964). Passively administered antibody will specifically depress the immune response and high affinity antibody is more effective in bringing about antibody mediated suppression than low affinity antibody (Siskind et al. 1968). However, there is no evidence that serum antibodies play a major part in most examples of immune tolerance.

Antigens must be 'seen' by the T cell in the

context of association with an MHC component on the surface of an antigen-presenting cell (APC). It has been speculated that the autoantigens do not have the opportunity or the mechanism to associate with the APC MHC in an effective manner. Thus autoimmunity would arise if the autoantigens could be adequately processed by the APC (Mitchison 1982). A situation might be envisaged in which unusual recruitment of APC to a given organ might increase the concentration of processed autoantigen to levels capable of triggering autoreactive lymphocytes. Alternatively autoimmunity might arise from derepression of genes coding for Ia-like (HLA-DR) antigens, so allowing their expression in the plasma membrane and thereby making the normal surface components potentially antigenic. Indeed Pujol-Borrell and colleagues (1983) have demonstrated that normal human thyroid follicular cells could be induced to express HLA-DR antigens by the lectins PHA and Con A.

Anti-idiotype antibodies can depress the production of antibodies bearing that idiotype and are therefore capable of maintaining a state of tolerance. Anti-idiotype antibodies can functionally delete clones of idiotype bearing B cells (Strayer et al. 1974, Kohler et al. 1974), and can also specifically inhibit secretion of that idiotype by mature antibody-secreting plasma cells (Schrater et al. 1979, Goidl et al. 1979, 1980). However, the action of anti-idiotype antibody, at least in adult animals, does not appear to result in irreversible clonal

deletion (Fernandez and Moller 1980).

The activity of lymphocyte effector cells of both T and B lineages is regulated by at least two types of lymphocytes: T helper cell and T suppressor cells. Containment of autoreactivity might be achieved either by (i) the activation of suppressor T cells or (ii) the inactivation of helper T cells. There is evidence for the existence of both mechanisms. Helper T cell tolerance can be induced by low doses of antigen (Weigle 1971), but can be terminated fairly easily by cross-reacting antigens and polyclonal activators (Elson et al. 1979). It has therefore been suggested that suppressor T cells (induced by antigen or anti-idiotypic interactions) also operate to ensure a lack of reactivity to self-antigens (Siskind 1984).

Two major mechanisms are thought to be responsible for the breakdown of self-tolerance in autoimmune diseases: T cell bypass and disordered immunological regulation. As discussed above, tolerance appears to be maintained at the T cell level rather than the B cell. Since B cells usually need the help of T cells to differentiate into antibody-secreting cells, the overall effect is tolerance. Thus any mechanism which can circumvent the T cell requirement, or cause T helper cell activation may lead to the activation of non-tolerant B cells. Such mechanisms include: drugs that bind to body constituents, partially degraded autoantigens, bacterial, viral and parasitic infections. Many drugs are known to

cause autoimmune thrombocytopaenia, haemolytic anaemia, leukopaenia and immune complex syndromes. Some autoimmune manifestations following drug administration are remarkably specific. In patients treated with alpha-methyldopa, haemolytic anaemias are not uncommon (Worlledge et al. 1966). Often the autoantibody is directed against the e antigen of the Rh series. The production of ANA is frequent in patients treated with procaineamide (Dubois 1969) and hydralazine (Alarcon-Segovia et al. 1967). It seems likely that these results follow from the coupling of the drug or a metabolite to an autoantigen. If host T cells can react with the antigenic determinants of the drug, autoantibodies could be formed through a helper effect. This has been demonstrated experimentally. Rabbit thyroglobulin coupled with arsanilic acid elicits the formation of autoantibodies in rabbits, even in the absence of adjuvants (Weigle 1965). Coupling of dinitrophenol (DNP) to myeloma proteins has been used to elicit the formation of anti-idiotypic antibodies in syngeneic mice sensitised to DNP (Iversen 1970). A lupus-like syndrome has been described in patients treated with nitrofurantoin. These patients show evidence of cell-mediated reactions to nitrofurantoin, but no antibody binding the drug has been observed (Pearsall et al. 1974), compatible with the drug functioning as a helper determinant.

Partially degraded self-components may expose

antigenic determinants to which the immune system may not be tolerant, and thus may elicit an immune response. This has been investigated using rabbit thyroglobulin digested by papain as an immunogen in rabbits (Anderson and Rose 1971). Intravenous injection of the cleavage products without adjuvant elicited the formation of autoantibodies against thyroglobulin, and mononuclear infiltrates were apparent in the rabbit thyroid gland. Partially degraded collagen is likewise immunogenic in experimental animals (Steffen 1969). The spleens of normal, non-immunised mice contain many cells that make plaques in target layers consisting of their own erythrocytes pre-treated with proteolytic enzymes (Cunningham 1976).

Many microorganisms possess or release substances (lipopolysaccharide, tuberculin, B.Pertussis components) that act as polyclonal B cell and T cell mitogens. That such substances induce autoimmune disease is demonstrated by the fact that Freund's adjuvant is widely used to induce experimental autoimmune diseases. As has been mentioned, LPS can stimulate murine lymphocytes to secrete autoantibodies *in vitro* and *in vivo* (Dziarsky 1982a, 1982b). Hammarstrom *et al.* (1976) found that bovine spleen cells cultured with polyclonal activators also produce antibodies to autologous RBC. Thus bacterial infections could produce polyclonal lymphocyte activation and autoimmunity. Moreover, bacteria might provide helper determinants, or might bear antigens eliciting antibodies capable of

cross-reacting with autoantigens.

Viral infections might be expected to elicit autoantibodies in two ways. Viral antigens and autoantigens may become associated to form immunogenic units. Viral antigens stimulating host T cells could then function as helper determinants. Host antigens are incorporated into the envelopes of some viruses, and virus antigens frequently appear on the surface of infected host cells (Burns and Allison 1975). Virus coded polymeráses can be immunogenic in animals, and these might function as helper determinants when bound to host nucleic acids.

It should be emphasised that such bypass mechanisms for the induction of autoimmunity do not by themselves ensure the continuation of the response. It seems likely that some defect in the regulatory control of self-reactive clones is required before an autoimmune response can become established. Several reports have documented defects in the T suppressor circuits in various autoimmune strains of mice (Cantor et al. 1978). There is evidence that the generation of non-specific suppressor T cells by polyclonal activators in patients with SLE is defective (Horowitz et al. 1977, Fauci et al. 1978). A significant proportion of clinically unaffected close relatives of these patients also demonstrate abnormally low levels of suppressor T cells (Miller and Schwartz 1979). This indicates that this deficit in SLE patients is not a result of the disease or its treatment,

and that additional factors must be involved in the causation of the disease.

A mechanism by which T suppressor cells might be deleted has been suggested by Alarcon-Segovia (1978, 1979). They demonstated that anti-RNP antibodies could penetrate live mononuclear cells having receptors for the Fc portion of IgG. T suppressor cells have such receptors, and this suppressor function was found to be abrogated by anti-RNP in vitro. Suppressor T cell loss or dysfunction caused by penetration of ANA into T suppressor cells may thus lead to a self-perpetuating autoimmune state. However, it is difficult to account for the antigenic specificity of different autoimmune disorders on the basis of a generalised depression in non-specific T suppressors without invoking defects in antigen-specific suppressors.

Chapter Two. SLE and MG.

1. Introduction.

SLE and MG are two autoimmune disorders which provide a number of interesting contrasts. In the spectrum of autoimmune diseases they are well separated, with MG near the organ specific end and SLE very clearly at the non-organ specific extreme. Only rarely are both diseases found in the same individual. In MG, although a number of autoantibody specifities may occur, anti- AChR antibodies predominate, whereas in SLE numerous autoantibody specifities abound. The pathological symptoms of MG are fairly clearly seen to be the result of anti-AChR antibodies interfering with the normal physiological functioning of its target antigen. In SLE, many of the symptoms are attributable to antibody-antigen complex deposition and consequential, local inflammatory tissue destruction. Autoantibodies are thus able to exert pathological effects without necessarily influencing the normal functioning of the antigen, and may produce lesions well away from the normal site of the target antigen. Some of the intracellular autoantigens in SLE are probably not even accessible to autoantibodies while functioning in viable cells. The function of certain nuclear and cytoplasmic antigens recognised by SLE sera (such as the ribonucleoproteins) are unknown, but their ubiquity would suggest they are probably of fundamental importance. The study of these antibody/antigen systems may provide insights into normal cell function.

2. Clinical manifestations of SLE.

SLE is characterised by inflammatory and destructive processes in a variety of tissues and organs including the skin, joints, kidney, heart, lungs, arteries and arterioles. The pathological changes occur primarily as a result of complement-fixing immune complexes (Brentjens et al. 1977, Koffler et al. 1982). The condition is more prevalent in women than men and has a peak incidence in the 25-29 year age group.

Clinical manifestations of the disease are extremely diverse. Lesions include erythemas of the skin (such as the classical facial butterfly rash), maculae, bullous and ulcerous lesions, arthritis, arthralgia, myositis, myalgia, damage to arteries, lachrimeal and salivary glands, pericarditis, interstitial pneumonia, glomerulonephritis, haemolytic anaemia, thrombocytopaenia and leukopaenia.

The clinical diagnosis of SLE is usually based on the American Rheumatism Association's preliminary and revised criteria for the classification of SLE (Cohen et al. 1971, Tan et al. 1982). In its revised form this consists of 11 clinical and serological criteria including a positive anti-nuclear antibody (ANA) fluorescent test, and the presence of anti-DNA or anti-Sm antibodies (see table 2.1). Four of the 11 criteria must be met for the diagnosis of SLE. The inclusion of the ANA

test in the revised criteria was found to increase the sensitivity of the diagnosis (Levin et al. 1984).

Table 2.1. The 1982 revised criteria for the classification of SLE.

1. Malar rash
2. Discoid rash
3. Photosensitivity
4. Oral ulcers
5. Arthritis
6. Serositis
7. Renal disorder: persistent proteinuria or cellular casts
8. Neurological disorder: seizures or psychosis
9. Haematological disorder: haemolytic anaemia or leukopaenia, lymphopaenia or thrombocytopaenia
10. Immunological disorder: positive LE, anti-DNA, anti-Sm or false positive serologic test for syphylis
11. Antinuclear antibody

(After Levin et al. 1984, Tan et al. 1982).

3. Aetiology of SLE.

The aetiology of SLE is uncertain and many factors are implicated, such as UV light, drugs and viral infection, with the involvement of a genetic predisposition for the disease and sex hormone levels.

A viral role for the onset of SLE has long been suspected. Paramyxovirus-like structures have been found in the kidney, skin and circulating lymphocytes of SLE patients, and a variety of serum antibodies have been found to viruses such as parainfluenza, rubella, reovirus, polio, adenovirus, cytomegalovirus, Herpes simplex and Epstein-Barr virus (Hurd et al. 1972, Pincus et al. 1978, Pincus 1982). A viral aetiology is also suspected in NZB mice which develop an SLE-like disease. A type C oncornovirus is specifically implicated, as a viral glycoprotein of Mr 20k is present in high concentration in the blood and spleen, and also deposited as an immune complex in the glomeruli (Yoshiki et al. 1974). However, the role of viruses in human SLE remains hypothetical, and no virus has been shown conclusively to be responsible for the disease.

Among the drugs which have been demonstrated to induce autoantibodies and a clinical SLE-like syndrome are procainamide, hydralazine, chloropromazine, isoniazid, trimethadone and alpha-methyldopa. These substances are able to interact with DNA and nucleoproteins in vitro, and induce ANA in individuals

receiving them. Unlike 'spontaneous' SLE, racial and sexual predispositions are not seen, the symptoms are generally milder and remission is usually complete on cessation of the drug.

That approximately 9 women contract the disease for every 1 man strongly suggests that sex hormones may play a role in the disease. During pregnancy, an exacerbation of the disease, which extends to the post partum period, is common. Talal et al.(1982) found that androgens relieve the symptoms in female NZB mice, and that castration of male NZB mice induced aggravation of the disease.

The incidence of SLE is higher in negro women than caucasians, and familial studies indicate a slight association of susceptibility to SLE with HLA-D2/D3 types (Estes and Christian 1971, Grigor et al. 1978, Fessell 1974, Inman 1978). First degree relatives have been reported to have a higher incidence of ANA (4.2%) than normals (0-1%) and also show a high incidence of Raynaud's phenomenon (Block et al. 1975). In many cases, members of the patient's family show other autoimmune manifestations such as hypergammaglobulinaemia, and a high frequency of rheumatoid arthritis (RA). Concordance of disease in monozygotic twins is high; 57% for SLE, 71% for ANA and 87% for hypergammaglobulinaemia (Block et al. 1975). Concordance is lower in dizygotic twins.

4. Autoantibodies in SLE.

The most prominent abnormality in patients with SLE is their ability to produce antibodies to a spectacular range of self-antigens. Some examples of autoantibody specificities found are given in table 2.2.

Table 2.2. Examples of autoantibody specificities found in SLE.

a. Cells:	Lymphocytes
	Erythrocytes
	Platelets
	Neutrophils
b. Tissues/organs:	Heart
	Neurons
	Collagen
	Muscle
c. Cytoplasm:	Ribosomes
	Mitochondria
	Lysosomes
	Cytoskeletons
d. Plasma proteins:	Ig
	Coagulins
e. Nuclear components:	DNA
(ANA)	Nucleoprotein
	Histone
	Ribonucleoprotein
	Nuclear matrix

The antinuclear antibodies have been particularly implicated in the pathogenesis of SLE and related diseases (Reichlin 1981). The number of distinct nuclear antigens recognised by SLE sera is large. Antibodies to various nuclear antigens differ in their diagnostic specificity for SLE, their relationship to disease activity and their postulated role in the disease.

4(i). Antibodies to DNA.

Anti-DNA antibodies occur frequently in active SLE with anti-dsDNA antibodies being nearly exclusively found in the disease (Tan et al. 1966, Koffler et al. 1971). They are closely related to disease pathogenesis through the process of immune complex formation. Exacerbation of the illness, with high fever and proteinuria coincides with the appearance of DNA in the serum and the disappearance of antibodies. Hence monitoring levels of anti-DNA antibodies has value in predicting disease flare-up (Swaak et al. 1982). A considerable heterogeneity in anti-DNA responses is seen. There are many differences between sera in the quantitative and qualititive features of these antibodies, in their ability to form immune complexes and cause inflammatory damage (Winfield et al. 1977, Miniter et al. 1979).

There are difficulties in studying antibodies

to DNA. As an antigen, DNA displays many structural forms and sequence patterns that can serve as antigenic determinants (Wells et al. 1980). Considerable attention has been devoted to the distinction between antibodies to dsDNA as opposed to antibodies to ssDNA. However, obtaining pure dsDNA is difficult and small breaks or gaps may allow the binding of anti-ssDNA antibodies. Even intact DNA may reveal regions of single-strandedness. Similarly, pure ssDNA may be difficult to obtain (Stoller and Papalian 1980). Regions of complementarity along two DNA molecules may generate duplex regions in ssDNA preparations. It should also be noted that components of sera may affect the conformation of DNA, so that even specifically defined DNA preparations may undergo changes during assay (Pisetsky 1983). With such heterogeneity, assessment of antibody response can be difficult.

4(ii). Antibodies to small nuclear and cytoplasmic ribonucleoproteins.

SLE serum antibodies are capable of binding a number of soluble nuclear and cytoplasmic ribonucleoproteins. These are complexes of small RNAs and protein found in eukaryotic cells. The RNA components are distinct from the three commonly encountered RNAs i.e. mRNA, tRNA and rRNA (Lerner and Steitz, 1981, Zieve 1981). Four principle ribonucleoproteins are bound by sera from SLE, MCTD, RA, Scleroderma and Sjogrens

Syndrome patients. These are Sm, nRNP, La and Ro. The presence of autoantibodies to these, together with their titre and specificity , is an important clinical feature for the diagnosis of these diseases.

The Sm antigen is a non-histone, nuclear, acidic protein. It is highly soluble in physiological salt solution and is insensitive to RNase, DNase and trypsin except on prolonged exposure. It is mostly, if not entirely found in the nucleus, and gives a speckled staining pattern by indirect immunofluorescence (Lerner et al. 1981). There has been little consistency in the literature regarding the protein composition of the Sm antigen. Several laboratories have found that the polypeptides associated with this antigen are within a range of Mr 12k-14k (Agelli et al. 1980, Takano et al. 1980, 1981). However, the lower Mr polypeptides are reported as representing either minor (Lerner et al. 1980), or major (Barque et al. 1981) components. MacGillivray et al. (1982) reported four components of Mr ~10k and one component of Mr~12k. Five similar polypeptides were found by Lerner and Steitz (1980), although the Mr estimates differed slightly. Billings and Hoch (1983) found the antigenic polypeptides to consist of a 26-27k doublet and one 13k polypeptide. Also present were non-antigenic components of 35k, 32k, 11k and <10k. There is also discrepancy in the analysis of the RNA content of Sm. Lerner and colleagues (1979, 1980, 1981), and Mount and Steitz (1981) reported the Sm antigen to

contain six RNA subunits designated Ula, Ulb, U2, U4, U5, U6. MacGillivray et al. (1982) found 10 RNA species migrating in PAGE between 4S and 5S. Similar species were reported by Takano et al. (1980, 1981) and White et al. (1981).

Anti-Sm antibodies are found in approximately 25-30% of SLE patients (Nakamura et al. 1978), and they occur almost exclusively in SLE. Their presence is taken as a diagnostic marker for the disease (Kurata and Tan 1976, Tan et al. 1982). Anti-Sm antibodies are primarily of the IgG class and are usually found with anti-nRNP antibodies. Where anti-Sm is found alone, there may be a greater chance of the patient suffering nephritis (Sabaharval et al. 1983).

The nRNP antigen is a nuclear, non-histone protein. Its antigenicity is destroyed by both RNase and trypsin (Takano et al. 1980, Douvas et al. 1979). The complex precipitated by anti-nRNP sera contains two RNA species, Ula and Ulb, both of which are also found in the Sm antigen. Other workers have reported the presence of smaller RNA species (MacGillivray and Carroll 1982, Takano et al. 1980, 1981). The Sm and nRNP antigens have independent antigenic determinants (Lerner et al. 1980), although both antigens can be found complexed to the same Ul RNA (Lerner et al. 1982). The number of polypeptides in nRNP has been reported to be between 5 and 9, and of Mr between 9k and 65k (Lerner and Steitz 1979, Gibbons et al. 1982, Takano et al. 1980, White et al. 1981,

Douvas and Tan 1981). Recently Billings and Hoch (1983) isolated Sm/nRNP from rabbit thymus by an affinity column of SLE IgG with both Sm and nRNP reactivities. The proteins isolated were run on reducing gels and analysed by immunoblot and Coomassie staining. The nRNP specific polypeptide was found to have a Mr of 73k. The conflicting data from the analyses of Sm and nRNP probably results from different methods of antigen detection, different cell and tissue sources and different extraction and isolation procedures.

Antibodies to nRNP are found in SLE, MCTD, Scleroderma and Sjogren's Syndrome, with the highest titres usually found in MCTD patients (Notman et al. 1975). In SLE, anti-nRNP antibodies are found in 23-50% of patients, often with antibodies of other specificities (Kurata and Tan 1976, Tan et al. 1976). By indirect immunofluorescence they give a speckled nuclear pattern (Provost 1979), and are of the IgG class. In SLE, anti-nRNP antibodies are usually found in conjunction with anti-Sm, whereas in MCTD anti-nRNP are found without anti-Sm. The presence of anti-nRNP as the sole precipitin is indicative of a subset of patients with low incidence of renal disease and CNS involvement (Notman et al. 1975, Provost 1979, Sharp 1982). When found in conjunction with other antibodies, the presence of anti-nRNP may be related to a high incidence of nephritis (Reichlin 1981). Anti-nRNP antibody has been found in complexes deposited in glomerular lesions (Kuraka 1979).

The La antigen is a soluble nuclear protein which is sensitive to trypsin but resistant to both DNase and RNase (Teppo et al. 1982). Although early reports suggested it was found in both nucleus and cytoplasm, it is now believed that the location of it in the cytoplasm was an artefact arising during the biochemical fractionation (Hendrick et al. 1981, Hardin et al. 1982). The Mr of the antigenic polypeptides have been variously estimated between 30k and 68k (Francoeur and Mathews 1982, Teppo et al. 1982). Antibodies to La are found in sera from patients with RA, SLE and Sjorgren's syndrome (Provost 1979). Sera with anti-La antibodies frequently contain antibodies to Ro.

The Ro antigen is a soluble, cytoplasmic, acidic glycoprotein which is resistant to both RNase and trypsin. The Ro protein is complexed with between 2 and 5 sRNAs, depending on the mammalian species (Hendrick et al. 1981, Reddy et al. 1983). These sRNAs vary in size from 800-110 nucleotides and are distinguishable from the U series which are components of Sm and nRNP (Lerner et al. 1980, Hendrick et al. 1981). By SDS-PAGE and immunoblotting, Lieu et al. (1984) have found the antigenic polypeptide to have a Mr of 61k. Wolin and Steitz (1984) similarly reported a Mr of 60k.

Antibodies to Ro are found in about 30% of SLE patients and 25% of patients with Sjogren's Syndrome (Scopelitis et al. 1980). Whereas sera with anti-La antibodies frequently contain anti-Ro antibodies, the

converse is not true in that there are many sera with anti-Ro activity which are free of anti-La activity (Tan 1982). When the antigens reactive with La and Ro antibodies are analysed, it can be shown that distinct sets of small RNAs are precipitated by each antibody (Lerner et al. 1981), a situation different from the Sm and nRNP situation described above. Warsicek and Reichlin (1982) found that patients with SLE who possessed antibodies to Ro without antibodies to La have a high incidence of antibodies to DNA and serious renal disease. Maddison and Reichlin (1979) have demonstrated that Ro-anti-Ro immune complexes participate in the development of nephritis. Patients with both anti-Ro and anti-La antibodies have a lower incidence of antibodies to DNA and a very low incidence of nephritis, and have milder disease symptoms (Warsicek and Reichlin 1982, Tan 1982). In addition to the relationship of anti-Ro antibodies to SLE and Sjogrens syndrome, many recent studies have shown that this antibody maybe related to neonatal lupus (Franco et al. 1981, Kephart et al. 1981, Weston et al. 1982). Infants with neonatal lupus are characterised by the presence of anti-Ro antibodies and occassionally with anti-La but without other types of ANA. These antibodies are transferred across the placenta from the mothers who have all been shown to have the antibody. The infants do not produce these antibodies as after a period of several months the antibodies disappear from the circulation. A high incidence of congenital

complete heart block has been observed in these infants and the correlation with anti-Ro is highly significant (Scott et al. 1983).

5. Clinical manifestations of MG.

MG is characterised by weakness and fatigability of skeletal muscles. This is frequently a progressive disease beginning with drooping of the eyelids, double vision, and impairment of speech and swallowing. The proximal muscles of the limbs and trunk are usually affected later, and respiration may be adversely affected in severe cases. The neuromuscular junction was implicated as the region of the defect by the observation that anticholinesterases could improve the strength of MG patients (Walker 1934). The nature of the defect was not elucidated until the snake toxin alpha-bungarotoxin (alpha-BTX) became available as a specific probe for AChR (Lee 1972). Neuromuscular junctions of MG patients were found tg bind far less radiolabelled alpha-BTX than normals, suggesting that the number of available AChR sites was far less (Fambrough et al.1973). In addition, the neuromuscular junctions of myasthenic patients were found to be considerably simplified morphologically (Engel and Santa 1971). The effect of these lesions is to immpair synaptic transmission, resulting in failure of nerve impulses to trigger muscle action potentials and hence muscle

contraction. An occurrence rate of 1 in 20,000 of the population has been suggested (Kurland 1957). The distribution of the disorder appears to be worldwide, and no ethnic groups seem to be particularly susceptible (Osserman 1958). The possibility that the CNS might be involved has been considered (Grashchenkov and Perelman 1966), although firm evidence is lacking. The EEG can be abnormal in MG (Hokkanen and Toivakka 1969, Levfert and Pirskanen, 1977). Papazian (1976) reported that MG patients had a significant disturbance of rapid eye movement (REM) sleep, which in normal individuals is thought to depend on acetylcholine as the transmitter. Anti-AChR antibody has been found in the cerebrospinal fluid of MG patients (Levfert and Piskanen 1977) but appeared to gain access by passive leakage rather than being synthesised locally (Keesey et al. 1978). Micro-infusion of MG serum or its IgG fraction into the caudate nucleus of rabbit brain provoked an abnormal EEG discharge and abnormal behaviour (Fontana et al. 1978). The degree of these abnormalities appeared to correlate with the titre of the antibody and could be corrected by anticholinesterases.

An association of particular HLA antigens with MG was recognised by Pirskanen et al. (1972) who found an increased frequency of HLA-B8 in young female patients. This has since been confirmed by several groups (Feltkamp et al. 1974, Fritze et al. 1974, Van den Berg-Loonen et al. 1977).

6. The nature of MG.

The autoimmune nature of MG was suspected because of its association with other autoimmune diseases (Simpson 1960), and the frequent structural abnormalities found in MG patients' thymus glands (Castleman 1966). That the AChR was the object of an immune attack was first indicated by a fortuitous experiment of Patrick and Lindstrom (1973). In attempting to raise rabbit antibodies to purified AChR from the electric organs of eels, they found that the immunised rabbits developed muscular weakness and other symptoms of MG, including a decremental muscle response on repetitive stimulation. The muscular weakness, as in human MG, was relieved by administration of anticholinesterases.

MG patients were then screened for the presence of serum anti-AChR antibodies. Approximately 90% of MG patients have detectable anti-AChR antibody, and a RIA which measures antibody binding to human muscle AChR has now become a standard diagnostic procedure (Lindstrom et al. 1976). Despite the common occurence of anti-AChR antibody, the serum levels of such antibody corresponds poorly to the severity of the patients' symptoms.

7. The role of anti- AChR antibody in MG.

Do these circulating antibodies represent a

secondary response to AChR damage caused by some other agent, or are they themselves the pathogenic agents? Immunoglobulin from MG patients, when repeatedly administered to mice, was found to induce characteristic features of MG. The component responsible was found to be IgG, and its effect was enhanced by complement (Toyka et al. 1977). Electron-microscope studies have identified IgG and complement at the postsynaptic membranes of MG patients' neuromuscular junctions (Engel et al. 1979). Plasma exchange, which temporarily lowers serum concentrations of anti-AChR antibodies, results in a concomitant improvement in the strength of MG patients (Pinching et al. 1976, Dau 1982).

Antibody induced reduction of available AChR could be produced by increasing the rate of degradation, decreasing the rate of synthesis, blocking the active site or by causing post synaptic damage in conjunction with complement fixation. There is evidence to support each of these possibilities. Probably the relative importance of each of these mechanisms depends upon the class, sub-class, fine specificity and affinity of the autoantibodies present.

Immunoglobulin from MG patients' sera has been found to increase the degradation of AChR of rat skeletal muscle in culture by 2-3 fold (Drachman et al. 1982). This was apparently due to antigenic modulation of the receptor, as the increased degradation was not seen when Fab fragment of myasthenic IgG was used. Autoradiography

and fluorescence microscopy have shown the addition of anti-AChR serum into muscle cultures to cause the AChR to aggregate into clusters (Lennon 1978, Tarrab-Hazdai et al. 1979). These are then believed to be endocytosed by coated pits in the muscle membrane and degraded in the interior of the cell (Pumplin and Drachman 1983). There is evidence that a similar increase in turnover of AChR occurs at intact neuromuscular junctions with the addition of MG IgG both in vivo and in vitro (Merlie et al. 1979, Reiness et al. 1978, Stanley and Drachman 1978).

Whether direct blockade of AChR by serum antibody is an important pathological feature in MG is under debate. Between 7% and 90% of MG sera have been reported to block the ACh binding site (Almon et al. 1974, Drachman et al. 1982). Where blockade does occur it is likely to be due to the attachment of antibody near to the ligand binding site and effecting blockade by steric hindrance (Drachman 1981.

There is only a weak correlation between the clinical severity of MG and the amount of antibody capable of binding to AChR as measured by RIA. The functional ability of antibody to reduce the available AChR might give a better correlation. Drachman et al. (1982) examined the ability of immunoglobulin from a large number of patients to induce degradation or blockade of AChR on cultured rat muscle. The ability to do this corresponded well with the clinical status of the

patients. Some immunoglobulins were better at blocking and some at degrading, indicating some functional heterogeneity in the autoantibody population. The potential of the antibody to produce disease may thus relate to epitopic specificities which either dispose to receptor blocking or degradation. Other properties are probably involved such as the ability to bind complement.

8. Aetiology of MG.

That marked differences exist between groups of patients suggests there are diverse origins of the disease even though the target of the autoimmune response is the AChR. Young females and older males are preferentially afflicted with the disease. Thymic abnormalities vary between groups; 25% have no abnormalities, 65% have hyperplasia and 10% have thymic tumours. Familial occurence of the disease is occasionally seen and there is association with other autoimmune diseases in some but by no means all patients.

The frequent occurrence of thymic abnormalites in MG patients suggests some involvement in the disease. Surgical thymectomy produces clinical remission in 35% of patients and improvement is seen in up to 50% (Buckingham et al. 1976). The thymus contains not only lymphocytes but other cells including myoid cells. These strongly resemble skeletal muscle when cultured in vitro (Kao and Drachman 1977, Wekerle et al. 1975), and also possess

surface AChR (Kao and Drachman 1977). This has been postulated as the source of antigen in MG. Newsome-Davis et al. (1982) have demonstrated that there are B cells in the thymus of MG patients capable of producing IgG, some of which is directed against AChR. In vitro, such lymphocytes can produce signficant amounts of anti-AChR antibody.

Several studies have examined the hypothesis that a virus may be the cause of MG. Antibodies to cytomegalovirus have been found in an increased proportion of MG patients (Tindall 1980), but neither this nor any other virus has been found in MG thymuses in systematic studies by electron microscopy (Drachman 1983).

A proportion of RA patients treated with penicillamine develop drug induced MG. This completely remisses with omission of the drug. Similarly the experimental administration of AChR initiates an MG-like syndrome in animals, but repeated administration is necessary to perpetuate the disease. Thus if the trigger in MG is an altered form of AChR, its continued presence would seem to be necessary. Alternatively the disease may result from a defect in immune regulation. There appears to be no overall deficiency of T suppressor cells in MG, although the possibility of a deficiency of suppressor cells specific for anti-AChR antibody has been proposed (Shinomiya et al. 1981).

Although our understanding of SLE and MG has

increased over recent years, clearly much remains to be learned. A precise understanding of the autoimmune response is necessary to discover how and why an autoimmune response is initiated and maintained in these disease states.

Chapter Three. Monoclonal antibodies and the study of autoimmune disease.

1. Introduction.

A detailed knowledge of the structural and functional properties of both the autoantigens and autoantibodies in an autoimmune disorder is clearly necessary for understanding the cause and management of the disease process. Studies of autoantibodies are complicated by the fact that the sera of autoimmune patients often contain a complex mixture of autoantibodies. Thus in functional studies of autoantibody containing sera there is always the possibility that quantitatively minor but functionally significant autoantibodies may confound the interpretation of data (Hough et al. 1982). Similarly it is very difficult to determine whether the observed multiple reactivities of autoimmune sera reflect a range of autoantibodies with different specificities, or autoantibodies with the same specificity reacting with a common determinant on different tissues. An homogeneous population of autoantibodies would theoretically allow both the types of problem to be resolved. Such populations of antibodies occassionally arise spontaneously. Monoclonal cold agglutinins (Dellagi et al. 1981), rheumatoid factors (Grey et al. 1968), and anti-nerve antibodies (Latov et al. 1980) are such examples. Unfortunately they occur too infrequently to be of general use.

2. Monoclonal antibodies (MCA).

Antibody-secreting lymphoid cells have a very short life when cultured in vitro. Certain myeloma cell lines have been adapted to permanent growth in culture, but the antibodies they produce are fixed and not of predefined specificity. When both types of cell are fused, hybrids can be derived which retain the highly desirable properties of immortality and secretion of antibody with a desired specificity. As the hybrid cells can be cloned, individual antibodies of exquisite specificity can be obtained indefinitely. The production of MCA in this fashion was first described by Kohler and Milstein (1975), and has made numerous complex biological systems amenable to precision analysis. In the area of autoimmunity this technology makes possible the study at the molecular level of both the autoantigens and the autoantibodies themselves. MCA lend themselves readily to the development of anti-idiotype antibodies. These are useful for detailed studies of the specificities and genetic origins of autoantibodies. In addition they may be used to manipulate the immune system and are thus potentially useful therapeutic agents.

3. MCA derived from immunised rodents.

A precise characterisation of the antigens involved in an autoimmune disease may be very important

to the understanding of its aetiology and pathogenesis. One approach to utilising MCA technology in the study of autoimmunity has been to isolate the relevant antigen, immunise rats or mice with it and raise a number of MCA. A large number of such MCA are useful as probes for the antigen and allow fine definition of its antigenic structure. These MCA can also be used as 'model' autoantibodies to determine the pathological significance of various specificities. This type of approach has been taken with the study of anti-islet antibodies related to diabetes (Eisenbarth et al. 1983) and the study of the thyrotropin receptor involved in Graves disease (Kohn et al. 1983). Probably the best example of this approach has been with the study of the AChR and its involvement in MG. These studies will be considered in detail as an example of this general approach.

3(i). Studies of autoantigens.

The AChR has been the subject of intensive study with MCA by a several groups of researchers. Lindstrom's group alone have generated a library of over 140 MCA to intact AChR and AChR subunits purified from the electric organs of Torpedo californica (Tzartos and Lindstrom 1980, Lindstrom 1983) and Electrophorus electricus (Tzartos et al. 1981), and to intact receptors purified from the muscles of cattle and humans (Tzartos and Lindstrom 1981).

While these MCA were produced exclusively in rats, other groups have utilised both rats and mice (Dwyer et al. 1981, Fuchs et al. 1981, Gomez et al. 1981, James et al. 1980, Lennon and Lambert 1981). About half the MCA raised against intact receptor react with denatured subunits prepared by dissociation in SDS (Gullick and Lindstrom 1982). When the subunit specificities of the MCA were examined, many were found specific for only one subunit, some weakly cross-reactive with one or more subunits, and a few strongly reactive with more than one subunit (Tzartos and Lindstrom 1980, Gullick and Lindstrom 1982). This cross-reaction probably reflects the extensive amino acid sequence homology between subunits (Raftery et al. 1980, Conti-Tronconi et al. 1982). MCA against Torpedo AChR often cross-react with receptors from other species and have been used to localise homologous subunits in receptors from other species (Einarson et al. 1982, Gullick and Lindstrom 1982, Lindstrom et al. 1980). A large number of MCA react with a small portion of the alpha subunit which Lindstrom has called the Main Immunogenic Region (MIR). This is highly conserved between species, is probably on the extracellular surface of alpha subunits and is distinct from the acetylcholine binding sites (Gullick et al. 1981). It is probably identical to the immunodominant, non-acetylcholine binding site found by Lennon and Lambert (1981).

Rodent MCA have been used to determine

anti-receptor specificities present in MG patients' sera (Tzartos et al. 1981). Six MCA of defined specificities were used to competitively inhibit the binding of serum anti-receptor antibodies to human muscle receptor. Two MCA to the MIR were used, one MCA to another site on the alpha subunit, one MCA to a site on the beta subunit, and two MCA to the gamma subunit. A large fraction of MG serum antibodies were found to be inhibited by MCA to the MIR, but very few by the MCA to the other alpha subunit site. MCA to determinants on both beta and gamma inhibited substantial amounts of serum antibodies. This strongly suggests that in MG, antibodies are made to many parts of the receptor molecule, and furthermore implicates the human muscle AChR as the immunogen rather than a cross-reacting antigen with limited structural similarity. The results also confirm that despite a predominant immune response to the MIR, the autoimmune response is polyclonal, involving anti-receptor antibodies of many specificities. This suggests that it might be difficult to achieve effective immune suppression with anti-idiotype antibodies to MCA.

3(ii)Use of MCA as model autoantibodies.

Monoclonal antibodies raised from immunised rodents have been used to examine some of the features which make auotantibodies pathological. Once again this will be discussed with reference to the AChR.

Theoretically, one would expect antibodies to the acetylcholine binding site to be of greatest pathological significance, because of inhibition of receptor function. Serum antibodies (Barkas et al. 1982) and MCA (Gomez et al. 1979) have been recognised that bind sufficiently near the acetylcholine binding sites to inhibit toxin binding. Some MCA have been found that bind directly on the acetylcholine site (James et al. 1980, Mochly-Rosen and Fuchs 1981). These compete with agonists and antagonists for binding and inhibit receptor function. However, there is little evidence that such antibodies play an important pathological role in most MG patients. Such antibodies represent a very small fraction of antibody specificities (Lindstrom et al. 1976) and their concentration correlates poorly if at all with severity of disease (Drachman et al. 1982, Compston et al. 1981). Electron microscopy studies reveal that in MG, receptors are lost rather than blocked by antibodies (Engel et al. 1979).

In contrast to MCA to the acetylcholine binding site, MCA to the MIR and most other sites so far studied do not inhibit receptor function (Lindstrom et al. 1983). However, a single MCA can passively transfer EAMG (Gomez et al. 1981, Kamo et al. 1982, Tzartos and Lindstrom 1980, Lennon and Lambert 1981). As with passive transfer by serum, passive transfer by MCA occurs by an acute process requiring complement (Lennon and Lambert 1981). Antibodies to the MIR seem to have a particular

pathological significance. Of 4 MCA tested that bound to the MIR, all transferred EAMG (Tzartos and Lindstrom 1980, Lindstrom 1983). None of 4 MCA reactive with other sites transferred EAMG even though one MCA was demonstrated to have bound the beta subunit in vivo (Lindstrom 1983). The relative importance of antigenic modulation and complement fixation in the ability to passively transfer EAMG is being studied using MCA with different specificities, and with MCA of similar specificities but of different classes and subclasses (Lindstrom 1983).

4. Monoclonal autoantibodies from mice with autoimmune disease.

Unlike active immunisation, where it may be assumed that elicited antibodies have been produced against the administered immunogen, the substances which give rise to a spontaneous autoimmune disease are not known. It has not yet been shown that the same antigenic determinants are recognised in spontaneous disease as those recognised by an animal immunised artificially with the antigen. As Eilat (1982) has pointed out , this is an important distinction, as it may turn out that whereas MCA raised by immunisation can be useful in studying some pathological consequences of autoantibody formation, they may have no relevance to mechanisms leading to the development of spontaneous autoimmune disease. This is

one reason why many workers have focussed their attention on raising MCA from mice which develop spontaneous autoimmune disease, and human autoimmune patients themselves. SLE is well represented by mouse models and many hybridomas have been derived from such animals without prior immunisation.

4(i). Studies of autoantibody specificities.

Pages and Bussard (1978) were the first to report the successful establishment of permanent murine hybridomas which secreted monoclonal autoantibodies. Peritoneal cells from an unimmunised NZB mouse (a strain which develops SLE) were fused with mouse myeloma cells and hybrids were isolated which secreted MCA to erythrocytes. Several IgM secreting clones were isolated which produced MCA which were haemolytic for bromelin treated mouse red blood cells.

Most monoclonal lupus antibodies studied have been directed against nucleic acid antigens. Eilat et al. (1980) obtained a hybridoma from an NZB/W F1 mouse which secreted a MCA to RNA. It was specific for ssRNA rather than dsRNA. It also reacted with random polymers poly(G,C) and poly(G,C,U), and apparently recognises an antigenic determinant containing (G),(C) and (U).

Further studies on epitope specificity of anti-DNA antibodies were undertaken by Lee et al. (1981). They examined 6 MCA, derived from NZB/W F1 mice, which

bound ssDNA. By experiments with synthetic homo and heteropolymers of purines and pyrimidines they found the binding of all 6 MCA to be influenced by the base sequence of the antigens. Other workers have studied MCA to DNA that were derived from lupus mice, including MRL-lpr/lpr (Andrzejewski et al. 1980, 1981), NZB/W F1 (Hahn et al. 1980, Marion et al. 1982) and BXSB (Hahn et al 1980). In most cases the MCA reacted preferentially with ssDNA rather than dsDNA. However Tron et al. (1982) have described 10 MCA from NZB/W F1 mice that react preferentially with dsDNA.

Andrzejewski et al. (1981), using MCA from MRL-lpr/lpr mice have demonstrated that a single autoantibody can bind to multiple nucleic acid acid antigens of widely different base composition. They suggest that these type of antibodies recognise the sugar phosphate backbone common to all nucleic acids. Further analyses have shown that some MCA to DNA can also bind phospholipids, including cardiolipin. Absorbtion of such autoantibodies with cardiolipin micelles inhibits their ability to produce the fluorescent ANA reaction. These results support the notion that some lupus autoantibodies can manifest a variety of serological abnormalities. The antibodies that binds to DNA and cardiolipin can explain the origin of the biological false positive test for syphilis in SLE. Also since anti-DNA autoantibodies can bind to other molecules, it is evident that DNA is not necessarily the immunogen or even the preferred target

antigen in SLE.

Lerner et al. (1981) produced MCA from MRL-lpr/lpr mice specific for either Sm, DNA or nRNP. In immunoprecipitation studies, the mouse anti-Sm gave an identical pattern of precipitation to a human anti-Sm serum. The monoclonal anti-Sm used in a competitive RIA has shown that at least one antigenic site, recognised by the monoclonal antibody, is distinct from nRNP (Lerner et al. 1982).

4(ii). Anti-idiotypic analyses of monoclonal autoantibodies.

Several groups have investigated the idiotypes of monoclonal anti-DNA antibodies. Rauch et al. (1982) prepared a polyclonal rabbit anti-idiotypic antiserum against a monoclonal anti-DNA antibody from an MRL-lpr/lpr mouse. This antiserum reacted with 8/12 other MRL derived anti-DNA MCA, and detected the presence of the idiotype in 40/40 MRL sera. Tron et al. (1982) prepared a monoclonal anti-idiotype antibody by immunising a mouse with a monoclonal anti-DNA derived from an NZB/W F1 mouse. This recognised the immunising idiotype in all 24 NZB/W F1 sera tested. Marion et al. (1982) analysed a rabbit antiserum directed to the idiotype of a monoclonal anti-DNA antibody of NZB/W F1 origin and found 8/13 MCA with similar ligand specificities shared a common idiotype.

5. Human monoclonal autoantibodies.

Establishing monoclonal autoantibodies directly from autoimmune patients has been an attractive proposition for a number of reasons. Certain autoantigens may be difficult to purify or poorly immunogenic in mice. Where they may be used as immunogens in rodents, the immune response may be very different to the autoimmune situation. Where murine MCA are available, it would be useful to compare their specificities directly with human monoclonal autoantibodies. Rodent models of human autoimmune diseases are not always available, and where they are, they may not be identical to the human counterpart. Thus autoantibodies may not have the same or even similar specificities as found in human disease. Human monoclonal autoantibodies would provide ideal reagents for studying idiotypes and hence the origins and diversity of the human autoimmune response.

5(i) Difficulties in the production of human MCA.

Human MCA have been the subject of some considerable interest in areas of research other than autoimmunity. These include blood group and tissue typing and diagnosis and therapy in oncology and infectious diseases. Despite an intensive research effort into the production of human MCA, progress has been very slow.

5(ii). Human-human fusions.

In 1980, Croce et al. and Olsson and Kaplan independently published work which suggested that human-human hybridoma technology might emulate the success of rodent monoclonal antibody production first reported by Kohler and Milstein five years earlier. However, two serious problems have yet to be overcome concerning each of the fusion partners required for fusion. The most readily available sources of human lymphocytes are peripheral blood and lymph node tissue. The percentage of B-lymphocytes producing specific antibody from these is likely to be very small compared to the cells available from the spleen of an optimally immunised mouse. To some degree this can be overcome by in vitro immunisation (Osband et al. 1981, Foon et al. 1983, Cavagnaro and Osband 1983), pre-stimulation with polyclonal activators (Chiorazzi et al. 1982, Shoenfeld et al. 1982) or pre-selection of antigen specific cells (Kozbor and Roder 1981).

Problems with the human cell lines were apparent from the start. It was two years before the success of 1980 was repeated, many workers being unable to produce hybridomas partly because of mycoplasma contamination of myeloma stocks (Sikora and Munro-Neville 1982). Different human myeloma lines and lymphoblastoid lines were adapted to continuous culture as HAT sensitive

variants. By 1983 Kozbor and Roder were able to review the performance of ten such lines as suitable partners for the production of hybridomas. The ideal cell line should have good growth characteristics, be robust in culture, fuse with high efficiency, support the secretion of large amounts of lymphocyte-derived immunoglobulin while secreting none of its own, and generate hybrids that clone easily and are stable over long periods of time. A number of such rodent lines are available but not one human line is available which meets these criteria (Foon et al. 1983, Kozbor and Roder 1983).

Table 3.1 Human and Rodent Fusion Partners.

Cell line	Cell Type	Ig secreted	Fusion Freq.($x10^{-6}$)
(Human)			
SKO-007	Myeloma	IgE	3.7
TM7-H2	Myeloma	IgG	N.D.
DHMC	Myeloma	IgG	1-10
GM1500	LCL	IgG	1.8
KR4	LCL	IgG	3-11
HMy-2	LCL	IgG	<1
GM0467.3	LCL	IgM	2.2
H351.1	LCL	IgM	2.2
UC729-6	LCL	IgM	N.D.
GM4672	LCL	IgG	N.D.
(Mouse)			
P3-X63Ag8	Myeloma	IgG	~10
NS1	"	light chains	"
X63	"	none	"
Sp2/0	"	"	"
NS0	"	"	"
(Rat)			
Y3.Ag.123	"	light chains	"
YB20.Ag20	"	none	"

(From Kozbor and Roder 1983, Galfre and Milstein 1981).

5(iii). Human-mouse fusions.

A number of workers have used murine myelomas in fusions with human lymphocytes to yield human-mouse heterohybrids which secrete human immunoglobulins. As early as 1973, Schwaber and Cohen reported the fusion of the mouse myeloma TEPC-15 with human PBL from which they cloned a hybrid secreting both human and mouse immunoglobulins. Levy and Dilley (1978) fused non-secreting, human neoplastic B-cells with the mouse myeloma P3-X63-Ag8 to produce hybrids which secreted, in large quantites, human immunoglobulin of the same light chain type as found on the surface of the neoplastic parent lymphocyte.

Human-mouse hybrids preferentially segregate human chromosomes (Croce et al. 1974, 1980), and as two human chromosomes carrying the rearranged genes for human light and heavy immunoglobulin chains must be retained, this technique is generally thought to be unsatisfactory. The instability problem may not be as great as is generally thought, for reasons discussed later in the thesis. In view of the disadvantages of human fusion partners, a degree of hybrid instability may be an acceptable compromise. Human monoclonal antibodies, using human-mouse fusions, have been successfully produced against the Forssman antigen (Nowinski et al. 1980), human cellular antigens (Schlom et al. 1980, Cote et al. 1983), keyhole limpet heamocyanin (Lane et al. 1982), and

tetanus toxoid (Kozbor et al. 1982, Butler et al. 1983).

5(iv). The EBV technique.

Although not involving fusion of cells to form hybrids, a third method of producing human MCAs should be mentioned here. This involves the transformation of lymphocytes by Epstein-Barr virus. EBV is a lymphotropic herpesvirus capable of polyclonal activation of normal human B-lymphocytes and converting them into immortal cell lines (Rosen et al. 1979, Miller and Lipman 1973). EBV transformed cells carry multiple copies of the viral genome and express an EBV specific nuclear antigen, EBNA. Steinitz et al. (1977) were the first to use EBV to produce B-cell lines secreting pre-defined antibody, in this case to the synthetic hapten NNP. They collected lymphocytes from normal volunteers who demonstrated high natural antibody titres to NNP, and selected for antigen receptor positive cells by rosetting with NNP-coupled erythrocytes. The lymphocytes were infected with EBV and after 4 weeks permanent lines were established which secreted anti-NNP antibodies. EBV transformed lines have now been made secreting antibodies to TNP (Kozbor et al. 1979), tetanus toxoid (Zurawski et al. 1978), Rhesus antigen D (Boylston et al. 1980, Crawford et al. 1983), phosphorylcholine (Yoshie and Ono, 1980), rheumatoid factor (Steinitz et al. 1980), and acetylcholine receptor (Kamo et al. 1982). After transformation the lines remain

oligoclonal for some months; they may be difficult to clone and often lose the antibody production (Zurawski et al. 1978, Kozbor and Roder 1981). Often only small amounts of antibody are secreted (Kozbor and Roder 1983). At the begining of this study the marmoset cell line B95-8 used as a source of EBV in many different laboratories was found to be contaminated with mycoplasma (Dr. B.Bradley, personal communication).

5(v). Successes in human monoclonal autoantibody production.

Comparatively little has been published on the successful production of human monoclonal autoantibodies. Hirano et al. (1980) established autoantibody producing B cell lines by transformation of autoimmune patient's PBL with EBV. Four lines were established, two of which produced anti-nuclear factor antibodies, and one produced anti ssDNA and anti dsDNA antibodies. It was not certain that the antibody products were monoclonal, however.

Shoenfeld et al. (1982) were the first to report the production of truly monoclonal human autoantibodies by the hybridization technique. They fused PBL from 2 SLE patients, 1 cold agglutinin patient and two patients with atopic allergy with the human cell line GM 4672. They isolated 6 hybrids secreting monoclonal anti-platelet antibodies, 7 secreting anti-ssDNA antibodies, and 7 hybrids secreting monoclonal cold

agglutinins.

Eisenbarth et al. (1982) fused PBL from a patient with type I diabetes mellitus with the human cell line GM 1500. They isolated a hybrid which secreted a MCA which had similar reactivity to pancreatic islet cells as found in the patients serum.

Kamo et al. (1982) established a human B cell line by EBV transformation which secreted a MCA to AChR. The B cells were obtained from the thymus of a patient with MG, and the MCA bound rat and human muscle receptor but not receptor from the electric organs of Narke japonica. Passive transfer of the MCA into rats was demonstrated to induce muscle weakness and electromyographic changes characteristic of MG.

Valente et al. (1982) obtained four human MCA against the receptor for thyroid stimulating hormone (TSH) by fusing the mouse myeloma NS1 with PBL from patients with Graves disease. Two of these were found to stimulate thyroid function in several assay systems. These 2 MCA , as well as the 2 non-stimulatory MCA, specifically inhibited TSH binding to cultured thyroid cells.

Satoh et al. (1983) fused both mouse and human myelomas with PBL from patients with insulin-requiring diabetes and other autoimmune abnormalities. The hybridoma products were screened by indirect immunofluorescence against tissue from a variety of human organs. They found 7 MCA which reacted with antigens in

multiple endocrine organs. This may partially explain multiple organ autoimmune diseases; an antigenic determinant that occurs in several different organs can account for the apparent multiplicity of the autoantibodies in such disorders.

The potential usefulness of human MCA in the study of autoimmune disease is only just starting to be realised. It seems likely that the keen interest being shown in human MCA in many fields of pure and applied immunobiology will overcome the technical problems that have plagued their production so far.

Aims of this study.

As has been discussed, human MCA have potential applications in many fields, including the diagnosis and treatment of infectious diseases, blood and tissue typing, oncology and the study of human immune responses. The technology for the production of human MCA lags behind that of its rodent counterpart. Optimal systems for the production of human MCA have yet to be established. The prospective producer of human MCA is faced with a number of alternative methods and variations. Antibody-secreting lines can be produced by EBV transformation of human lymphocytes, by cell fusion to form hybridomas, or by a combination of the two (Kozbor and Roder, 1983). If a fusion method is chosen, the choice between human and mouse fusion partner has to be made, and then one selected from the many now available. The first aim of this project was to evaluate the performance of one human fusion partner, LICR-LON-HMy2, and two mouse partners, NS1 and X63-Ag8.653, in the production of human MCA by the PEG-induced fusion method. The LICR-LON-HMy2 line was chosen as it had been shown to successfully produce human hybrids (Edwards et al. 1982), and was the only HAT-sensitive human lymphoblastoid or myeloma line freely available at the beginning of this study. NS1 and X63 were chosen as well established mouse myelomas of proven use in the production of murine MCA. In addition, NS1 had

been shown to be useful in the production of human immunoglobulin-secreting hybrids (Schlom et al. 1980, Nowinsky et al. 1980, Wunderlich et al. 1981).

The production of human MCA can be divided into two component problems; the first is that of establishing stable, human immunoglobulin-secreting cell lines, and the second of establishing lines secreting antibodies of pre-defined specificity. Establishing specific antibody-secreting lines from rodents is critically dependent on an effective immunisation schedule and timing of fusion following a final boost. Whereas this can be completely controlled in animal work, there are far fewer circumstances in humans where this is possible. Autoimmune patients provide a population of 'naturally immunised' subjects which overcome ethical problems of deliberate immunisation. In addition, MCA have potential uses in the study of both the autoantigens and the autoantibodies in autoimmune disease. In MG, murine MCA to the AChR have been extremely useful in the detailed study of the auto-antigen. Human MCA to the AChR would be useful to study the human response to the autoantigen in terms of antibody specificity, idiotypic diversity and the relative pathogenicity of individual antibodies. In SLE, in addition to the interest in the autoantibodies, MCA would be of considerable use in probing the structure and function of many autoantigens. The ribonucleoproteins recognised by certain SLE sera are of particular interest, and murine MCA have not been readily

forthcoming, probably because of the poor immunogenicity of these molecules.

The second aim of this project was to examine the possibility of producing human MCA by fusion of PBL from autoimmune patients with demonstrable serum autoantibodies. Specifically the the questions asked were could hybrids be made from MG PBL which secreted anti-AChR antibodies, and similarly could anti-nRNP, Sm, Ro and anti-La secreting hybrids be formed from PBL of SLE patients? If such MCA could be made they would then be characterised.

Chapter Four. Materials and Methods.

Materials.

1. Cells and cell culture materials.

The HAT sensitive, human lymphoblastoid cell line LICR-LON-HMy2 was a gift from Dr P.A.W. Edwards, Ludwig Institute for Cancer Research, Surrey.

The HAT sensitive mouse myeloma NSI/1.Ag4.1 was a gift from Dr G. Farrar, Biochemistry Dept. University of Bath.

The HAT sensitive mouse myeloma X63-Ag8.653 and the human epithelial cell line HEp-2 were obtained from Flow Laboratories, Scotland.

Normal human skin fibroblasts from human foreskin, supplied at passage 11, were a gift from M. McAleer and Dr S. Moss, Dept. of Pharmacy, University of Bath.

Mycoplasma-free, marmoset cell line B95-8 and EBV transformed human tonsil B cells were a gift from Dr M.D.Melamed, M.R.C. Cambridge.

Sterile media and supplements; RPMI 1640, BME (modified) with Earle's salts, 'myoclone' FCS, NCS, L-glutamine (200mM), sodium pyruvate (100mM), 50X HAT, 50X HT concentrates, and disposable plasticware were obtained from Nunc Gibco, Flow Laboratories and Sterilin.

2. Immunological reagents.

Alkaline phosphatase conjugated antibodies and human IgA (from colostrum) were obtained from Sigma.

Goat anti-whole human serum and goat anti-whole mouse serum were obtained from Miles.

Sheep anti-human IgG and sheep anti-human IgM were a gift from Prof. G. Stevenson, Tenovus Laboratories, Southampton.

Goat anti-polyvalent human immunoglobulins was a gift from H. Lotwick, Dept. of Biochemistry, University of Bath.

3. Radio-isotopes.

^{125}I (carrier free) was obtained from the Radiochemical Centre, Amersham, Bucks.

4. Chromatography media.

Protein A Sepharose CL-4B, Sepharose 4B and DEAE Sepharose CL-6B were obtained from Pharmacia. AcA 34 Ultrogel was obtained from LKB.

5. Buffers and solutions.

Buffers and solutions were made with Analar grade reagents and double distilled water. Where not

detailed in the text, buffers and solutions were prepared according to 'Data for Biochemical Research' (Dawson et al. 1974).

6. Instruments.

Ultracentrifuge model L5-65 was supplied by Beckman. Bench centrifuges and micro-centaur centrifuge were from M.S.E. UV absorbtions were measured by a Cecil CE 212 variable wavelength monitor and a manual ELISA reader fitted with a 405 nm filter, from Gilford Instruments. A model 1280 Ultrogamma from LKB was used for gamma radiation counting. A Zeiss Orthoplan light microscope with incident UV facility was used for cell counting and immunofluorescent studies.

7. General reagents.

Trypsin (type X1) prepared from bovine pancreas, DNA (from calf thymus), Heparin, 2-mercaptoethanol, 8-azaguanine, DMSO, Cyanogen bromide, Colcemed, Morpholinopropanesufonic acid, DTT, Vinblastine sulphate, Cytochalasin B, Hoechst 33258, DNase 1, BSA and P-nitrophenyl phosphate were from Sigma.Iodoacetimide, Nigrosine, Nonided P-40 were from B.D.H. chemicals. Aprotinin was from Bayer, Germany. PEG 4000 (GK) was from Merck. Benzoquinonium chloride was a gift from Sterling Winthrop, Renssalaer, N.Y., U.S.A.

All other reagents were from Sigma, B.D.H., Fisons or Aldrich.

8. Patients and antigens.

Peripheral blood samples from SLE and MCTD patients were obtained with the co-operation of Dr P. Maddison, Royal National Hospital for Rheumatic Disease, Bath.

Peripheral blood was collected from myasthenic patients with the co-operation of Dr T.Wallington, Blood Transfusion Centre, Southmead Hospital, Bristol, Dr Cambell, Bristol Royal Infirmary and Dr Wakefield, Royal United Hospital, Bath.

Ro and La antigens, purified by affinity chromatography from human spleen extract, calf thymus respectively, were gifts from Dr P. Skinner, R.N.H.R.D. Bath. Sm and nRNP antigens, purified by affinity chromatography from calf thymus extract were gifts from D. Brennand, Dept. Biochemisty, University of Bath.

Methods.

Cells:

1. Isolation of peripheral blood lymphocytes.

20ml of freshly drawn venous blood was diluted 1:1 with PBS containing 40 I.U. ml^{-1} heparin. 10 ml

volumes were layered onto 5 ml volumes of "Lymphoprep" in 15 ml siliconised glass centrifuge tubes, according to the makers' instructions. The gradients were centrifuged at 200xg (at the interface) for 30 min at room temperature. Mononuclear cells were aspirated from the interface and washed twice by centrifugation in PBS (20 min at 400xg, then 5 min at 200xg). Cells were resuspended in complete RPMI 1640 (see 'maintenance of cell lines') and a small sample was stained with an equal volume of 10mg% Gentian Violet in 3% Glacial acetic acid. After counting in a haemocytometer counting chamber, the remaining cells were diluted to 10^6 ml^{-1} and stored for up to 24 h at 37°C in 5% CO_2 until use.

2. Depletion of T-lymphocytes by AET-SRBC rosette gradient centrifugation.

T-cell depleted PBL were obtained by the method of Madsen et. al.(1980). SRBC of less than two weeks of age were suspended in 140 mM AET, pH 9.0 for 15 min at 37°C. They were then washed by centrifugation five times in isotonic saline at 500xg. AET-SRBC were stored up to one week in PBS containing 40% heat inactivated FCS at 4°C.

3 ml of cell suspension in PBS at a density of $3x10^6$ ml^{-1} was added to 3 ml of 1% v/v AET-SRBC in 40% FCS and cooled to 4°C for 30 min. They were then centrifuged at 200xg for 15 min at room temperature. The supernatant was

removed and the pellet overlaid with 5 ml of Percoll adjusted to a specific gravity of 1.078 with PBS. 1 ml of PBS/FCS was then layered over the Percoll. The tubes were centrifuged at 550xg for 20 min at room temperature. The non-rosetted cells (non-T cells) were collected from the interface and washed. These were found upon re-rosetting to be contaminated with < 3% rosetting cells.

3.Transformation of lymphocytes by EBV.

Isolated human PBL, depleted of T cells, were suspended in undiluted B95-8 culture supernatant at 1 x 10^6 ml^{-1}. After incubation for 1 h at 37°C with constant agitation, the cells were spun down and resuspended in fresh, complete RPMI, at a concentration of 2 x 10^5 ml^{-1}. Cells were then cultured at 37°C in 5% CO_2 and fed and expanded when necessary.

4.Maintenance of cell lines.

Three cell lines were used as fusion partners; two mouse myelomas (NS1, X63-Ag8.653.) and one human lymphoblastoid line (LICR-LON-HMy2). They were all grown in RPMI 1640 supplemented with 10% FCS, 2mM. glutamine, $5x10^{-5}$M 2-mercaptoethanol, and 60 μg ml^{-1} tylocine, in an atmosphere of 5% CO_2 in air, at 37°C. Under these conditions the cell lines had doubling times of 18-24 h, and were maintained in log phase growth by daily

sub-culturing. Prior to fusions, the viability of the cells (determined by nigrosine dye exclusion) exceeded 95%. Periodically 8-Azaguanine was included in the culture medium at 20 μg ml^{-1} to eliminate any HAT insensitive variants.

Human epithelial cell line HEp-2 and normal human fibroblasts were grown as monolayers in B.M.E. with Earle's salts supplemented with 10% NCS, 2mM glutamine and 60 μg ml^{-1} tylocine, in an atmosphere of 5% CO_2 at 37°C. When cells had grown to confluency, they were harvested by brief trypsinisation with 0.025% crude trypsin in PBS, and new cultures initiated at a split ratio of 1:40 for HEp-2 and 1:4 for fibroblasts.

B95-8 cells were grown as monolayers in complete RPMI. Cells were seeded at 1 x 10^5 ml^{-1}. For the production of virus, cells were allowed to grow for 7-10 days and the supernatant harvested, filtered through a 0.45 m filter, and stored at 4°C for up to 1 week. New cultures were initiated from confluent, but not overgrown flasks following brief trypsinisation as for human fibroblasts.

5. Screening cell lines for Mycoplasma contamination.

Cell lines were periodically checked for mycoplasma contamination by Hoechst 33258 staining as described by Freshney (1983). This stain binds specifically to DNA, and mycoplasmas can be detected

under a UV microscope by their characteristic particulate or filamentous pattern on the cell surface.

(a). Monolayer cultures:

Cells in mid-log phase growth were seeded on multispot microscope slides and allowed to attach overnight at 37°C in a humidified, gassed incubator. The slides were then rinsed in PBS followed by acetic acid-methanol (1:3) diluted with an equal volume of PBS. The cells were fixed for 10 min in pure acetic acid-methanol, then rinsed thoroughly with distilled water. Slides were immersed for 10 min in PBS containing 50 ng ml^{-1} Hoechst 33258. After rinsing with water, the cells were mounted in buffered glycerol (Glycine 0.42g, NaOH 0.02g, NaCl 0.51g, H_2O 30 ml, Glycerol 70 ml), and examined under an epifluorescent microscope, using a Zeiss UG-1 filter and dichroic mirror for excitation, and K400, K460 filters for emission.

(b) Suspension cultures:

$5x10^6$ cells in mid log phase growth were washed with PBS by centrifugation (5 min 200xg). The pellet was resuspended in 5 ml of PBS and 5 ml of acetic acid-methanol added with constant stirring. The cells were pelleted by centrifugation then resuspended in pure acetic acid-methanol and fixed for 10 min. The cells were

pelleted by centrifugation and resuspended in 300 µl of fresh fixative. 50 µl of this suspension was dropped onto a cold microscope slide soaked in methanol. After drying in air, the cells were stained as for monolayer cultures.

6. Viability of cells.

Cell viability was determined by nigrosine dye exclusion. Cells in suspension were mixed with an equal volume of 0.2% nigrosine in PBS and incubated for 5 min at room temperature. The cells were counted in the counting chamber of a haemocytometer. Dead cells took up the stain and appeared black.

7. Preparation of mouse peritoneal cell feeder layers.

Mice were killed by cervical dislocation and the abdominal skin removed to reveal the peritoneal wall. 5 ml of sterile PBS was injected into the peritoneal cavity and, after gentle massage, removed with a pasteur pipette via a small incision above the liver. Cells were irradiated with 2000 Rads from a ^{60}Co source and centrifuged for 5 min at 200xg. The cells were resuspended in complete RPMI medium, diluted appropriately and transferred to 96 well trays such that each well received 10^4 cells in 50 µl. The trays were kept at 37°C in a humidified atmosphere of 5% CO_2 in air, for 24 h before use.

8. Fusions.

PBL and myeloma cells were added together in a 40 ml siliconised glass centrifuge tube, diluted with PBS and spun to a common pellet for 10 min at 200xg. The ratio of PBL to myelomas was between 1:1 and 2:1. After loosening the pellet, 1 ml of 50% PEG in PBS was added dropwise over one min with constant agitation. Agitation was continued in a water bath at 37°C for 90 s, after which the cells were slowly diluted with PBS at room temperature, then gently resuspended in complete RPMI 1640, and distributed in 96 well trays pre-seeded with mouse peritoneal cells, such that each well received $1x10^5$ myeloma cells in 100 µl of medium. After 24 h incubation, 100 µl of medium containing 2 x HAT was added. In fusions with EBV-transformed lymphocytes, 1 µM oubain was included in the medium to prevent the outgrowth of unfused lymphoblastoid lines. Wells were examined after 7 days when feeding was started by replacing half the medium with fresh 1 x HAT. Feeding was repeated every two or three days. Wells positive for growth were screened for immunoglobulin or antibody production when cells covered half the bottom of the well. Hybrids of interest were cloned directly from the 96 well trays. HAT selection was maintained for three weeks post fusion and the cells passaged through HT medium before culturing in standard medium.

9. Cloning of hybrids.

Hybrids were transferred singly from a culture in log phase growth, by use of a micromanipulator and microscope, to individual wells of a 96 well tray pre-seeded with feeder cells. To ensure only single cells were transferred, cells were only picked up when alone in the field of a high power lens, and expelled into an intermediate vessel for examination before being moved to the final well.

10. Cryopreservation of cells.

Cells in log phase growth were pelleted by centrifugation. The pellet was resuspended in complete medium such that the concentration did not exceed $2x10^7$ ml^{-1}. The suspension was then chilled on ice for 15 min and an equal volume of cold freezing mixture (20% DMSO, 40% FCS, 40% RPMI) was added with stirring. 1 ml aliquots were transferred to freezing ampoules, placed in the vapour phase of liquid nitrogen overnight at a freezing rate of -1^oC per min, then stored in the liquid phase until required. For recovery of frozen cells, ampoules were rapidly thawed at 37^oC, washed once by centrifugation, then cultured at high density until fully recovered.

11. Preparation of cell monolayers for immunofluorescence.

Confluent cultures of HEp-2 or human fibroblasts were trypsinised and suspended in complete medium. 40 µl aliquots were then transferred to each well of a 10 spot microscope slide, previously heat sterilised. The slides were placed in clean petri dishes and incubated overnight at 37°C in a humidified incubator. After a 5 min wash in PBS the slides were immersed in ice cold acetone for 1 min and allowed to dry in air. Alternatively some slides were fixed in 2% paraformaldehyde in PBS for 15 min at room temperature immediately before the acetone step. Fixed slides were stored dessicated at -20°C until required.

12. In situ fractionation of cells.

Cells grown as monolayers on microscope slides were fractionated in situ by the method of Staufenbiel and Deppert (1984). Cell monolayers were washed three times with 10mM Morpholinopropanesulfonic acid, pH 6.2; 10mM NaCl; 1.5mM $MgCl_2$; 30 µg ml^{-1} Aprotinin; 10% glycerol (Kern matrix buffer, KM). For the first extraction step, the cells were incubated at 4°C with KM buffer containing 1% NP40, 1mM EGTA and 5mM DTT. After 3 min the extraction buffer was replaced with fresh extraction buffer and incubation continued for 27 min.

The slides were washed three times with buffer A and incubated for 15 min at 37°C with KM buffer containing 50 μg ml^{-1} DNase 1. After washing, the slides were incubated for 30 min at 4°C in KM buffer containing 2M NaCl, 1mM EGTA and 5mM DTT. The slides were then washed in KM buffer. Slides were fixed after each step of the extraction procedure for 1 min in cold acetone and stored at -20°C until required.

13. Drug disarrangement of cytoskeletons.

HEp-2 cells or fibroblasts were grown overnight on microscope slides. The medium was aspirated from the slides and the cells were treated with medium containing either 0.5 μg ml^{-1} Colcemid for 24 h, 10 μg ml^{-1} vinblastine sulphate for 4 h, or 10 μg ml^{-1} cytochalasin B for 30 min. The cells were rinsed with PBS and fixed in cold acetone for 1 min. The slides were stored at -20°C until required.

14. Chromosome Analysis of cells.

Hybrids were harvested in log phase growth and suspended at 10^6 cells ml^{-1} in complete medium containing 0.1 μg ml^{-1} Colcemid for 4 h. Hypotonic treatment with 0.075M KCl for 12 min was followed by fixation with 1:3 acetic acid:methanol. After two changes of fixative, the cells were dropped on cold, methanol soaked slides and

air dried. Chromosomes were counted after staining for 10 min with 2% Giemsa in PBS, or following Hoechst staining as described under 'Screening for Mycoplasma'. At least forty mitotic figures were counted for each line.

General Methods:

15. Preparation of immunoglobulins.

Human IgM was prepared from Waldenstrom macroglobulin sera according to the method of Jehanli and Hough (1981). Serum samples were applied to a column of Ultrogel AcA 34 and eluted with 0.1M Tris/HCl, 0.15M NaCl, 0.02% NaN_3, pH 8.0. IgM containing fractions were located by double diffusion with sheep anti-human μ chain antiserum. Fractions containing IgM were pooled, concentrated by ultrafiltration and dialysed against 0.05M Na_2HPO_4/citric acid buffer, pH 6.8. Ion-exchange chromatography of the partially purified IgM was carried out on a 2.5 x 40 cm column of DEAE Sepharose CL6B, equilibrated with 0.05M Na_2HPO_4/citric acid buffer, pH 6.8. The column was eluted with 300 ml of starting buffer followed by a linear gradient made from starting buffer (300 ml) and 0.1M Na_2HPO_4/citric acid, pH 5.0 (300 ml). Residual bound protein was removed from the column with 0.4M NaCl. Fractions were examined by immunoelectrophoresis using sheep anti-whole serum proteins. Fractions giving a single precipitin arc

corresponding to IgM were pooled, dialysed against 0.1M Tris/HCl, 0.15M NaCl buffer pH 8.0, and concentrated to 10 mg ml^{-1}.

Human IgG was prepared from pooled normal human serum by affinity chromatography on protein-A sepharose CL4B. Serum was applied to the column of sepharose and washed with PBS until the optical density was back to baseline. IgG was then eluted with 0.2M citric acid/phosphate buffer, pH 3.2 containing 0.5M NaCl. The elution was monitored by UV absorbtion at 280 nm. The IgG was collected and dialysed against PBS and stored at $-20^{o}C$.

Mouse IgG was prepared from serum by affinity chromatography on protein-A sepharose CL4B. The pH of the serum was adjusted to 8.0 with 0.1M NaOH, and diluted 1:1 with 0.1M phosphate buffer, pH 8.0. The serum was applied to the column and washed until no more protein was washed through. The procedure was then identical to the preparation of human IgG.

Purity of immunoglobulins was confirmed by immunoelectrophoresis and SDS-PAGE.

16. Protein determination.

Protein concentrations were determined by UV absorbtion at 280 nm. The extinction coefficients (E_{280}) were taken from Johnstone and Thorpe (1982): IgG 13.6, IgM 11.8, secretory IgA 12.6. For other proteins the

following formula (taken from the same source) was used:

Protein conc.(mg ml^{-1})= 1.55 x absorbance at 280nm - 0.77 x absorbance at 260nm.

17. Affinity columns.

Sepharose 4B was activated by cyanogen bromide according to the method of March et al. (1974). 20g of washed sepharose was suspended in 40 ml of 2M K_2CO_3 and stirred slowly. 2g of cyanogen bromide, dissolved in 2 ml of acetonitrile was added at once to the slurry on ice, and stirring was continued for 2 min. The mixture was then poured into a coarse sintered-glass funnel and washed with cold water and 0.1M $NaHCO_3$. Protein to be coupled was dissolved in 20 ml of 0.1M sodium bicarbonate buffer, pH 8.0, and added to the activated gel. The suspension was stirred overnight at 4^oC, washed with PBS and unreacted sites blocked by incubation in 1M ethanolamine, pH 8.0 for 1 h. After washing in PBS, 0.5M acetic acid and PBS again, the coupled gel was stored in PBS containing 0.02% sodium azide at 4^o.

18. Affinity isolation of antibodies.

(a) Anti- μ chain antibodies:

Sheep antiserum to human IgM was passed down a column of IgM-sepharose. Anti-IgM antibodies were eluted

with ice cold 0.5M acetic acid. The protein was immediately dialysed against PBS. Denatured protein was removed by centrifugation (30 min at 10,000xg). Anti-light chain activity was removed by two passages through an IgG-sepharose column. The pure antibody was stored at $4^{o}C$ in PBS at 1.0 mg ml^{-1}.

(b) Anti- γchain antibodies:

Sheep antiserum to human IgG was passed down a column of IgG-sepharose. Anti-IgG antibodies were prepared as described above. Anti-light chain activity was removed by passage down an IgM(κ) column and an IgM(λ) column. The pure antibody was stored as above.

19. SDS Polyacrylamide gel electrophoresis.

SDS-PAGE was performed according to the method of Laemmli (1970) using a slab gel apparatus. Standards used were alpha-2-macroglobulin (Mr 174,000), human chain (Mr 74,000), BSA (Mr 68,000), human chain (Mr 50,000), ovalbumin (Mr 43,500), human light chain (Mr 25,000), and ribonuclease A (Mr 13,500).

20. Radio-iodination of α-BTX.

α -BTX was labelled to a high specific activity with ^{125}I by the method of Urbaniak et al. (1973). All

procedures were carried out at room temperature, and unless otherwise stated all solutions were made in 0.05M potassium phosphate pH 7.5. Carrier free $Na^{125}I$ was stored at ambient temperature, and was used for up to three weeks after the quoted reference date. $Na^{125}I$ (100 mCi ml^{-1}, 10 µl) in dilute NaOH was added to α-BTX (1 µg, 20 µl). Chloramine T (0.5% w/v, 10 µl) was then added and the solution stirred for 1 min, after which sodium metabisulphate (0.016% w/v, 750 µl) and potassium iodide (1% w/v, 10 µl) were added. The reaction mixture was immediately applied to a Sephadex G25 column equilibrated with 0.01M potassium phoshate pH 7.4 containing 1% BSA, and eluted with the same buffer. 1 ml fractions were collected and 5 µl aliquots counted in an LKB Ultrogamma counter. The radioactive protein peak fractions were pooled, and the specific activity of the labelled toxin calculated assuming 100% recovery of protein.

21. Preparation of crude human AChR.

A modification of the method described by Stephenson et al. (1981) was used. Adult human limb muscle was obtained from patients undergoing amputation because of vascular abnormalities or diabetic gangrene. In the latter case only limbs in which gangrene was confined to the toes were used. The muscle, mainly gastrocnemious, was coarsely chopped and frozen immediately in liquid nitrogen. All subsequent operations were carried out at

4°C and buffers contained 1 mM PMSF, 0.02% sodium azide and 1 mM EDTA in 0.01 M potassium phosphate pH 7.4. Thawed muscle (200-300 g) was homogenised in a Waring blender for 1 min at full speed in 5 vol of buffer containing 0.1 mM benzethonium chloride, 10 μg ml^{-1} soyabean trypsin inhibitor, 1 mM benzamidine hydrochloride, 100 μg ml^{-1} bacitracin, 50 mM NaCl and 10 mM iodoacetamide. After centrifugation at 20,000xg for 1 h, the pellet was homogenised in 1 - 2 vol of the above buffer containing 2% v/v Triton X-100, and was extracted by paddle stirring for 3 h. The extract was centrifuged for 1 h at 100,000xg and the supernatant containing solubilised receptor stored at 4°C until assay and use.

Assays.

22.Assay for human immunoglobulin.

Supernatants were screened for production of human immunoglobulin by an enzyme-linked immunoassay. Gilford EIA cuvettes were coated with 250 μl of PBS containing 10 μg ml^{-1} anti-human immunoglobulin heavy chain specific antibodies for 24 h at 4°C. Cuvettes were then blocked by incubation with PBS containing 1.0% casein for 15 min. After washing with PBS containing 0.05% Tween 20, 50 μl of culture supernatant and 200 μl of PBS-Tween were added to each well and incubated for 1 h at 37°C. The cuvettes were washed and 250 μl of

alkaline phosphatase conjugated goat anti-human μ, γ, α, κ, or λchain specific antibody (diluted 1:1000 in PBS-Tween) was added to each well. After 1 h incubation at 37°C, the cuvettes were washed and 250 μl of p-nitrophenyl phosphate (1mg ml^{-1}) in 10% diethanolamine buffer (pH 9.6) was added to each well. Colour changes were monitored by a Gilford EIA reader with a 405nm filter.

23. CELIA for estimation of human immunoglobulins.

To determine immunoglobulin concentrations, a competitive enzyme-linked assay was employed. Cuvettes were coated overnight at 4°C with human IgM, IgG or IgA at 200 ng ml^{-1} in 0.05M carbonate buffer pH 9.6. Serial dilutions of standard immunoglobulins and appropriately diluted culture supernatants in PBS-Tween were transferred to LP3 tubes (Luckhams). An equal volume of alkaline phosphatase conjugated antibody (1:1000 in PBS-Tween) was added and the tubes incubated for 1 h at 37°C. 250 μl of the mixture was then added to the cuvettes previosly blocked and washed as before. The cuvettes were incubated, washed and substrate added as above. A standard curve was constructed by expressing the A_{405} of the wells containing standard Ig as a percentage inhibition when compared to the control wells incubated with conjugate alone.

24. ELISA for RF and antibodies to Ro, La, nRNP, Sm, DNA.

Gilford cuvettes were coated overnight at 4°C with poly-L-lysine in PBS at 50 μg ml^{-1}. After washing three times with PBS-Tween, antigen was added at 10 μg ml^{-1} in PBS. Cuvettes were incubated overnight at 4°C. After washing three times with PBS-Tween, unoccupied sites were blocked by incubation for 30 min at room temperature with 1% casein in PBS. 250 μl of culture supernatant diluted 1:1 in PBS-Tween was added to each well and incubated overnight at 4°C. The cuvettes were washed three times with PBS-Tween and then 250 μl of the appropriate anti-human heavy chain specific antibody-alkaline phosphatase conjugate was added. After 2 h incubation at 37°C, the cuvettes were washed as before an 250 μl of substrate was added. Colour changes were monitored on a Gilford EIA reader. Controls included standard human reference sera and normal human sera diluted $1:10^4$ in PBS-Tween, irrelevant monoclonal immunoglobulins in culture medium and culture medium from unfused X63-Ag8.653 cells.

25. Assay for AChR.

For the assay of crude AChR preparations, an ammonium sulphate precipitation assay was used, based on the method of Meunier et al. (1972). Crude receptor (100 μl) was mixed with ^{125}I-α-BTX (0.5-1.5 nM in 50 μl) and

incubated for 45 min at room temperature. Saturated ammonium sulphate solution (133 µl) was added to give a 40% v/v final concentration. The samples were left for 16 h at 4°C, then 40% ammonium sulphate (1ml) was added and the samples filtered on Whatman GF/C filter discs. Each filter disc was washed with 3 ml of 40% ammonium sulphate and counted on an LKB 1280 Ultrogamma counter. The specific binding was measured by carrying out all assays in the presence of 0.01M benzoquinonium chloride (50 µl). The ^{125}I-α-BTX that remained bound in the presence of this competing ligand was defined as non-specifically bound. The specific binding was defined as that proportion of total radioactivity that was displaced by the ligand. Receptor activity was expressed as the molarity of ^{125}I-α-BTX binding sites.

26. RIA for antibodies to human AChR.

The radioimmunoassay for the detection of anti-acetylcholine receptor antibodies was a modification of the method of Lindstrom (1977). Solubilised acetylcholine receptor from human muscle was incubated with a five fold excess of ^{125}I-α-BTX for 45 min at room temperature. 0.1 pmol. samples of labelled receptor were then transferred to LP3 tubes and each incubated with 100 µl of culture supernatant for 2 h at room temperature. 5 µl of normal human serum was added to each tube followed by 50 µl of goat anti-polyvalent human immunoglobulins. A

precipitate was allowed to form for 2 h at room temperature. Samples were then centrifuged at 3000xg for 10 min, and the pellet washed three times by centrifugation with 0.01M potassium phosphate buffer pH 7.4, containing 0.15M NaCl. The pellet was counted in an LKB 1280 Ultrogamma counter. Control samples were pre-incubated with a five fold excess over α-BTX of benzoquinonium. The resulting counts were subtracted from the test values to give specific counts of Ab-toxin-receptor complex precipitated.

27. Indirect immunofluorescence.

Forty µl of culture supernatant were applied to a well of fixed HEp-2 cells or human fibroblasts on a multispot slide. Control wells received either ANA positive or normal serum diluted 1:40 in PBS. Slides were incubated for 30 min in a humidified chamber at 37°C. After three washes of 5 min in PBS, 20 µl of goat anti-polyvalent human immunoglobulins (Sigma), diluted 1:20 in PBS, was added to each well. The slides were incubated for 30 min as before, washed three times in PBS, and counterstained for 5 min in 0.01% Evans Blue. The cells were mounted in glycerol and observed immediately under a UV microscope fitted with Zeiss filter set: (2xKP409/K510/K110).

Chapter 5. The production of human immunoglobulin-secreting hybridomas.

1. The fusion of HMy2 with normal PBL.

Six fusions were performed to investigate the suitability of this cell line for the production of human monoclonal antibodies. PBL were isolated from 20 ml volumes of blood, freshly drawn from healthy individuals and fused to HMy2 cells as described (chapter 4). Hybrids were visible to the naked eye from three weeks after fusion, and wells were scored for hybrid growth at five weeks. The results are summarised in table 5.1.

Eleven out of the 20 wells positive for growth produced IgM and none produced IgA. All hybrids were positive for IgG. As HMy2 produces IgG it was not possible from these assays to tell whether any of the secreted IgG was derived from the lymphocyte fusion partner.

Table 5.1. Success of fusion of HMy2 with normal PBL

Fusion	Lymphocytes fused	Wells seeded	Wells with growth
1	2.1×10^7	192	0
2	2.4×10^7	192	2
3	1.8×10^7	180	0
4	2.0×10^7	192	17
5	1.5×10^7	150	1
6	2.6×10^7	192	0
Totals	12.4×10^7	1098	20

2. The fusion of HMY2 with T-cell depleted PBL.

Immediately after fusion, the cell population plated out is a mixture of autologous, semi-allogeneic, and allogeneic components, which might be expected to generate cytotoxic immune reactions of parental lymphoid cells against the HMy2 line and hybrids. Three fusions were carried out to see if the frequency of hybrid outgrowth could be improved using T-cell depleted PBL. Cells were isolated from 40ml samples of blood, half were T-cell depleted and the other half untreated. Both populations were separately fused, and wells positive for growth counted after five weeks. Four wells contained hybrids from the unfractionated samples and no viable hybrids resulted from the T-cell depleted fractions.

3. Fusion of NS1 and X63 with normal PBL

A series of fusions were performed to examine the fusion frequency of NS1 and X63 myeloma cell lines with normal PBL. The results are summarised in tables 5.2 and 5.3. All fusions yielded hybrids and many hybrids produced human immunoglobulins. As the X63 line fused with the higher frequency, this line was used to fuse with a series of SLE and MG PBL.

Table 5.2. Success of fusion of NS1 with normal PBL.

Fusion	Lymphocytes fused	Wells seeded	Wells +ve growth	Wells +ve human Ig
1	2.4×10^7	192	25	11
2	1.9×10^7	190	33	9
3	1.7×10^7	170	34	5
Totals	6.0×10^7	552	92	25

Table 5.3. Success of fusion of X63 with normal PBL.

Fusion	Lymphocytes fused	Wells seeded	Wells +ve growth	Wells +ve human Ig
1	2.2×10^7	192	111	22
2	1.6×10^7	160	96	16
3	2.6×10^7	192	121	30
4	2.0×10^7	192	119	33
Totals	8.4×10^7	736	447	101

4. The fusion of X63 with SLE and MG PBL.

Twelve fusions were performed with SLE PBL and ten with MG PBL. The results are presented in tables 5.4 and 5.5. An estimate of the number of hybrids resulting from each fusion can be obtained from the observed proportion of plated wells which show growth:

$$m = - \log_e (1 - p)$$

where m = mean number of hybrids per well.

and p = proportion of wells showing growth

(Galfre and Milstein 1981).

The number of hybrids produced by fusion expressed as a fraction of the number of lymphocytes used is known as the fusion frequency. This was calculated from the data. The mean fusion frequency of X63 with normal PBL was 8.4×10^{-6} (S.D. 1.1×10^{-6}), with MG PBL 4.8×10^{-6} (S.D. 4.5×10^{-6}), and with SLE PBL was 4.5×10^{-6} (SD 2.8×10^{-6}).

Table 5.4. Success of fusion of X63 with SLE PBL.

Patient	Lymphocytes fused	Wells seeded	Wells +ve growth	Wells +ve human Ig.
H.S.	1.7×10^7	170	34	5
J.B.	2.0×10^7	192	108	28
D.T.	3.0×10^7	192	140	30
N.G.	1.3×10^7	130	32	12
E.A.	3.0×10^7	192	119	22
M.W.	1.2×10^7	120	10	1
C-S.*	3.8×10^7	192	85	22
C-S.*	3.4×10^7	192	87	18
C-S.*	3.0×10^7	192	104	24
S.Wo.	1.4×10^7	140	7	1
S.Wa.	2.0×10^7	192	102	46
E.C.	0.9×10^7	90	25	11
Totals	2.7×10^8	1994	853	220

* Repeat samples from the same patient

Table 5.5. Success of fusion of X63 with MG PBL.

Patient	Lymphocytes fused	Wells seeded	Wells +ve growth	Wells +ve human Ig.
J.R.	1.1×10^7	96	20	4
J.Ro.	2.5×10^7	192	26	5
R.H.	2.5×10^7	192	61	13
S.P.	1.6×10^7	160	10	5
Sh.*	10×10^7	480	408	153
Sh.*	10×10^7	480	421	211
M.P.	2.7×10^7	192	38	3
S.T.	1.5×10^7	150	10	0
J.A.	1.6×10^7	160	84	24
J.S.	3.5×10^7	192	174	41
Totals	3.7×10^8	2294	1252	459

* PBL obtained from leukapheresis packs.

5. The fusion of X63 with EBV-transformed lymphocytes.

Two fusions were performed using X63 and EBV-transformed PBL, and one fusion using X63 and EBV transformed tonsil B cells. The results are shown in table 5.6.

Table 5.6 Success of fusion of X63 and EBV-transformed B cells.

Fusion	Lymphocytes fused	Wells seeded	Wells +ve growth	Wells +ve human Ig
1-PBL	1.0×10^6	96	59	51
2-PBL	"	"	64	52
3-Tonsil	"	"	66	61

The calculated fusion frequencies were 105×10^{-6} and 92×10^{-6}, for EBV-PBL and 112×10^{-6} for EBV-tonsil cells.

6. Cloning and stability of hybrids.

Thirteen hybrids were selected for cloning, including two from MG patients, eight from SLE patients and three from normal subjects. Ten produced IgM, 2 produced IgG and one produced IgA. All except the IgA producing hybrid were selected on the basis of an antibody specificity.

Hybrids were cloned directly from the 96 well trays. The percentage of seeded wells which gave rise to daughter clones (i.e. the cloning efficiency) varied between 4% and 22%, with a mean of 9.6%. The fastest growing immunoglobulin positive clone for each hybrid was immediately re-cloned. The cloning efficiencies improved to a mean of 28% (range 6.3% to 46%).

Immunoglobulin synthesis was not detected in any daughter clones for one of the hybrids. However, for the remaining 12 hybrids between 29% and 90% of the daughter clones (mean 65%) were found to be producing human immunoglobulin after the first cloning.

On recloning, between 90% and 100% of the daughter clones were found to be producing immunoglobulin for all hybrids except one. This hybrid yielded only 74% immunoglobulin secreting clones. After recloning a third time, 92% of the daughters from this hybrid were positive for immunoglobulin secretion.

All 12 cloned hybrids continued immunoglobulin secretion through 6-8 months of continuous culture.

7. Chromosome content of hybrids.

Chromosome numbers of unfused X63 and four cloned hybrids are presented in table 5.7. The hybrids were randomly selected and include two from normal subjects (MR and RH), one from an MG patient (JA) and one from an SLE patient (SW). Representative metaphase spreads are shown in fig 5.1.

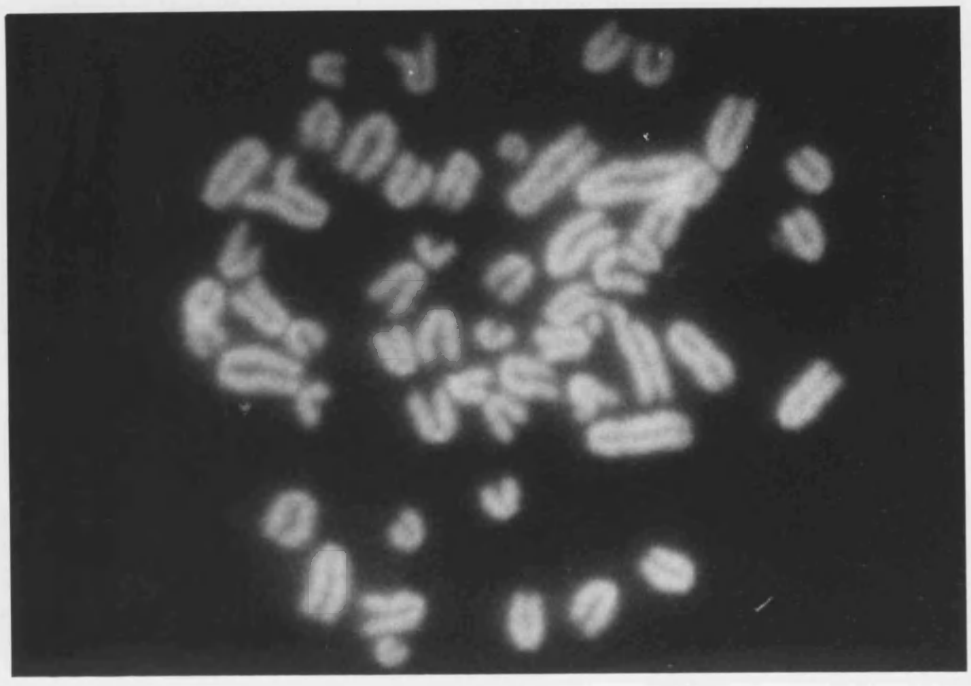
Table 5.7 Chromosome content of hybrid cells.

Cell line	No. of chromosomes (mode and range).
X63	49 (45-53)
MRB1	54 (44-61)
RHB6	91 (80-122)
JAE6	88 (69-113)
SWF2	95 (72-110)

Fig. 5.1 Representative metaphase spreads of X63 and derived hybrids.

(a) Unfused X63, (b) Hybrid RHB6, (c) Hybrid JAE6. Chromosomes are stained with Hoechst 33258. Hybrid spreads are shown at lower magnification than X63.

(a)



(b)

(c)

8. Heavy chain and light chain isotype distribution of secreted immunoglobulin.

All hybrids obtained from fusions of NS1 and X63 with PBL from normals, autoimmune patients and from fusions with EBV transformed cells, were screened for production of human IgG, IgA and IgM. The proportion of the classes detected in the different groups is presented in table 5.8.

Table 5.8 Distribution of immunoglobulin class secreted by mouse-human hybrids.

Fusion partners:	% Hybrids secreting: IgG	IgA	IgM	No. screened
NS1-normal PBL	16	0	84	25
X63-normal PBL	7	2	91	101
X63-SLE PBL	19	10	71	220
X63-MG PBL	18	5	77	459
X63-EBV PBL	12	3	85	96
X63-EBV tonsil	11	8	81	61

Supernatants of immunoglobulin secreting hybrids cultured for periods over two months were randomly selected and screened for kappa and lambda light chains. The results are presented in table 5.9.

Of 12 cloned hybrids cultured for periods of at least six months, 10 were found to produce lambda chains and 2 to produce kappa chains.

The kappa:lambda ratio was tested for association with fusion partner (tonsil vs PBL) by X^2. There was found to be evidence of an association (X^2= 4.856, d.f.= 1, $p < 0.05$).

Table 5.9 Light chain isotype frequencies of immunoglobulins secreted by hybrids.

Fusion partners:	% Hybrids secreting: Kappa	Lambda	No. screened
X63-PBL	32	68	43
X63-EBV PBL	27	73	96
X63-EBV tonsil	46	54	61

8. Quantity of immunoglobulin secreted by hybrids.

Standard curves for the CELIA for human IgG, IgM, IgA and mouse IgG (whole molecule) are shown in figs. 5.2 - 5.6. Each point represents the mean of triplicate determinations; the standard deviations were within $\pm$ 2.5%. The assays were specific and linear for each class of immunoglobulin over a range of approximately 10 ng ml^{-1} to 100 ng ml^{-1}.

The effect of mouse immunoglobulin on the assays for human immunoglobulin was investigated using dilutions of mouse serum. No inhibition was seen even at immunoglobulin concentrations of 100 μg ml^{-1} (assuming IgG to be 11.7 mg ml^{-1}, IgM to be 1.0 mg ml^{-1} and IgA to be 0.26 mg ml^{-1}, Hudson and Hay 1980).

Culture supernatants were collected as cells needed feeding. Pooled samples from 11 cloned and 24 uncloned hybrids were assayed for human immunoglobulin concentration. This was found to vary between 1 μg ml^{-1} and 12 μg ml^{-1} for the different lines. There was no apparent difference in the quantity secreted between hybrids secreting different classes of immunoglobulin. No mouse immunoglobulin was detected in any of these supernatants, in an ELISA sensitive to 10ng ml^{-1} of mouse IgG.

Fig. 5.2 CELIA for IgM. Standard curve and inhibition by other Igs.

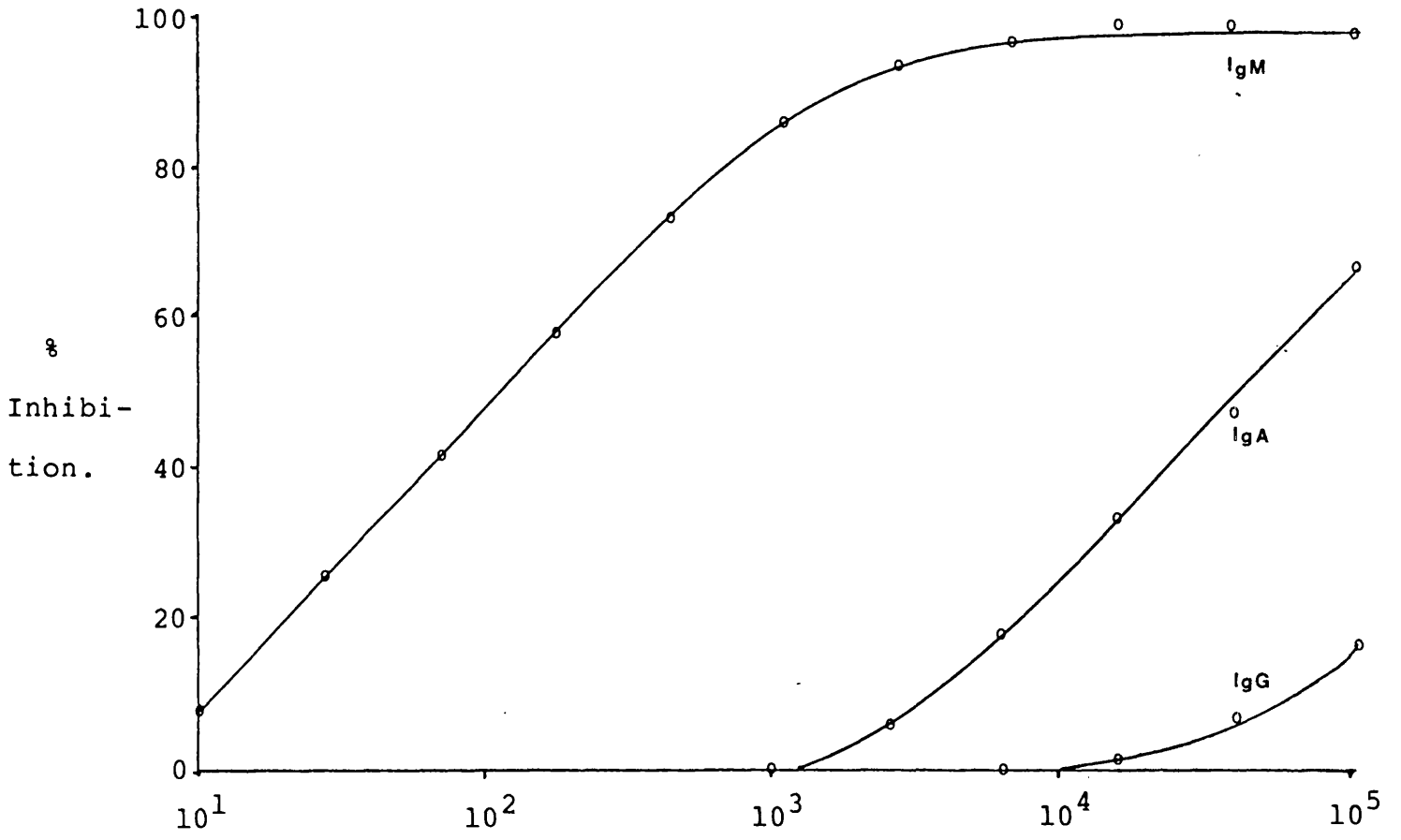


Fig.5.3 CELIA for IgG. Standard curve and inhibition by other Igs.

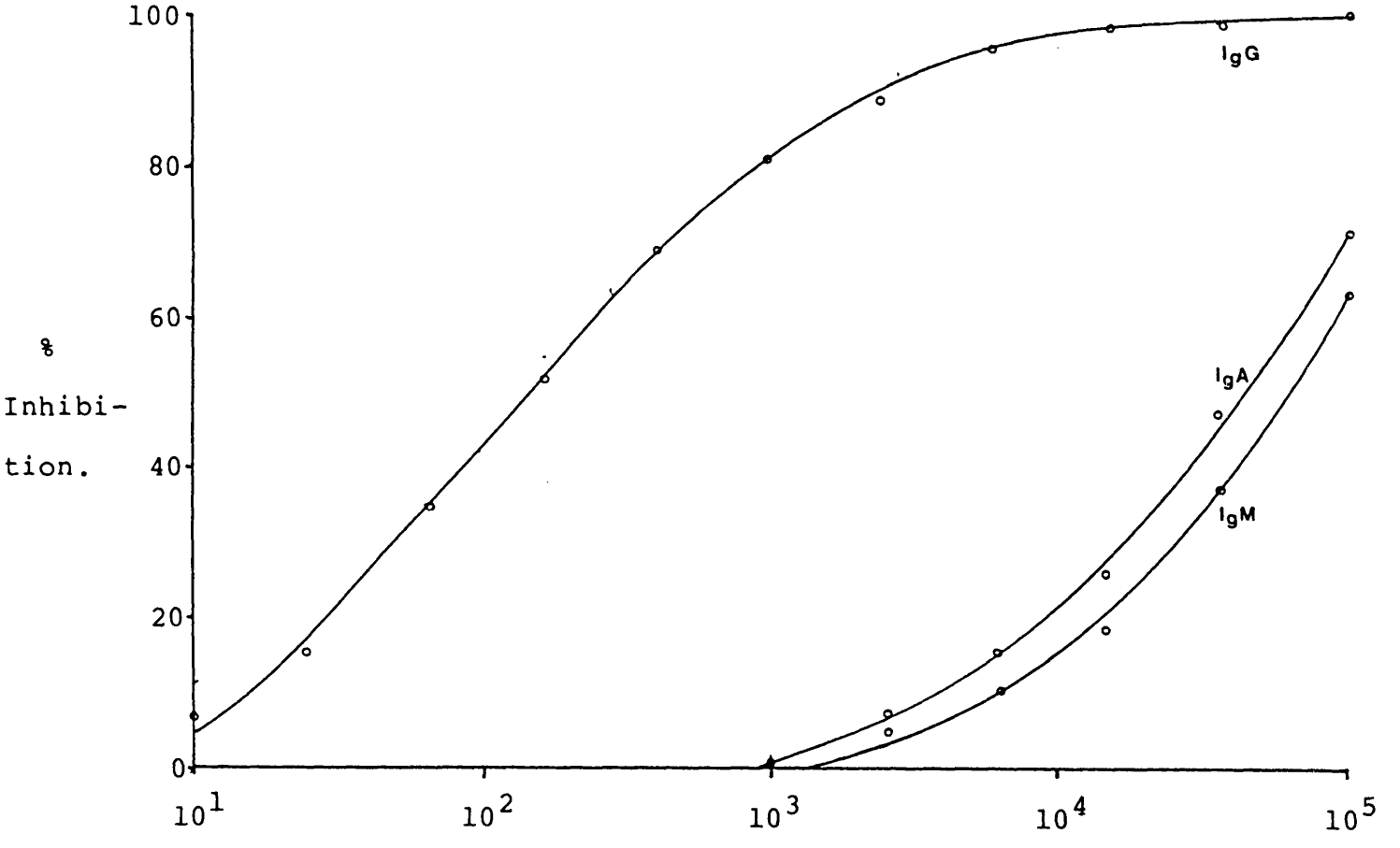


Fig.5.4 CELIA for IgA. Standard curve and inhibition by other Igs.

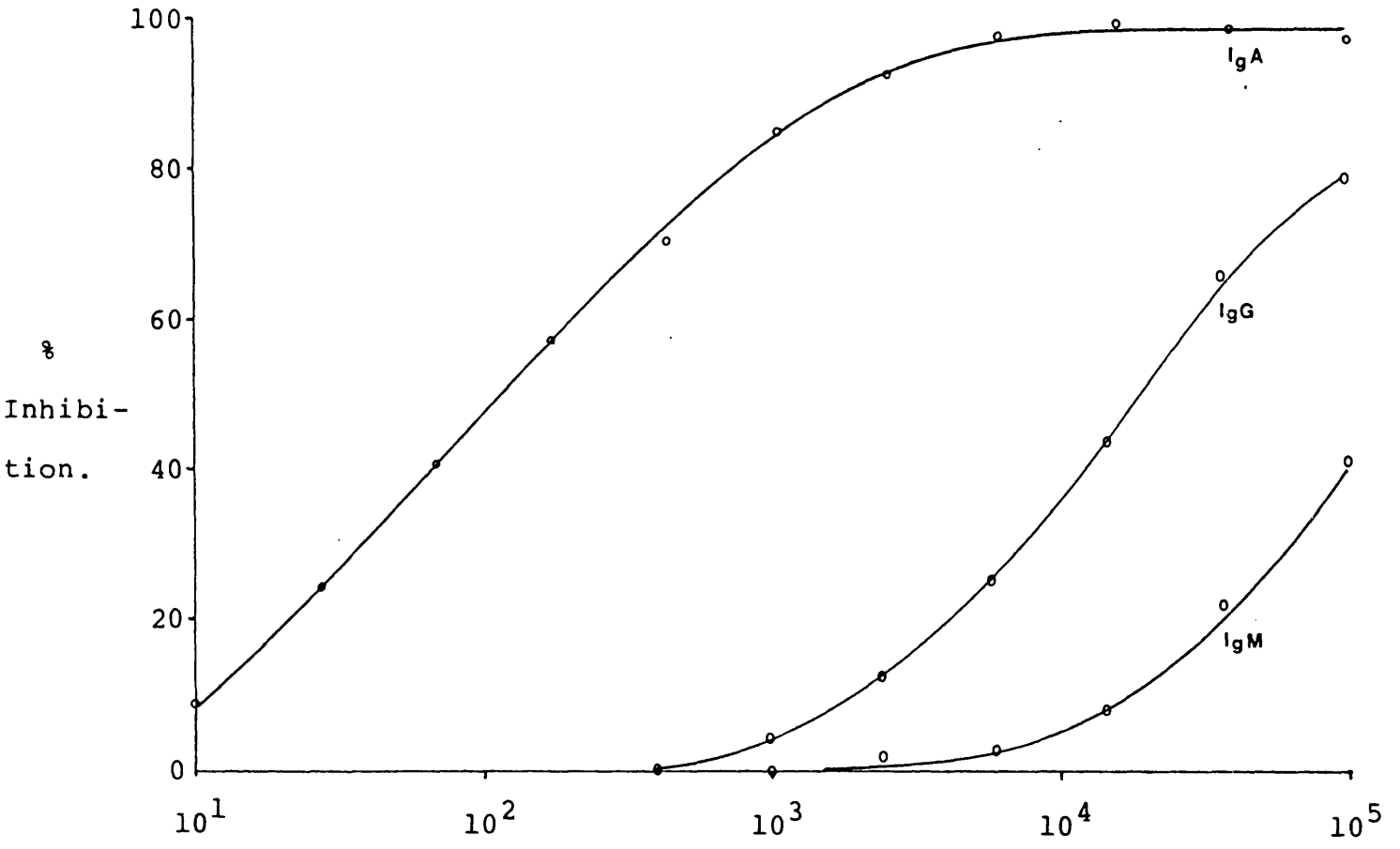
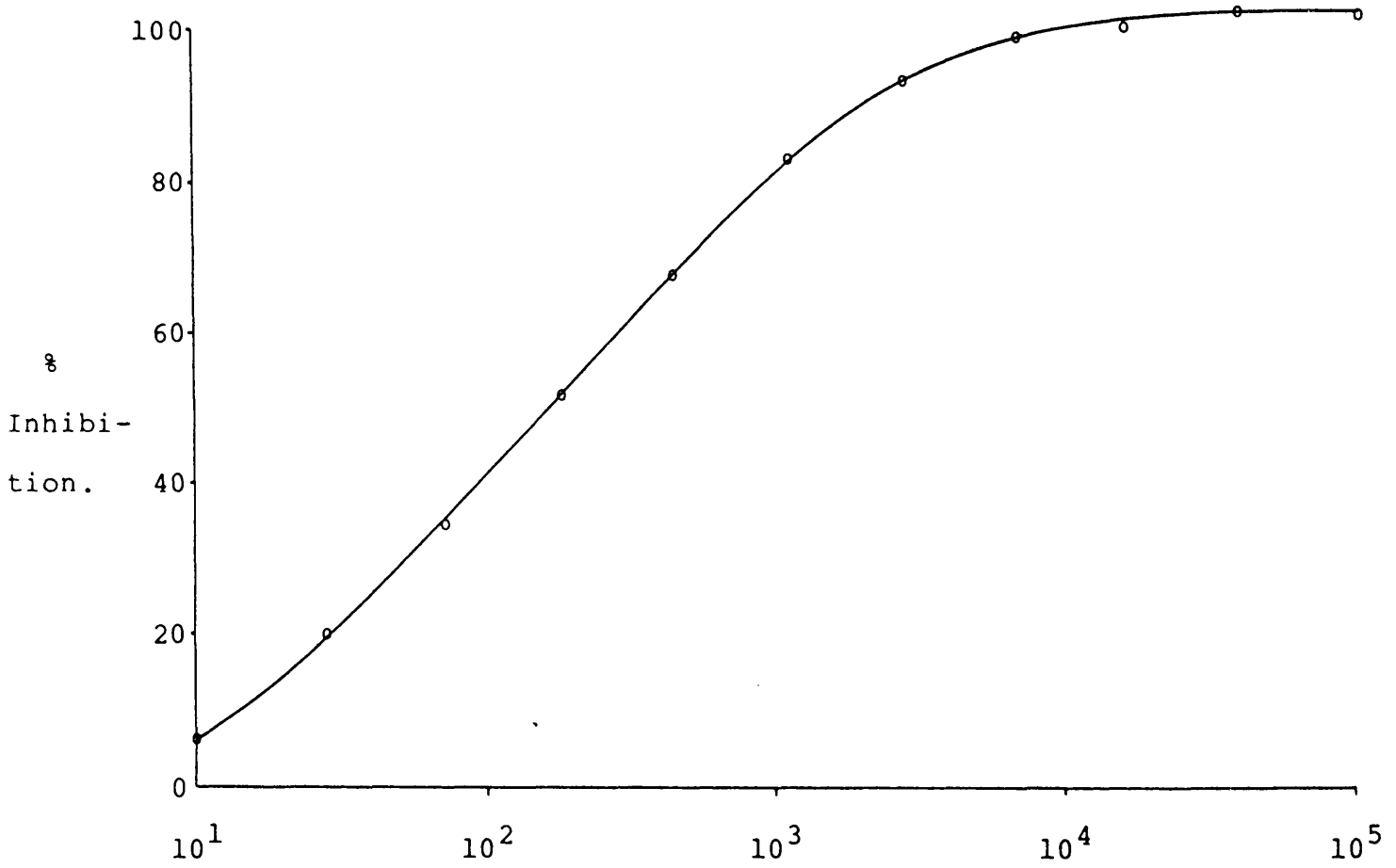


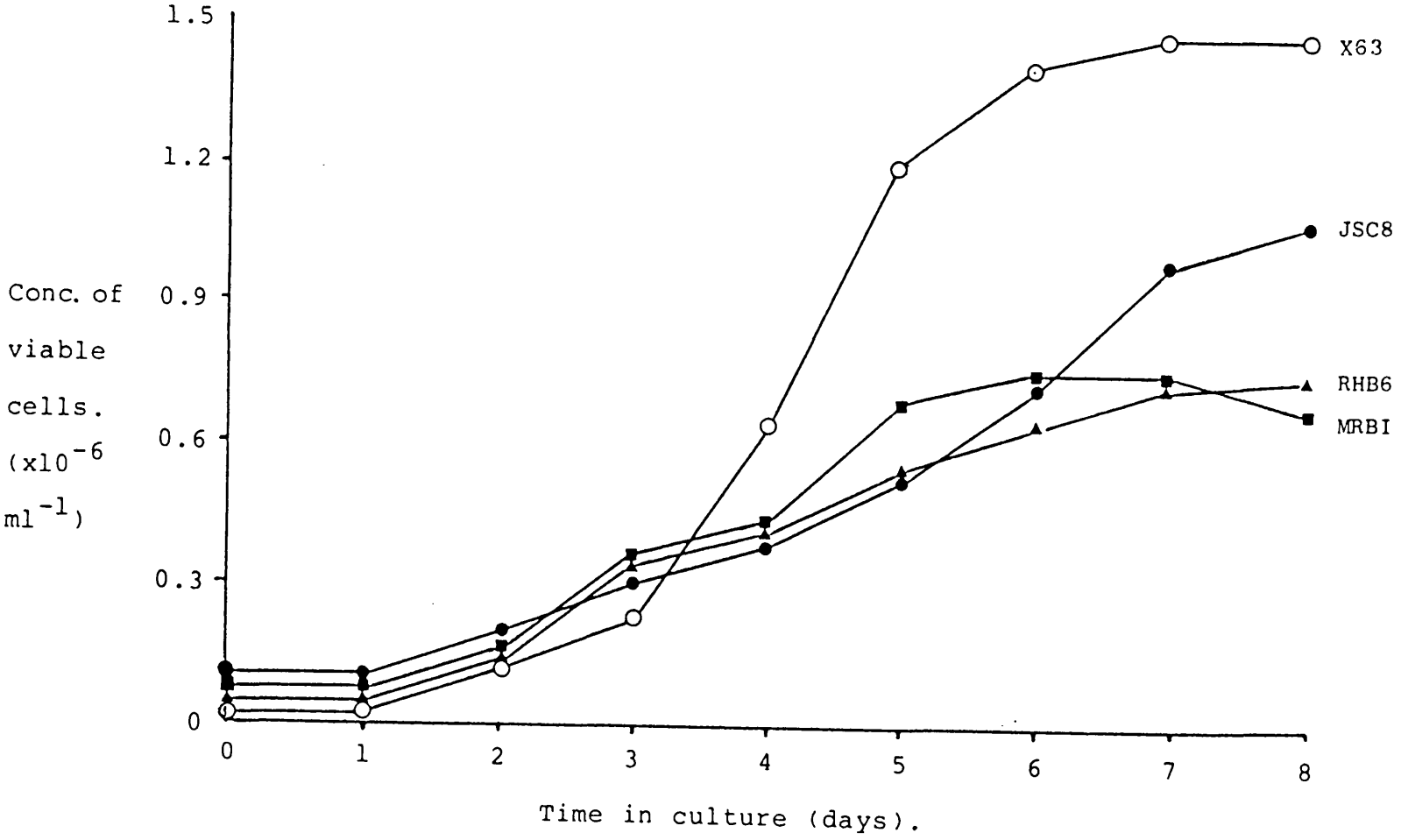
Fig.5.5 CELIA for mouse Ig. Standard curve.



10. Growth characteristics of Hybrids.

Culture flasks were seeded with unfused X63 or hybrid cell lines JSC8, RHB6 and MRB1 at a concentration of approximately 50,000 ml^{-1}. Small aliquots were removed at 24 h intervals and viable cells counted. The results are plotted in fig 5.7.

Fig.5.7 Growth curves of X63 and hybrid cell lines.



Discussion

The first critical step in the production of monoclonal antibodies is the generation of hybridomas from the two fusion partners with the highest possible efficiency. Reported fusion procedures vary enormously between laboratories. The technique used in this study was loosely based on the work of Fazekas de St. Groth and Scheidegger (1980), who attempted to optimise the fusion technique for rodent MCA production. In this study, the HMy2 line was clearly inferior in generating hybrids to either the NS1 or X63 mouse myelomas. Half the fusions failed to produce any hybrids and the overall fusion frequency was only 1.6×10^{-7}. This was significantly lower than NS1 (1.7×10^{-6}) which in turn was significantly lower than X63 (8.2×10^{-6}, Student's t-test $p < 0.01$ for both comparisons). HMy2 has now been sucessfully fused with lymphocytes from peripheral blood and lymph nodes as well as chronic leukaemic lymphocytes (Edwards et al. 1982, Sikora et al. 1982, Abrams et al. 1983). Reported fusion frequencies are between 10^{-6} and 10^{-8}, with 10^{-7} being the usual.

Although depleting the T-lymphocytes before fusion resulted in no improvement, it is possible that the fusion frequency of HMy2 could be increased by manipulating the variables involved in the fusion procedure. This was not attempted as the results with the murine lines were encouraging enough to merit pursuing.

The fusion frequency of X63 with normal PBL

approached that commonly achieved with mouse-mouse fusions, but was both lower and more variable with PBL from autoimmune patients. This is perhaps not surprising as the normal group were a small group of laboratory workers all in their mid-twenties. The autoimmune group were considerably more heterogenous in age (mid twenties to seventies) and general state of health. In addition there were variable delays between taking blood samples and fusing the isolated lymphocytes.

Fusion frequencies of X63 with EBV transformed PBL or tonsil cells were remarkably high. Table 5.10 shows a comparison of the fusion frequencies for human and mouse cell lines with human lymphocytes as reported by the authors, or where authors have supplied enough information to enable fusion frequencies to be calculated.

Table 5.10 Fusion frequencies of human and mouse cell lines with human lymphocytes.

Cell line	Fusion partner	Fusion freq. ($\times 10^{-6}$)	Ref.
X63 (mouse)	EBV-B-cells	92-112	(Present study)
X63 (mouse)	EBV-B cell line	20.0	Kozbor et al. (1982a)
KR-4 (human)	EBV-B cell line	11.2	Kozbor et al. (1982b)
LTR 228 (human)	PWM stim. PBL	10.0	Larrick et al. (1983)
X63 (mouse)	normal PBL	8.4	(Present study)
UC 729-6 (human)	CLL lymphocytes	3-10	Glassy et al. (1983)
NS1 (mouse)	PBL (normal+ renal cancer)	6.1	Cote et al. (1982)
NS1 (mouse)	CCL lymphocytes	5.1	Foon et al. (1983)
X63 (mouse)	SLE and MG PBL	4.7	(Present study)
KR-4 (human)	Immune PBL	3.6	Kozbor et al. (1982b)
GM 1500 (human)	PBL (diabetics)	2.5	Eisenbarth et al. (1982)
HF2 (human)	CCL lymphocytes	2.2	Foon et al. (1983)
GM 0767.3 (human)	PWM stim. tonsil	2.2	Chiorazzi et al. (1982)
GM 1500 (human)	Immune PBL	1.8	Croce et al. (1980)
GM 4672 (human)	PWM stim. SLE PBL	1.7	Shoenfeld et al. (1982)
NS1 (mouse)	normal PBL	1.7	(Present study)
UC 729-6 (human)	CLL lymphocytes	1.4	Foon et al. (1983)
U-266 (human)	CLL lymphocytes	1.0	Foon et al. (1983
HMy2 (human)	CLL lymphocytes	0.5	Foon et al. (1983)
UC 729-6 (human)	normal PBL	0.5	Glassy et al. (1983)
KR-4 (human)	normal PBL	0.4	Kozbor and Roder (1983)
TM-H2 (human)	normal PBL	0.1-0.7	Sullivan et al. (1982)
HMy2 (human)	PBL (normal+ renal cancer)	0.3	Cote et al. (1982)
HMy2 (human)	normal PBL	0.2	(Present study)
HMy2 (human)	normal PBL	0.1	Edwards et al. (1982)
SKO-007 (human)	PBL (normal+ renal cancer)	0.1	Cote et al. (1982)

A number of conclusions can be drawn. For a particular cell line the fusion frequency is greater if the lymphocyte partner is EBV transformed (Kozbor 1982a, 1982b, Kozbor and Roder 1983), of CLL origin (Glassy et al. 1983,) or PWM stimulated (Shoenfeld et al. 1982, Larrick et al. 1983) rather than normal PBL. The fusion frequency achieved in this study with X63 and untransformed PBL compares favourably with the best two human cell lines KR-4 and LTR 228 even though they were fused with EBV transformed B cells and PWM stimulated PBL B cell blasts respectively. The performance of X63 looks even more impressive if one considers that probably only ~10% of the fusion partners were B cells. When X63 cells are fused with EBV transformed B cells, the fusion frequencies are by far the highest. HMy2 fuses poorly compared to both other human and mouse cell lines, in this and other studies.

The fusion frequency of a particular cell line obviously needs to be considered with regard to the proportion of derived hybrids which secrete human immunoglobulin. Although X63 is an efficient fusion partner, only 10%-30% of the hybrids were found to produce human immunoglobulin when the fusion partner was untransformed PBL. This presumably reflects the fusion of X63 with non-B cells in the population. When fused with transformed B cells only, 81% - 92% of the hybrids were found to secrete human immunoglobulin.

Comparative data on the percentage of

immunoglobulin secreting hybrids from human cell-lines are shown in table 5.11. As well as fusing with relatively high efficiency, 96% of hybrids derived from KR-4 fused with a B cell line secreted immunoglobulin derived from the latter. No data are available from fusion of this line with PBL. Similarly, almost half the hybrids obtained from the human line LTR 228 produced immunoglobulin derived from the B cell blast fusion partners. HMy2 and SKO-007 generate a fairly high percentage of immunoglobulin-secreting hybrids but their fusion frequencies are both very low.

Table 5.11 New immunoglobulin secretion in hybrids derived from human cell partners and X63.

Cell line	% hybrids secreting new Ig.	Ref.
KR-4(i)	96%	Kozbor et al.(1982b)
X63(i)	81%-92%	Present study
HMy2	65%-67%	Foon et al.(1983)
		Cote et al.(1982)
LTR 228	<50%	Larrick et al.(1983)
SKO-007	47%	Cote et al.(1982)
UC729-6	30%-60%	Glassy et al.(1983)
		Foon et al.(1983)
X63(ii)	35%	Kozbor et al.(1982a)
X63(iii)	22%	Present study
HF2	24%	Foon et al.(1983)
U-266	1%	Foon et al.(1983)

(i) Fused with EBV-transformed B cells.

(ii) Fused with B cell line.

(iii) Fused with normal and autoimmune PBL.

One major criticism of human-mouse hybrids has been that they are unstable. In this study, long term hybrid instability was not a problem. Of thirteen hybrids selected for intensive study, twelve were successfully cloned and continued to secrete immunoglobulin over at least a six month period of observation. There is a general feeling that such heterohybrids are unsatisfactory for producing human MCA as they preferentially lose human chromosomes and thus the ability to produce human immunoglobulin. It is not immediately apparent why stability was not a problem with the hybrids studied here; it may be a property of the X63 line, the autoimmune origin of the lymphocytes or some technical factor in the propogation of the hybrids. There is no reason to suspect that PBL from autoimmune patients are especially likely to confer stability in hybrids, but this can only be established by fusing X63 to PBL of other origins. Any peculiarities of the X63 line which enable it to produce stable hybrids need be established by comparative studies. Kozbor (1982a) found hybrids generated from this myeloma and an B cell line to be stable for periods of >6 months and that hybrids retained a mean of 24 human chromosomes. Most hybrids examined in the present study contained many more chromosomes than the X63 line, but it was not established how many were of human origin.

There are very many technical variables which may contribute to the stabilty of the hybrids, such as

type of feeder layer, serum factors, culture conditions etc. Although it seems unlikely that these are of significance, it is of interest that Astaldi et al. (1980) found that human endothelial cell culture supernatant was able to increase the stability of mouse-human heterohybrids derived from the mouse myeloma SP2/0.

Perhaps the most important reason for the present success was the cloning of hybrids directly from the post-fusion trays as early as possible, and the re-cloning until >90% of sub-clones produced immunoglobulin. Cote et al. (1983) found that 38% of uncloned hybrids from NS1 lost immunoglobulin production in 2 to 3 months after fusion. This rose to 42% after 4 months. Butler et al. (1983) found that when antigen specific clones derived from PBL and the mouse myeloma SP1 were identified at 3 weeks and maintained without cloning, production invariably ceased in the following 6 weeks. Stable hybrids could be rescued by cloning at 3 weeks however. Where mouse myelomas have been employed for generating stable immunoglobulin secreting human-mouse hybrids, early, aggressive cloning seems a common denominator (Nowinski et al. 1980, Schlom et al. 1980, Butler et al. 1983).

The main and often quoted reason for developing a human myeloma hybrid partner for human MCA production is that human-human hybrids are stable with respect to chromosome loss (Weiss et al.1967, Croce et al.1974),

whereas mouse-human hybrids are are unstable (Croce et al. 1974, 1980). Both these statements need some qualification. Many people are familiar with the production of mouse-human hybrids which segregate human chromosomes. These fusions have been utilised with great success in the area of somatic cell genetics for the mapping of human chromosomes. Heterohybrids are scored for the presence or absence of a human gene product and the presence or absence of each of the human chromosomes. Concordance between these is taken as evidence for the assignment of a marker to a particular chromosome (Goodfellow and Solomon 1982). It has been known for a long time that in such mouse-human somatic hybrids (usually fibroblasts), human chromosomes are preferentially eliminated (Weiss and Green 1967, Matsuya and Green 1969, Nabholz et al. 1969). However, preferential loss of human chromosomes is not a universal feature of mouse-human heterohybrids. Hybrids have been produced in which mouse chromosomes are preferentially lost (Jami and Grandchamp 1971, Minna and Coon 1974, Ringertz and Savage 1976). Such reverse chromosome segregation can also be induced by pre-treating the mouse cells with BrdU or radiation before fusion (Pontecorvo 1971). Hybrids between human diploid fibroblasts and mouse RAG cells randomly lose chromosomes from both species (Chen 1979).

In mouse-human fibroblast hybrids human chromosome loss is not random. Chromosomes 7, 11, and 12

are preferentially retained, while others e.g. 9, are lost (Croce et al. 1973, Norum and Migeon 1974, Ruddle and Creagan 1975). When Croce et al. (1980) examined the retention of chromosomes in hybrids derived from the mouse myeloma cell lines P3X63.Ag8 and P326Bu4 with human lymphocytes, they found human chromosomes to be lost, but in a different pattern to the fibroblasts. Chromosomes 14, 22, and 5 were retained in more than 50% of independent hybrids with chromosome 14 being retained in 100%. Chromosomes 1 and 2 were found in less than 10% of hybrids. This is of some significance as human immunoglobulin heavy chain genes have been assigned to chromosome 14 (Croce et al. 1979, Hobart et al. 1981), lambda light chain genes to 22 and kappa light chain genes to chromosome 2 (Erikson et al. 1981). Thus if this is generally true of mouse myelomas one would expect hybrids producing lambda light chain immunoglobulin to be much more stable than hybrids producing kappa chain immunoglobulin. There was no suggestion from the present study that kappa chain producing hybrids were more unstable than lambda producers. Whereas there does seem to be a preference in the production of lambda secreting hybrids, the twice cloned hybrids cultured for over 6 months included two kappa producers. The stable hybrids produced from this myeloma by Kozbor (1982a) were kappa secretors, so the production of MCA with kappa isotype is not precluded. The one hybrid which lost immunoglobulin production was initially producing IgM(lambda).

If the problem of chromosome instability in mouse-human hybrids is not as bad as it has seemed, there is accummulating evidence that it is more of a problem with human-human hybrids than originally envisaged. While some hybrids derived from some human lines have been reported to retain most of the parental chromosomes (KR-4 Kozbor et al.1982, UC729-6 Glassy et al.1983, LTR228 Larrick et al.1983), there have also been reports of chromosome loss. The modal chromosome numbers in most hybrids derived from HMy2 are only 17 to 27 more than the parent myeloma (Edwards et al. 1982). Similarly Olsson et al. (1983) report a rapid loss of chromosomes from hybrids of both RH-L4 and SKO-007. During 40 days after fusion, the chromosome content of most hybrids fell from near tetraploid to between diploid and triploid. Many hybrids lost immunoglobulin production during this period. Cote et al. (1983) found the loss of production of immunoglobulin by uncloned hybrids from the human lines HMy2 and SKO-007 to be comparable to immunoglobulin loss in hybrids derived from murine NS1. Foon et al., examining hybrids from the four human lines U-226, HMy2, HF2 and UC729-6, found that the majority of hybrids lost immunoglobulin production at the first cloning stage. The authors suggest that this may have been due to switching off of gene function, or loss of secretion rather than production. In the absence of any karyotypic analysis however, it cannot be ruled out that chromosome loss may have been a factor.

The isotype distribution of MCA derived from human lymphocytes has not been systematically researched. A predominance of hybrids secreting human immunoglobulin of the IgM class has been reported elsewhere for fusions with PBL (Glassy et al. 1983, Chiorrazzi et al. 1982) and with fusions of EBV transformed PBL (Kozbor and Roder 1983). This presumably reflects the predominance of circulating B cells which are surface IgM positive (Pernis et al. 1971, Froland and Natwig 1972, Preud'Homme and Seligman 1972). Although IgG producing cells predominate in tonsil B cells, mainly IgM producers are selected for following transformation by EBV (Yoshie et al. 1980). The kappa:lambda ratio of immunoglobulins produced by hybrids derived from human lymphocytes has not been well documented. It was surprising to find the kappa:lambda ratio of hybrids in this study to be ~1:2.5 (PBL derived) and 1:1 (tonsil derived). The ratio of kappa:lambda bearing B cells circulating in the peripheral blood has been reported to be ~2:1 (Seigmann et al. 1973). More carefully controlled studies have shown this to be ~1:1 (Vossen and Hijmans 1975). In cloned EBV-transformed tonsil cells Yoshie et al. (1980) found the ratio of kappa:lambda secreting clones to be 2:1. Although the light chain ratios of immunoglobulin-secreting hybrids derived from PBL and tonsil were significantly different, there would seem a lambda bias from the expected ratio in each case. The reason for this is not clear.

The quantity of immunoglobulin secreted by X63-human hybrids is comparable to that reported for other fusion partners. Human cell lines typically generate hybrids which secrete between 0.1 and 10 $\mu g\ ml^{-1}$ (Kozbor and Roder 1983, Foon et al. 1983). Cote et al. found 70% - 75% of all hybrids generated with NS1 or the two human lines HMy2 and SKO-007 to produce between 1 and 10 $\mu g\ ml^{-1}$, with the remaining hybrids secreting up to 100 $\mu g\ ml^{-1}$. No mouse immunoglobulin was detected in hybrids generated from X63 and thus one can assume that all secreted immunoglobulin was derived from the lymphocyte fusion partner. This is not the case for most of the human cell lines. When both fusion partners produce immunoglobulin of their own the resulting hybrids continue to synthesise both immunoglobulins. Hybrid immunoglobulin molecules can thus be secreted, effectively diluting the desired antibody and making isolation and idiotypic studies a problem. Even when the parent cell line only synthesises trivial amounts of immunoglobulin, the resulting hybrids produce much higher quantities (Kozbor et al.1982). HF2 is the only human cell line which produces no immunoglobulin of its own.

In conclusion, X63 was found to be superior to either the mouse myeloma NS1 or the human lymphoblastoid line HMy2 in generating immunoglobulin-secreting hybrids. Many of the useful characteristics of the X63 line were found to be transferrable from the mouse-mouse fusions to the fusion with human PBL. The line fused with high

efficiency and hybrids were robust in culture, cloned easily and were recovered without difficulty from storage in liquid nitrogen. Stable hybrids were formed which secreted useful quantities of human IgM, IgG, and IgA of both lambda and kappa isotypes. The X63 myeloma produces no immunoglobulin of its own and no murine immunoglobulin could be detected in the supernatants of human immunoglobulin-secreting hybrids. In comparison to human cell line fusion partners, only LTR 228 and KR-4 generate human immunoglobulin secretors with an efficiency which approaches that of X63, but both have the disadvantage of producing their own immunoglobulin. The only human non-producing cell line HF2 generates immunoglobulin-secreting hybrids at a far lower efficiency than X63. If the stability of the heterohybrids produced in the present study can be repeated in other situations, the X63 line would seem to be by far the most suitable line currently available for the production of human MCA.

Chapter Six. The production of human autoantibodies by human-mouse hybrids.

Results.

Results.

1. The RIA for antibodies to AChR.

Details of the RIA for anti-AChR antibodies have been published (Carter et al. 1981). This assay is used routinely in this department as a diagnostic aid for MG. The range of anti-AChR antibodies in normal human sera is 0-1 x 10^{-10} mol l^{-1} of α-bungarotoxin binding sites. The titres of antibodies usually found in MG sera are in the range 0-1500 x 10^{-10} mol l^{-1} α-bungarotoxin binding sites. The antibody titres in the sera of the patients used in this study are presented in table 6.1.

Table 6.1. Anti-AChR antibody titres in MG patients' sera.

Patient	Titre (10^{-10}M).
JR	1556
JRo	5087
RH	161
SP	158
Sh	73
MP	240
ST	94
JA	1430
JS	257

2. Failure to detect MCA to AChR.

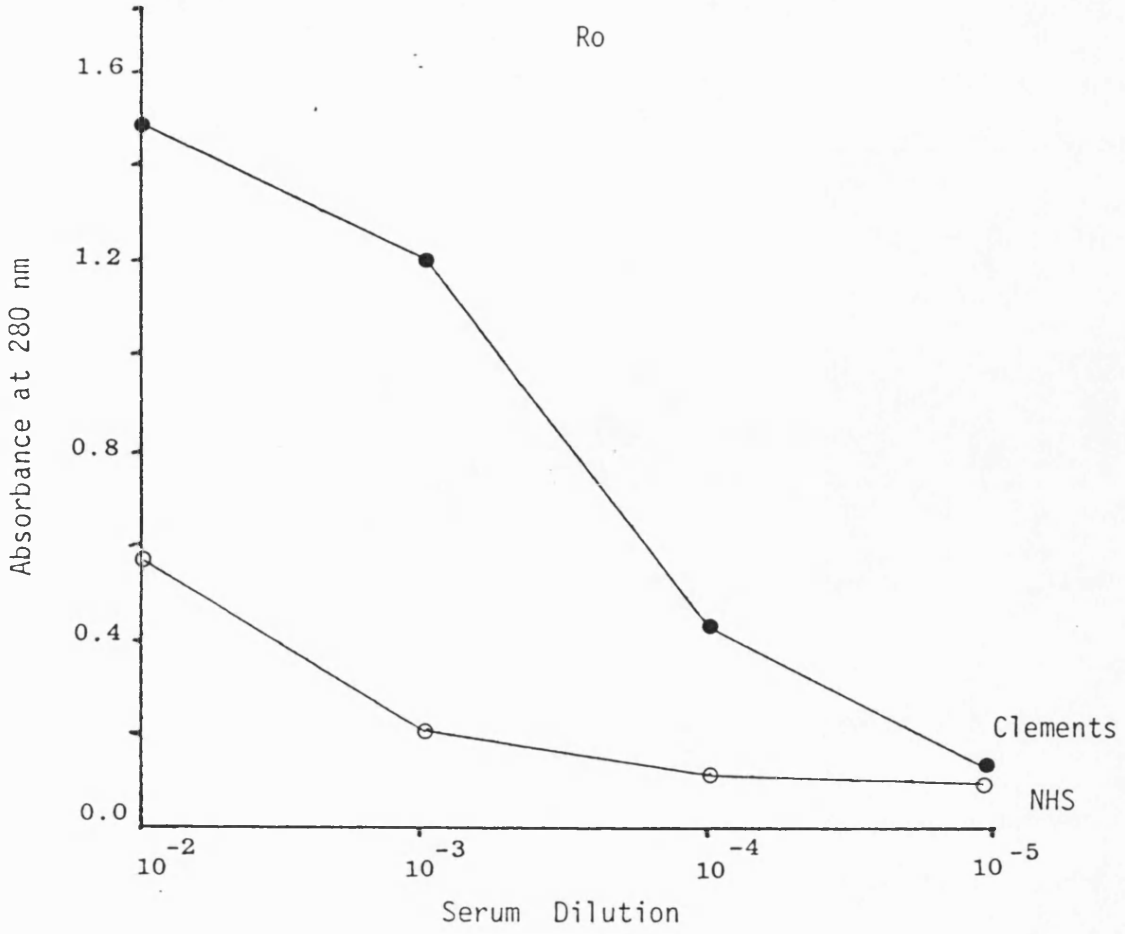
459 human immunoglobulin-secreting hybrids made from MG patients were screened by RIA for anti-AChR activity. They were derived from 10 fusions using PBL from 9 different patients. 353 supernatants contained IgM, 23 contained IgA and 83 contained IgG. No antibody activity to AChR was detected in any of the supernatants.

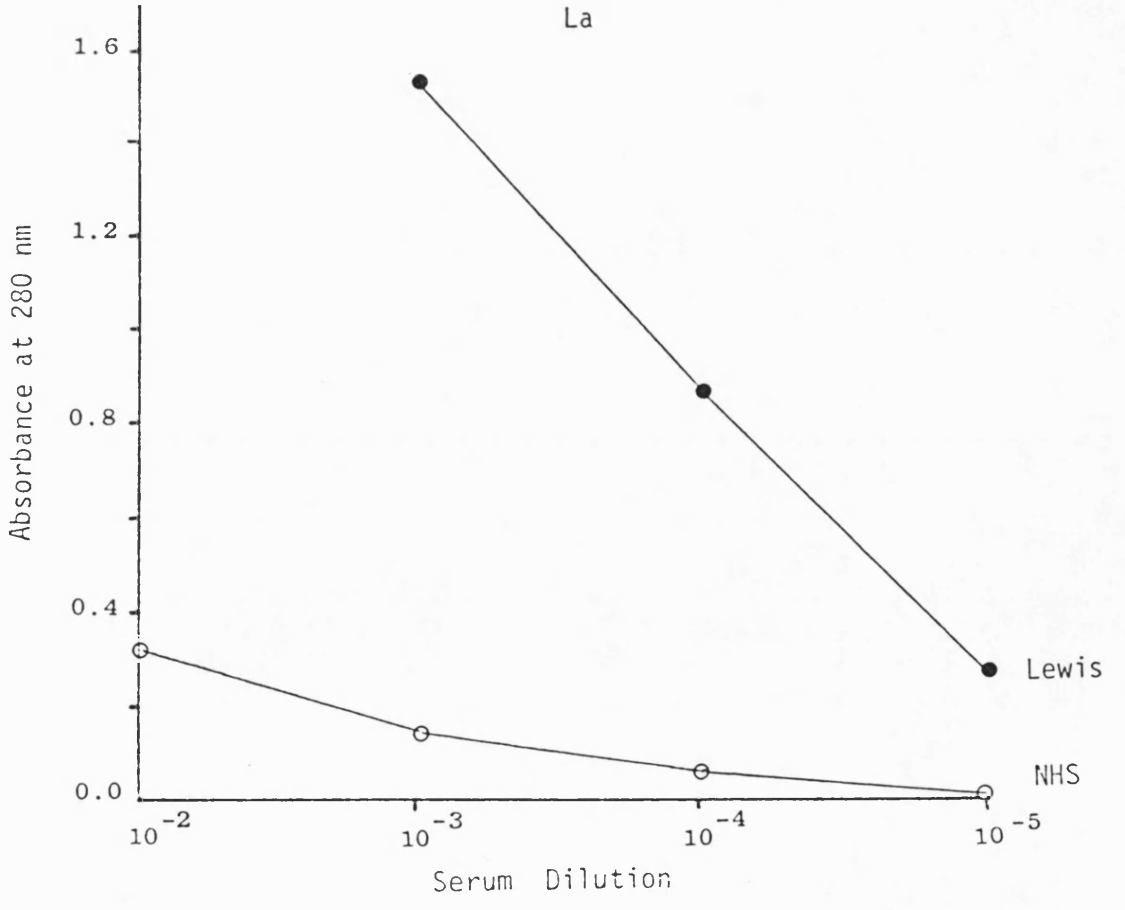
3. ELISA for antibodies to nuclear and cytoplasmic antigens and rheumatoid factor.

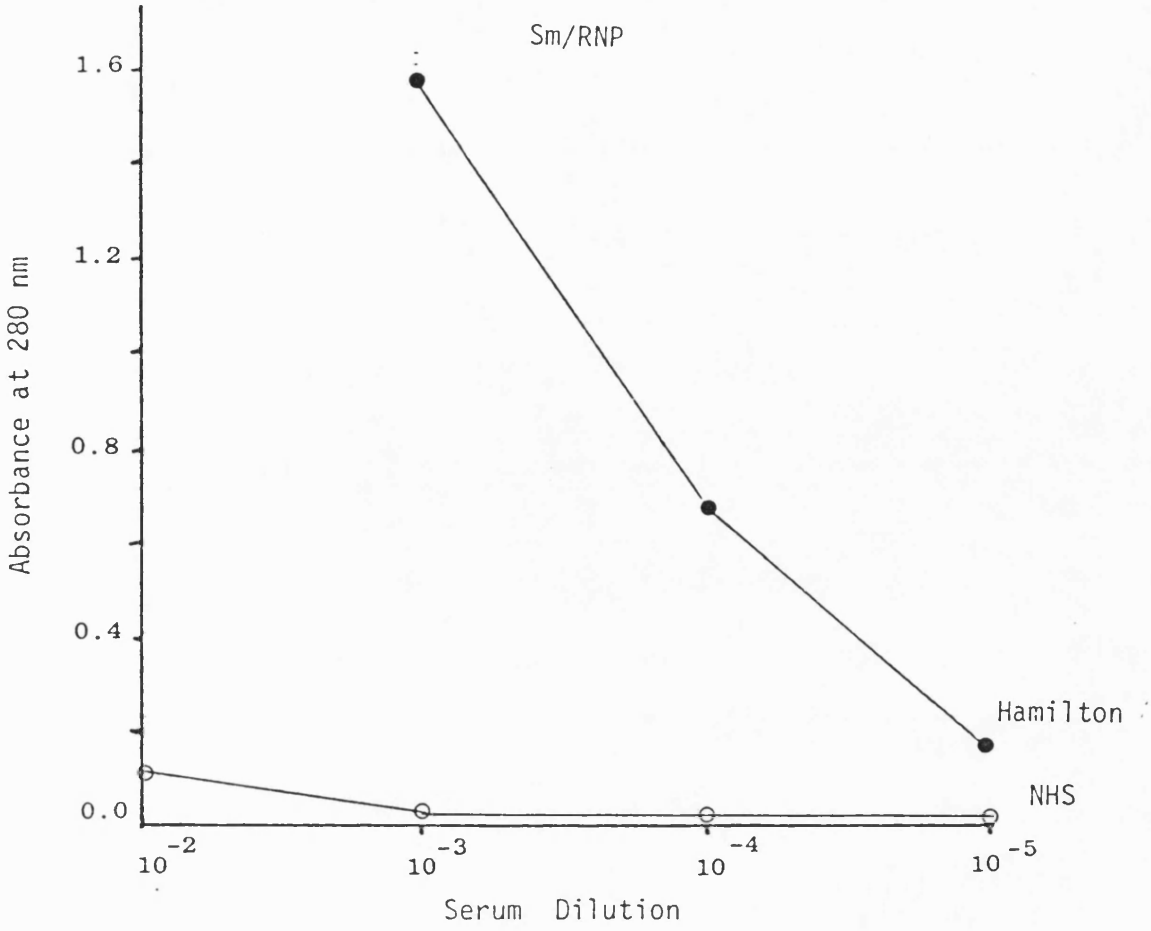
These assays have been the subject of previous studies establishing their sensitivity and specificity (Maddison et al, in press, Brennand 1983). The specificities of the ELISAs were confirmed using known standard monospecific sera. Typical binding curves are shown in fig 6.1. Positive and normal sera controls were included in all assays. The ELISAs confirmed the individual serological data obtained by double diffusion for antibodies to soluble ribonucleoprotein antigens, and RIA for anti-DNA antibodies. (Table 6.2). All patients gave clear positive ELISA results at serum dilutions of at least 1:10,000.

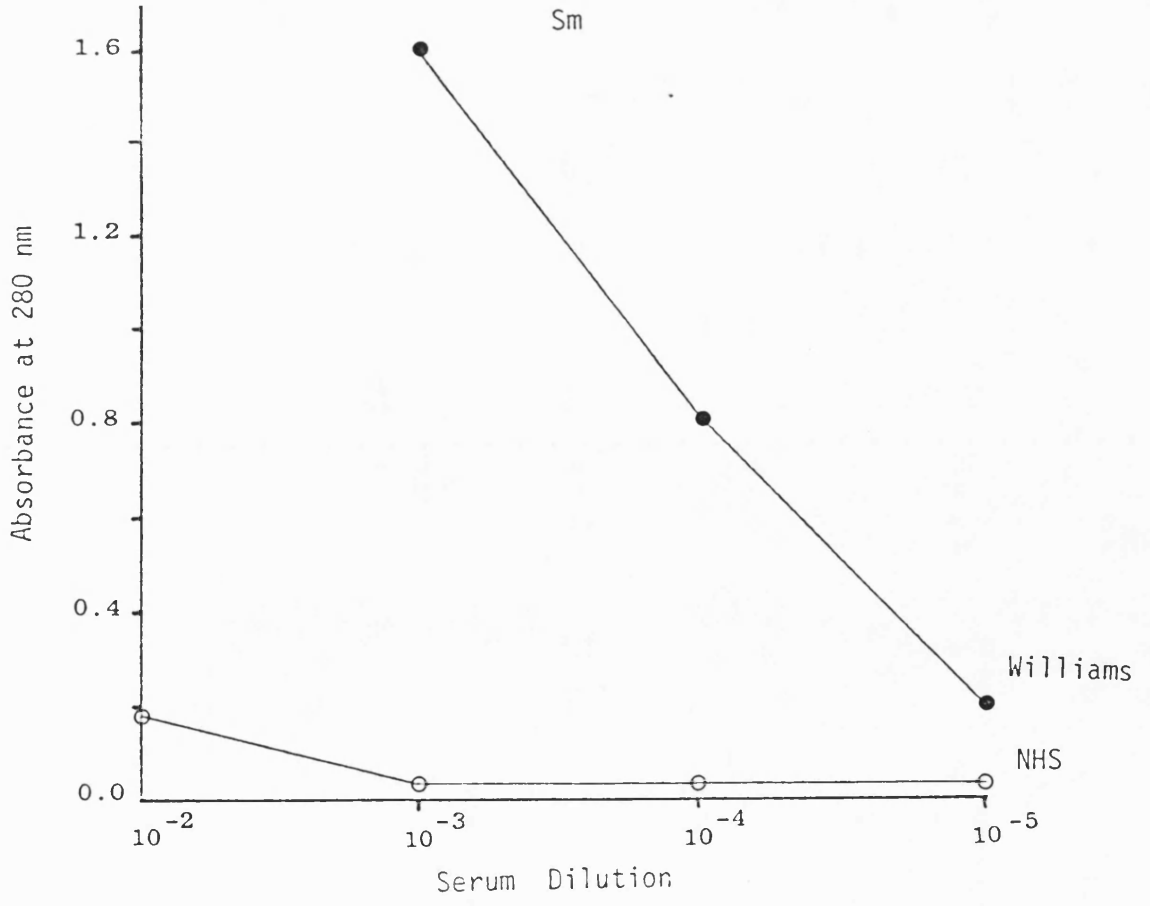
Fig. 6.1 Typical binding curves of ELISAs using standard sera.

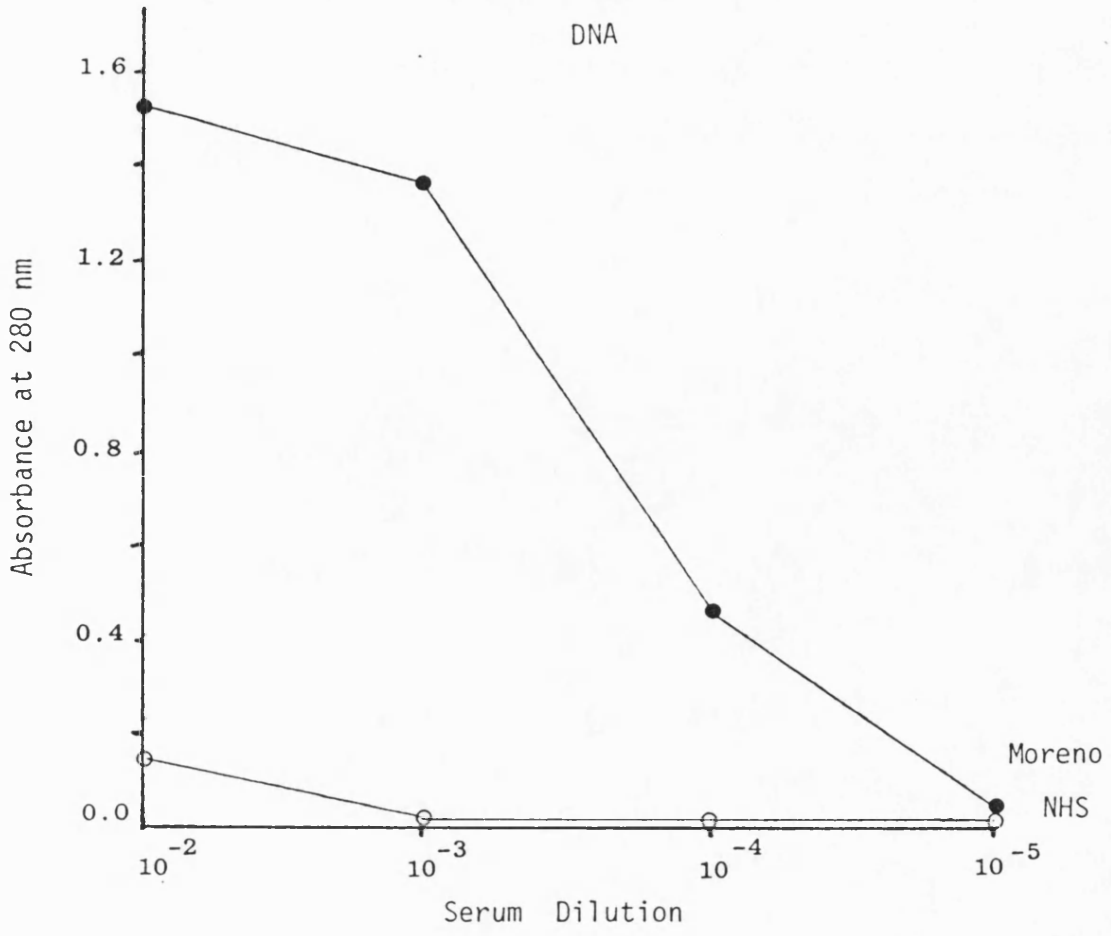
Points shown are means of triplicates. Variation between replicates was less than 0.1 O.D. units. In each case, the positive serum is an accepted reference standard containing precipitating antibodies against the antigen in question. Pooled normal human sera (NHS) was used as a negative control.


Ro
Absorbance at 280 nm
1.6
1.2
0.8
0.4
0.0
Clements
NHS
10-2
10-3
10-4
10-5
Serum Dilution


La
Absorbance at 280 nm
1.6
1.2
0.8
0.4
0.0
Lewis
NHS
10-2
10-3
10-4
10-5
Serum Dilution


Sm/RNP
1.6
1.2
0.8
0.4
0.0
Absorbance at 280 nm
Hamilton
NHS
10 -2
10 -3
10 -4
10 -5
Serum Dilution


Sm
1.6
1.2
0.8
0.4
0.0
Absorbance at 280 nm
Williams
NHS
10 -2
10 -3
10 -4
10 -5
Serum Dilution


DNA
1.6
1.2
0.8
0.4
0.0
Absorbance at 280 nm
Moreno
NHS
10 -2
10 -3
10 -4
10 -5
Serum Dilution

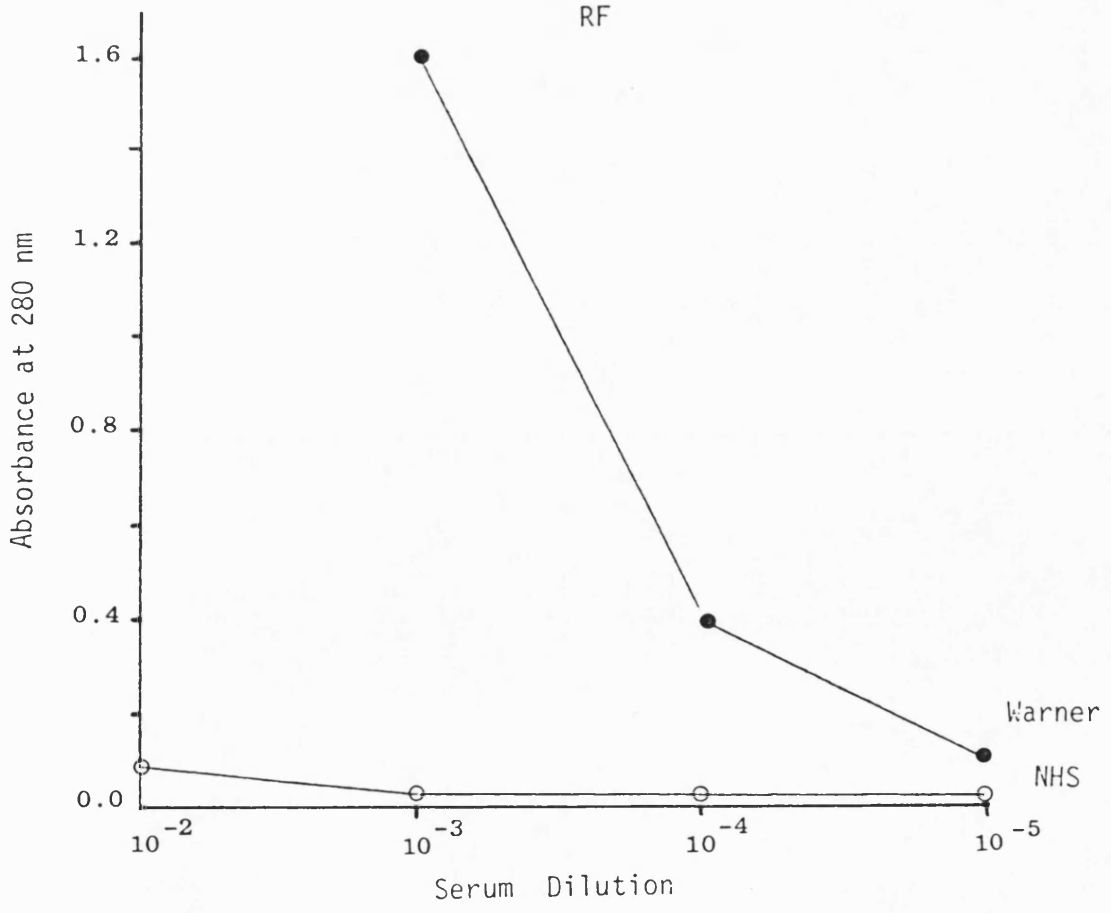

RF
1.6
1.2
0.8
0.4
0.0
Absorbance at 280 nm
Warner
NHS
10 -2
10 -3
10 -4
10 -5
Serum Dilution

Table 6.2. Antibody specificities present in SLE patients' sera.

Patient	Diagnosis	Precipitins	Anti-DNA
HS	SLE	Ro & La	+
JB	SLE	Ro & La	+
DT	MCTD	nRNP	+
NG	Polymyositis and SS	Ro	-
EA	SS	Ro	-
MW	MCTD	nRNP	-
CS	SLE/SS	Ro	-
SWo	MCTD	nRNP	+
SWa	MCTD	nRNP	+
EC	SLE	Ro & La	+

4.Characterisation of patients' sera by indirect immunofluorescence.

The sera of the patients used in this study were screened by indirect immunofluorescence against HEp-2 cells and normal human fibroblasts. Titres were obtained by serial dilution of the serum samples and were defined as the reciprocal of the highest dilution of serum giving visible fluorescence under a UV microscope. Titres were obtained using both an anti-polyvalent human immunoglobulin-FITC conjugated second antibody, and an IgM specific conjugate. Under the conditions described in the methods section, no staining was observed in a series of 10 normal sera diluted 1:8 or greater. Titres of 8 or greater were accordingly considered positive. There was little difference when either HEp-2 cells or fibroblasts were used as a substrates. Results for both polyvalent and IgM serum antibodies against human fibroblasts are presented in table 6.3. All SLE patients gave positive results as did one MG patient (JA). IgM autoantibodies were either absent or present at very low titre.

Table 6.3. Characterisation of sera by indirect immunofluorescence.

Patient	Cytoplasmic staining			Nuclear staining		
	Pattern	Polyvalent titre	IgM titre	Pattern	Polyvalent titre	IgM titre
HS	hazy	16	8	fine speckled	32	8
JB	-	-	-	fine speckled	1024	64
DT	hazy	8	-	fine speckled	1024	32
NG	hazy	32	8	fine speckled	32	8
EA	hazy & specks	64	16	fine speckled	512	16
MW	-	-	-	fine speckled	16	-
CS	-	-	-	coarse speckled	256	64
SWo	hazy	8	-	fine speckled	256	8
SWa	fine specks	8	8	fine speckled	1024	128
EC	hazy	16	-	coarse speckled	1024	16
JA*	-	-	-	fine speckled	64	-

*MG patient.

5. The detection of MCA to nuclear and cytoplasmic antigens by ELISA.*

220 human immunoglobulin-secreting hybrids from SLE patients were screened for antibody activity against Ro, La, nRNP, Sm and IgG. They were derived from 12 fusions using PBL from 10 different patients. 156 of the supernatants screened contained IgM, 22 contained IgA and 42 contained IgG. No antibodies were found that reacted specifically with any of these antigens. Fourteen MCA were found that apparently reacted with more than one of these antigens.

When these 14 supernatants were screened against ELISA cuvettes coated without antigen but with casein blocking protein, 10 demonstrated binding. This binding was unaffected when BSA was substituted for casein. The four MCA which were not 'false positives' were all comparatively weak by ELISA and needed extended substrate incubation times ($\sim$ 2 hours) to be demonstrated. Their reactivities were to both Ro and La (CSD4 and NGB1), to La and nRNP (SWG10) or to La, Ro and nRNP (HSE7).

6. The detection of MCA to nuclear and cytoplasmic antigens by indirect immunofluorescence.

The 14 supernatants showing reactivity in the

* SEE APPENDIX

ELISAs were screened by indirect immunofluorescence against fixed human fibroblasts and HEp-2 cells. In addition, the 124 human immunoglobulin positive wells from four SLE fusions (CS, SWo, SWa and EC), together with 65 immunoglobulin positive wells from two MG fusions (JA and JS), 54 immunoglobulin positive wells from two healthy subject fusions (RH and MR) and 128 immunoglobulin positive wells from fusions with EBV transformed lymphocytes were similarly screened. Results were similar using either fibroblasts or HEp-2 cells as targets. The results are shown in table 6.4.

Table 6.4 Detection of MCA by indirect immunofluorescence against human fibroblasts.

Hybrids	No screened	No +ve	IIF Pattern
SLE PBL(false +ve ELISA)	10	10	Cytoplasmic filaments (10)
SLE PBL (+ve ELISA)	4	2	Even cytoplasm (2)
SLE PBL (-ve ELISA)	124	2	Cytoplasmic filaments (2)
MG PBL	65	3	Cytoplasmic filaments (2) " and nucleus (1)
Normal PBL	54	3	Cytoplasmic filaments (3)
EBV-PBL	80	5	Cytoplasmic filaments (4) Even cytoplasm (1)
EBV-tonsil	48	3	Cytoplasmic filaments (2) Even cytoplasm (1)

Fluorescent staining was seen in hybrids from all sources. Four hybrid supernatants (CSD4, and HSE7 from SLE patients, and 1 each from the EBV-tonsil and EBV-PBL fusions) showed an even cytoplasmic/membrane staining pattern (fig 6.2). Twenty-four others showed a filamentous cytoplasmic staining pattern (fig 6.3). In addition to a cytoplasmic filament staining pattern, one hybrid supernatant (JAE6) stained the entire nucleus (fig 6.4).

All 10 of the MCA giving false positive reactions by ELISA showed clear filamentous staining of fibroblasts.

Fig. 6.2 Even cytoplasmic/membrane immunofluorescent staining pattern shown by four MCA (CSD4, HSE7, E2A7, T1F7) against acetone-fixed human fibroblasts.

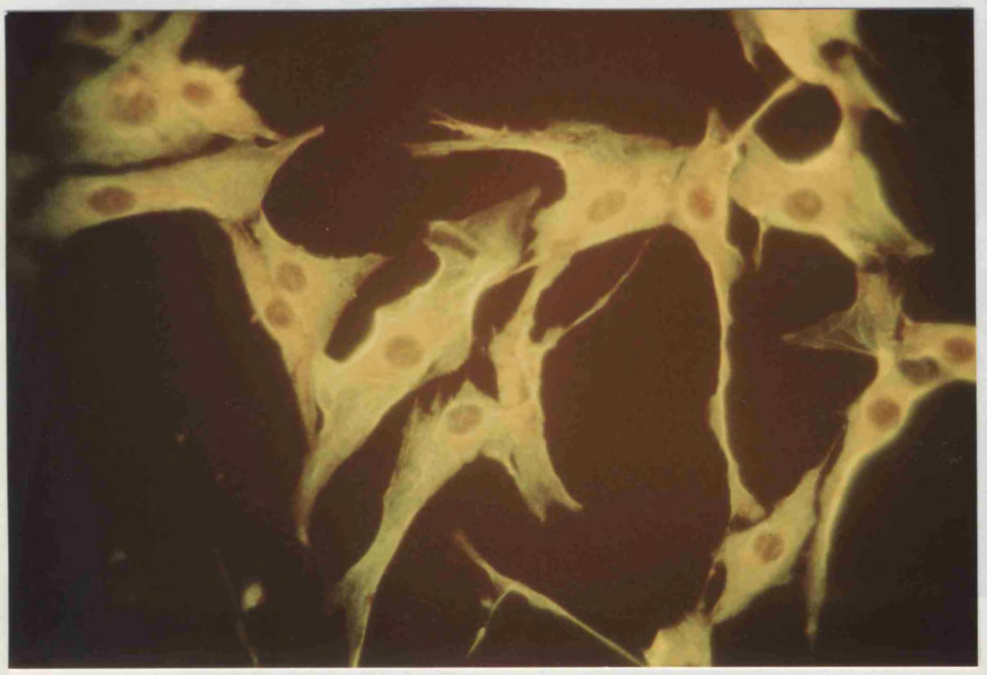


Fig. 6.3 Cytoplasmic filament staining pattern shown by twenty-three MCA against acetone fixed human fibroblasts.

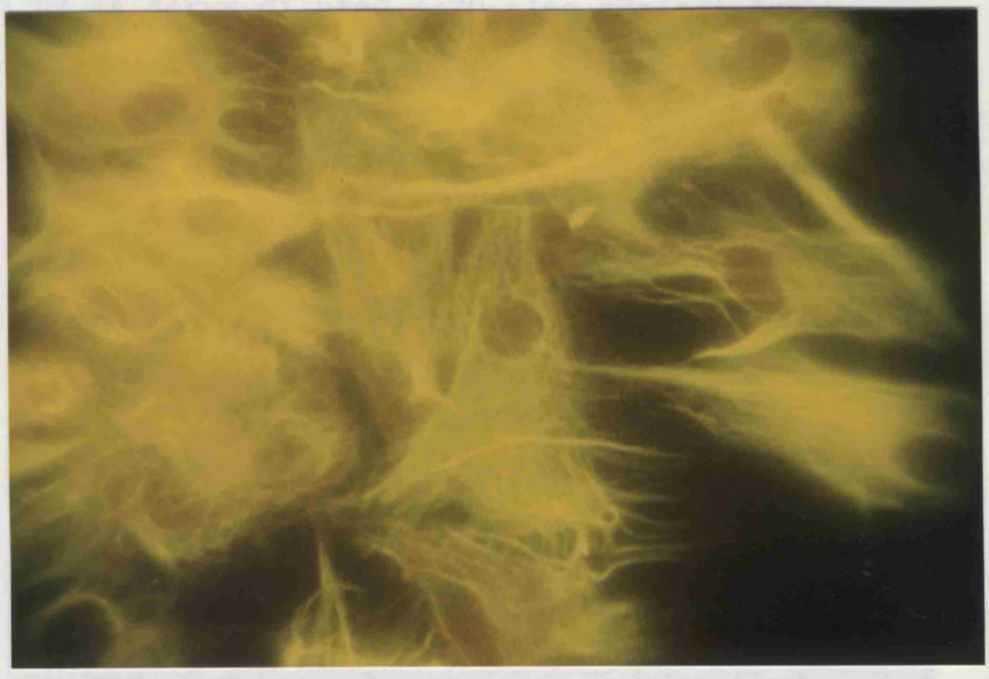
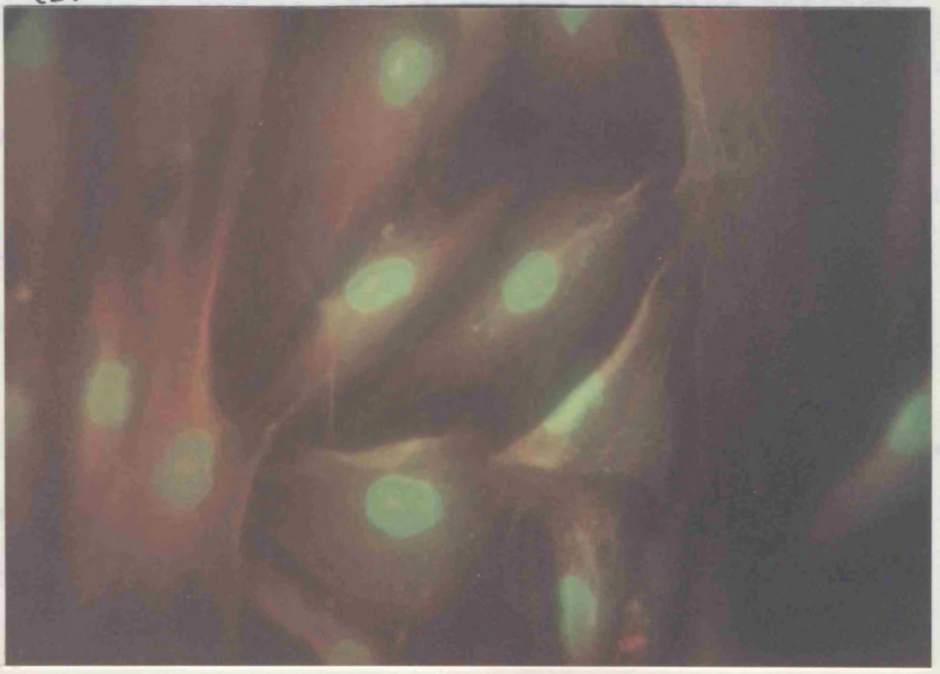


Fig. 6.4 (a) Cytoplasmic filament and nuclear staining pattern shown by MCA JAE6 against acetone-fixed human fibroblasts. Compare with (b) nuclear staining pattern shown by JA serum diluted 1:20 also against human fibroblasts.

(a)

(b)



7. The characterisation of MCA:

(i) Drug disarrangement of cytoskeletal structures.

Eleven hybrids showing positive reactions against fixed human cells by indirect immunofluorescence were twice cloned and characterised further. Staining patterns against fixed fibroblasts were compared with fibroblasts pre-treated with either Colcemid, vinblastine or cytochalasin B. Sixteen uncloned cell supernatants were similarly screened.

Seven of the cloned MCA showing cytoplasmic filament staining patterns on HEp-2 and human fibroblasts (CSH11, SWF2, SWD10, JAA10, SWH10, MRD12, RHB6) showed a pattern of collapsed filaments around the nucleus when the cells had been pre-treated with either Colcemid or vinblastine (fig 6.5). This is typical of vimentin-type intermediate filaments. Pre-treatment of cells with cytochalasin B had no effect on the staining pattern. This was seen with 14 filament-staining supernatants from uncloned hybrids.

The 2 cloned MCA (CSD4 and HSE7) and 2 uncloned supernatants showing even cytoplasmic staining demonstrated no change following any drug treatment.

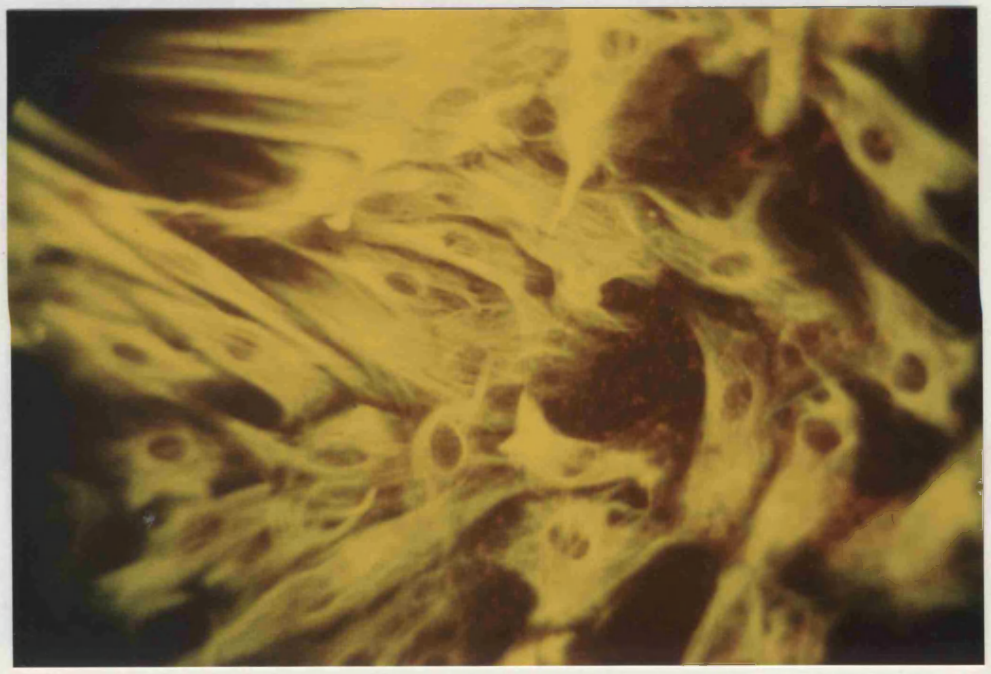
One cloned MCA (CSD1) giving a cytoplasmic filament staining pattern, showed a collapsed perinuclear filament pattern following either Colcemid or vinblastine treatment, but with parallel bundles remaining stained

throughout the cytoplasm. Although treatment with cytochalasin B alone did not make any obvious difference, when fibroblasts were treated with both Colcemid and cytochalasin B, the perinuclear filaments remained visible but no parallel bundles could be seen (fig 6.6). This is consistent with both the intermediate filaments and microfilaments being bound by the MCA.

The 1 cloned MCA showing nuclear and cytoplasmic filament staining (JAE6) demonstrated no change after treatment of the cells with cytochalasin B. The cytoplasmic filaments collapsed after treatment with either Colcemid or vinblastine, but the nuclear staining remained unaltered (not shown).

Fig. 6.5 The effect of Colcemid treatment on the staining patterns seen with MCA to cytoplasmic filaments. (a) Staining seen with untreated fibroblasts. (b) Following treatment of fibroblasts with Colcemid, the filaments are collapsed around the nucleus.

(a)



(b)

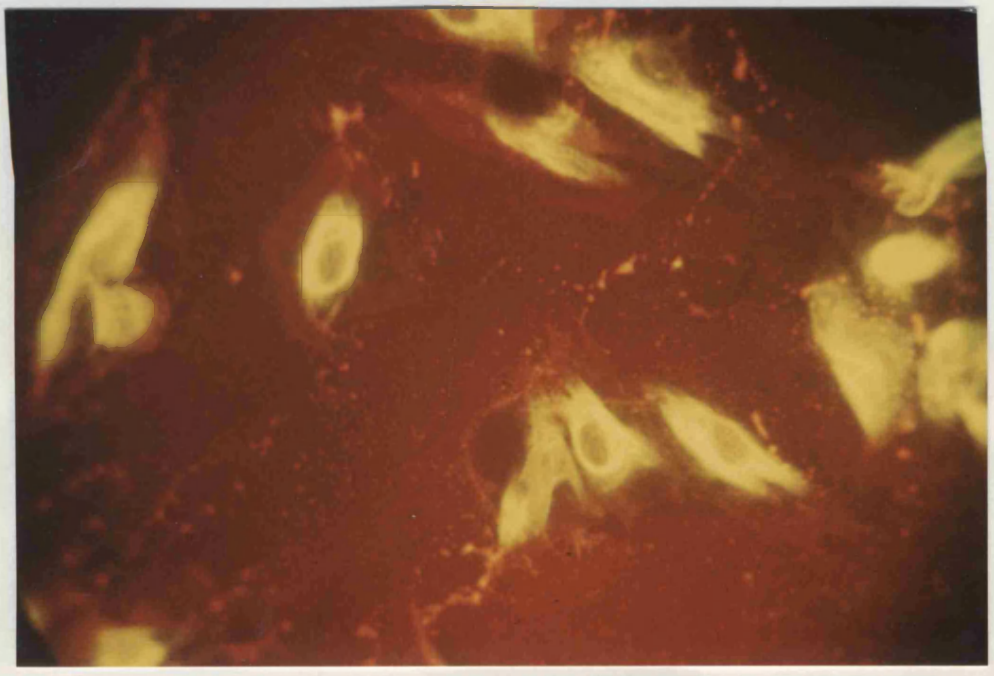
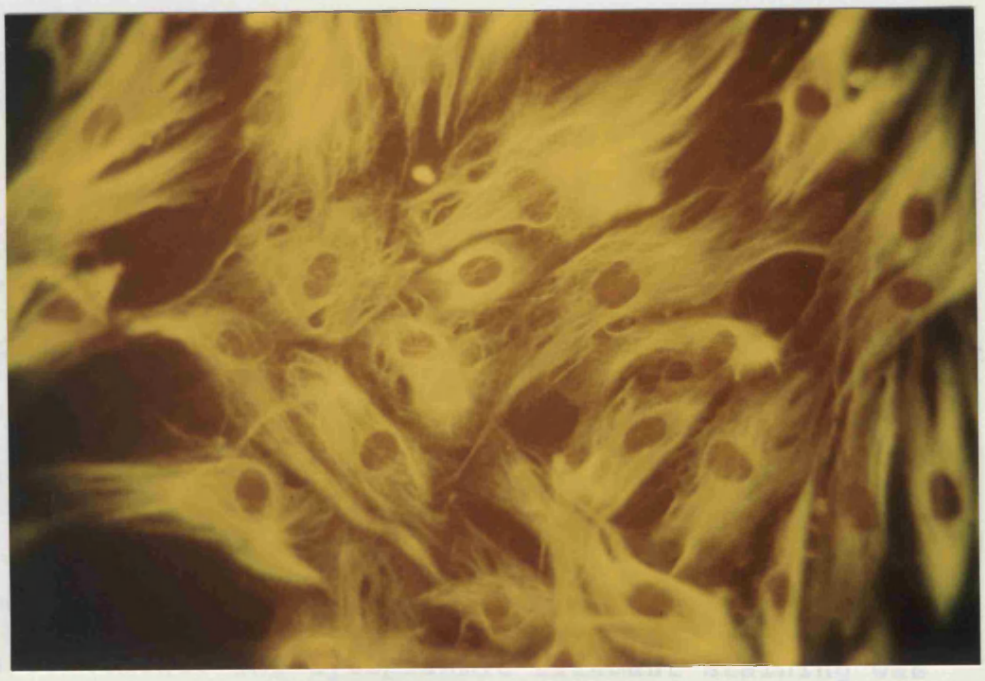
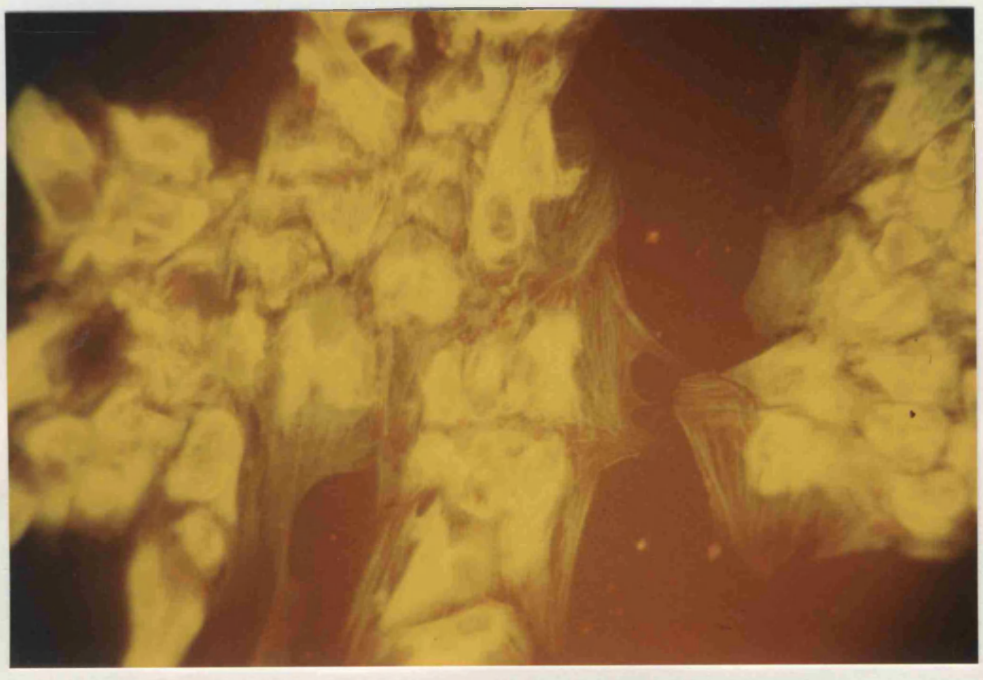


Fig. 6.6 Effect of Colcemid on the staining pattern shown by one MCA, CSD1. Following Colcemid treatment (b), collapsed filaments can be seen around the nucleus and also parallel bundles in the cytoplasm. The parallel bundles disappeared following treatment with cytochalasin B.

(a)



(b)



(ii) *In situ fractionation of cell substrates.*

MCA staining patterns were observed after each step of the *in situ* fractionation procedure described in chapter 4, i.e. detergent extraction, DNase treatment and high salt extraction.

The 8 cloned MCA showing cytoplasmic filament reactivities showed very little change of staining after each of these steps (fig 6.7).

The 2 cloned MCA showing an even staining pattern showed a more filamentous pattern following treatment of the fibroblasts with NP-40, but this was not altered following the subsequent DNase and high salt extractions (fig 6.8).

The 1 cloned MCA (JAE6) showing cytoplasmic and nuclear staining lost the nuclear staining following the DNase treatment. The cytoplasmic filament staining was resistant to all treatments (fig 6.9).

Fig.6.7 Staining pattern of cytoplasmic filament-reactive MCA (CSHl1) against fibroblasts treated with detergent, DNase and high salt extractions.

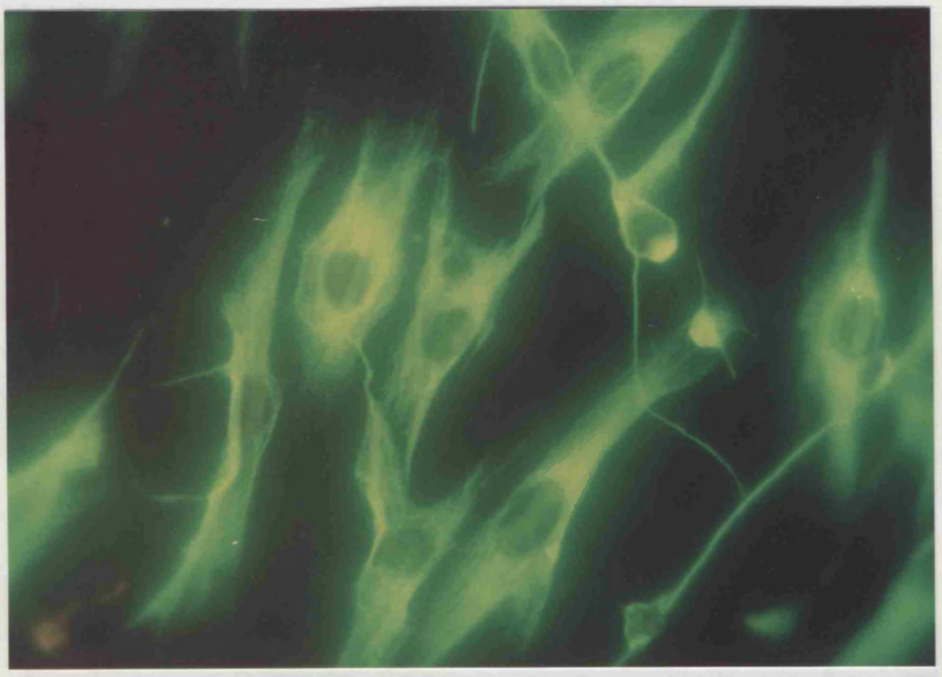


Fig. 6.8 Staining pattern of even cytoplasm/membrane-reactive MCA (HSE7) following extraction of fibroblasts with detergent, DNase and high salt.

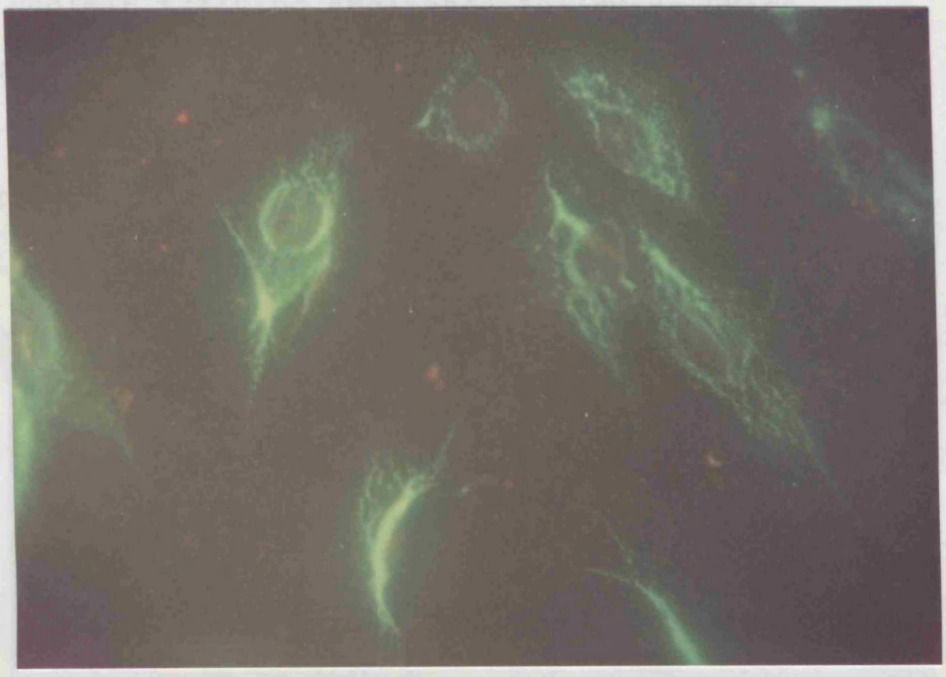


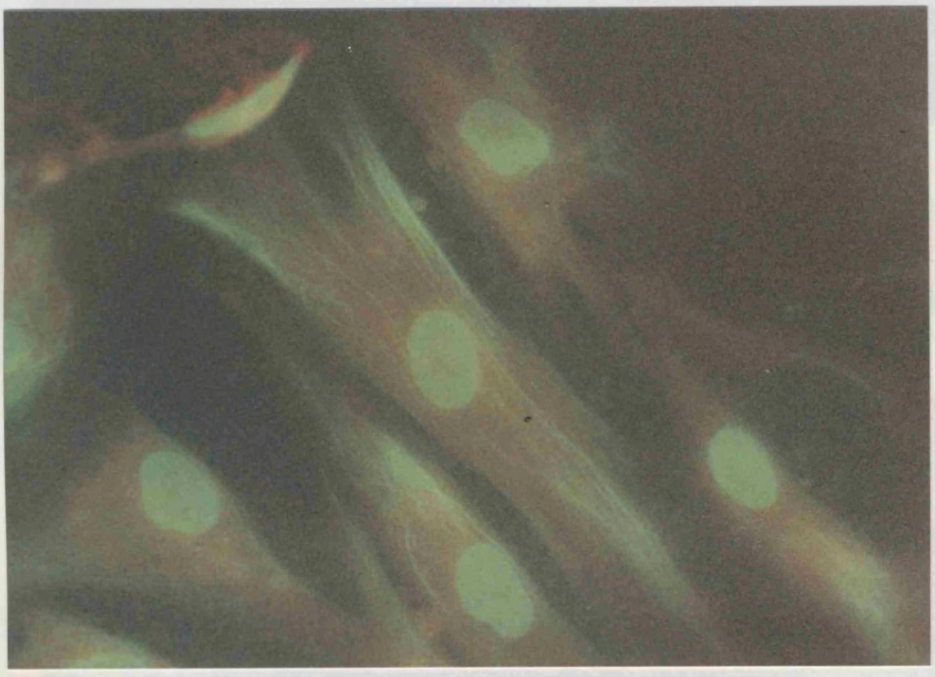
Fig. 6.9 Effect of *in situ* fractionation on the staining pattern of JAE6.

(a) Filament and nucleus staining unaffected following detergent extraction.

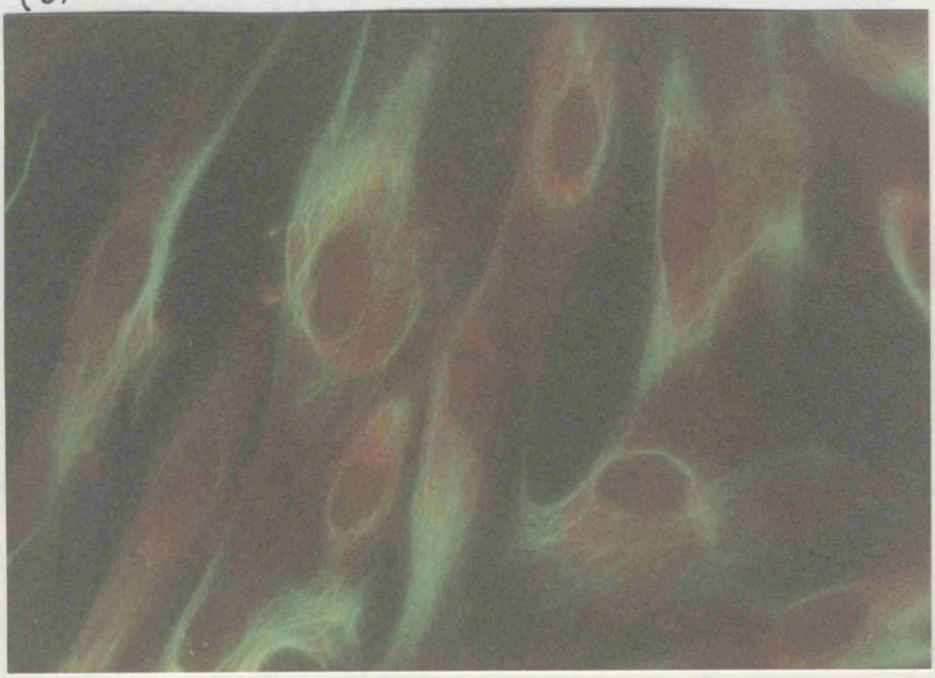
(b) Nuclear staining abolished following DNase extraction.

(c) No further change apparent following high salt extraction.

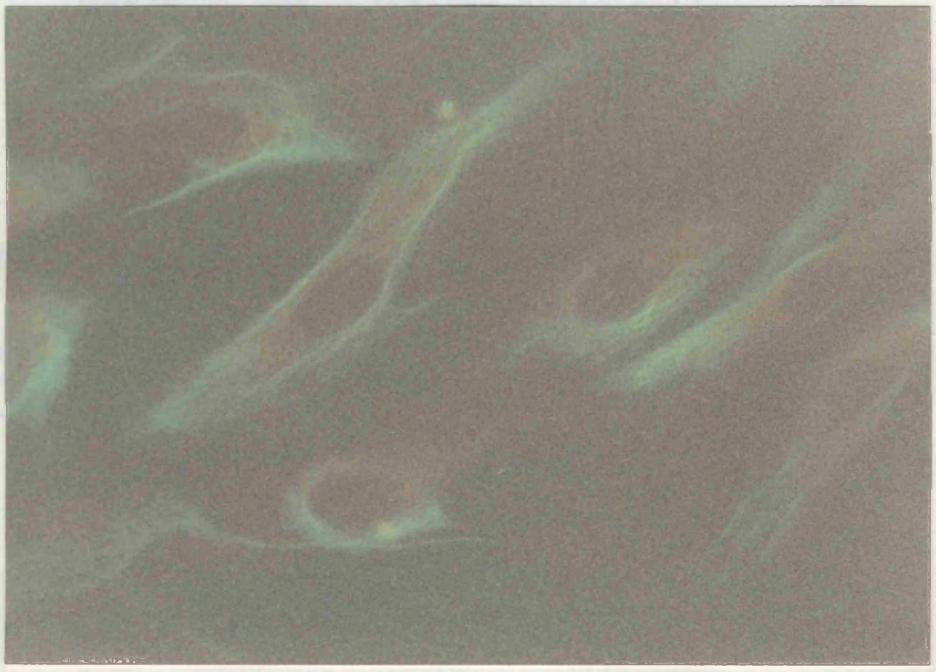
(a)



(b)



(c)



The data on the monoclonal autoantibodies derived from all sources are summarised in table 6.5.

Table 6.5

(a) Summary table of monoclonal autoantibodies derived from SLE patients.

Patient	Hybrid	Ig class	Elisa reactivity	Specificity
CS (anti-Ro)	CSD4*	IgG	Ro+La	cytoplasm/membrane
	CSH5	IgG	'false positive'	IF
	CSH11*	IgM	'false positive'	IF
	CSA8	IgM	'false positive'	IF
	CSD1*	IgM	'false positive'	IF + microfilaments
NG (anti-Ro)	NGB1	IgM	Ro+La	cytoplasm/membrane
HS (anti-Ro+La)	HSE7*	IgM	Ro,La,RNP	cytoplasm/membrane
SWa (anti-nRNP)	SWE3	IgM	'false positive'	IF
	SWF2*	IgM	'false positive'	IF
	SWD10*	IgM	'false positive'	IF
	SWG10	IgM	weak La+nRNP	cytoplasm/membrane
	SWH10*	IgM	'false positive'	IF
	SWC9	IgM	'false positive'	IF
	SWH1	IgM	'false positive'	IF
SWo (anti-nRNP)	SWA3	IgM	negative	IF
EC (anti-Ro+La)	ECD2	IgM	negative	IF

* Twice-cloned hybrids.

(b) Summary table of monoclonal autoantibodies derived from MG patients.

Patient	Hybrid	Ig class	Elisa reactivity	Specificity
JA (anti-DNA)	JAA10*	IgM	N.D.	IF
	JAE6*	IgM	strong DNA	IF + DNA
JS	JSC8	IgM	N.D.	IF

* Twice-cloned hybrids.
N.D. Not done.

(c) Summary table of monoclonal autoantibodies derived from normals.

Source	Hybrid	Ig class	Elisa reactivity	Specificity
Normal PBL	MRD12*	IgM	N.D.	IF
	RHB6*	IgM	N.D.	IF
EBV-PBL 1	E1D2	IgM	N.D.	IF
	E1F6	IgM	N.D.	IF
	E1G2	IgM	N.D.	IF
EBV-PBL 2	E2A7	IgM	N.D.	cytoplasm/membrane
	E2E1	IgM	N.D.	IF
EBV-tonsil	T1B6	IgM	N.D.	IF
	T1F7	IgM	N.D.	cytoplasm/membrane
	T1E11	IgM	N.D.	IF

N.D. Not done.
* Twice-cloned hybrids.
IF-intermediate filaments.

8. Discussion

A striking feature of these results is that while both the MG and SLE groups were selected on the basis of high titres of serum antibodies (extremely high titres of autoantibodies were found in some of the patients), of 679 immunoglobulin-secreting hybrids screened, not one was detected with a specificity which was anticipated from the data on the patients' sera. This is in contrast to what is usually found in mouse-mouse fusions using spleen cells from an immunised animal, when frequently a large percentage of hybrids secrete MCA specific for the immunogen. The early reports of success in human-human fusions showed a high percentage of hybrids to make antibody of desired specificity (Olsson and Kaplan 1980, Croce et al. 1980).

One possibility to explain why no MCA of desired specificity were found is that the assays were not sensitive enough to detect them. The RIA for antibodies to AChR is dependent upon the integrity of a protein which may have been subject to considerable proteolysis. Muscle was obtained from legs amputated because of either vascular disorders or diabetic gangrene. It would seem likely that the leg muscles were probably subjected to varying periods of ischaemia with attendant partial autolysis. During the extraction steps considerable quantities of proteolytic enzymes were probably released and the enzyme inhibitors included in

the extraction buffers may not have completely protected the receptor. Thus there may have been receptor present with intact antigenic determinants, but unable to bind labelled toxin. Conversely, receptor may have been present which bound labelled toxin, but had certain antigenic determinants destroyed by proteolysis. A polyvalent antiserum might be expected to show considerable reactivity in such a situation of partial degradation of receptor, whereas the binding of a MCA would show an all or nothing binding. Carter et al. (1981) reported that most myasthenic sera were capable of precipitating only 46-86% of toxin-labelled AChR under conditions of excess serum. This may support the notion that much of the receptor is indeed degraded.

However, the RIA was shown to be adequate, at least for certain specificities of MCA, by raising murine MCA against electric eel AChR and demonstrating that some of these could cross react with human AChR in such a RIA and give a clear positive result (data not shown).

Loss of certain antigenic determinants may have occurred during the purification of the ribonuclear proteins, again by proteolysis or denaturation during acid elution from the affinity columns. However, positive ELISA reactions could be demonstrated for some of the SLE patients' sera at dilutions as high as 1 : 100,000, which suggests that the assays should have been sensitive enough to detect at least some MCA.

Human or rodent MCA may demonstrate "assay

specificity". That is they may perform excellently in one assay but give mediocre or even negative results in another (Haaijman et al. 1984). It was for this reason that indirect immunofluorescence was additionally used as a screen. Although certain antigenic determinants may have been lost during acetone fixation, extensive proteolysis should have been avoided. That no expected specificities of MCA were demonstrated by either type of assay indicates that the failings were not due to "assay specificiy".

Another possible explanation of why no expected MCA specificities were made concerns the source of the lymphocyte fusion partner. The peripheral circulation may be a poor source of lymphocytes of relevant specifities as compared to either spleen or lymph node tissue. The immunoglobulin class and subclass distribution of immunoglobulin-positive, circulating lymphocytes does not reflect the relative concentrations of serum immunoglobulins (McConnell 1975). IgM positive cells account for about 82% of immunoglobulin positive B cells but only 8% of serum immunoglobulin. Conversely, IgG1 and IgA positive cells form less than 2% of circulating B cells but account for 72% of the total serum immunoglobulin. It is possible that predominant antigen binding specificities of serum immunoglobulins may be similarly poorly represented in the circulating blood lymphocyte population.

In this context it should be noted that the

predominant class of immunoglobulins made by the hybrids was IgM, yet very little of the serum autoantibodies were found to be of this class in the SLE patients' sera, and IgG is by far the most prevalent class in which anti-AChR antibodies are found in MG sera (Lefvert 1981).

Although little work has been done to compare lymphocytes from different sources, Olsson et al. (1983) compared the fusion of spleen cells and PBL of mice immunised with SRBC. Fusions with PBL yielded far fewer growing hybrids and far fewer immunoglobulin-secreting hybrids. However, when the percentage of immunoglobulin-secreting hybrids which make anti-SRBC antibodies is compared there is very little difference between the two sources of lymphocytes (table 6.6).

Table 6.6 Comparison of lymphocyte source in the production of anti-SRBC MCA from immunised mice.

Lymphocyte source	% wells +ve growth	% secreting Ig	% of Ig +ve secreting anti-SRBC antibody
SRBC	64%	50%	16%
PBL	5%	2.5%	20%

Extrapolating to the human situation, one might expect that where antigens have either been administered or the subject is undergoing an immune response to an infectious agent, fusions with PBL should yield antibody secretors with reasonable frequency. Indeed many of the successes in producing human MCA by fusion with PBL have followed immunisation or infection with e.g. tetanus toxoid (Butler et al. 1983, Larrick et al. 1983), Keyhole limpet haemocyanin (Lane et al. 1982), 2,4-dinitrochlorobenzene (Olsson and Kaplan 1980), Rhesus D positive cells (Bron et al. 1984) and measles virus (Croce et al. 1980).

Haskard and Archer (1984) studied another autoimmune disease, rheumatoid arthritis, and found only one hybrid from 530 human-human hybrids to make rheumatoid factor. It seems probable that during chronic autoimmune responses, as seen in the patients in this study and the RA patients of Haskard and Archer's study, the appropriate B cell specificities or B cells at the appropriate state of activation for fusion are not as well represented in the peripheral circulation as the cases outlined above. Although the ELISAs for antibodies to La, Ro, Sm, nRNP and IgG have been demonstrated to be both sensitive and specific, no MCA from the SLE fusions were found which were specific for a single antigen. Four were found which reacted weakly with 2 or 3 antigens. Ten were found which apparently reacted with the ELISA cuvettes, even though the latter had been

blocked by either casein or BSA. Only one MCA (JAE6 from an MG patient) demonstrated a specific reactivity by ELISA, in this case anti-DNA.

It seems unlikely that the 4 MCA from SLE patients which reacted with 2 or 3 antigens by ELISA are representative of circulating antibodies peculiar to SLE, and certainly do not represent the specific ANA characteristic of this disease. Whereas these MCA clearly gave higher ELISA readings compared to irrelevant MCA of even 10 fold higher concentration, both SLE and normal sera diluted 1:1000 (i.e. to levels of immunoglobulin comparable to the MCA in the supernatant) demonstrated equal or higher binding to the immobilised antigens. It seems doubtful that such weak binding is of significance. It may represent the binding of antibodies to minor impurities in the ribonuclear antigen preparations.

In this context it should be noted that only two of the four MCA showing 'restricted' ELISA reactivities demonstrated binding to cells by immunofluorescence. The even staining patterns shown by these and two similar MCA from normals are not typical of patterns given by ANA positive sera. The antigen(s) involved are insensitive to cytoskeleton-disrupting drugs, and are resistant to detergent, DNase and salt extraction. Their location is thus more likely to be the cytoplasm than the membrane. Cote et al. (1983) described human anti-cytoplasmic MCA derived from both cancer patients and normals which gave a similar

immunofluorescence pattern against cultured cells, but the authors reported no attempt to further characterise the antigen(s) involved. While more work needs to be done on characterising the specificity of these MCA, they do not seem to be related to overt disease.

The most suprising finding of this study was the high percentage of human MCA which bound to cytoskeletal structures. They were found in MCA derived from SLE PBL (9%), MG PBL (5%), normal PBL (6%), EBV-transformed PBL (5%) and EBV-transformed tonsil B cells (4%). The structures recognised form perinuclear coils after treatment with Colcemid or vinblastine, but are not affected by cytochalasin B, and they are resistant to extraction by high salt, DNase and detergent. The drug treatment and *in situ* fractionation data are thus consistent with the cytoskeletal structures being intermediate filaments of the vimentin type (Virtanen, 1984). As has been discussed in Chapter One, intermediate filament antibodies have been frequently reported in human sera. Antibodies predominantly of the IgM class are found to be elevated during acute viral infections such as hepatitis, chicken pox, measles, mumps and infectious mononucleosis (Toh *et al.* 1979). Similarly, in 82% of sera from patients with acute malaria, IgM antibodies to intermediate filaments could be demonstrated, compared to only 8% of normal sera (Mortazavi-Milani *et al.* 1984a). Elevated anti-intermediate filament antibodies have been reported

in a number of autoimmune diseases; Angioimmunoblastic lymphadenopathy (Dellagi et al. 1984), RA (Osung et al. 1982), CREST and SLE (Senecal et al. 1982, Alcover et al.1984, Kurki et al. 1983). Normal PBL can be induced to produce anti-vimentin intermediate filament antibodies in vitro by polyclonal activators such as EBV (Whitehouse et al. 1974, Mortazavi-Milani et al. 1982). In MCA production in mice, Dales et al. (1983) found that after immunisation of Balb/c mice with vaccinia virus, fusions preferentially yielded hybrids secreting antibodies with binding activity to intermediate filaments of mouse and human cells. Recently Haskard and Archer (1985) have reported that 10 of 425 human-human hybrids from RA patients produced anti-intermediate filament MCA.

The presence of antibodies to intermediate filaments has been taken to indicate the presence of a viral infection. This is partly because of the large number of viral infections described in which anti-intermediate filament antibodies have been found, and also because a cross-reaction of measles virus phosphoprotein and herpes simplex virus protein with intermediate filaments has been described (Fujiyami et al. 1983). The presence of anti-intermediate filament antibodies has been taken as evidence of a viral aetiology for SLE (Alcover et al. 1984), and angioimmunoblastic lymphadenopathy (Dellagiet al. 1984). Allison et al. (1971), and Toh et al. (1979) suggested that viral antigens and cytoskeletal structures may

facilitate autoimmunisation by stimulating helper or by-passing suppressor T cells.

An alternative explanation for the appearance of anti-intermediate filament antibodies is that they are produced not by antigenic stimulation, but by polyclonal activation. This explains their appearance in situations where viruses are not implicated, such as malaria and trypanosome infections (Mortazavi-Milani et al 1984), and in vitro responses of lymphocytes to polyclonal activators (Mortazavi-Milani et al 1982). The finding of this study that MCA to intermediate filaments can be produced with high frequency not only from autoimmune patients but also from healthy subjects supports this idea. If the high frequency of MCA with specificities for intermediate filaments reflects a similar proportion of circulating B cells with such specificities, then it is not suprising that anti-intermediate filament antibodies appear in the serum in diverse situations which have in common a polyclonal activation component.

As no anti-intermediate filament antibodies were detected in the sera of the subjects in this study, it seems probable that the population of B cells which were fused represented a precursor or possibly memory population. That the majority of hybrids secreted IgM is consistent with this idea. It could be argued that such a high proportion of anti-intermediate filament specific B cells reflects a history of viral infection. However, the presence of such large numbers in normal subjects, and

the ease in which they can be elicited by polyclonal activators warns against attributing too great a significance to their appearance in certain diseases or taking their presence as indicative of a viral aetiology for any of the autoimmune diseases.

It is very suprising that such a large percentage of B cells are programmed to produce antibodies to intermediate filaments. It is possible that certain B cells are preferentially fused, and these include a misleadingly high proportion of anti-intermediate filament reactive cells. However, the widespread occurrence of serum antibodies with this specificity argues that the B cell repertoire is indeed biased to produce such antibodies. It may be that autoantibodies of this specificity may be involved in the normal physiological removal of tissue degredation products as suggested by Grabar (1983). Circulating levels of such autoantibodies would conceivably be controlled to such levels as necessary for this function. Thus anti-intermediate filament antibodies may be present in normal sera in their complexed form and hence not detected until such circumstances (e.g. polyclonal activation) that they are in excess over their antigen concentrations. If this is so there must be something peculiar to the removal of intermediate filaments that merits so much of the immune system's attention, and why this should be so is not clear. Another problem with this explanation (and Grabar's theory in general) is that

agammaglobulinaemia patients should be expected to show some symptoms resulting from inadequate clearance of intermediate filaments (and other tissue degradation products), and no such symptoms have been recognised.

The anti-intermediate filament MCA isolated in this study demonstrated some interesting cross-reactions. JAE6 from a myasthenic patient stained both intermediate filaments and the entire nucleus. The nuclear staining but not the intermediate filament staining was abolished by DNase treatment, and by ELISA a very strong reaction was demonstrated to DNA, but not RNA, IgG or any nucleoproteins. Such a cross reaction has recently been recognised by Andre-Schwartz et al. (1984) The authors described 4 human monoclonal antibodies isolated from SLE patients which give very similar staining patterns and sensitivities to both cytoskeletal-disrupting drugs and DNase, as does JAE6. By immunoblot tequniques they found the MCA to recognise a protein of Mr 54,000 consistent with the vimentin polypeptide.

One MCA, CSD1, from an SLE patient, recognised both Colcemid and cytochalasin B-sensitive cytoskeletal structures. This is consistent with the MCA recognising both intermediate filaments and microfilaments. The microfilaments were visibly stained after disruption of the intermediate filaments by Colcemid and displayed a highly characteristic pattern. Although the main protein of microfilaments is actin, several other proteins are also present (Schliwa 1981, Korn 1982). The exact protein

recognised in the microfilaments by CSD1 needs to be determined by immunoblotting. The demonstration of a cross reaction between intermediate filaments and microfilaments by a MCA has not to the author's knowledge been reported before.

The 10 MCA which bound to both blocked ELISA trays and intermediate filaments represents a cross-reaction only very recently recognised in the literature. Haskard et al. (1985) reported 5 of 383 IgM-secreting hybrids derived from RA patients gave a similar 'false positive' reaction on blocked 'Falcon' ELISA trays. Of these, 4 gave immunofluorescence patterns expected of anti-intermediate filament antibodies in that they stained filaments which collapse on treatment with Colcemid. This cross-reaction may occur through either common epitopes being present on the assay plates and intermediate filaments, or through binding of MCA by non-idiotypic sites i.e. not conventional antibody-antigen combinations.

A feature of experiments with MCA in general has been the repeated demonstration of cross-reacting sites on molecules in which conventional serology has failed to detect homology (Lane and Koprowski, 1982). Many of these cross-reactions are between unrelated molecules. Thus Hannestad and Stollar (1978) reported a MCA which bound to both the Fc portion of IgG and chromatin, Jacob et al . (1984) found a MCA which reacted to both DNA and a cell surface protein, and Wood et al.

(1982) found a MCA to a surface antigen of Trypanosoma cruzi which also reacted with neurons and cardiac muscle. In the literature of cross-reacting MCA, anti-intermediate filament specificities are becoming pre-eminent. Dulbecco et al. (1981) found 3 of 13 rodent monoclonal anti-Thy-1 antibodies (2 IgM and 1 IgG) to react with vimentin-type intermediate filaments. Blase et al. (1982) reported a MCA-defined common determinant between tropomyosin and vimentin. Dales et al. (1983) found among many IgM, anti-intermediate filament MCA derived from Balb/c mice immunised with vaccinia virus, 1 IgM MCA which also recognised the virally-coded haemagglutinin glycopeptide. Fujinami et al. (1983) described two rodent MCA; one which bound the phosphoprotein of measles virus and intermediate filaments, and another which bound both a protein of herpes simplex virus and intermediate filaments. Andre-Schwartz et al. (1984) found 4 IgM MCA from SLE patients which were selected as anti-DNA which also bound vimentin intermediate filaments. Rubin et al. (1984) found 4 of 23 mouse monoclonal IgM anti-IgG antibodies which also bound to intermediate filaments. It seems very unlikely that a single cross-reacting epitope can explain all these observations. As these MCA recognise intermediate filaments and a single other antigen (DNA or IgG, or plastic rather than all 3 for example), one would have to suppose that intermediate filaments carry a large number of epitopes with fortuitous cross-reactivities on

unrelated molecules. A more reasonable explanation is that two distinct regions of the antibodies are involved in binding; the variable region to the antigen and either a part of the variable region or a completely distinct region of the immunoglobulin concerned with binding to intermediate filaments. The amino acid sequence of the immunoglobulin concerned with intermediate filament binding may be a fairly frequent event independent of antigen-driven, clonal expansion. It is perhaps significant that the overwhelming majority of anti-intermediate filament antibodies are of the IgM class, suggesting that perhaps the binding is dependent on a structural feature usually restricted to a proportion of this class of molecules.

In conclusion, this study has found that MCA with autoantibody specificities are made with only very low frequency when PBL are fused from SLE and MG patients with extremely high titres of serum autoantibodies. Expansion of the PBL with EBV prior to fusion would allow many more immunoglobulin-secreting hybrids to be screened without increasing the absolute frequency of relevant clones. Solid lymph tissue from autoimmune patients may be a better source of lymphocytes with autoantibody specificities than the peripheral circulation. MCA with specificities for intermediate filaments were a common feature of hybrids produced from PBL and tonsil from normals and autoimmune patients. This is consistent with a large percentage of B cells being present in the normal circulation reactive with intermediate filaments and

capable of secreting antibodies in the diverse situations reported. The finding here and elsewhere that very many of these anti-intermediate filament MCA show unexpected cross-reactivities with unrelated molecules, and their class almost always being IgM, suggests that the intermediate filament binding may not be a conventional antigen-antibody binding. This should warn against attaching to much significance to their appearance in viral or autoimmune diseases.

Conclusions.

The first aim of this project was to evaluate the performance of one human fusion partner, HMy2, and two mouse partners, NS1 and X63, in the production of human MCA by the PEG-induced fusion method. Serious problems were encountered with the human fusion partner. Half the fusions performed with the HMy2 line failed to produce any hybrids, and overall only one hybrid was formed per 1.6×10^7 lymphocytes fused. This is approximately one thousand times less efficient than fusions involving rodent-rodent fusion partners. As only limited numbers of lymphocytes were available from autoimmune patients, such a low fusion frequency made it impractical to attempt making human MCA with this line. The HMy2 line also suffers from the disadvantage of secreting IgG, and thus hybrids formed from it are likely to secrete mixed molecules resulting in considerable dilution of the desired antibody. These two problems have been reported by other workers with this line and almost all other fusion partners. Despite considerable effort, no human fusion partner has been made which compares favourably in performance with rodent cell lines used for making rodent MCA.

Both murine fusion partners, NS1 and X63 were found to fuse with human PBL with higher efficiency than did HMy2. All fusions with these lines yielded hybrids, and the fusion frequencies were approximately 15-50 times

higher than achieved with HMy2. As the X63 line fused with the highest frequency and produces no immunoglobulin of its own, this line was selected for intensive study. As well as fusing efficiently, the hybrids derived performed excellenty. Hybrids were made which supported the secretion of all major classes of human immunoglobulins of both kappa and lambda isotype. The hybrids cloned efficiently, were robust in culture and were easily recovered from storage in liquid nitrogen. By cloning early and repeatedly until all daughter clones were positive for immunoglobulin secretion, no problems of hybrid instability were encountered. For the majority of hybrids only two cloning steps were required to achieve stability.

The transformation of lymphocytes by EBV prior to fusion was found to be extremely advantageous. The fusion frequency was found to be even greater (approximately 1×10^{-4}) and -85% of the hybrids secreted human immunoglobulin compared to 10-30% when untransformed lymphocytes were fused. This represents a 50 fold increase in efficiency of generation of immunoglobulin-secreting hybrids. A population of dividing, transformed B cells also permits repeat fusions to be made from a single sample, and thus alows selection and expansion of cells making desired antibodies prior to fusion. The first problem which has plagued human MCA production, i.e. the efficient establishment of human immunoglobulin-secreting cell lines has thus been

overcome. The method outlined above represents a major improvement over currently used techniques, in terms of the number of immunoglobulin-secreting hybrids which can be made from small samples of blood, and the excellent characteristics of the hybrids obtained.

There still remains the problem of establishing lines of pre-defined specificity. At first sight the results from this study do not look very encouraging. No human immunoglobulin-secreting hybrids were derived from autoimmune patients which produced antibodies with specificities anticipated from the data on the patients' sera. However, there are a number of points to take into consideration. All fusions with B cells derived from autoimmune patients were performed without EBV transformation. The 679 human MCA screened may just have been too small a sample. This can easily be determined by performing another series of fusions following EBV transformation. This would allow many thousands of hybrids to be screened from each blood sample.

Another problem concerns the detection of MCA to the AChR. The raising of rodent MCA to the human AChR in this department has also proved to be extremely difficult. Although mice can be immunised to produce high serum antibody titres, no MCA-secreting hybrids were identified after screening nearly 1000 hybrids using a RIA analagous to the assay used in this study. No problems were encountered, however using AChR from the electric organ of Torpedo Electrophorus as the immunogen

and target in the assay. Thus there may have been a problem peculiar to the assay using human AChR for screening MCA (as discussed in Chapter 6). More confidence could be placed in the results of the remaining 220 MCA from SLE patients. However, this represents a very small sample from which to draw conclusions about the feasibility of obtaining monoclonal autoantibodies.

The choice of autoimmune patients as "naturally immunised" subjects may not have been an appropriate model to evaluate the production of human MCA. There remains the possibility that the relevant antigen specific B cells are not activated or mobilised in the same way in autoimmune patients as following deliberate immunisation. Once again, this can be determined by screening a far greater number of hybrids, or by using lymphocytes obtained from other lymphoid tissue.

EBV has been shown in other studies to be clearly capable of activating and transforming circulating B cells with specificities to administered antigens (Kozbor et al. 1979, Zurawski et al. 1978, Crawford et al. 1983, Yoshie and Ono 1980) and to autoantigens (Steinitz et al. 1980, Kamo et al. 1981). In view of the high fusion frequency of EBV transformed cells with X63, the rescue of specific antibody-secreting cells following transformation should be straightforward. Thus the production of human MCA of pre-defined specificity from PBL, following EBV transformation and

fusion looks extremely promising.

The most suprising finding concerning the MCA made in this study was the high percentage of MCA which demonstrated binding to intermediate filaments. Although the presence of serum autoantibodies to intermediate filaments has been described in SLE, they were not found in the sera of the patients studied here. Furthermore, similarly high proportions of anti-intermediate filament MCA were made following fusions with MG PBL, normal PBL and normal, EBV transformed tonsil B cells. Their frequent occurence cannot therefore be associated with overt disease. B cells have been demonstrated, in non-autoimmune subjects, which synthesise antibodies to autoantigens. It may be that the fusion process (with or without EBV transformation) rescues quiescent B cells, and thus the MCA made reflect the antibody potential of circulating B cells rather than the serum antibodies synthesised by mature plasma cells. The normally controlled autoreactivity present in us all is thus revealed. The fusion process may have something in common with the polyclonal activation of B cells. The high percentage of anti-intermediate filament MCA found is consistent with the frequent demonstration of anti-intermediate filament antibodies made following stimulation of B cells with PCA both <u>in vitro</u> and <u>in vivo</u>. The presence of anti-intermediate filament antibodies in many diverse infectious and autoimmune diseases may also a consequence of polyclonal activation

known to occur in these situations. It is difficult however, to understand why such a high percentage of B cells are present which produce anti-intermediate filament antibodies. It is not convincing to explain this in terms of a physiological function for such antibodies. It is also difficult to imagine the fusion process, EBV transformation and polyclonal activation all selecting for B cells making antibodies of this specificity. That these antibodies may not be binding to intermediate filaments by conventional antibody-antigen interactions is a distinct possibility. There is evidence which supports this idea. Many rodent MCA have been described which have been raised to very different molecules and then found to also bind to intermediate filaments. It would seem that many MCA with different antigen binding sites share a common feature of being able to bind intermediate filaments. The nature of the binding of these MCA needs to be examined by using immunoglobulin fragments rather than whole molecules, looking at fine specificities, and by comparison with antibodies raised by deliberate cross-species immunisation with intermediate filaments.

Although the findings of this study throw no light on the autoimmune diseases SLE and MG, the MCA reactive with intermediate filaments may contribute to a better understanding of the common occurence of such antibodies in a number of different disease situations. The efficient technique developed here of making human

MCA promises to finally bring human MCA technology onto a level with that of its rodent counterpart. The contributions of human MCA to the understanding of human immune responses and the understanding and treatment of human disease, promise to be equally as exciting as the revolution brought about by rodent MCA technology.

References.

ABRAMS,P.G., KNOST.J.A., CLARKE,G., WILBURN,S., OLDHAM,R.K., FOON,K.A., (1983)
J.Immunol. 131, No.3, 1201-1204.

AGELLI,M., BOMBARDIERI,S., IOPOLO,S. (1980)
IRCS Med. Sci. Biochem. 8, 754.

ALARCON-SEGOVIA,D., RUIZ-ARGUELLES,A., FISHBEIN,E. (1978)

Nature 271, 67-69.

ALARCON-SEGOVIA,D., RUIZ-ARGUELLES,A., LLORENTE,L. (1979)

J.Immunol. 122, 1855-1862.

ALARCON-SEGOVIA,D., WAKIM,K.G., WORTHINGTON,L.W., WARD,C.G. (1967)
Medicine 46, 1.

ALCOVER,A., MOLANO,J., RENART,J. (1984)
Arthritis Rheum. 27, 922-928.

ALLISON,A.C., DENMAN,A.M., BARNES,A.D. (1971)
Lancet i, 135.

ALMON,R.R., ANDREW,C.G., APPEL,S.H. (1974)
Science 186, 55-57.

ANDERSEN,C.L., ROSE,N.R. (1971)
Clin.Expt.Immunol. 107, 1341.

ANDRZEJEWSKY,C. STOLLAR,B.D., LALOR,T.N., SCHWARTZ,R.S. (1980)
J.Immunol. 124, 1499-1502.

ANDRE-SCHWARTZ,J., DATTA,S.K., SHOENFELD,Y., ISENBERG,D.A., STOLLAR,B.D., SCHWARTZ,R.S. (1984)
Clin.Immunol.Immunopath. 31, 261-271.

ANDRZEJEWSKY,C., RAUCH,J., LAFER.E., STOLLAR,B.D., SCHWARTZ,R.S. (1981)
J.Immunol. 126, 226-231.

ASTALDI,G.C.B., JANSSEN,M.C., LANSDORP,P., WILLEMS,C., ZEIJLEMAKER,W.P., OOSTERHOF,F. (1980)
J.Immunol. 125, No.4. 1411-1414.

BARKAS,T., GAIRNS,J., KERR,H., COGGINS,J., SIMPSON,J. (1982)
Eur.J.Immunol. 12, 757-761.

BARQUE,J.P., YENI,P., PERAUDEAU,L. (1981)
Biochem. Biophys. Res. Commun. 99, 284-291.

BARTOS,D., BARTOS,F. CAMPBELL,R.A. (1980)
Science 208, 1178.

BILLINGHAM,R.E., BRENT,L., MEDEWAR,P.B. (1953)
Nature 12, 602.

BILLINGHAM,R.E., BRENT,L., MEDEWAR,P.B. (1956)
Philos. Trans. R. Soc. Lond. 239, 357.

BILLINGS,P.B., HOCH,S.O. (1983)
J.Immunol. 131, 347-351.
BLASE,S., MATSUMARA,F., LIN,J. (1982)
Cold Spring Harbour Symp. Quant. Biol. 46, 455.

BOYLSTON,A.W., GARDNER,B., ANDERSON,R.L.,
HUGHES-JONES,N.C. (1980)
Scand.J.Immunol. 12, 355-358.

BRENNAND,D. (1983)
MSc Thesis, Universty of Bath.

BRENTJENS,J., OSSI,E., ALBIN,B., SEPULVEDA,M., KANO,K.,
SHEFFER,J., VALSILION,P., MARINE,E., BALIAH,T.,
JOCKIN,H., ANDREWS,G. (1977)
Arthritis Rheum. 20, 962-968.

BRUYNES,C., URBAIN-VANSANTEN,G., PLANNARD,C.,
DEVOS-CLEOTENS,C., URBAIN,J. (1976)
Proc.Natl.Acad.Sci. U.S.A. 73, 2462-2466.

BUCKINGHAM,J.M., HOWARD,F.M., BERNATZ,P.E., PAYNE,W.S.,
HARRISON,E.C., O'BRIEN,P.C., WEILAND,L.H. (1976)
An. Surg. 184, 453-458.

BURNET,F.M. (1962)
'The Integrity of the Body'. O.U.P.

BURNET,J.L., FENNER,F. (1949)
In 'The Production of Antibodies.' Macmillan Co.,
Melbourne.

BURNS,W.H., ALLISON,A.C. (1975)
In 'The Antigens' Ed. Sela,M. p479. Acad. Press.

BUTLER,J.L., LANE,C., FAUCI,S. (1983)
J.Immunol. 130, 165-168.

CANTOR,H., BOYSE,E.A. (1978) Cold Spring Harbour Symp.
Quant. Biol. 41, 23-32.

CARTER,B., HARRISON,R., LUND,G.G., MORRIS,H.,
SAVAGE-MARENGO,T., STEPHENSON,F.A. (1981)
Ann.Clin.Biochem. 18, 146-152.

CASTLEMAN,B. (1966)

Ann.N.Y.Acad.Sci. 135, 496-503.

CAVAGNARO,J., OSBAND,M.E. (1983)
Biotechniques 3, 30-36.

CHEN, T.R. (1979)
Cytogenet.Cell.Genet. 23, 221-230.

CHIORAZZI,N., WASSERMAN,R.J., KUNKEl,H.G. (1982)
J.Exp.Med. 156, 930-935.

COHEN,A.S., REYNOLDS,W.E., FRANKLIN,E.C., KULKA,J.P., ROPES,M.W., SHULMAN,L.E., WALLACE,S.L. (1971)
Bull. Rheum. Dis. 21, 643-648

COMPSTON,S., VINCENT,A., NEWSOME-DAVIS,J., BATCHELOR,J. (1981)
Brain 103, 579-601.

CONTI-TRONCONI,B.M., HUNKAPILLER,M., LINDSTROM,J.M., RAFTERY,M.A. (1982)
Proc.Natl.Acad.Sci. U.S.A. 79, 6489-6483.

COTE,R.J., MORRISSEY,D.M., HOUGHTON,A.N., BEATTIE,E.J., OETTGEN,H.F., OLD,L.J. (1983)
Proc.Natl.Acad.Sci.U.S.A. 80, 2026-2030.

CRAWFORD,D.H., HARRISON,J.F., HARLOW,M.H., WINGER,L., HUEHNS,E.R. (1983)
Lancet Feb. 19, 386-388.

CROCE,C.M., KNOWLES,B.B., KOPROWSKI,H. (1973)
Exp.Cell.Res. 82, 457.

CROCE,C.M., LINNENBACH,A., HALL,W., STEPIEWSKI,Z., KOPROWSKI,H. (1980)
Nature 288, 488-489.

CROCE,C.M., SHANDER,J.M., CICUREL,L., D'ANCONNA,G.G., KOPROWSKI,H. (1980)
Eur.J.Immunol. 10, 486-488.

CROCE,C.M., SHANDER,M., MARTINIS,J., CICUREL,L., D'ANCONA,G.G., DOLBY,T.W., KOPROWSKI,H. (1974)
Proc.Natl.Acad.Sci.U.S.A. 76, 3416-3419.

CUNNINGHAM,A.J. (1974)
Nature 252, 749.

CUNNINGHAM,A.J. (1976)
Transplant. Rev. 31, 23.

DALES,S., FUJINAMI,R.S., OLDSTONE,M.B.A. (1983)
J.Immunol. 131, 1546-1553.

DARR,A.S., FABRE,J.W.(1981)

Clin.Expt.Immunol. 45, 37-47.

DAU,P. (1982)
Ann.N.Y.Acad.Sci. 377, 700-708.

DAWSON,R.M.C., ELLIOT,D.C., ELLIOT,W.H., JONES K.M. (1974)
Data for Biochemical Research. 2nd Edit. O.U.P.

DELLAGI,K., BROUET,J.C., PERREAU,J., PAULIN,D. (1982)
Proc.Natl.Acad.Sci. U.S.A. 79, 446-450.

DELLAGI,K., BROUET,J.C., SCHENMETZLER,C. (1981)
Blood 47, 1875-1884.

DELLAGI,K., BROUET,J.C., SELIGMAN,M. (1984)
New Eng.J.Med. 310, No.4, 215-218.

DIGHIERO,G., GUILBERT,B., AVREAMAS,S. (1982a) J.Immunol. 128, 2779-2787.

DIGHIERO,G., GUILBERT,B., AVREAMAS,S. (1982b) J.Immunol. 128, 2788-2792.

DIXON,F.J., MAURER,P.H. (1955)
J.Exp.Med. 101, 245.

DOUVAS,A.S., STUMPH,W.E., REYES,P., TAN,E.M. (1979)
J.Biol.Chem. 254, 3608.

DOUVAS,A.S., TAN,E.M. (1981)
In 'The Cell Nucleus', Ed. Busch, H. Vol.8, p369.
Acad.Press, N.Y.

DRACH,G.W. (1973) Surg.Forum. 24, 35-37.

DRACHMAN,B.D. (1983)
Trends in Neurosci. Nov. p446-452.

DRACHMAN,D.B., ADAMS,R.N.,JOSIFEK,L., SELF,S. (1982)
New Eng.J.Med. 307, 769-775.

DRACHMAN,D.B., ANGUS,C.W., ADAMS,R.N., MICHELSON,J., HOFFMAN,G.J. (1978)
New Eng.J.Med. 298, 1116-1122.

DRESSER,D.W., (1978)
Nature 274, 480.

DRESSER,D.W., GOWLAND,G. (1964)
Nature 203, 733.

DRESSER,D.W., POPHAM,A.M. (1976)
Nature 264, 552.

DULBECCO,R., UNGER,M., BOLOGNA,M. (1981)

Nature 292, 772.

DUBOIS,E.L. (1969)
Medicine. 48, 217.

DWYER,D., KEARKNEY,J., BRADLEY,R., KEMP,G., OH,S., (1981)

Ann.N.Y.Acad.Sci. 377, 143-157.

DZIARSKI,R. (1982a)
J.Immunol. 128, 1018-1025.

DZIARSKI,R. (1982b)
J.Immunol. 128, 1026-1030.

EDINGTON,T.S., DELESSIO,D.J. (1970)
J.Immunol. 195, 248.

EDWARDS,P.A.W., SMITH,C.M., MUNRO NEVILLE,A. O'HARE,M.J.
(1982)
Eur.J.Immunol., 12, 641-648.

EILAT,D. (1982)
Molecul.Immunol. 19 NO.7., 943-955.

EILAT,D., ASAFSKY,R., LASJOV,R. (1980)
J.Immunol. 124, 766-768.

EINARSON,B.L., GULLICK,W.J., CONTI-TRONCONI,B.M.,
LINDSTROMJ.M. (1982)
Biochemistry 21, 5295-5302.

EISENBARTH,G.S., JACKSON,R., SRIKANTA,S. (1983)
In 'Monoclonal Antibodies' p 197-221. Eds. Haynes and
Eisenbarth, Academic Press.

EISENBARTH,G.S., LINNENBACH,A., JACKSON,R., SEARCE,R.,
CROCE,C.M.
(1982)
Nature 300, 264-267.

ELSON,C.J. (1977) Eur.J.Immunol. 7, 6-10.

ELSON,C.J., NAYSMITH,J.D., TAYLOR,R.B. (1979)
Int. Rev. Exp. Pathol. 19, 137-203.

ENGEL,A.G., SAHASHI,K., LAMBERT E.H., HOWARD,F. (1979)
Int. Cong. Ser. Excerpta Med. 455, 111-122.

ENGEL,A.G., SANTA,T. (1971)
ANAS 377, 110-124.

ERIKSON,J., MARTINIS,J., CROCE,C.M., (1981)
Nature 294, 173-175.

ESTES,D., CHRISTIAN,C.L. (1971)

Medicine, 50, 85.

FAMBROUGH,D.M. DRACHMAN,D.B., SATYAMURTI,S. (1973)
Science 182, 293-295.

FAUCI,A.S., STEINBERG,A.D., HAYNES,B.F. (1978)
J.Immunol. 121, 1473.

FAZEKAS DE ST.GROTH, SCHEIDEGGER,S. (1980)
J.Immunol.Meth. 35, 1.

FELTKAMP,T.E.W., VAN DEN BERG-LOONEN,P.M.,
NIJINHUIS,L.E., ENGELFRIET,C.P. (1974)
Br.J.Med. 1, 131.

FERNANDEZ,C., MOLLER,G. (1980)
J.Exp.Med. 11, 53.

FESSEL,G.J. (1974)
Arch.Int.Med. 134, 1027.

FONTANA,A., GROB,P.J., SANTHER,R., DUBS,R., MOTHERSILL,I.
(1978)
In 'Biochemistry of Myasthenia Gravis.' p.183. Eds. Lant
and Mashbanks. Acad. Press.

FOON,K.A., ABRAMS,P.G., ROSSIO,J.L., KNOST,J.A.
OLDHAM,R.K. (1983)
In Monoclonal Antibodies and Cancer pp. 143-155. Eds.
ROSS,B.D., LANGMAN,R., TROWBRIDGE,I., DULBECCO,R.
Academic Press.

FOURNEY,G.J., LAMBERT,P.H., MIESCHER,P.A. (1974)
J.Exp.Med. 140 1189.

FRANCO,H.L., WESTON,W.L., PEEBLES,C., FORSTOT.S.L.,
PHANUPHAK,P. (1981)
J.Am.Acad.Dermatol. 4, 67.

FRANCOEUR,A.M., MATHEWS,M.B. (1982)
Proc.Natl.Acad.Sci. U.S.A. 79, 6772.

FRESHNEY,R.I., (1983)
Culture of Animal Cells. Alan.R.Liss, Inc., N.Y. U.S.A.

FRITZE,D., HERMANN,C., FARRAMARZ,N. (1974) Lancet i
240-243.

FROLAND,S.S., NATWIG,J.B. (1972)
Scand.J.Immunol.I 1, 267.

FUCHS,S., BARTFIELD,D., MOCHLY-ROSEN,D., SOURVIYON,M.,
FEINGOLD,C. (1981)
Ann.N.Y.Acad.Sci. 377, 110-124.

FUJIYAMI,R.S., OLDSTONE,M.B.A., WROBLEWSKA,Z. (1983)

Proc.Natl.Acad.Sci. U.S.A. 80, 2346-2350.

GALFRE,G., MILSTEIN,C. (1981)
Methods in Enzymology 73, 3-46.

GIBBONS,J.J., AUGUSTYNEK,D., TSAI,C.C., ROODMAN,S.T.
Molecul.Immunol. 19, 765.

GLASSY,M.C., HANDLEY,H.H., HAGIWARA,H., ROYSTON,I. (1983)

Proc.Natl.Acad.Sci. U.S.A. 80, 6327-6331.

GOIDL,E.A., SCHRATER,A.F., SISKIND,G.W., THORBECKE,G.L.
(1979)
J.Exp.Med. 150, 138.

GOIDL,E.A., SCHRATER,A.F., SISKIND,G.W., THORBECKE,G.L.
(1980)
Eur.J.Immunol. 10, 810.

GOLDMAN,R.D., KNIPE,D.M. (1973)
Cold Spring Harb. Symp. Quant. Biol. 37, 523.

GOMEZ,C., RICHMAN,D., BERMAN,P., BARNES,S., ARNASON,B.,
FITCH,F. (1979)
Biochem. Biophys. Res. Commun. 88, 575-582.

GOMEZ,C., RICHMAN,D., BURNES,S., ARNASON,B. (1981)
Ann.N.Y.Acad.Sci. 377, 97-109.

GOODFELLOW,P., SOLOMON,E. (1982)
In Monoclonal Antibodies in Medicine. Eds. McMichael and
Fabre. Academic Press.

GORBARS,M., DHERMY,D., BOURNIER,O., BEZEAUD,A., BOIVIN,P.
(1981)
Clin.Expt.Immunol. 43, 87

GRABAR,P. (1975)
Clin.Immunol.Immunopath. 4, 453.

GRABAR,P. (1983)
Immunology Today 4, No. 12 337-340.

GRASHCHENKOV,N.I., PERELMAN,L.B. (1966)
Ann.N.Y.Acad.Sci. 135, 398.

GREY,H.M., KOHLER,P.F., TERRY,W.D., FRANKLIN,G.C. (1968)
J.Clin.Invest. 47, 1875-1884.

GRIBOR,R., EDMONDS,J., LEWKOMA,R., BRESNIHAN,B.,
HUGHES,G.R.V. (1978)
Annal.Rheum.Dis. 37, 121.

GUILBERT,B., DIGHIERO,G., AVRAMEAS,S. (1982)
J.Immunol. 128, 2779.

GULLICK,W.J., LINDSTROM,J.M. (1982)
J.Cell.Biochem. 19, 223-230.

GULLICK,W.J., TZARTOS,S.J., LINDSTROM,J.M. (1981)
Biochemistry 20, 2173-2180.

HAAIJMAN,J.J., DEEN,C., KROSE,C. (1984) Immunol.Today 5, 56-58.

HAHN,B.H., EBLING,F., FREEMAN,S., CLEVINGER,N., SAVIE,J. (1980)
Arthritis Rheum. 23, 942-945.

HAMMARSTROM,C., SMITH,E., PRINII,D. (1976) Nature 263, 60.

HANAN,R., OYAMA,J. (1954)
J.Immunol. 73, 49.

HANNESTAD,K., STOLLAR,B.D. (1978)
Nature 275, 671.

HARDIN,J.A., RAHN,D.R., SHEN,C., LERNER,M. (1982)
J.Clin.Invest. 70, 141.

HASKARD,D.O., ARCHER,J.R. (1984)
J.Immunol.Meth. 74, 361-367.

HASKARD,D.O., GUL,V., ARCHER,J.R.(1985)
J.Immunol.Meth. 77, 291-295.

HAWKINS,B.R., CHEAH,P.S., DAWKINS,R.L. (1980)
Lancet ii 1957-1059.

HENDRICK,J.P., WOLIN,S.L., RIMKE,J., LERNER,M.R., SEITZ,J.A. (1981)
Molecul.Cell.Biol. 1, 1138-1149.

HIRANO,T., TERAOKA,O., TERNSISHI,T., TSUYUGUCHI,I., TOHDA,H., OIKAWA,A. (1980)
Microbiol. Immuno. 24, 879-886.

HOBART,M.J., RABBITTS,T.H., GOODFELLOW,P.N. (1981)
Ann.Hum.Genet. 45, 331-5.

HOFFMAN,G.W. (1975)
Eur.J.Immunol. 5, 638-647.

HOKKANEN,E., TOIVAKKA,E. (1969)
Acta Neurol. Scand. 45, 556.

HOOPER,B., WHITTINGHAM,S., MATHEWS,J.D. (1972)
Clin.Expt.Immunol. 12, 79-87.

HOROWITZ,S., BORCHERDING,W., MOORTHY,A.V. (1977)

Science 197, 999.

HOUGH,D.W. MILLER,N.I., MADDISON,P.J. (1983)
Immunol.Letters 6, 123-127.

HUDSON,L., HAY, F.C. (1980)
Practical Immunology. Blackwell Scientific Publications.
Oxford.

HURD,E.A., DOURDLE,W., CASEY,H., ZIFF,M. (1972)
Arthritis Rheum. 15, 267-274.

INMAN,R.D. (1978)
Arthritis Rheum. 21, 849.

IVERSEN.G.M. (1970)
Nature. 227, 273.

IZUI,S., EISENBERG,R.A., DIXON,F.J. (1979a)
J.Immunol. 122, 2096.

IZUI,S., KOBAYAKAWA,T., LOUIS,J., LAMBERT,P.H. (1979b)
Eur.J.Immunol. 9, 338.

IZUI,S., ZALDIVAR,N.M., SCHER,I., LAMBERT,P.H. (1977)
J.Immunol. 119, 2151.

JACOB,L., TRON,F., BACH,J-F. (1984)
Proc.Natl.Acad.Sci. U.S.A. 81, 3843-3845.

JAMES,R., KATO,A., REY,M., FULPIUS,B. (1980)
FEBS LETT. 120, 145-148.

JAMI,J., GRANDCHAMP,S. (1971)
Proc.Natl.Acad.Sci. U.S.A. 68, 3097-3101.

JERNE,N.K. (1973)
Sc. Am. 229, 52-60.

JERNE,N.K. (1974a)
In 'Cellular Selection and Reguation in the Immune
Response'. Ed. Edelman,G.M. Raven, New York.

JERNE,N.K. (1974b)
Ann. Immunol. (Paris). 125, 373-389.

JOHNSTONE,A., THORPE,R. (1982)
Immunochemistry in Practice. Blackwell Scientific
Publications. Oxford.

KAMO,I., FURUKAWA,S., TADA,A., MANO,Y., IWASAKI,Y.,
FURUSE,T., ITO,N., HAYASHI,K., SATOYOSHI,E. (1982)
Science 215, 995-997.

KAO,I., DRACHMAN,D.B. (1977)
Science 195, 527-529.

KAPLAN,H.S., OLSSON,L., RAUBITSCHEK,A. (1982)
In Monoclonal Antibodies in Clinical Medicine. p 17-35.
Eds. McMichael and Fabre. Academic Press.

KEESEY,J.C., TOURTELLE,W.W., HERNMAN,C., ANDREWS,J.M., LINDSTROM,J. (1978)
Lancet i, 777.

KEPHART,D.C., HOOD,A.F., PROVOST,T.T. (1981)
J.Invest.Dermatol. 77, 331.

KLUSKENS,L., KOHLER,H. (1974)
Proc.Natl.Acad.Sci. U.S.A. 715083-5087.

KOFFLER,D. CARR,R., AGNELLO,V., THOBURN,R., KUNKEL,H.G. (1971)
J.Exp.Med. 134, 294-312.

KOFFLER,D., BIESECKER,G., KATZ,S.M. (1982)
Arthritis Rheum. 25, 858-861.

KOHLER,G., MILSTEIN,C. (1975)
Nature 256, 495-497.

KOHLER,G., MILSTEIN,C. (1976)
Eur.J.Immunol. 6, 511-519.

KOHLER,H. (1975)
Transplant Rev. 27, 24-56.

KOHLER,H., KAPLAN,D.R., STRAYER,D.S. (1974)
Science 186, 643.

KOHN,L.D., YAVIN,E., YAVIN,P., LACCETTI,P., VITTI,E., VALENTE,W. (1983)
In 'Monoclonal Antibodies' p222-259. Eds. Haynes and Eisenbarth, Academic Press.

KORN,E.D. (1982)
Physiol. Rev. 62, 672.

KOSKIMIES,S. (1979)
Scand.J.Immunol. 10, 371.

KOZBOR,D., LAGARDE,A.E., RODER,J.C. (1982b)
Proc.Natl.Acad.Sci. U.S.A. 79, 6651-6655.

KOZBOR,D., RODER,J.C. (1981)
J.Immunol. 127, No.4. 1275-1280.

KOZBOR,D., RODER,J.C. (1983)
Immunol. Today 4, No.3, 72-79.

KOZBOR,D., RODER,J.C., CHANG,T.H., STEPLEWSKI,Z., KROPROWSKI,H. (1982a)

Hybridoma 1, 323-328.

KOZBOR,D., STEINITZ,M., KLEIN,G., KOSKIMIES,S., MAKELA,O. (1979)
Scand.J.Immunol. 10, 187-194.

KURATA,N., HARA,I., MYAWAKI,S., OFIYI,T. (1979)
Arthritis Rheum. 22, 632.

KURATA,N., TAN,E.M. (1976)
Arthritis Rheum. 19, 574-579.

KURKI,P., HELVE,Y., VIRTANEN,I. (1983)
J.Rheumatol. 10, 558-562.

KURKI,P., MIETTINEN,A., LINDER,E., PIKKARAINEN,P., SALASPURO,M. (1980)
Gut 21, 878.

KURKI,P., VIRTANEN,I. (1984)
J.Immunol.Meth. 67, 209-223.

KURLAND,L.T. (1957) Quoted by Osserman and Genkins (1972)

Mt. Sinai J. Med. 38,497.

KUZNETSOV,S.A., RODINOV,V., BERSHADSKY,A.D., GELFAND,V.I., ROSENBLAT,V.A. (1980)
Cell Biol.Int.Rep. 4, 1017.

LAEMMLI,U.K. (1970)
Nature 227, 680-685.

LANE,D., KOPROWSKI,H. (1982)
Nature 296, 200.

LANE,H.C., SHELAMER,J.H., MOSTOWSKI,H.J., FAUCI,A.S. (1982).
J.Exp.Med. 155, 333-338.

LARRICK,J.W., TRUITT,K.E., RAUBITSCHEK,A.A., SENYK,G., WANG,J.C.N. (1983)
Proc.Natl.Acad.Sci. U.S.A. 80, 6376-6380.

LATOV,N., SHERMAN,W.H., NEMNI,R. (1980)
New Eng.J.Med. 303, 618-621.

LEE,C.Y. (1972)
Ann.Rev.Pharmacol. 12, 265-286.

LEE,J.S., LEWIS,J.R., MORGAN,A.R., MOSMAN,T.R., SINGH,B. (1981)
Nucleic Acid Res. 9, 1499-1721.

LEFVERT,A.K. (1981)
Ann.N.Y.Acad.Sci. 377, 125-142.

LEFVERT,A.K., PIRSKANEN,R., (1977).
Lancet ii, 351.

LENNON,V.A. (1978)
In Cholinergic Mechanisms and Psychopharmacology (Ed Jenden), p77-92. Plenum Press N.Y.

LENNON,V.A., LAMBERT,E.H. (1981)
Ann.N.Y.Acad.Sci. 377, 77-96.

LERNER,E.A., LERNER,M.R., HARDIN,J.A., JANEWAY,C.A., STEITZ,J.A. (1982)
Arthritis Rheum. 25, 761-766.

LERNER,E.A., LERNER,M.R., JANEWAY,C.A. STEITZ,J.A. (1981)

Proc.Natl.Acad.Sci. U.S.A. 78, 2737-2781.

LERNER,M.R., BOYLE,J.A., MOUNT,S.M., WOLIN,S.C., STEITZ,J.A. (1980)
Nature 283, 220-224.

LERNER,M.R., STEITZ,J.A. (1979)
Proc.Natl.Acad.Sci. U.S.A. 76, 5495-5499.

LERNER,M.R., STEITZ,J.A. (1981)
Cell 25, 298-300.

LEVIN,R.E., WEINSTEIN,A., PETERSON,M., TESTA,M.A., ROTHFIELD,N.F. (1984)
Arthritis Rheum. 27, 530-538.

LEVY,R., DILLEY,J. (1978)
Immunol. 75, No.5, 2411-2415.

LIDMAN,K., BIBERFELD,A., FAGRAEUS,R., NORBERG,R., TORSTENSSON,R., UTTER,G., CARLSSON,J., LINDBERG,U. (1976).
Clin.Expt.Immunol. 24, 266.

LIEU,T.S., JIANG,M., STEIGERWALD,J.C. TAN,E.M. (1984)
J.Immunol.Meth. 71, 217-228.

LINDSTROM,J.M. (1977)
Clin.Immunol.Immunopath. 7, 36-43.

LINDSTROM,J.M. (1983)
In 'Monoclonal Antibodies' p 259-296. Academic Press. Eds. Haynes B.F. and Eisenbarth G.S.

LINDSTROM,J.M., COOPER,J.F., TZARTOS,S.J. (1980)
Biochemistry 19 1454-1458.

LINDSTROM,J.M., ENGEL,A.G., SEYBOLD,M.E., LENNON,V.A., LAMBERT,E.H. (1976)

J.Exp.Med. 144, 739-753.

LINDSTROM,J.M., SEYBOLD,M.E., LENNON,V.A., WHITTINGHAM,S., DUANE,D.D. (1976) Neurology 26, 1054-1059.

LINDSTROM,J.M., TZARTOS,S.J., GULLICK,W.J. (1981) Ann.Natl.Acad.Sci. 377,1-19.

LUDUENA,R.F., FELLOUS,A., FRANCON,J., NUNES,J., McMANUS,L. (1981) J.Cell.Biochem. 89, 680.

MacGILLIVRAY,A., CARROLL,A.R. (1982) In 'Biochemistry and Biology of the Cell Nucleus' (Hnilica,L.S. Ed.) CRC Press, Boca-Ratori FL.

MacGILLIVRAY,A., CARROLL,A.R., DAHI,S. (1982) Febbs. Lett. 141, 139.

MADDISON,P.J., REICHLIN,M. (1979) Arth. Rheum. 22, 858.

MADDISON,P.J., SKINNER,R.P., VLACHOYIANNOPOULOS,P. BRENNAND,D., HOUGH,D. (In Press) Clin.Exp.Immunol..

MADSEN,M., JOHNSTON,H.E., HANSEN,P.W., CHRISTIANSEN,S.E. (1980) J.Immunol.Meth. 33, 323-326.

MARCH,S., PARIKH,T., CUATRECASCAS,P. (1974) Anal.Biochem. 60, 149-152.

MARION,T.N., LAWTIN,A.R., KEARNEY,J.R., BRILES,D.E. (1982) J.Immunol. 128, 668-674.

MATSUYA,Y. GREEN,H., (1969) Science 163, 697-698.

McCONNELL,I. (1975) The Immune System. Blackwell. London.

MERLIE,J.P., HEINEMANN,S. LINDSTROM,J.M. (1979) J.Biol.Chem. 254, 6300-6327.

METCALF,E.S., KLINMAN,N.R. (1976) J.Exp.Med. 143, 1327-1340.

MEUNIER,J.C., OLSEN,R.W., MENEZ,A., FROMGEOT,P., BOQUET,P., CHANGEUX,J.P. (1972) Biochem. 11, 1200-1210.

MIGGIANO,V., NABHOLTZ,M., BODME,W. (1969) Wister Inst. Symp. Monogr., 9, 61-76.

MILSTEIN,C.,(1982)

In Monoclonal Antibodies in Clinical Medicine. Eds. McMichael and Fabre. Academic Press.

MILLER,G., LIPMAN,M. (1973)
Proc.Natl.Acad.Sci.U.S.A. 70, 190-194.

MINITER,M.F., STOLLAR,B.D., AGNELLO,V. (1979)
Arthritis Rheum. 22, 959-968.

MINNA,J., COON,H.G. (1974)
Nature 252, 401-404.

MITCHISON,N.A. (1982)
In 'Receptors, antibodies and disease'. Ciba Foundation Symposium 90, p57 Pitman.

MOCHLY-ROSEN,D., FUCHS,S. (1981)
Biochemistry 20, 5920-5924.

MOROI,Y., PEEBLES,C., FRITZLER,M.J., STEIGERWALD,J., TAN,E.M. (1980)
Proc.Natl.Acad.Sci. U.S.A. 77, 1627.

MORTAZAVI-MILANI,S.M., BADAKERE,S.S., HOLBOROW,E.J. (1984a)
Clin.Exp.Immunol. 55, 177-182.

MORTAZAVI-MILANI,S.M., FACER,C.A., HOLBOROW,E.J. (1984b)
Immunology 52, 423-426.

MORTAZAVI-MILANI,S.M., STIERLE,H.E., HOLBOROW,E.J. (1982)
Immunol.Letters 5, 203-205.

MOUNT,S.M., STEITZ,J.A. (1981) Nucleic Acids Res. 9, 6351-6368.

NABHOLTZ,M., MIGGIANO,V., BODMER,W. (1969)
Nature 223, 358-363.

NAGAYAMA,A., DALES,S. (1970)
Proc.Natl.Acad.Sci. U.S.A. 60, 464.

NAKAMURA,R.M., PEEBLES,C.L., TAN,E.M. (1978)
Amer.J.Clin.Pathol. 70, 800-807.

NEWSOM-DAVIS,J., VINCENT,A., WILCOX,N. (1982)
In 'Receptors, Antibodies and Disease', p225-238. Pitman Press.

NISONOFF,A., BANGASSER,S.A. (1975)
Transplant. Rev. 27, 263-283.

NORBERG,R., THORSTEINSSON,T., UTTER,G., FAGRAEUS,A. (1979)
Eur,J.Biochem 100, 575.

NORUM,R., MIGEON,B. (1974)
Nature 251, 72.

NOSSAL,C.J.V., PIKE,B.L. (1975)
J.Exp.Med. 141, 904-917.

NOSSAL,C.J.V., PIKE,B.L., TEALE,J.M., LAYTON,J.E.,
KAY,T.W., BATTYIE,F.L. (1979) Immunol.Rev 43, 18-26.

NOTMAN,D.D., KURATA,N., TAN,E.M. (1975)
Ann.Int.Med. 83, 464-469.

NOWINSKI,R., BERGLUND,C., LANE,J., LOSTROM,M.,
BERNSTEIN,I., YOUNG,W., HALOMORI,S., HILL,L., COONEY,M.
(1980)
Science 210, 237-239.

OLSSON,L., KAPLAN,H.S. (1980)
Proc.Natl.Acad.Sci.U.S.A. 77, No.9, 5429-5431.

OLSSON,L., KRONSTIM,H., CAMBON DE MOUZON,A. (1983)
J.Immunol.Meth. 61, 17-32.

OSBAND,M., CAVAGNAW,J., KUPCHIK,H.G. (1981)
Blood 60, (5) Suppl.81a.

OSSERMAN,K.E. (1958)
Myasthenia Gravis. Grune and Stratton, N.Y.

OSUNG,O.A., CHANDRA,M., HOLBORROW,E.J. (1982)
Ann.Rheum.Dis. 41, 69.
56, 281-284.

PAGES,J.M., BUSSARD,A.E. (1978) Cell.Immunol. 41, 188-94.

PAPAZIAN,D. (1976) Neurology 26, 311-316.

PATRICK,J., LINDSTROM,J. (1973)
Science 180, 871-872.

PEARSALL,H.R., EWALT,J., TSOI,M.S., SUMIDA,S. (1974)
J. Lab. Clin. Med. 83, 728.

PEDERSEN,J.S., TOH,B.H., MACKAY,I.R., TAIT,B.D.,
GOST,I.D., KASTELAN,A., HADZIC,N.Z. (1982)
Clin.Exp.Immunol. 48, 527.

PERNIS,B., FERRARINI,M., FORNI,L. (1971)
In 'Progress in Immunology', Ed.Amos,B. p.95. Academic
Press.

PINCHING,A.H., PETERS,D.K., NEWSOME-DAVIS,J. (1976)
Lancet ii, 1373-1376.

PINCUS,T. (1982)
Arthritis Rheum. 25, 847-856.

PINCUS,T., STEINBERG,A.D., BLACKLOW,N.R., DECKER,J.L. (1978)
Arthritis Rheum. 21, 873-879.

PIRSKANEN,R., TILLIKAINEN,A., HOKKANEN,E. (1972)
Ann. Clin. Res. 4, 304.

PISETSKY,D.S. (1983)
In 'Monoclonal Antibodies' p153-172. Academic Press.

POLLARD,T.D. (1981)
J.Cell.Biochem. 91, 156s-165s.

PONTECORVO,G. (1971)
Nature 230, 367-369.

PREUD'HOMME,J.L., SELIGMAN,M. (1972)
J.Clin.Invest. 51, 701.

PROVOST,T.T. (1979)
J.Invst.Dermatol. 72, 110-113.

PUJOL-BORRELL,T., HANAFUSA,L., CHIOVATO,L. (1983)
Nature 304, 71-73.

PUMPLIN,D.W., DRACHMAN,D.B. (1983)
J.Neurosci. 3, 576-584.

RAFF,M.C., OWEN,J.T., COOPER,M.D. LAWTON,R., MEGSON,M., GATHINGS,W.E. (1975)
J.Exp.Med. 142, 1952-1064.

RAFTERY,M.A., HUNKAPILLER,M., STRADER,C., HOOD,L. (1980)
Science 208, 1454-1457.

RAUCH,J., MURPHY,E., ROTHS,J.B., STOLLAR,B.D., SCHWARTZ,R.S. (1982)
J.Immunol. 129 236-241.

REDDY,R., TAN,E.M., HENNING,D., NOHGA,K. (1983)
J.Biol.Chem. 258, 1383.

REICHLIN,M. (1981)
Clin.Exp.Immunol. 44, 1-10.

REINESS,C.G., WEINBERG,C.B., HALL,Z.W. (1978)
Nature 274, 68-70.

RICHTER,P.H. (1975)
Eur.J.Immunol. 5, 350-354.

RINGERTZ,N.R., SAVAGE,R.E. (1976)
In Cell Hybrids. Academic Press N.Y.

ROBERTS,I.M., WHITTINGHAM,S., MACKAY,I.R. (1973)

Lancet ii 936-940.

ROITT,I. (1980) Essential Immunology. Blackwell. London.

ROSEN,A., GERGELY,P., JONDAL,M., KLEIN,G., BRITTON,S. (1979)
Nature. 267, 52.

RUBIN,R.J., BALDERAS,R.S., TAN.E.M. (1984)
J.Exp.Med. 159, 1429-1440.

RUDDLE,F., CREAGAN,R. (1975)
Arthritis Rheum.G 9, 407.

SABAHARVAL,U.K., FONG,S., HOCH,S., COOK,R., VAUGHAN,J., CURD,J. (1983)
Clin.Exp.Immunol. 51, 317-324.

SATOH,J., PRABHAKAR,B.S., HASPEL,M.V. (1983) N.Eng.J.Med. 309, 217-20.

SCHLIWA,M. (1981)
Cell 25, 587.

SCHLOM,J., WUNDERLICH,D., TERAMOTO,Y.A. (1980)
Proc.Natl.Acad.Sci.U.S.A. 7, 6841-6845.

SCHRATER,A.F., GOIDL,E.A., THORBECKE,G.W., SISKIND,G.W. (1979)
J.Exp.Med. 150, 138.

SCHWABER,J., COHEN,E.P., (1973)
Nature. 244, 444-446.

SCOPELITIS,E., BUAIDO,J.J., ALSPAUGH,M.A. (1980) Arth. Rheum. 23, 287.

SCOTT,J.S., MADDISON,P.J., TAYLOR,P.V., ESSCHER,E. SCOTT,O., SKINNER,R.P.
(1983)New Eng.J.Med. 309, 209.

SELIGMAN,M., PREUD'HOMME,J.L., BROUET,J.C. (1973)
Transplantation Reviews 16, 85-113.

SENECAL,J.C., ROTHFIELD,N.F., OLIVER,J.M. (1982)
J.Clin.Invest. 68, 716.

SHARP,G.C. (1982)
Arthritis Rheum. 25, 757-760.

SHINOMIYA,N., YATA,J., SEGAWA,M. (1981)
Ann.N.Y.Acad.Sci. 377, 882-883.

SHOENFELD,Y., HSU-LIN,S.C., GABRIELS,J.E., SILBERSTEIN,L.E., FURIE,B.C., FURIE,B., STOLLAR,B.D., SCHARTZ,R.S. (1982)

J.Clin.Invest. 70, 205-208.

SIKORA,K., ALDERSON,T., PHILLIPS,J., WATSON,J.V. (1982)
Lancet i, 11-14.

SIKORA,K., MUNRO NEVILLE,A. (1982)
Nature. 300,316-317.

SIMONSEN,M. (1955)
Nature 175, 763.

SIMPSON,J.A. (1960)
Scott,Med.J. 4, 419-436.

SISKIND,G.W. (1984)
In 'Fundamental Immunology' Ed.W.E.Paul. Raven Press.

SISKIND,G.W., DUNN,P., WALKER,J.G. (1968)
J.Exp.Med. 127, 55.

SMITH,R.T., BRIDGES,R.A. (1958)
J.Exp.Med. 108, 227.

STANLEY,E.F., DRACHMAN,D.B. (1978)
Science 200, 1285-1287.

STAUFENBIEL, M., DEPPERT, W. (1984)
J.Cell.Biol. 98, 1886-1894.

STEFFEN,C. (1969)
Ann. Immunol. 1,47.

STEINITZ,M., KLEIN,G., KOSKIMIES,S. (1977) Nature 269,
420-422.

STEINITZ,M., IZAK,G., COHEN,S., EHRENFELD,M., FLECHNER,I.
(1980)
Nature. 287, 443-445.

STEPHENSON,F.A., HARRISON,R., LUNT.G.G. (1981)
Eur,J.Biochem 115, 91-97.

STOLLAR,B.D., PAPALIAN,M. (1980)
J.Clin.Invest. 66, 210-219.

STRAYER,D.S., COSENZA,H., LEE,W.M., ROWLEY,D.A. KOHLER,H.
(1974)
Science 186, 640.

SULLIVAN,A.K., TESKEY,L.M., CAMPLING,B., PRICE,G.B.
(1982)
In 'Hybridoma Technology', pp 63-68, Ortho Pharm Inc.,
Canada.

SWAAK,A.J., GROENWALD,J., AARDEN,L.A.,
SOATIUS-VAN-EPS,L.W., FELTKAMP,T.E. (1982)

Ann.Rheum.Dis. 42, 388-395.

TAKANO,M., AGRIS,P.F., SHARP,G.C. (1980)J.Clin.Invest. 65, 1449.

TAKANO,M., GOLDEN,S.S., SHARP,G.C., AGRIS,P.F. (1981) Biochemistry 20, 5929-5936.

TALAL,N. (1977)
Autoimmunity. Academic Press.

TALAL,N. (1982) Clin.Rheum.Dis. 8, 23-28.

TAN,E.M. (1982)
Adv. Immunol. 33, 167.

TAN,E.M., COHEN,A.S., FRIES,J.F., MASI,A.T., McSHANE,D.J., ROTHFIELD,N.F., SCHALLER,J.G., TALAL,N., WINCHESTER,R.J. (1982)
Arthritis Rheum. 25, 1271-1277.

TAN,E.M., KUNKEL,H.G. (1966)
J.Immunol. 96, 464-471.

TAN,E.M., ROBINSON,H., ROBITAILLE,P. (1976)
Scand.J.Immunol. 5, 811-818.

TARRAB-HADZAI,R., YAFFE,D., PRIVES,Y., AMSTERDAM,A. FUCHS,S. (1979)
In Plasmapheresis and the immunobiology of Myasthenia Gravis (Ed. Dau, P.C.), chapt. 3. Houghton Mifflin, London.

TEPPO,A.M., GRIPENBERG,M., KURTI,P., BAKLEIN,K. (1982)
Scand.J.Immunol.I 15, 1.

TINDALL,R.S.A. (1980)
Neurology 30, 554-557.

TOH,B.H., YILDIZ,A., SOTELO,J., OSUNG,O., HOBOROW,E.J., KANAKOUDI,F., SMALL,J.V. (1979) Clin.Exp.Immunol. 37, 76-82.

TOH,B.H., YILDIZ,A., SOTELO,J., OSUNG,O., HOBOROW,E.J., KANAKOUDI,F., SMALL,J.V. (1979) °CEI 37, 76-82.

TOYKA,R.V., DRACHMAN,D.B., GRIFFIN,D.B. (1977)
N.Eng.J.Med. 296, 125-131.

TRENKER,E., RIBLET,R. (1975) J.Exp.Med. 142, 1121-1132.

TREVES,A.J., FUKS,Z., VOSS,R., TAL,T., BARAK,V., KONIJN,A.M., KAPLAN,R., LASKOV,R. (1984)
J.Immunol. 132, NO.2, 690-694.

TRON,F., LE GUERN,C., CAZENAUE,P.A., BACH,J.F. (1982)

Eur.J.Immunol. 12 761-766.

TZARTOS,S.J., LINDSTROM,J,M. (1980)
Proc.Natl.Acad.Sci. U.S.A. 77, 755-759.

TZARTOS,S.J., LINDSTROM,J.M. (1981)
In 'Monoclonal Antibodies in Endocrine Research'. Eds.
Fellows,R. and Eisenbarth G. Raven Press N.Y.

TZARTOS,S.J., RAND,D.E., EINARSON,B.L., LINDSTROM,J.M.
(1981)
J.Biol.Chem. 256, 8635-8645.

VALENTE,W.A., VITTI,P., YAVIN,Z., YAVIN,E., ROTELLA,C.M.
GROLLMAN,E.F., TOCCAFONDE,R.S., KOHN,L.D. (1982)
Proc.Natl.Acad.Sci. U.S.A. 79, 6680-6684.

VAN DEN BERG-LOONEN,E.M., NIJENHUIS,L.E.,
ENGELFRIET,C.P., FELTKAMP,T.E.W. (1977)
J.Immunogenetics 4, 331.

VENABLES,P.J.W., SMITH,P.R., MAINI,R.N. (1983)
Clin.Exp.Immunol. 54, 731.
VOSSEN.J.M., HIJMANS,W. (1975)
Ann.N.Y.Acad.Sci. 254, 262.

WADA,K., VENO,S., HAZAMA,T., OGASAHARA,S., KANG,J.,
TAKAHASHI,M., TARVIS,S. (1983)
Clin.Exp.Immunol. 52, 297.

WALKER,M.B. (1934)
Lancet i, 1200-1201.

WARSICEK,C.A., REICHLIN,M. (1982)
J.Clin.Invest. 69, 835.

WEIGLE,W.O. (1971) Clin.Exp.Immunol. 9, 437.

WEIGLE,W.O. (1965)
J.Exp.Med. 122, 1049.

WEIGLE,W.O., DIXON,F.J. (1959)
In 'Mechanisms of Hypersensitivity' Eds. Shaffer, Lo
Grippo and Chase, p529. Little-Brown, Boston.

WEISS,M.C., GREEN,H., (1967)
Proc.Natl.Acad.Sci. U.S.A. 58, 1104-1111.

WEKERLE,H., PATERSON,B., KETELSEN,U.P., FELDMAN,M. (1975)

Nature 256, 493-494.

WELLS,R.D., GOODMAN,T.C., HILLER,W. (1980)
Prog. Nucleic Acid Res.Mol.Biol. 24, 167-267.

WESTON,W.L., HARMON,C., PEEBLES,C., MANCHESTER,D.,

FRANCO,H.L.HUFF,J.C., NORISS,D.A. (1982)
Br.J.Dermatol. 107, 377.

WHITE,P.J., GARDNER,W.D., HOCH,S.O. (1981)
Proc.Natl.Acad.Sci. U.S.A. 78, 626.

WHITEHOUSE,J.M.A., FERGUSON,N., CURRIE,G.A. (1974)
Clin.Exp.Immunol. 17, 227-235.

WILLIAMS,R.C. (1977)
In Autoimmunity, Ed. Talal,N. Academic Press.

WINFIELD,J.B., FAIFERMAN,J., KOFFLER,D. (1977)
J.Clin.Invest. 59, 90-96.

WOLIN,S.L., STEITZ,J.A. (1984)
Proc.Natl.Acad.Sci. U.S.A. 81, 1996-2000.

WOOD,J.N., HUDSON,L., JESSEL,T.M. (1982)
Nature 296, 34-38.

WORLLEDGE,S.M., CARSTAIRS,K.C., DACIE,J.V. (1966)
Lancet 2,135.

YOSHIE,O., ONO,Y. (1980)
Cell.Immunol. 56, 305-315.

YOSHIE,O., TADA,K., ONO,Y. (1980)
J.Exp.Med. 132, 397.

YOSHIKI,T. (1974) Proc.Natl.Acad.Sci. 70, 1878-1882.

ZIEVE,G. (1981)
Cell 25, 501-523.

ZURAWSKI,V.R., HABER,E., BLACK,P.M. (1978)
Science 199, 1439-1441.

APPENDIX

The detection of MCA to nuclear and cytoplasmic antigens by ELISA.

Culture supernatants were judged positive or negative by comparison to culture supernatants containing irrelevant monoclonal antibodies. These negative controls gave less than 0.2 O.D. units above the background shown by wells receiving no immunoglobulin, even after 2 hours of substrate incubation. The culture supernatants judged to be negative gave O.D. values between 0.0 and 0.3 above the irrelevant MCA controls. The four culture supernatants judged to be positive gave optical densities between 0.8 and 1.6 above the irrelevant MCA controls after 2 hours of substrate incubation. It should be noted that after this length of incubation, the normal human sera controls (diluted 1:1000) gave O.D. values in the same range as these four positives.