INVESTIGATION OF THE NORMAL B-CELL COUNTERPARTS OF NEOPLASTIC CELLS WHICH SECRETE COLD AGGLUTININS OF ANTI-I AND ANTI-i SPECIFICITY

submitted by

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for the degree of Master of Philosophy

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ABSTRACT

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INVESTIGATION OF THE NORMAL B-CELL COUNTERPARTS OF NEOPLASTIC CELLS WHICH SECRETE COLD AGGLUTININS OF ANTI-I AND ANTI-i SPECIFICITY

by Geoffrey John Smith

Cold agglutinin disease is characterised by the production of monoclonal antibodies which can bind to and agglutinate red cells in the cold. Similar autoantibodies are found at low levels in normal sera and are transiently raised following certain infections. A subset of these autoantibodies, specific for the Ii carbohydrate antigens, bears cross-reacting idiotypic determinants. A monoclonal antibody recognizing one of these idiotypic determinants has been produced and found to inhibit specifically the cold agglutination of red cells, suggesting that the antibody is recognizing an idiotope on the cold agglutinin molecule closely involved in binding to the red cell antigen.

It has been possible using the anti-idiotypic antibody to identify cells which synthesis immunoglobulins bearing the cold agglutinin-associated idiotope, both in patients with CAD and in normal individuals.

The normal counterparts of the neoplastic cells were found throughout the normal adult lymphoid tissue where they account for 2.9-10.8% of the B lymphocyte population. They are also present in fetal spleen at only 15 weeks gestation, suggesting that immunoglobulins bearing this sequence form part of the immature B-cell repertoire.

Using the anti-idiotypic antibody and magnetic beads, it has been possible to isolate the idiotope positive cells in good yield and purity from normal tonsil tissue where they constitute 1-2% of the normal lymphoid cells. Cells released from the magnetic beads have been transformed with EB virus, to produce an idiotope-bearing cell line.

The IgM secreted by the EBV cell line was found to agglutinate red cells in the cold, indicating that normal cold agglutinin producing cells were among this idiotope-positive population. The cell line and its secreted IgM have been compared to a similar cell line established from the neoplastic B-cells of a patient with cold agglutinin disease.

CHAPTER 1

Introduction

The complementary systems of innate and adaptive immunity have evolved to protect us from the numerous pathogens present in the environment. Innate immunity involves non-specific mechanisms of antigen clearance, exemplified by phagocytic cells and the complement cascade. In contrast, the adaptive immune response features memory, specificity and the ability to recognise "non-self".

Antigen recognition can result in two classically described pathways; firstly the cell mediated response where the T cell through its antigen receptor recognizes antigen on the surface of other body cells in conjunction with molecules encoded by the major histocompatibility complex; secondly, the humoral response where B cells recognize antigen by virtue of their surface immunoglobulin, and differentiate to produce antibody as the effector molecule. The basis of the immune response is to recognize foreign antigens and to react to them, while tolerating molecules of the body's own tissue. When the finely balanced control mechanisms of the immune response are disturbed autoreactive T and B cells may be produced and this may in turn lead to autoimmune disease.

This project is concerned with human B cells and their production of autoantibodies to red cells in normal individuals and in autoimmune disease.

1.1 B cell development

B cells derive from pluripotent haemopoietic stem cells but as yet no specific stem cell for the B cell series has been identified. B cell precursors first appear in the fetal liver at the twelfth week of gestation and seed in the bone marrow around the fifteenth to sixteenth week of gestation (1,2). The fetal spleen does not appear to be an important site for the production of B cell precursors, since only relatively mature cells are found in this organ (2). From

sixteen to nineteen weeks (2) the bone marrow is the primary site of B cell production (3) and retains this function throughout life.

Information on the differentiation sequence of the B-cell lineage has been obtained by the study of B-cell tumours which are considered to be "frozen" at a particular point in differentiation. However, caution has to be applied here as tumour cells often display aberrant phenotypes and are therefore not strictly analogous to normal cells.

The earliest identifiable normal bone marrow B cell precursor is a cell expressing nuclear terminal deoxynucleotidyl transferase (TdT) and surface MHC class II antigens (4) (Fig 1.1). Ig heavy chain gene rearrangements can be demonstrated at this stage (5,6) showing commitment to the B cell lineage. Further antigen-independent differentiation gives precursors expressing the common acute lymphocytic leukaemia antigen (CD10), HLA-Dr and in a large proportion of cases nuclear TdT activity (4,7). They are CD19+, CD24+ (1,8,9), in variable proportions express the CD22 antigen in the cytoplasm (8) and do not produce detectable levels of Ig (7). This normal B-cell precursor phenotype is reproduced by most cases of common acute lymphocytic leukaemia (10).

The synthesis of Ig μ chains and their cytoplasmic expression characterizes the next pre-B cell stage of differentiation (Fig 1.2). About 15% of cases of CD10+,CD19+ ALL are able to express μ chains in the cytoplasm and are defined as pre-B ALL (11,12). At this stage CD22 expression is switched from the cytoplasm to the surface membrane (8). The subsequent rearrangement of light chain genes in pre-B cells gives rise to immature B cells which incorporate monomeric IgM into their plasma membranes (13). CD10 expression and TdT activity are lost during this development (9) and the expression of CD20 and CD37 on the membrane increases (14). Approximately 80% of the immature B cells in the peripheral blood are sIgM+, sIgD+ and the remainder express sIgG or sIgA (15). The tumour cells found in chronic lymphocytic leukaemia (CLL) are thought to comprise a similar immature B cell population (16), again the sIg heavy

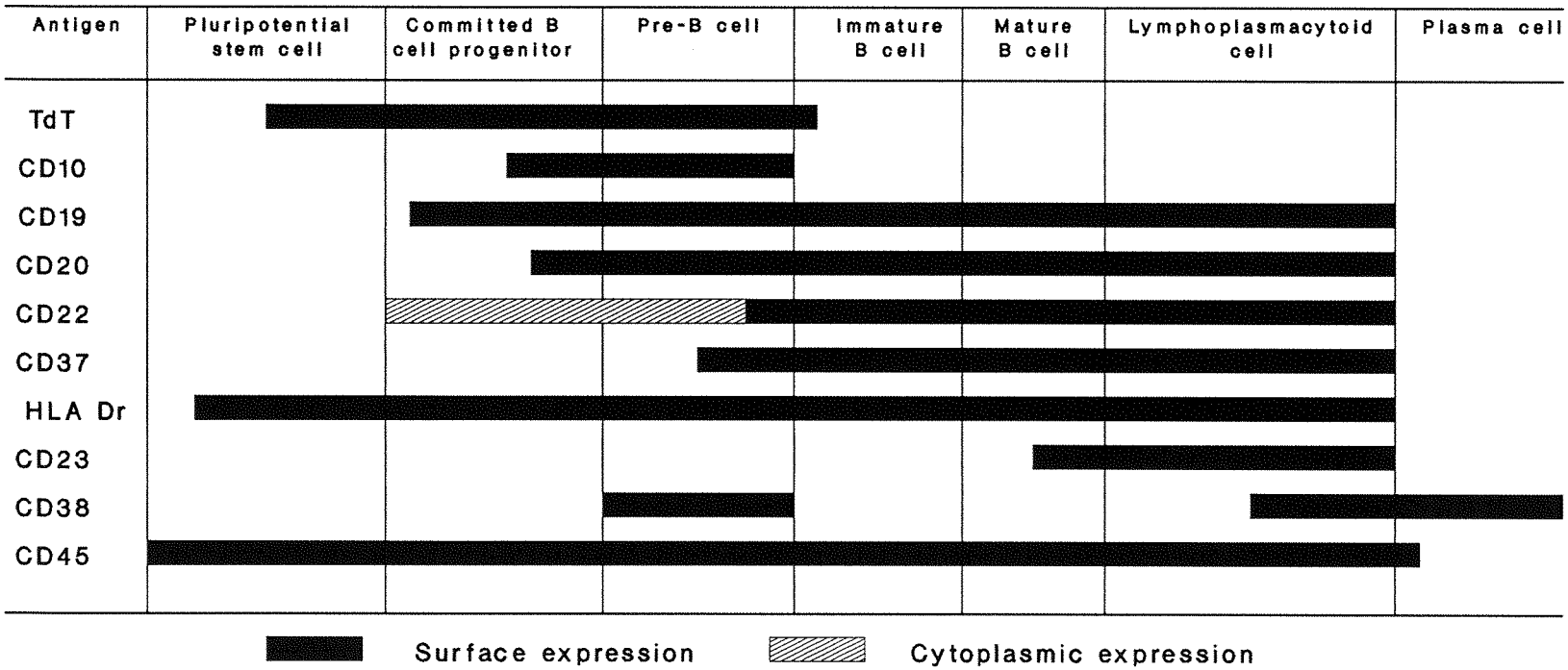


Fig 1.1 Immunologically defined antigen expression during B cell differentiation

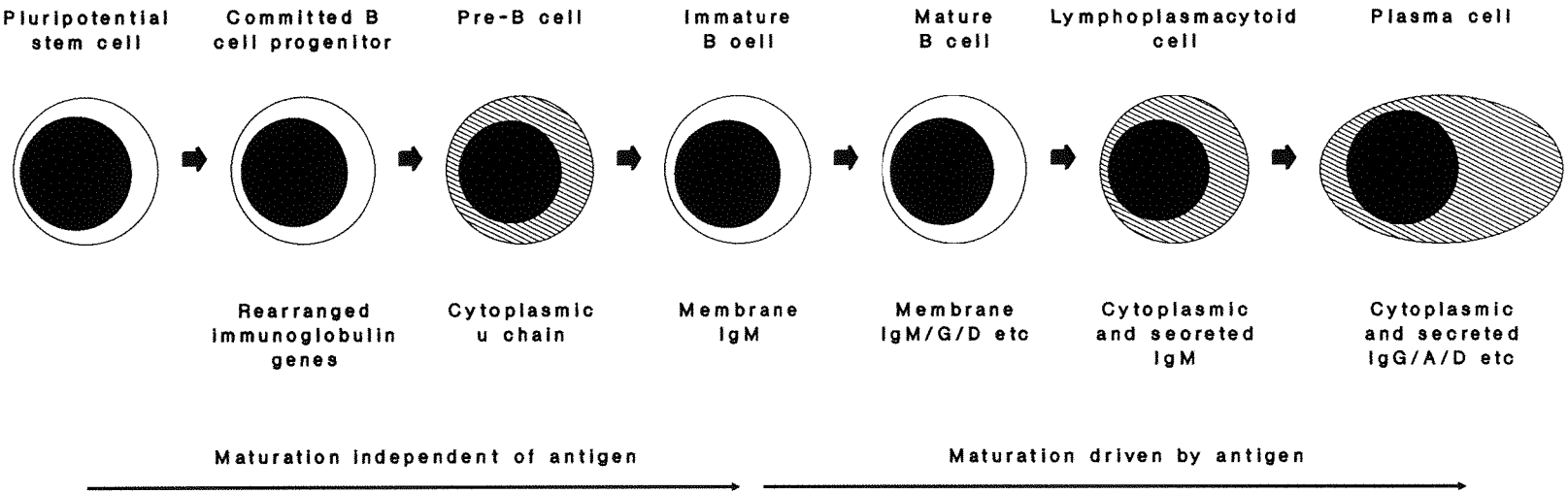


Fig 1.2 Normal B lymphocyte differentiation and immunoglobulin expression

chains are predominantly of μ and δ type with only a minority showing μ or δ alone (17). However CLL cells differ from normal resting B cells in their expression of the CD5 antigen (18) and by virtue of their sIg which is monoclonal and present typically in low density.

Following antigenic or mitogenic stimulation resting B cells transform into large dividing B immunoblasts which are capable of secreting small amounts of Ig (19). B immunoblasts mature through several mitotic phases to give lymphoplasmacytoid cells which have cIgM (20) and in some cases sIgM of the same light chain type (21); these cells secrete low affinity 19S IgM. Following immunoglobulin heavy chain class switching, plasma cells develop with 7S cIg and these can secrete 7S Ig (G,A,M,D or E). At this terminal stage of B cell differentiation sIg and class II antigens are usually absent (22). B cell stimulation can also produce memory B cells through intermediary centroblastic and centrocytic stages in the germinal centres of lymphoid organs (23).

1.2 Immunoglobulin structure

Immunoglobulins have a basic polypeptide chain structure consisting of two identical heavy chains and two identical light chains held together by non-covalent interactions and covalent interchain disulphide bonds, the number of which is related to the Ig class and subclass. The basic subunit of Ig is a sequence of about 110 amino acids known as a domain. Ig heavy chains have four or five domains (V_H+C_H1-C_H3 or C_H4) and light chains two domains (V_L+C_L). (see Fig 1.3)

The heavy chain C regions in man have nine alternative amino-acid sequences constituting the various classes (IgG, IgA, IgM, IgD and IgE) and subclasses (IgG1-4 and IgA1-2). These constant domains determine the effector properties of the Ig molecules e.g complement fixation. Two types of light chain κ and λ are found associated with all the heavy chain classes or subclasses but only one type of light chain κ or λ is expressed on a single Ig molecule. A single variable region of an Ig may be expressed on a B cell in combination with different heavy chain constant regions.

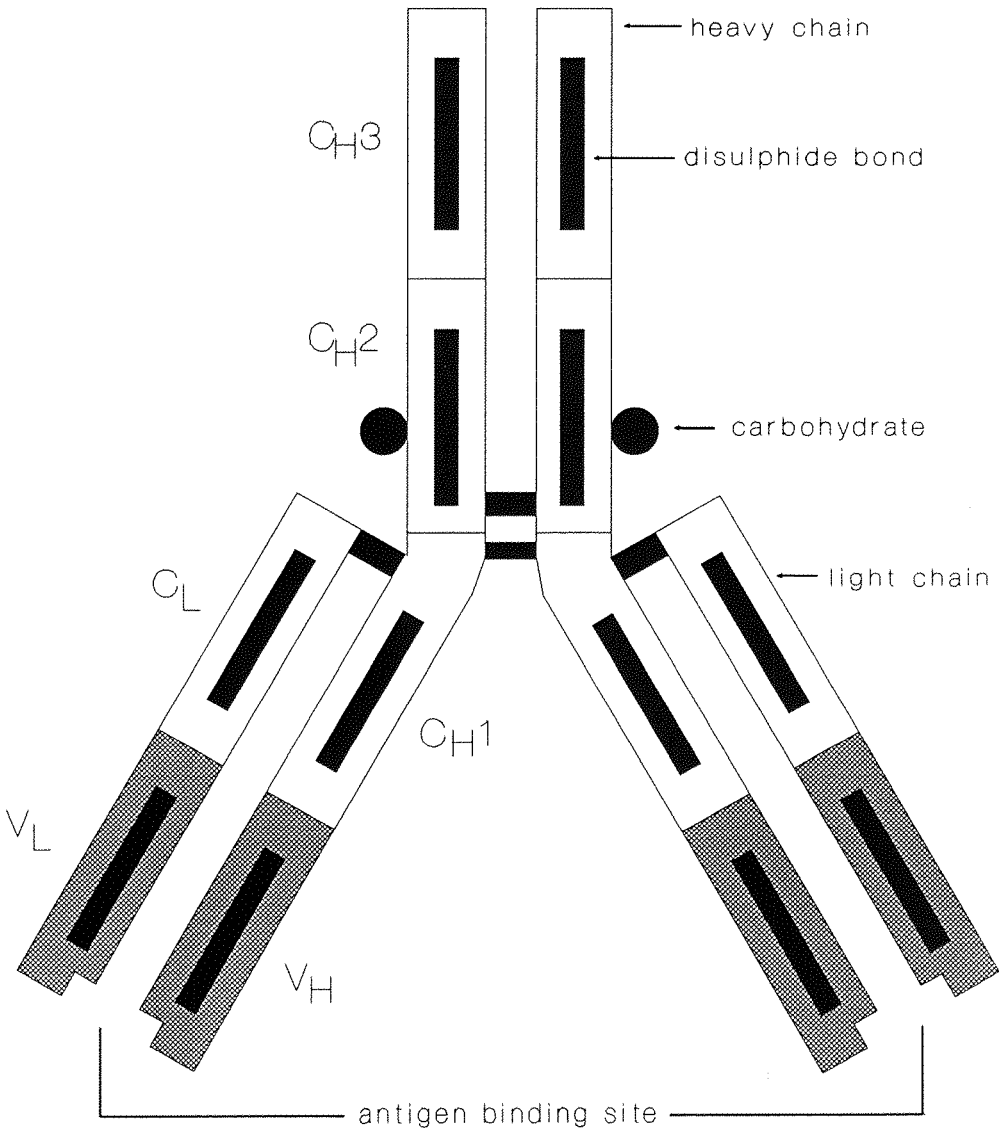


Fig 1.3 The basic structure of IgG

The N-terminal or variable region domains of each polypeptide chain (V_H and V_L) show greater amino-acid sequence variation than the constant region domains and contain the antigen binding site. This sequence variability is not random and Ig V domains contain hypervariable regions where amino acid sequence variation between proteins is most prominent. Three segments of the heavy chain V region are hypervariable as are three segments of the κ and λ light chain V regions (near positions 30, 50 and 95). These hypervariable regions determine antigen binding specificity and are termed complementary determining regions (CDR). The relatively invariant intervening peptide sequences are termed framework determining regions (FDR). Based on framework amino-acid sequence homology and serological data κ, λ, and heavy chain V regions can be divided into subgroups. In man, six λ subgroups (V_L I-VI), three κ subgroups (V_K I-III) and six heavy chain subgroups have been described.
Many reviews of immunoglobulin structure are available (24-27).

1.3 **Idiotypes**

The antigenic individuality of myeloma proteins was first described in 1955 (28) and the concept was later extended to include induced antibodies (29,30). It was shown that antibodies raised to a myeloma protein could still bind to the immunogen after exhaustive absorption with serum or other myeloma proteins (29,30). Each immunizing protein therefore has unique antigenic determinants, and the term idiotype was introduced (31) to designate these determinants. The first evidence that idiotypic determinants might relate directly to the antibody combining site came from a study of human IgM cold agglutinins (32). Idiotypes can be defined as a set of idiotopes where idiotopes are unique antigenic determinants on V_H, V_L or are formed by combinations of V_H and V_L. These determinants may be be postulated to show a continuum of specificity, from more or less public determinants present on a significant proportion of normal polyclonal Ig, to more or less private

determinants not detectable in normal Ig. Private idiotypic determinants appear to be related to the CDRs determining the shape of the antibody combining site, while public idiotypic determinants lie outside the antibody combining site (33) and are thought to relate to FDRs. Idiotypic determinants usually require heavy and light chain association for full expression although some isolated polypeptide chains do express idiotypic reactivity (34).

1.4 **The Idiotypic Network**

In 1973 Jerne proposed the network theory of the immune system (35) in which Id-anti Id interactions played a central role in the regulation of the immune response. Experimentally a number of model antigen systems have supported Jerne's theory (36,37) and have shown that anti-Ids may either enhance (38,39) or suppress (40) the immune response. The factors determining whether anti-Id enhances or suppresses are not fully understood but include: the dose of anti-Id (41); the nature of the anti-Id (internal image versus regulatory); the timing of anti-Id administration (42); and the isotype of the anti-Id used (43).

The network theory also describes the hierarchical organisation of antibodies via Ids. Antibodies reacting with external antigen are called Ab1, anti-idiotypic antibodies generated by Ab1 are Ab2's, antibodies generated by Ab2 are Ab3, and so on. If two different antibodies express a cross reactive idiotope but react to different antigen they belong to parallel sets. When an anti-Id mimics the antigens structure it is called an internal image because it is generated by the immune system as opposed to the antigen.

T cells are also involved in the network theory and shared idiotypic determinants have been identified on T and B cells (44,45). Id specific T suppressor cells (46,47) and Id specific T helper cells (48) have been reported to be generated by the idiotypic network. Thus a complex network of T and B cells exists apparently controlled by idiotypic interactions.

1.5 Genetic control of immunoglobulin synthesis

Ig genes comprise 3 unlinked loci each containing both variable and constant regions. In man the heavy chain gene is on chromosome 14 (49), the κ chain gene on chromosome 2 (50) and the λ cain gene on chromosome 22 (51).

The overall structure of the human Ig gene segment is illustrated in Fig 1.4. The κ gene locus consists of about fifty V_κ genes (52,53) and a cluster of J_κ segments linked to a single C_κ segment (53). In man the λ gene locus is poorly characterised and the total number of V_λ segments is not known. The heavy chain gene locus contains multiple V, D, J and C gene segments. There are nine J_H segments including three defective pseudogenes in a cluster 5′ to the nine functional C_H genes (54,55), in addition there are three C_H pseudogenes. The total number of V_H and D segments is not clear although five D segments have been cloned and experiments suggest that the human V_H is comparable to that in the mouse i.e, one hundred (56,57). A fourth type of segment, the leader (L) segment, is located upstream of each V gene segment and encodes most of the short hydrophobic leader peptide that marks the protein for transport across the endoplasmic reticulum.

The formation of an active heavy chain gene involves firstly the integration of one of several D gene segments with a single J_H followed by the integration of a V_H segment to the DJ_H to give the V_HDJ_H unit (58,59). A B cell differentiation stage preceding the pre-B cell with DJ_H generally on both chromosomes has been demonstrated (58,59). T cell lines frequently contain integrated DJ_H units, though not V_HDJ_H units (60) suggesting that V_H to DJ_H joining is B cell specific and could be connected with the commitment to either the T cell or B cell lineage. After V gene integration the V_HDJ_H unit is located next to the C_μ gene segment and is transcribed with the C_μ segment to produce μ heavy chains. This stage is analogous to the pre-B cell stage of differentiation, which expresses cytoplasmic μ chains but no light chains (61,62).

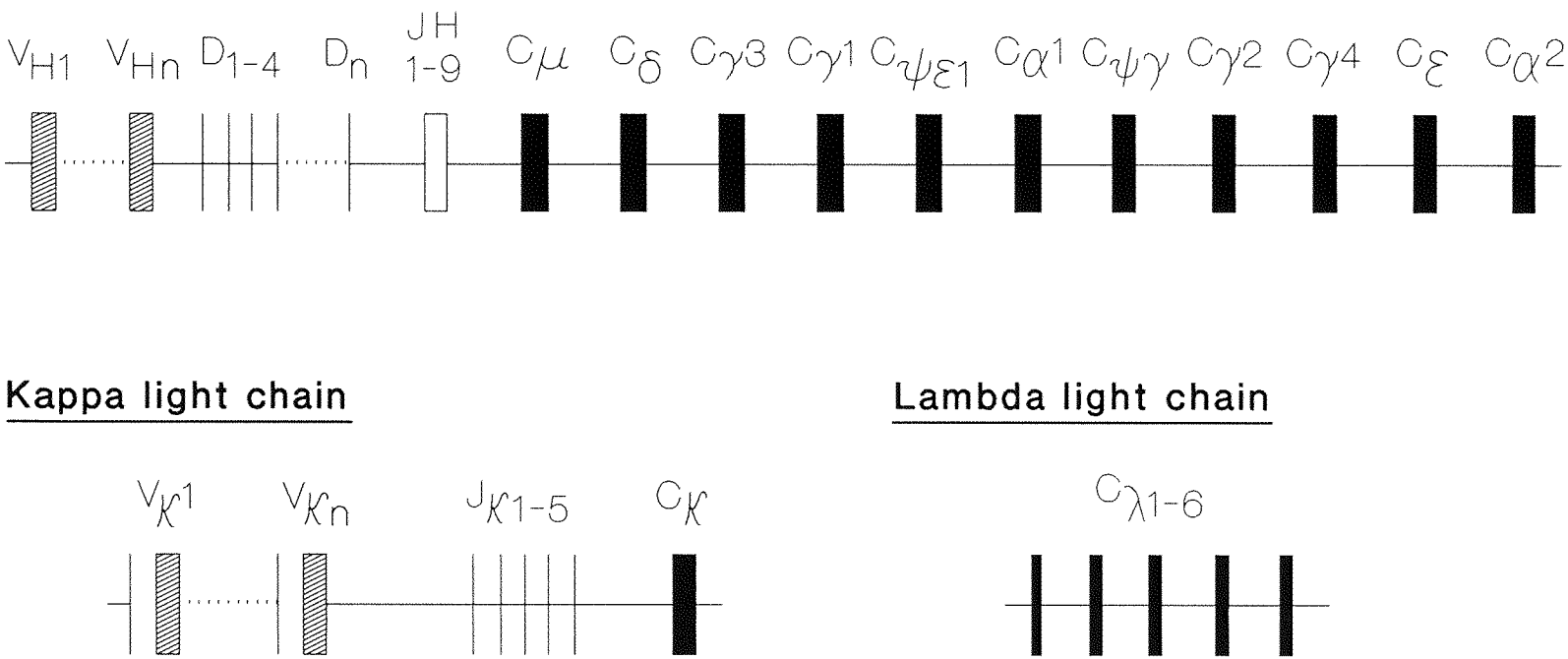


Fig 1.4 Organisation of gene segments at the human immunoglobulin loci

Active light chain genes are formed by a DNA rearrangement that integrates one of several V_L gene segments with one of several J_L gene segments and the appropriate C gene segment. κ gene rearrangement usually occurs before λ chain rearrangement, suggesting there is a regulatory mechanism that permits λ chain rearrangement only after non functional rearrangement at both κ alleles (63). The C_κ segment is deleted from the unexpressed κ loci and may serve to ensure allelic and isotype exclusion (64). The light chain produced is assembled in the endoplasmic reticulum with heavy chain to give whole Ig (firstly IgM) which may be inserted into the cell membrane or secreted.

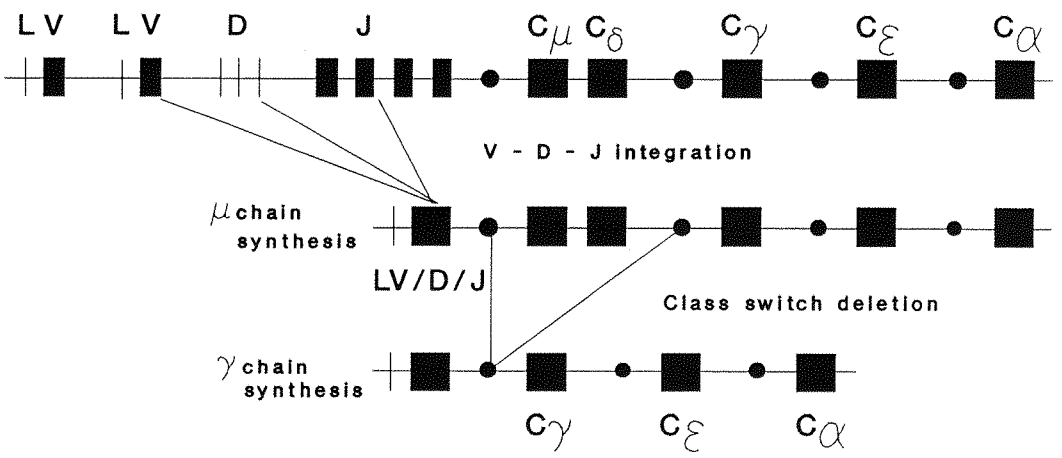
1.6 **Heavy chain class switching**

During the development of B cells the V_H region is first expressed with the C_μ region resulting in the production of IgM. Subsequently a switch to other Ig classes can occur, containing the same V_H region but a different C_H region.

The deletion model for class switching (65) is now widely accepted. The C_H genes are clustered and the integrated V_H gene is initially located next to the C_μ gene segment. These genes are expressed together resulting in the production of μ polypeptide chains. Subsequently a heavy chain class switch can occur by a DNA recombination event that brings the V_H gene next to a different C_H gene, with deletion of the intervening C_H gene segments. (see Fig 1.5)

Support for the deletion model comes from work showing that the J_H gene segments are located next to the C_μ gene in the germ line configuration whereas in plasmacytomas secreting IgA or IgG, the V_HDJ_H unit is adjacent to the C_H expressed (66,67,68). Switching by DNA deletion has been found universally in plasma cells but another mechanism may occur in less differentiated lymphocytes expressing surface IgM and IgD. The co-expressed μ and δ chains share the same V_H regions suggesting they are produced from the same chromosome (69), making it difficult to explain by the deletion model. In both humans and mice the C_μ gene is nearest to the C_δ, and they are separated by only a few kilobases (70,71). It thus appears that the integrated V_H ,

Heavy chain



Light chain

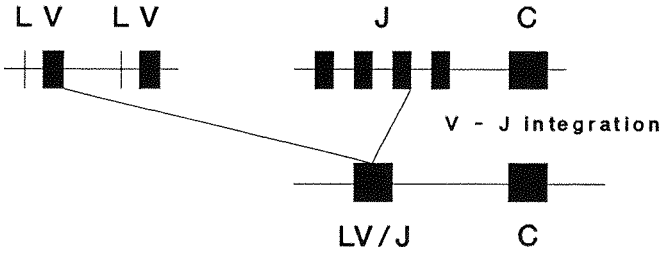


Fig 1.5. Immunoglobulin gene rearrangements: V gene integration and heavy chain class switch deletion.
(Circles indicate switch regions)

C_μ and C_δ genes form a single transcription unit that can give rise to μ or δ mRNAs by alternative RNA processing mechanisms (72,73).

1.7 Generation of antibody diversity

The total antibody repertoire in the mouse has been estimated to be about 10^6 to 10^8 molecules (74). Some of this diversity is generated by different combinations of heavy and light chain polypeptides. Some pairing of heavy and light chain may be incompatible, but it is known that individual heavy chains can associate in different cells with several different light chains, and vice versa (75). At the genetic level three mechanisms can contribute to V region diversity: (1) combinatorial joining of light-chain V and J gene segments, and heavy chain V, D and J gene segments; (2) variation in the sequence at the joining sites; and (3) somatic mutation. Taking into account the combinational association of heavy- and light-chain polypeptides and combinational joining of V, D and J segments, the estimated total diversity produced in the mouse is approximately 4×10^6 antibody molecules. Although the human Ig genes are not yet fully characterized a similar figure may be suggested.

Somatic mutations have been found in κ, λ and heavy chain genes (76,77,78), occurring not only in the V gene segment, but also in the J segments and probably the D segments. In the majority of cases, the changes are single base substitutions. Somatic mutations are more common in IgG and IgA molecules than in IgM, in both V_H and V_L regions (77,79,80) and these observations are compatible with the idea that the immune response may initially employ unmutated V genes, somatic mutations only occurring later. Further it has been suggested that somatic mutation could be activated by the heavy chain class switch or mutations may simply accumulate as a result of repeated cell divisions (77,79). The very high frequency of mutations, often a few per cent of the nucleotides sequenced suggests also that a specialised hypermutation mechanism is operative. It appears that the pool of antibodies available at the start of an

immune response is created by gene integration mechanisms acting on the library of germ line gene segments, without somatic mutation. Then, during immune response, antibodies with higher antigen binding affinities are selected following somatic mutation, together with recruitment of different germ line genes.

1.8 **Autoimmunity**

Autoimmunity still remains a little understood phenomena and yet the number of diseases for which an autoimmune origin is suspected continues to increase. The complex humoral and/or cellular mechanisms involved in the effector arm of autoimmune reactions have been partly elucidated yet little is known of how autoreactivity is triggered and what keeps most people free from its deleterious consequences.

Ehrlich's 'Horror autotoxicus' theory that the immune system does not as a rule react against its own body components (81) has been abandoned, to be replaced with the notion that self-recognition may be a normal event and have a physiological role. Autoreactive B cells (82,83) and probably T cells exist in normal individuals, particularly the elderly (84) and there is evidence that autoreactivity of this type may exist even at birth preceding ontogenic development pressures.

1.9 **Etiology of autoimmune disease**

Spontaneous autoimmune disorders have a multifactorial basis, with immunological, virologic, hormonal and other factors playing essential roles, each acting singly or synchronously with the others.

Most autoimmune conditions are associated with particular HLA profiles (85) the most common being systemic lupus erythematosus (SLE) with DR2 and DR3, rheumatoid arthritis with DR4 and Graves disease with DR3. The importance of genetic background in autoimmunity is shown by the increased incidence of autoimmune diseases within families, particularly in monozygotic twins with diabetes or SLE,

where the concordance rate is around fifty per cent (86,87). This implies genetic factors are important but that other precipitating factors are also required.

It seems without some basic immune defect self reactivity remains under tight control. Autoimmune diseases in animal models or man may have quantitative and/or qualitative defects in suppressor T cells, but it is not clear whether these defects are simply secondary to other processes (88). However it is easy to see how such defects would contribute to enhanced B cell autoantibody production. Similarly, deficiencies of complement components, especially C2 and C4 (89) and of IgA (90,91,92) are also strongly associated with autoimmune diseases.

The hormonal impact in autoimmunity is well known and it includes not only the sex hormones, but also the thymic hormones (93), corticosteroids and vitamin D (94). It seems that the effect of both sex and thymic hormones is mediated at least partially by receptors on CD8+ cells (95) and these effects are reflected in the high incidence of autoimmune diseases among females during puberty (93), while using oral contraceptives (96) and during pregnancy.

Environmental factors probably act as the inducing agents for autoimmune diseases only in individuals with an appropriate immune-genetic-hormonal background (97). The major environmental agents include the sun (98), infections (97) and drugs, pre-eminent among which is penicillamine which is known to induce SLE, RA, pemphigus and myositis (99).

The mechanisms by which drugs induce autoimmune disease can include, altering antigen (98), immune complex formation (100) and effects on T cell function (101). Some drugs possess a chemical structure resembling a self antigen (102). Infectious agents probably have a major role in triggering autoimmunity. For example, hepatitis B infection has been linked to chronic active hepatitis, cryoglobulinaemia and polyarteritis nodosa. The postulated mechanisms by which viruses, bacteria and even parasites may act as inducing agents are as diverse as the agents themselves and may include: molecular mimicry (103);

increased HLA expression (104); polyclonal B cell activation (105); alteration of self and interestingly idiotypic interactions.

Regulatory anti-Ids with autoreactivity may arise as a normal consequence of the idiotypic network (106). A pathological response would be prevented by the network and suppressor T cells, but in susceptible individuals autoimmune disease may develop. Secondly, idiotypic antibodies may arise which bind cell membrane receptors (107) and therefore act as autoantibodies. Anti-Ids of this nature have been demonstrated to insulin and acetylcholine receptors (108).

1.10 Cellular origin of autoantibodies

It has been suggested that B cells expressing the CD5 antigen (equivalent to Ly-1 in mice) might represent a distinct B cell lineage (109) and be the origin of natural autoantibodies. The CD5 antigen is a glycoprotein MW 67,000 and was thought to be T cell specific (110). Subsequently, CD5 has been demonstrated on some neoplastic B cells in humans and mice (111,112,113), and on a small number of normal B cells in human lymph nodes, tonsil, spleen and blood (114). In humans as in mice, CD5+ B cells occur at a much higher frequency in fetal and neonatal tissues and in cord blood (115), suggesting CD5+ B cells are produced early in ontogeny, and then form a self renewing population (109). Where present, the CD5 antigen is expressed at low density on human B cells, as is Ly-1 on mouse B cells (115).

The association between Ly-1+ B cells and autoantibody production in mice is not disputed and is based on the following findings. Ly-1 B cells occur with increased frequency in certain mouse strains predisposed to develop autoimmune syndromes, such as NZB mice (116), where they are largely responsible for the production of antibodies to single-stranded DNA and to thymocytes (117). In the motheaten mouse strain, which also develops a severe autoimmune syndrome and has a high level of spontaneous IgM production (118) virtually all the B cells are of the Ly-1+ phenotype (119). Ly-1+ B cells produce autoantibodies but

generally do not respond to exogenous antigen challenge (117) suggesting that Ly-1+ cells use only a restricted number of V_H genes. Not all autoimmune strains of mice show increased numbers of Ly-1+ B cells (117). Autoantibody secretion has also been demonstrated by Ly-1+ B cells from normal mice, which secrete antibodies to bromelin treated mouse RBCs in culture (120,121).

As yet there is little evidence for implicating CD5+ B cells in the autoimmune diseases of man. Increased numbers of CD5+ B cells have been demonstrated in patients with rheumatoid arthritis (122), but as yet not in patients with other connective tissue disorders such as systemic lupus erythematosus (123). The analysis of cultured EBV infected CD5+ and CD5- B cells from healthy individuals has shown that the CD5+ population is mostly committed to the production of IgM class polyreactive antibodies that react with a variety of self antigens, for example the Fc portion of IgG, ssDNA, thyroglobulin and insulin, and foreign antigens such as tetanus toxoid, bacterial lipopolysaccharides and saccharides. In contrast the CD5- B cell population produces monoreactive, mainly IgG antibodies in immunized subjects (124). The relationship of these polyreactive antibodies to the autoantibodies that are characteristic of the various autoimmune diseases is still unclear and awaits further detailed analysis.

1.11 **Cold Agglutinins**

Autoagglutination of chilled blood was first described in 1903 by Landsteiner as occurring in various mammalian species. The phrase "cold agglutination" was introduced in 1925 by Amzel and Hirzfeld (125), to describe autoantibodies binding to RBCs which reacted optimally in the cold (0°C). These autoantibodies are termed cold agglutinins (CA).

Low titre CAs can be demonstrated in all healthy adults, the titres ranging from less than 1/2 to 1/64 (126,127), and are usually clinically insignificant because they do not act at body temperatures. Only on rare occasions have low titre CA with raised thermal amplitudes capable of RBC destruction in vivo been described. These normal CA have specificities

for the Ii blood group antigens with a preponderance for the I antigen. The sera of newborns do not contain CA, they are not produced by the fetus nor are they transferred from the mother because they are of IgM type (128). Naturally occurring CA appear to reflect a population of autoreactive B cells which are kept in control by T cells and/or the idiotypic network.

As with autoimmune diseases in general the effector mechanisms leading to RBC destruction by CA are well documented but the etiology of cold agglutination, post infection and in chronic cold agglutinin disease is poorly understood.

1.12 **Post Infectious Cold Agglutination**

Isolated incidents of patients with respiratory infections and an unusual degree of autohaemagglutination had been first noted in 1918 by Clough and Richter (129). The presence of CAs in patients suffering from viral pneumonia was first conclusively demonstrated in 1943 (130).The CA titre was within the range 1/512-1/32,000 at 2-4°C (131) and appeared to be transient in nature (132) falling to normal levels as the pneumonia resolved. Mycoplasma pneumoniae causes approximately twenty per cent of the pneumonia in the general population and at least fifty per cent of cases produce CA during the first two to three weeks following respiratory symptoms. The transient autoimmune haemolytic anaemia caused by the CA is an example of infection induced autoimmunity (133). The CA are usually IgM type showing restricted L chain heterogeneity and are mostly directed against the I antigen (134).

One suggested mechanism for the production of CAs following M.pneumoniae infection, involves the formation of a complex between the organism and the I antigen. The complex may then be presented in an immunogenic form to the hosts immune system (207). This concept is supported by the finding that M.pneumoniae adheres to RBCs via receptors consisting of sialic acid α2-3 linked to long carbohydrate chains of Ii type (135). The binding of M.pneumoniae to erythrocytes of I and i types has been shown to be

comparable (136), indicating that M.pneumoniae in its adhesive specificity may not be able to distinguish between the branched structures of I type and the linear structures of i type. It is intriguing therefore, that anti-I antibodies rather than anti-i antibodies are elicited following infection with this agent. This finding may simply reflect the greater abundance of I antigen molecules on the surface of the host cells with which the mycoplasma forms a complex, an idea supported by the fact that mycoplasma infection usually occurs in children and adults rather than in neonates (208) where the I antigen is poorly developed.

A second mechanism for CA production has been postulated (137) whereby the M.pneumoniae passively absorbs glycolipids of the I antigen type to again form an immunogenic complex capable of triggering CA production.

The occurrence of CA in EBV induced infectious mononucleosis is well established, with the incidence ranging from eight to seventy per cent of cases (138). The CA are of typically anti-i specificity although other specificities may be found, and are of IgM or IgG type. Cytomegalovirus may also induce CA which are IgM proteins with predominant anti-I specificity. Similarly HIV (139) and rubella have been reported to cause CA and the list of CA inducing agents may well include other viruses, bacteria and parasites but the evidence is not as clear as for the above agents.

1.13 Chronic cold agglutinin disease

The first case reports of CAD are credited to Iwai and Mei-Sai in 1925 (140), who described patients with high titre CA, chronic haemolytic anaemia and associated Raynauds phenomenon. CAD is a disease of the elderly with a peak incidence in the seventh and eighth decades of life (132,141) although cases have been reported in childhood (142). The clinical manifestations are due to vascular disturbances or haemolysis and reflect the functional properties of the patients CA (143). The circulatory changes are described as acrocyanosis or Raynauds phenomenon and are caused by intracapillary agglutination of RBC, most commonly

in the distal extremities. Acrocyanosis may be aggravated by CA acting as cryoglobulins and gangrene may result (144). CAD patients develop a chronic haemolytic process of moderate severity, it being rare for the haemoglobin concentration to fall below 7g/dl (145).

Idiopathic CAD may be considered a form of benign monoclonal gammopathy in that high titres of CA are produced without an underlying lymphoproliferative disorder. CAD has many features of Waldenstroms macroglobulinaemia, with the very high titre CA in this disease usually occurring due to monoclonal IgM proteins, and lymphoproliferative disorders may develop as terminal events in the disease (146). The CA are invariably monoclonal IgM proteins with kappa L chains predominating and usually have I or i specificity. Other specificities do occur eg, anti-Pr (138) or anti- Gd (147). The titres of the CA range from 1/1000 to > 1/1,000,000 at 4°C although titres around 1/32,000 are more usual (148) and the agglutination persists up to temperatures of at least 28-32°C(132), causing serious intravascular agglutination and haemolysis at body temperatures.

Patients with CAD require no specific therapy usually only having a mild, chronic haemolytic process. When exacerbations of moderate severity occur they often respond to the simple measure of keeping the patient warm, and blood transfusion is rarely necessary or advisable. Patients not infrequently manage to live with their disease for periods of 10 years or more (132). If the antibody titre is very high, usually associated with a lymphoproliferative disorder, agents such as cyclophosphamide (141) or chlorambucil (149) have been used to drastically reduce the antibody production. Plasmaphoresis may also be used if the patient is suffering from severe haemolytic crises.

In common with almost all autoimmune diseases the etiology of CAD is unknown and it is not known if it is inevitable that CAD transforms into a malignant lymphoproliferative form.

1.14 **The Ii Blood Group Antigens**

CA were originally thought to be directed against an antigen common to all human RBCs (150), until Wiener in 1956 (151) found five blood samples out of 22,000 that were not agglutinated by a potent CA serum. The term 'I' was introduced for the commonly occurring antigen and 'i' to denote the lack of I antigen. A differential expression of I and i on adult and cord blood RBCs has been noted (152,153); all infants at birth are phenotype i and the phenotype changes to predominantly I within eighteen months of birth.

The expression of I and i antigens is reciprocal. Fetal and newborn RBCs express the I antigen weakly and adult RBCs express the i antigen weakly. The theory (152) that a very common gene Z, is necessary for the conversion of i into I and that a very rare adult might be homozygous for a recessive gene z , which would not change i into I holds up remarkably well in view of recent structural data.

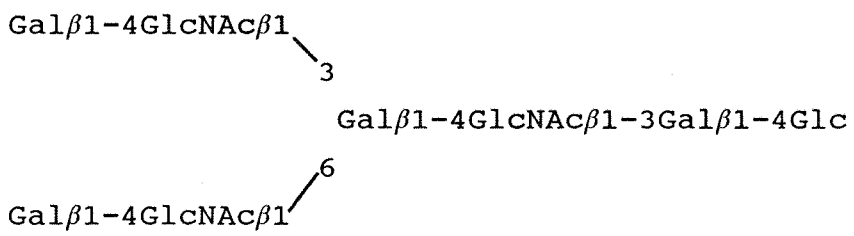
The number of I antigen sites on adult RBCs has been estimated as 1-5 $\times 10^5$ (155,156), and the number of i antigen sites on cord blood at between 0.2-0.65 $\times 10^5$ (156). I and i antigens can also be demonstrated on leucocytes by agglutination tests (157) and in many species of animal (158); anti-I is thus an heterophil antibody. On RBCs the I antigen is expressed on glycolipids and glycoproteins while in body fluids such as saliva (159) or milk (160) it is glycoprotein.

I and i antigens are carbohydrates (161,162,163) and are the internal structures of the major blood group antigens A, B and H. The blood group precursor chains can be divided into type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) oligosaccharides (164). The Ii antigens use exclusively the type 2 oligosaccharides (165), and are built of repeating N-acetyl-lactosamine units (reviewed 134,166). Anti-i recognises a linear chain while anti-I recognises a branched chain.

i antigen

Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc

I antigen



The branched chain is generated by the addition of a further lactosamine unit in 1-6 linkage to the penultimate Gal of the i chain. The enzyme capable of converting i to I is termed the branching enzyme and may be coded by a Z or 'I' gene. It is known that the oligosaccharide chains of neonatal erythrocytes are simpler and contain fewer branched structures than those of adult erythrocytes (167,168). Thus the antigenic change from i to I in the first year of life fits in well with the increase in branched oligosaccharide chains.

Both the linear and branched chains are converted into blood group A, B and H active structures (128) by the addition of appropriate monosaccharides under the control of A, B and H genes.

H substance is generated by substitution of the terminal Gal with Fucα1-2. H substance is the precursor for A and B, which are generated by a further substitution of Gal with GalNAcα1-3 or Galα1-3, respectively (Fig 1.6). Similarly Le^a and Le^b active substances can be built from these basic oligosaccharide chains (169).

The minimum structure for an i antigen site is two N-acetyllactosamine units and de-N-acetylation by hydrazinolysis abolishes i activity (170). Anti-I antibodies recognise various oligosaccharide domains on the branched structure (171,172). Group 1 antibodies recognise the Galβ1-4GlcNAcβ1-6 branch point sequence, group 2 recognise

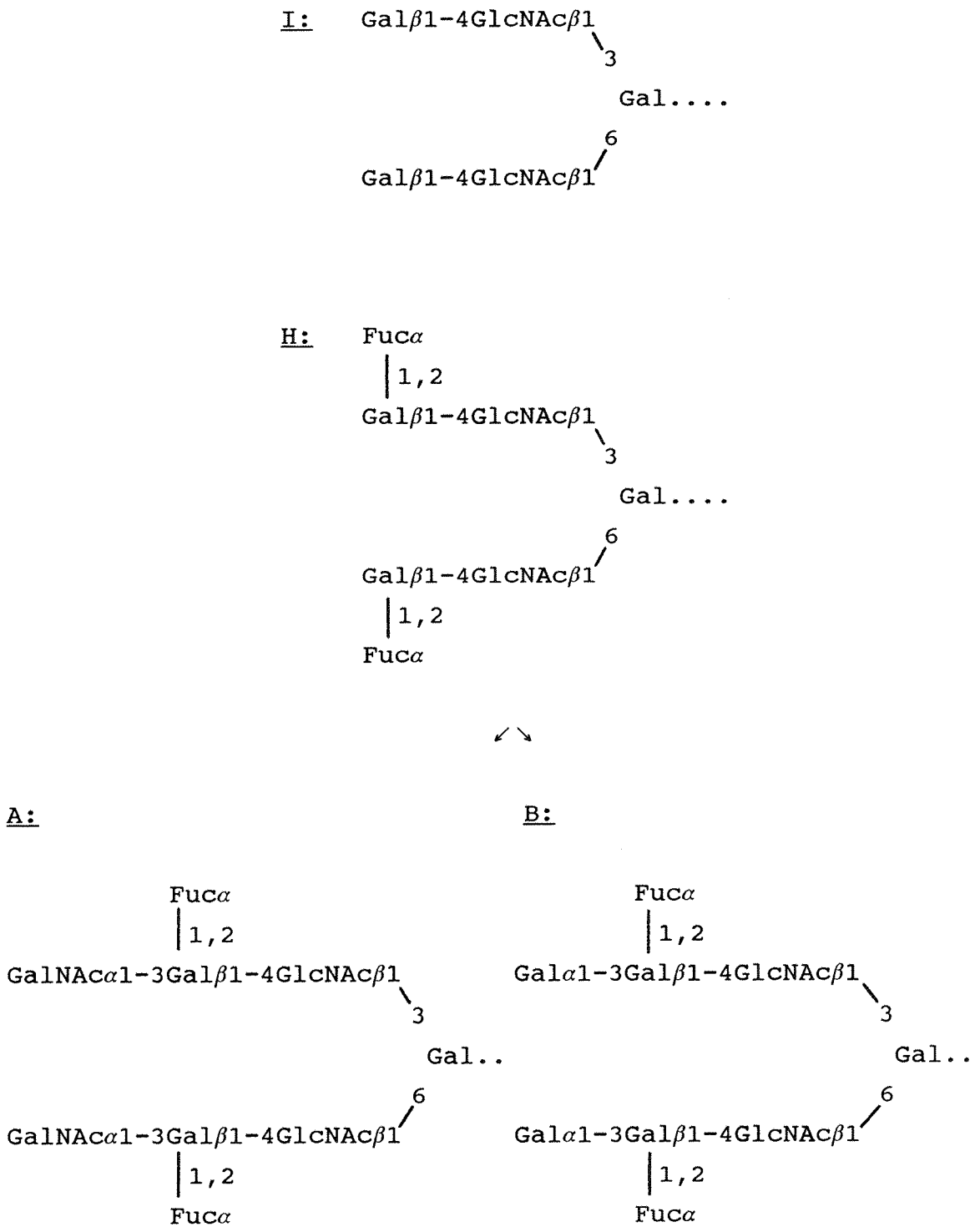


Fig 1.6. The branched I antigen is shown as the basic sequence of the H and AB antigens on adult red blood cells. The linear i antigen is similarly the base sequence of the H,A or B antigens on fetal red cells.

Galβ1-4GlcNAcβ1-3 sequences and group 3 antibodies require both chains of the branched polylactosamine structure. The I antigens can be masked by fucosylation (H substitution) and to a lesser extent by NeuAc and Gal (128). These observations provide an explanation for the marked variations among the anti-I and anti-i CAs in their reaction patterns with complex structures such as erythrocytes.

1.15 **Anti-I and Anti-i cold agglutinins.**

Some of the earliest studies of idiotypes demonstrated cross reactive determinants on anti-I CAs (32). Monoclonal anti-I CAs have been shown to be highly kappa L chain restricted (173), and serologic studies show the majority belong to the minor KIIIb sub group (174). Few CAs use either KI or KII L chains (175), and sequence analysis of these L chains suggests they have greater homology to the germ line Humkv 325 gene, than to the other twelve sequenced KIII genes (176). The Humkv 325 gene has also been linked to monoclonal RFs and to the L chains of antibodies to intermediate filaments or low density lipoprotein (177). KIIIb L chains are also utilised by RFs expressing the Wa CRI (178).

While anti-I CAs generally use Kappa L chains and anti-i can use lambda L chains idiotypes common to both types have been demonstrated (179) and subsequent peptide mapping has suggested that similar H chains may be used in these antibodies (180). Isolated H chains from anti-I or anti-i CAs share idiotypic determinants (181). Early work (179,182) using polyclonal antibodies suggested that the VHI subgroup was being used by CAs. A preliminary study using sub-class specific antibodies and Western blot analysis (183) suggested that the VHII subgroup was preferentially used in CAs. Subsequent work from the same laboratory has now demonstrated that cold agglutinins exclusively use heavy chains that derive from the VHIV gene family (202,205).

This pattern of restricted VHIV-VKIIIb pairing in anti-I CAs is different from the VHI preference demonstrated in a collection of thirty KIIIb monoclonal RFs (linked to the Humkv325 gene) (183), suggesting the binding specificities may be determined by VH sequences.

1.16 Background and design of project.

While raising a panel of rat monoclonal anti-idiotypic antibodies against the IgM expressed on the cells of a patient with B-NHL, an antibody (9G4) recognizing shared idiotypic determinants was produced (184). The patient had no monoclonal serum IgM or evidence of CAD. However the antibody recognised a cross reacting idiotypic determinant present on IgM CAs of anti-I and anti-i type. The reactivity was similar to that of a monoclonal antibody reported previously which was specifically raised against a CA (185), in that it reacted with 7/8 IgM CAs but failed to react with IgM Ma, which is known to lack the majority of idiotypic determinants shared by this group of proteins (186).

Initial studies by ELISA showed that 9G4 reacted with 0.2% and 0.6% of normal human IgM and IgG respectively and detected a minor population of cells in a lymph node.

The first part of this study was designed to extend the characterisation of the anti-idiotypic antibody with particular regard to its specificity and to the location of the idiotypic determinant.

Secondly blood and bone marrow cells from a patient (R.H) with frank cold agglutinin disease where examined to determine the distribution of idiotope expression in the malignant cells. This work was then extended to look for the expression of idiotope in other B-cell malignancies, which showed no evidence of cold agglutinin disease.

Thirdly, normal and immature lymphoid tissues where examined for the presence of the idiotype positive cells. Idiotope bearing cells have been purified in good yield and viability from normal tonsil tissue using magnetic beads, phenotyped and then transformed with EBV to give stable cell lines. The Igs produced by these lines have been examined for their ability to agglutinate RBCs in the cold in comparision to CAs from CAD patients. The phenotype of the tonsil cell line has been compared to that of an idiotope positive line raised from a patient with cold agglutinin disease.

CHAPTER 2

Materials and methods

2.1 Buffers and solutions

Buffers and solutions were prepared using glass distilled water and AnalaR grade biochemicals. The pH of each buffer was checked before use, and minor adjustments if necessary were made with the appropriate acid or base. The composition of each buffer is listed below.

Phosphate buffered saline (PBS), pH 7.2.

NaCl	8.0g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g
$d.H_2O$	1 Litre

PBS (pH 7.2)-azide

NaN_3	0.65g/l of PBS

PBS (pH 7.2)-paraformaldehyde

2% solution in PBS

PBS (pH 7.2)-glycerol

PBS	10ml
Glycerol	90ml

PBS, pH 7.0.

Solution A	0.1M Na_2 HPO_4 (14.2g/l)
Solution B	0.1M KH_2PO_4 (13.6g/l)
Solution C	0.85% NaCl (8.5g/l)

Add 60mls solution A, 40mls solution B and 900mls solution C. Store at 4°C.

PBS, pH 7.3.

NaCl	35.06g
Na_2HPO_4	17.2g
KH_2PO_4	3.95g
d.H_2O	5 Litres

PBS (pH 7.3) -Bovine serum albumin (BSA)

BSA	10g
PBS	1 Litre

PBS (pH 7.3) -Tween

Tween 20	1ml
PBS	1 Litre

Citrate Phosphate buffer, pH 5.5.

Citric acid	2.34g
Na_2HPO_4	3.65g
d.H_2O	500ml

ELISA coating buffer, pH 9.5.

Na_2CO_3	0.79g
$NaHCO_3$	1.46g
d.H_2O	500ml

ELISA substrate solution

Orthophenylene diamine (OPD)*	10mg
H_2O_2	20μl
Citrate phosphate buffer	50ml

* OPD is a potential carcinogen and should be handled accordingly.

TRIS buffered saline, pH 7.6.

NaCl	40.0g
TRIS	3.025g
d.H_2O	1 Litre

Adjust pH to 7.6 with 0.1M HCl.

APAAP substrate

Naphthol AS-MX phosphate solution	2ml.
Levamisole	12.5mg
Fast Red TR salt	25.0mg
d.H_2O	96ml

Mix naphthol phosphate and water, dissolve Levamisole and Fast Red and filter (Whatman No1). Prepare immediately before use.

Mayers haemotoxylin

Haemotoxylin	1.0g
Sodium iodate	0.2g
Potassium alum	50.0g
Citric acid	1.0g
Chloral hydrate	50.0g
d.H_2O	1 Litre

The haemotoxylin, alum and sodium iodate are allowed to dissolve overnight, the chloral hydrate and citric acid are then added and the solution brought to the boil for 5 minutes. When cool the solution is filtered (Whatman No1) and is ready for use.

Glycerine jelly

Gelatine	15g
Glycerol	100ml
d.H_2O	100ml

Dissolve gelatine in distilled water by careful boiling, add to glycerol and allow to cool, remelt as required.

Cell media

Eagles minimal essential medium with Earle's salts in 20mM Hepes buffer (MEM), foetal calf serum (FCS), and newborn calf serum (NCS) were supplied by Gibco Ltd, Glasgow. The FCS was heat inactivated by swirling at 56°C for 30 minutes to remove complement activity before use. RPMI 1640 medium containing 25mM Hepes and L-Glutamine was supplied by Flow Laboratories Ltd, Ayrshire.

Cell culture media

RPMI 1640	500ml
FCS	25-100ml (5-20%)
Streptomycin	0.1mg/ml
Penicillin	0.06mg/ml
Fungizone	0.002mg/ml
Gentamycin*	50μg/ml
Ciprofloxacin*	0.01mg/ml

* only used when Streptomycin/Penicillin/Fungizone was ineffective.

MEM-NCS

MEM	500ml
NCS	25ml (5%)

2.2 General methods

2.2.1 Protein concentration

Concentration of proteins was by ultrafiltration under nitrogen pressure using Diaflow membranes (PM10) in Amicon chambers (Amicon Ltd, High Wycombe , Bucks.)

2.2.2 Enzyme linked immunosorbent assay (ELISA)

The ELISA technique was used to measure low levels of specific proteins, dependent on the primary capturing antibody used.

A 96 well plate (Nunc-Immuno Plate II) was loaded with 200μl of primary antibody diluted to give 50μg/ml in coating buffer. The plate was covered and incubate for 1 hour at 37°C followed by an overnight incubation at 4°C. The coating antibody was shaken from the plate and the wells filled with 200μl of PBS-BSA for a 1 hour incubation at 37°C which prevented further non-specific sticking of protein to the plate. Extensive washing of the plate with PBS-Tween was carried out using a cell harvester (Titretek, Norway). The test antigen plus appropriate standards and controls was added to the wells for a further incubation of 90 minutes at 37°C, followed after washing by addition of horse radish peroxidase labelled antibody for a similar incubation. The plate was washed and substrate added for colour development (approximately 30 minutes in the dark). The reaction was stopped by the addition to each well of 80μl 2.5M H_2SO_4 and the optical density read at 492nm on a Dynatech Micro ELISA autoreader (Dynatech Ltd).

2.3 **Cell preparation**

2.3.1 Peripheral blood and bone marrow preparation

Venous blood was collected into sterile plastic or glass tubes containing 25 units/ml heparin (CP Pharmaceuticals Ltd) and bone marrow aspirated into sterile MEM containing 25 units/ml heparin. A mononuclear cell suspension was prepared by layering the blood/bone marrow onto Lymphoprep (Nyegaard) and centrifuging at 1000g for 20 minutes at room temperature. The cells were harvested from the interface washed twice in PBS and resuspended in MEM-NCS. An incubation for 1hr at 37°C was used to remove extrinsically bound immunoglobulin from the cell surface, this was followed by a wash in MEM-NCS at room temperature before storage on ice at 4°C.

2.3.2 Preparation of cells from solid lymphoid tissue

Solid lymphoid tissue e.g. tonsil, was collected into sterile MEM and processed as soon as possible after collection. The tissue was teased through a fine wire mesh using a rubber tipped syringe plunger, large lumps being removed by filtration through a tea strainer mesh. The cells were washed and incubated as in section 2.3.1 before storage on ice at 4°C. Where the cells were to be subsequently cultured this procedure was carried out aseptically in a Class II microbiological safety cabinet.

2.3.3 Tissue sections

Tissue for sectioning was taken and immediately snap frozen in liquid nitrogen for storage. For cutting the tissue was embedded in O.C.T compound (BDH Ltd) and 5μm sections cut at a cabinet temperature of -15°C using a Frigo Cut 2700 cryostat (Reichert Jung). After air drying the sections were used immediately or stored at -20°C wrapped in aluminium foil.

2.3.4 Cell counting, viability and morphological examination

Leucocyte counts on blood samples or cell preparations were performed on a 1/500 dilution of sample in Isoton II using Zaponin detergent as a membrane lyzing agent and a Coulter Model ZM counter (Coulter Electronics Ltd).

The cell viability was determined by Trypan Blue dye exclusion (Flow Laboratories). Two drops of 0.02% trypan blue were added to one drop of cells at approximately 10^6/ml and mixed gently. A drop was transferred to a haemocytometer chamber, allowed to settle and 100 cells counted. Those excluding the dye were considered viable.

The cells morphology was assessed after air dried blood films or cytospin preparations were automatically stained using Wright's stain (Miles Scientific Ltd).

2.3.5 Cell freezing and thawing

Cells requiring long term storage were frozen. Freezing involved diluting cells to approximately $2x10^6$/ml in RPMI 1640 containing 20% FCS and 10% dimethylsulphoxide, then using a controlled cell freezer unit (Cryoson). Storage was then in liquid nitrogen vapour.

For further analysis the cells were rapidly thawed by resuspending the cell pellet in a large volume of RPMI 1640/20% FCS at 37°C. The cells were washed twice in medium and then incubated at 37°C for 1hr to remove extrinsically bound immunoglobulin. This was followed by a wash at room temperature before storage on ice at 4°C.

2.4 **Cell Analysis**

The monoclonal antibodies used in this study are described according to their cluster of differentiation (CD) number where known, or by their clone number (see appendix 1).

2.4.1 Alkaline phosphatase anti-alkaline phosphatase (APAAP) staining

This immunocytochemical method uses monoclonal antibodies to detect cell surface, cytoplasmic and nuclear antigens. To visualise the primary antibody a complex of monoclonal mouse anti-alkaline phosphatase and alkaline phosphatase is employed. The complex is bound to the detecting antibody using an excess of a bridging antibody; sheep anti-mouse immunoglobulin. Finally, the enzyme is reacted with an appropriate substrate to give a coloured reaction product visible microscopically.

The APAAP technique was used to stain tissue sections or cytospin preparations made from cell suspensions at approximately $1x10^6$ cells/ml using a Cytospin 2 cytocentrifuge (Shandon Southern Ltd). Cytospin preparations were dried overnight before use. Cryostat sections were used immediately after brief air drying or were stored foil wrapped at -20°C until required. On removal from -20°C storage the sections must be rapidly air dried to prevent condensation forming on the slides.

Although the APAAP technique is sensitive it has two drawbacks; firstly, many antigens are destroyed by acetone fixation and secondly, it is difficult to distinguish between surface and cytoplasmic staining, particularly when there is a high nuclear to cytoplasmic ratio and strong staining.

1. Using a diamond marker, draw a tight ring around the cells or section.
2. Fix cells in dry acetone.
   Sections: 10 minutes in acetone, remove and allow acetone to evaporate for 10 minutes.
   Cytospins: 5 minutes in acetone, remove and immediately immerse in TBS (DO NOT ALLOW SLIDE TO DRY OUT).
3. Wash slides x3 in TBS, 2 minutes per wash.
4. Apply sufficient primary monoclonal antibody* to cover cells for 30 minutes. Do not allow evaporation of antibody solution.

5. Wash slides x3 in TBS, 2 minutes per wash.
6. Apply sheep anti mouse immunoglobulin solution for 30 minutes.
7. Wash slides x3 in TBS, 2 minutes per wash.
8. Apply APAAP complex (Dako UK Ltd) at recommended dilution, for 30 minutes.
9. Wash slides x3 in TBS, 2 minutes per wash.
10. Add substrate to slides and leave in the dark for 30 minutes.
11. Wash slides in TBS, then tap water and counterstain in Mayer's haematoxylin, sections: 30 seconds.
cytospins: 7 minutes.
12. Wash slides in tap water, air dry and mount in glycerine jelly.

* Appropriate mouse and/or rat monoclonal antibodies must always be used as negative primary antibody controls. See Appendix 1 for monoclonal antibodies used.

2.4.2 Cytoplasmic immunoglobulin

A direct immunofluorescence technique on cytospin preparations was used to detect cytoplasmic immunoglobulin, primarily in lymphoplasmacytoid or plasma cells.

A tight ring was drawn around the cells with a diamond marker, and the slides fixed in methanol for 5 minutes followed by a 10 minute wash in PBS (pH 7.2). After carefully wiping around the cells, fluorescein conjugated goat anti human immunoglobulin heavy chain (GAMD) or light chain specific reagents (Atlantic Antibodies Ltd) were applied for 20 minutes, followed by 2 washes in PBS for 10 minutes. Finally the slides were mounted in PBS/glycerol and sealed with glycerine jelly.

The cells were examined with a Leitz fluorescence microscope fitted with an HBO-50 mercury vapour ploem epi-illuminator (Leitz Gmbh, Wetzler, West Germany). One hundred cells were scored and the percentage showing cytoplasmic fluorescence noted. The type of cell i.e lymphoplasmacytoid or plasma cell was also noted.

2.4.3 Dual labelling for cytoplasmic 9G4 and immunoglobulin

As previously, cytospin preparations were fixed in methanol and washed in PBS, 9G4 at 5μg/ml was then applied to the cells for 20 minutes, followed by two 10 minute washes in PBS. To detect the unlabelled 9G4, rhodamine conjugated goat anti mouse immunoglobulin ($F(ab')_2$ fragment) (Sigma Chemical Co Ltd) was applied for 20 minutes and then washed in PBS. Fluorescein conjugated goat antibodies to the Fc portions of human IgG or IgM (Atlantic Antibodies Ltd) were finally added to the cells and after thorough washing and mounting the slides were examined as previous, using appropriate excitation and emission filters for rhodamine and fluorescein. The goat anti IgG and IgM conjugates were absorbed prior to use on a Sepharose column to which a mouse monoclonal IgG_1 had been linked, so as to remove any cross reaction between the antibodies and the primary 9G4 monoclonal antibody. Also the anti IgG antibody was diluted for use in a 1 in 5 dilution of normal goat serum to block any reaction with the goat IgG in the rhodamine conjugated goat anti mouse immunoglobulin.

2.4.4 Direct immunofluorescence

Direct immunofluorescence was used to detect cell surface immunoglobulin. 250μl of cell suspension at a concentration of approximately $2x10^6$/ml was pelleted in a V bottomed microtitre plate by centrifugation at 200g for 3 minutes. The supernatant was removed and the pellet resuspended by gentle agitation. 10μl of fluorescein conjugated goat anti human immunoglobulin heavy chain (GAMD) or light chain specific reagent (Atlantic Antibodies Ltd) was added and incubated for 20 minutes at 4°C, followed by two washes in cold PBS-azide, spinning at 200g between each wash. The relevant control of fluorescein conjugated normal IgG was always included. If the final analysis was to be by fluorescence microscopy, the pellet was resuspended in 1 drop of PBS-glycerol and a slide of each sample prepared.

For analysis by fluorescent activated cell sorter (FACS III/FACScan, Becton-Dickinson Ltd) the pellet was resuspended in 100μl PBS-azide and kept on ice before analysis. If immediate analysis was not available a further 100μl of PBS-paraformaldehyde was added to give a final concentration of 1% paraformaldehyde. The samples could then be kept for up to 1 week at 4°C before analysis.

Using the fluorescent microscope 100 cells were scored noting the number that were positively stained and the degree of positivity on an arbitrary scale from negative to bright positive (+++). The FACS has the advantage of analysing at least 10,000 cells, giving an objective result and giving a quantative estimate of the fluorescence intensity.

2.4.5 Indirect immunofluorescence

Indirect immunofluorescence was used to detect unlabelled rat or mouse monoclonal antibodies (see Appendix 1) bound to cells using fluorescein conjugated second antibodies, goat anti-rat Ig $F(ab')_2$ fragment and sheep anti-mouse Ig $F(ab')_2$ (Sigma Chemical Co Ltd) respectively.

10μl of primary monoclonal antibody was incubated with approximately $2x10^6$ cells and washed as for the direct florescence technique. 10μl of conjugated secondary antibody was then added to the resuspended cell pellet and incubated for 20 minutes at 4°C, followed by two washes in cold PBS-azide before analysis. Appropriate primary and secondary antibody controls were always run with each sample.

2.5 **Red cell agglutination and agglutination inhibition**

2.5.1 Agglutination

Venous blood was taken into tubes containing 25 units/ml heparin and group O adult I or cord blood i cells were used. After centrifugation at 1000g for 5 minutes, the plasma was removed and the RBCs washed x3 in PBS (pH 7.0) and kept on ice at 4°C as a 2% cell suspension. Test sera were double

diluted in PBS using 51x7mm precipitin tubes (Epson Glass Industries Ltd), the final volume in each tube being 25μl. The diluted sera were cooled on ice and 25μl of the 2% RBCs added and incubated for 1hr at 4°C on ice.

The results were scored microscopically (Appendix 2), using a x10 objective, pre-cooled microscope slides and pasteur pipettes. It is important to avoid any heating of the cells which may cause reversal of weak agglutination reaction. The results were expressed as a titre i.e: the reciprocal of the last dilution showing weak agglutination.

2.5.2 Agglutination inhibition

For inhibition studies, adult I RBCs were used and a suitable dilution of a known patients anti I serum was chosen, usually the last dilution giving maximum 4+ agglutination. Varying amounts of competing test or control antibody was added to this constant dilution of agglutinin and allowed to stand on ice at 4°C, before addition of indicator RBCs as above.

2.6 Isolation of idiotope-positive tonsil cells

2.6.1 Magnetic bead separation

The rat monoclonal anti-cross reacting idiotypic antibody (9G4) which recognizes cold agglutinins with anti-I or anti-i specificity is of IgG2a subclass and available as a concentrated hybridoma culture supernatant. As a control of the same subclass, a rat monoclonal antibody specific for a private idiotypic determinant on a patient's tumor was used.

A cell suspension was prepared aseptically from bilateral tonsillectomy tissue as previously described and washed twice in culture medium. At this stage a cell count and viability test were performed and cytospin preparations made to assess the number of idiotope positive cells in the original tonsil tissue. The cells were pelleted and resuspended in 5mls of a saturating concentration of 9G4 (35μg/ml), for 30 minutes at 4°C with gentle agitation.

After washing x3 in cold PBS-azide, the cells were resuspended in 15mls cold culture medium. Magnetic beads, M-450, (Dynal U.K Ltd) precoated with sheep anti-rat IgG were washed x3 in cold culture medium using a magnet (Dynal MPC-1) to collect the beads. This was to remove any trace of preservative (0.01% thimerosal) from the beads. The beads were added to the cells and left on ice for 15 minutes with regular mixing. Following binding of the cells to the beads, a magnet was applied to the outside of the tube for 5 minutes so that beads plus rosetted cells became attached to the side of the tube. Non-rosetted cells were poured off and the rosetted cells were washed in cold medium using the magnet for collection. Rosettes were finally resuspended in medium and stored on ice.

A preliminary titration using various ratios of beads to target cells was used to establish an optimum for cell recovery. The efficiency of idiotope positive cell removal was estimated by staining both the cells in the rosettes and the residual non-rosetted population using the previously described APAAP technique. If cytospin preparations were made rosetted cells could be stained to assess their morphology and/or cytoplasmic immunoglobulin content. Similarly direct and/or indirect immunofluorescence could be performed on the cells using the magnet to collect the rosettes during the washing stages.

2.6.2 Elution of cells from magnetic beads

Cells rosetted with anti-idiotype and magnetic beads were resuspended in 100ml of culture medium and incubated at 37°C in an atmosphere of 5% CO_2, with gentle agitation. The cell-bead suspension was then drawn slowly up and down through a fine bore pasteur pipette to remove loosely bound cells. The magnetic beads plus any residual rosettes were collected by applying the magnet for 5 minutes. Released cells were poured off, washed in medium, counted, viability tested and further analysed as required.

Preliminary experiments were performed to determine the optimal incubation time required for removal of cells from the magnetic beads.

2.6.3 Cell immortalization with EB virus

The Epstein-Barr virus (EBV) is a B lymphotropic herpes virus which causes infectious mononucleosis in man (190). Infectious EBV can be isolated from infected patients, but a more reliable source for laboratory use is the cell line B95-8 (191). This is derived from Cotton-topped marmoset mononuclear cells which had been infected in vitro by wild type virus from an infectious mononucleosis patient. Polyclonal activation of B cells with EBV results in proliferation and differentiation giving rise to IgM, IgG and IgA secreting lymphoblastoid cell lines, with a predominance of IgM producing cells (192).

Active EB virus was harvested from the marmoset cell line B95/8 by centrifuging the culture supernatant at 400g for 15 minutes at 20°C to remove most of the cells, then passing it through a 0.45μm filter, which retains cells and large particles but not the enveloped virus particles. Supernatants from healthy cultures of B95/8 cells (at 1-2x10^6/ml) should have a immortalizing titre of around 10^{-3} when used to infect human cells. Supernatant was stored in 1ml aliquots at -20°C before use.

Purified idiotope positive cells were centrifuged at 100g for 10 minutes, the supernatant removed and the cells resuspended in EBV culture supernatant to give 2x10^6 cells per ml. After incubation for 1 hour at 37°C, the cells were centrifuged, resuspended in culture medium (containing 5x10^{-5}M 2-mercaptoethanol) to give 5x10^5 cells per ml and plated in 200μl volumes in a 96 well plate. After 4 days the cells were fed by removing half of the old medium and replacing it with fresh medium. The cells were monitored by microscopy until 30-40 colonies were visible, cells were then transferred to a 24 well plate. After a further growth period, cytocentrifuged preparations were made from the cells in each well and these were examined for the presence

of idiotope using the APAAP technique. All the wells were found to contain >95% idiotope positive cells and were pooled for further expansion in tissue culture flasks. This pooled polyclonal cell line was used in all subsequent analyses. During the initial cell growth the medium was supplemented with 20% FCS, and this percentage was gradually lowered until 5% FCS was used to maintain the cultures.

CHAPTER 3

RESULTS

3.1 The nature of the 9G4 idiotype

9G4 is a rat IgG2a monoclonal anti-idiotypic antibody which reacts with an idiotypic determinant present on IgM cold agglutinins of anti-I and anti-i specificity. Its pattern of reactivity is similar to that of a monoclonal antibody (185) specifically raised against IgM Mar known to express such cross-reacting idiotypic determinants (206). The anti-idiotypic antibody recognises a conformation dependent heavy chain determinant, but is highly specific for cold agglutinins and does not react with a panel of 74 myeloma proteins or macroglobulins (184). Studies using an ELISA assay have shown that 9G4 also reacts with a small component of normal IgM and IgG (184) and with cold agglutinins which arise following infection (F.K Stevenson and D. Roelcke, unpublished observations).

3.1.1 Agglutination inhibition

Sera from three patients with CAD (R.H, M.O and D.F) with titres of 1/8192, 1/2048 and 1/512 respectively against adult I antigen-bearing red cells at 4°C were used in this assay. For each serum a graph of serum dilution against strength of agglutination was plotted and from the curve obtained, the dilution corresponding to the steepest part of the curve, i.e the greatest rate of change in agglutination, was selected. This dilution is the most sensitive at which to assess the ability of the anti-idiotypic antibody to inhibit agglutination of I antigen bearing RBCs by the CA sera.

To assess the ability of 9G4 to inhibit agglutination by these CA sera, various concentrations of monoclonal antibody where added to the selected dilution of patient's serum, followed by an assessment of agglutination at 4°C. Monoclonal antibodies against the κ and μ chain constant

regions were used in comparison to 9G4. Controls of an irrelevant rat IgG2a monoclonal antibody (5H7), and culture medium concentrated x10, had no effect on the agglutination reaction.

9G4 gave similar results with the three patient's sera, showing complete inhibition of agglutination at 50μg/ml in all cases which decreased to zero at between 1.56 and 0.39μg/ml. The anti-κ and anti-μ monoclonal antibodies showed no inhibition of agglutination up to concentration of 300μg/ml. The results using serum from patient R.H are shown in Fig 3.1.

3.2 9G4 expression in cold agglutinin disease

Three patients with CAD associated with IgM paraproteinaemia were studied. Patient R.H. (age 69 years) presented with hepatosplenomegaly and haemolytic anaemia, and was diagnosed as having Waldenstroms macroglobulinaemia/non-Hodgkins lymphoma (NHL) involving blood and bone marrow. Patient D.F. (age 79 years) presented with Raynauds phenomenon with a serum IgM paraprotein and, although there was no evidence of lymphoma initially, eventually developed an immunoblastic lymphoma which failed to respond to aggressive chemotherapy. Patient B.R. (age 70 years) presented with painful extremities and haemoglobinuria when cold, as well as a detectable IgM paraprotein. No clinical signs of NHL were observed in this patient. In all three cases the IgM paraprotein was shown to cause preferential agglutination of adult rather than cord group O red cells at 4°C, indicating a probable anti-I specificity.

Blood mononuclear cell preparations from the three patients were investigated for cell surface markers (Table 3.1). In two patients, R.H. and D.F. there was a marked peripheral lymphocytosis due to neoplastic B cells, with low numbers of residual T cells. In each case the B cells expressed a monotypic κ light chain and the idiotypic determinant recognised by 9G4.

Fig 3.1 The effect of 9G4 and of monoclonal antibodies to the constant regions of μ and κ chains on cold agglutination. The monoclonal antibodies were mixed with a 1/256 dilution of serum from patient R.H (22μg/ml of IgM) and the agglutination of adult red cells at 4°C was assessed.

Fig 3.1

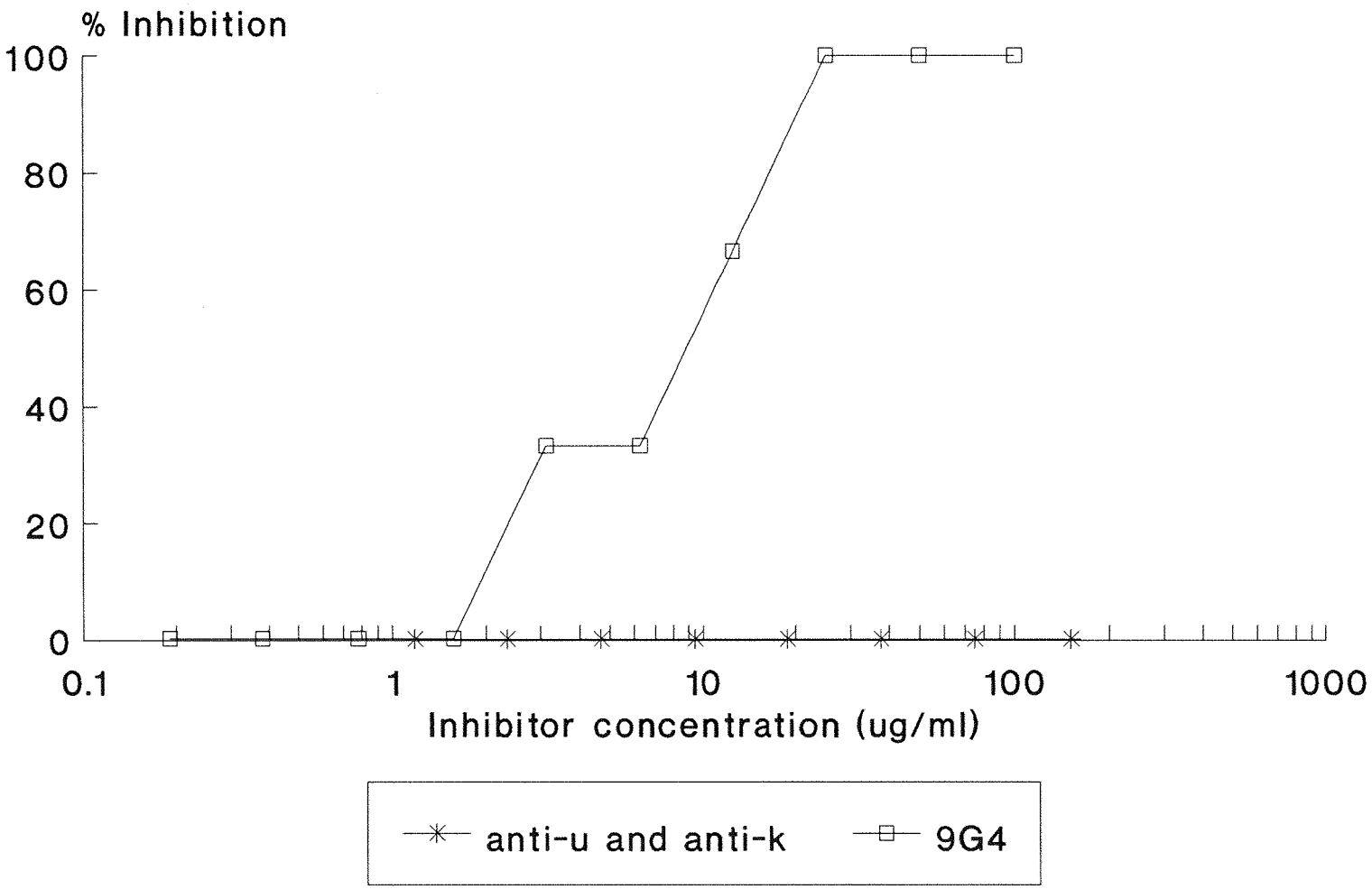

% Inhibition
100
80
60
40
20
0
0.1
1
10
100
1000
Inhibitor concentration (ug/ml)
anti-u and anti-k
9G4

Table 3.1 Cell and serum markers in three patients with cold agglutinin disease

	Patient		
	R.H.	D.F.	B.R.
WBC ($x10^9$/L)	44.0	62.5	11.0
Cell markers*			
CD3	13	2	89
CD5	8	92	-
μ	77	-	18
κ	77	71	3
λ	-	6	27
Id	77	82	21
Serum paraprotein			
Level (g/l)	54	12.2	7.8
Type	IgMκ	IgMκ	IgMλ
C.A titre**	8192	512	4096

* % of total lymphoid cells.

** Cold agglutination titre, as reciprocal of the last dilution causing agglutination of adult group O red cells at 4°C.

- Not detected

In patient B.R. there was only a minor population of neoplastic B cells in the blood: these expressed IgMλ and reacted with 9G4.

For patient R.H. a bone marrow aspirate heavily infiltrated with tumour cells was obtained and it was possible to compare cell populations from this with a matched blood sample. Morphological examination of the stained bone marrow smear showed lymphoplasmacytoid cells (38%), plasma cells (2%) and small lymphocytes (1%); the

residual cells were myeloid (33%) and erythroid precursors (26%). After Ficoll-Triosil separation, the lymphoplasmacytoid and plasma cell populations were enriched to 80% and 10% respectively and all these cells stained for IgMκ and 9G4 (see Fig 3.2). The plasma cells showed a strong uniform cytoplasmic staining on cytospin preparations whereas the lymphoplasmacytoid cells contained accumulations of stained material in a "dotted" pattern suggestive of vesicular inclusion.

3.3 **9G4 expression in B cell malignancy**

A previous limited study of frozen tissue sections (184) had demonstrated the presence of 9G4 positive cells in 2/12 cases of B cell non Hodgkins lymphoma, with strong cell surface staining in each case. This study extends this work to look at 9G4 expression in further cases of B-cell malignancy, all of which where selected at random from the routine laboratory workload.

Of fifty seven cases of CLL examined for surface idiotope expression using sensitive FACS analysis, only three cases gave a positive result. None of these patients had evidence of cold agglutinin disease.

Because of the limited cell numbers available, a further fifty four blood and bone marrow samples from patients with immunologically defined B cell malignancy where examined by the APAAP technique which will detect surface and cytoplasmic 9G4 expression. The twenty seven blood samples consisted of fifteen patients with chronic lymphocytic leukaemia (CLL) and twelve with non Hodgkins lymphoma (NHL). In all but one case the 9G4 reactive cells formed <0.1% of the total lymphoid population. The exception was a case of kappa positive B-NHL in which 82% of the lymphocytes showed strong surface(?) 9G4 expression (see Fig 3.3). This case had no laboratory or clinical evidence of cold agglutinin disease.

Twenty seven bone marrow aspirates were obtained from different patients to the above blood samples, these consisted of six cases of CLL, eleven of NHL and ten of

Fig 3.2. Mononuclear cells from the bone marrow of a patient with cold agglutinin disease (R.H). The cells were cytocentrifuged and stained with anti-idiotype, by the APAAP technique.
The majority of the cells are lymphoplasmacytoid cells showing a dotted pattern of cytoplasmic idiotope expression. One idiotope positive plasma cell with strong diffuse cytoplasmic staining is shown.
Magnification x 1550.

Fig 3.3. Mononuclear cells from a patient with B-NHL. The cells were cytocentrifuged and stained with anti-idiotype, by the APAAP technique.
The cells in this case expressed only surface idiotypic immunoglobulin.
Magnification x 1550.

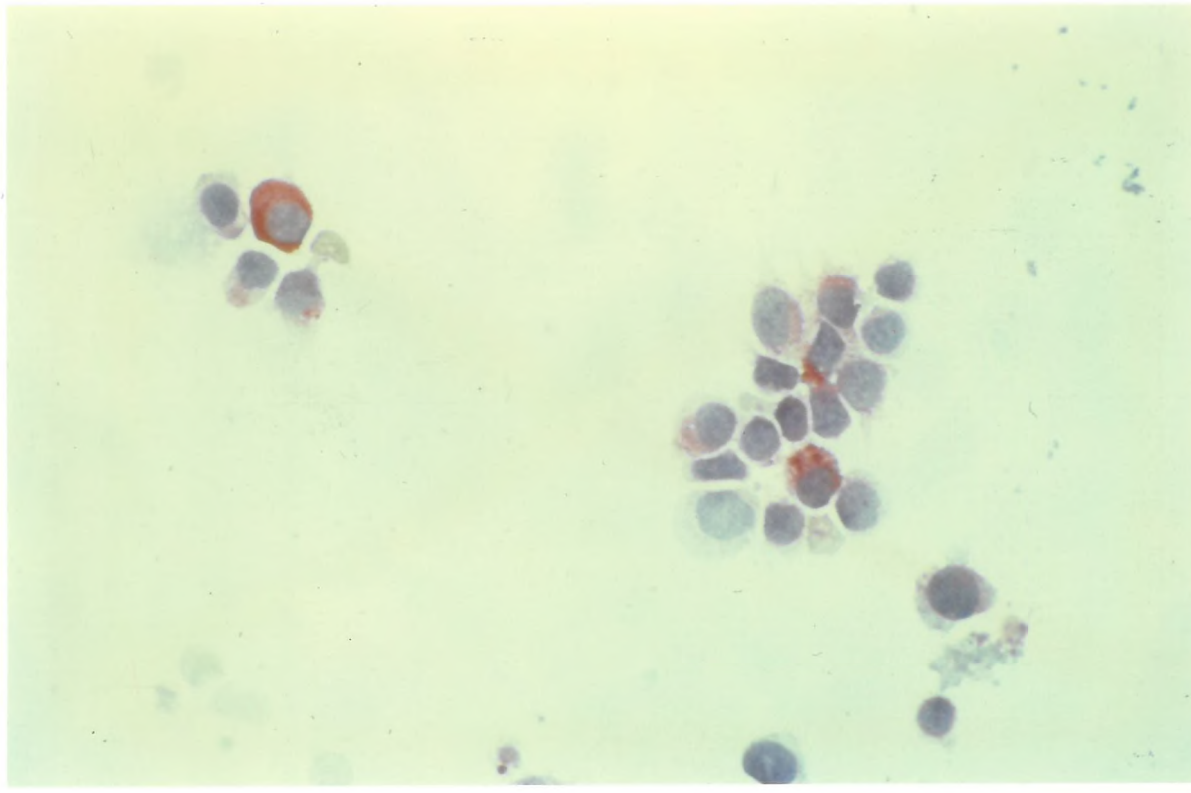


Fig 3.2

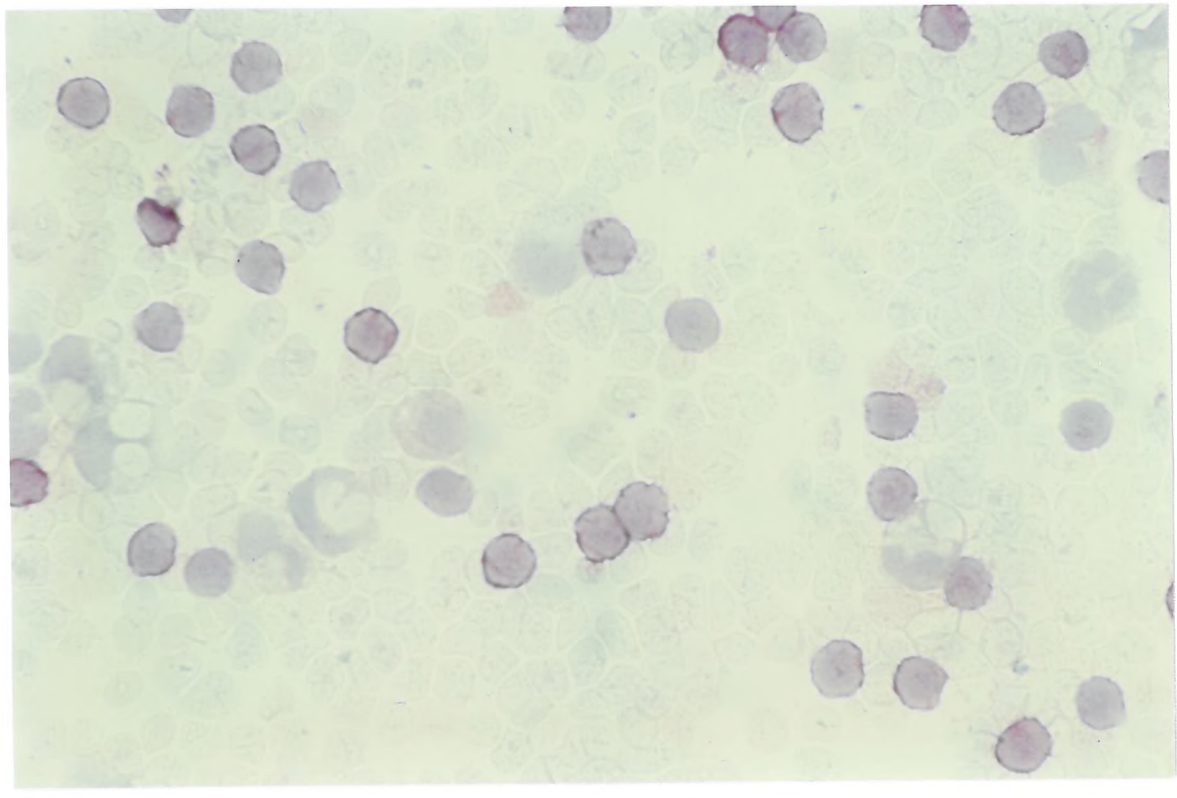


Fig 3.3

myeloma. In all the cases the 9G4 reactive cells formed <0.1% of the total lymphoid population and no positively staining plasma cells were seen.

100 cases of immunohistologically defined B cell NHL were assessed for the presence of 9G4 positive cells using the APAAP technique in tissue sections. Only one case was 9G4 positive and this was classified as follicle centre cell centrocytic/centroblastic NHL. All the other cases had <1% 9G4 reactive cells. (personal communication, Dr.D.B.Jones).

3.4 **9G4 expression in normal adult lymphoid tissues**

Using mononuclear cell cytospin preparations and the APAAP technique, normal adult blood and bone marrow, was examined for the presence of cells expressing 9G4. Cell preparations from normal tonsil, spleen and lymph node were similarly examined. An irrelevant rat IgG2a monoclonal antibody (5H7) used as a control was negative throughout all the tissues. The results are shown in Table 3.2.

As previously, 9G4 gave strong surface/cytoplasmic staining. In the blood samples the positive cells were again small morphologically normal lymphocytes (see Fig 3.4). In the bone marrow and more prominently in the spleen, tonsil (see Fig 3.5) and lymph node tissues, there were also small numbers of lymphoplasmacytoid and plasma cells staining for 9G4. In the plasma cells the staining was more easily discernible as cytoplasmic as opposed to surface.

3.4.1 Cytoplasmic expression of 9G4 in normal tonsil cells

To determine whether cells containing cytoplasmic 9G4 were present in normal tonsil, cytospin preparations were made from two tonsilectomy samples. These were stained, after fixation for IgG or IgM using directly labelled FITC conjugates, and on the same slides after washing with 9G4, the 9G4 reactivity being detected using a goat anti mouse immunoglobulin antibody conjugated with TRITC (Sigma Ltd).

Table 3.2. Incidence of 9G4 reactive cells in cord blood and normal adult lymphoid tissue

Tissue*	Cell populations	
	B cells$^{\phi}$	9G4-positive (% of B cells)
Blood (10)	10.1 (±6.2)	6.9 (±3.0)
Bone marrow (10)	6.5 (±4.6)	10.8 (±4.6)
Tonsil (14)	51.5 (±17.0)	3.6 (±2.5)
Spleen (11)	34.0 (±15.0)	5.0 (±2.9)
Lymph node (6)	25.0 (±13.0)	3.2 (±2.4)

* - Numbers in parentheses refer to numbers of samples tested for 9G4 expression.

$^{\phi}$ - B cell populations are given as percentages of total lymphoid cells, and are the accumulated working normal ranges in the laboratory. 9G4 positive cells are expressed as percentages of the B cells. All are means (±1SD).

Control slides where stained with, 1; the TRITC conjugate alone to check for non-specific binding to human Ig in the plasma cells and 2; with the direct FITC conjugates followed by the TRITC conjugate to check for non-specific binding of TRITC conjugate to the Ig of the FITC conjugate (this should not in theory occur as the primary FITC conjugates where both raised in a goat). All the control slides gave the expected results, showing an absence of non-specific binding by either labelled conjugate.

Because of the very small numbers of positive cells in the tonsil at least 80 fields at a x600 magnification (≈15,000 cells) had to be examined and the cytoplasmic staining cells are counted as a percentage of the total lymphoid cells. The results are shown in Table 3.3. From the results there would appear to be an overlap population of 9G4 positive cells

Fig 3.4. Mononuclear cells from normal blood. The cells were cytocentrifuged and stained with anti-idiotype, by the APAAP technique.
Magnification x 1550.

Fig 3.5. Normal tonsil cells. The cells were cytocentrifuged and stained with anti-idiotype, by the APAAP technique.
Magnification x 1550.

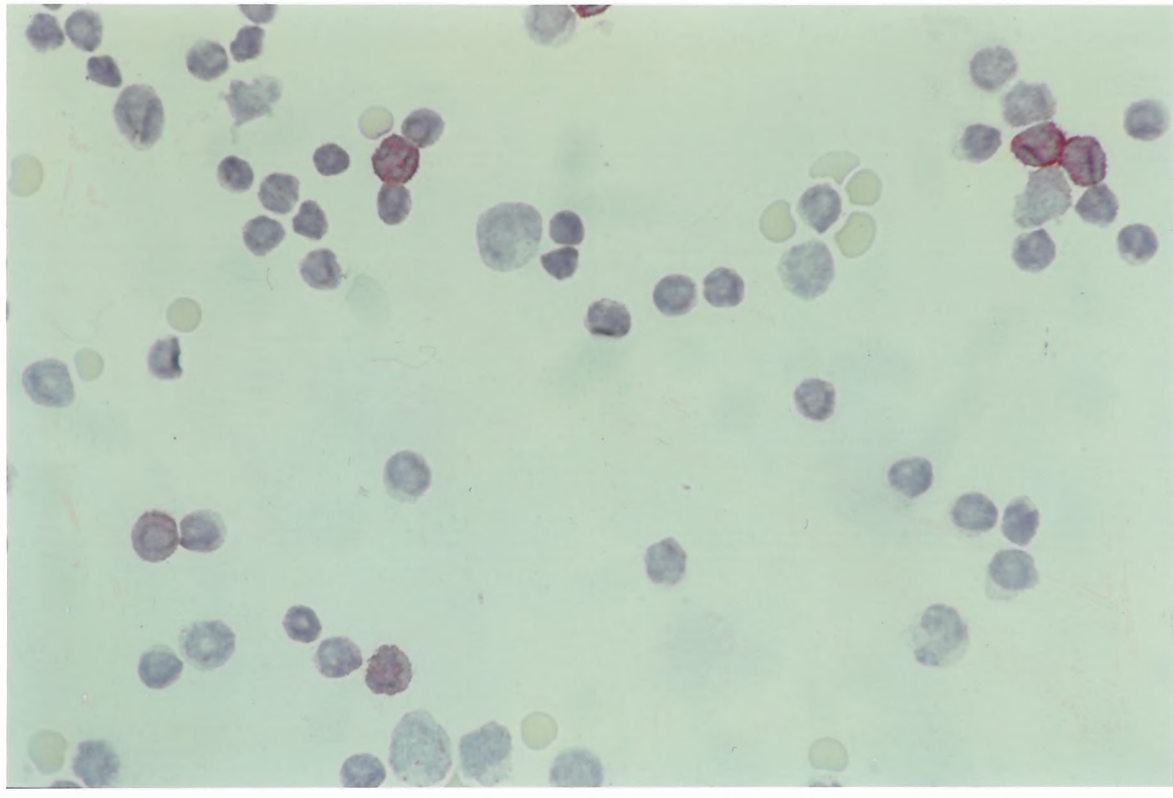


Fig 3.4

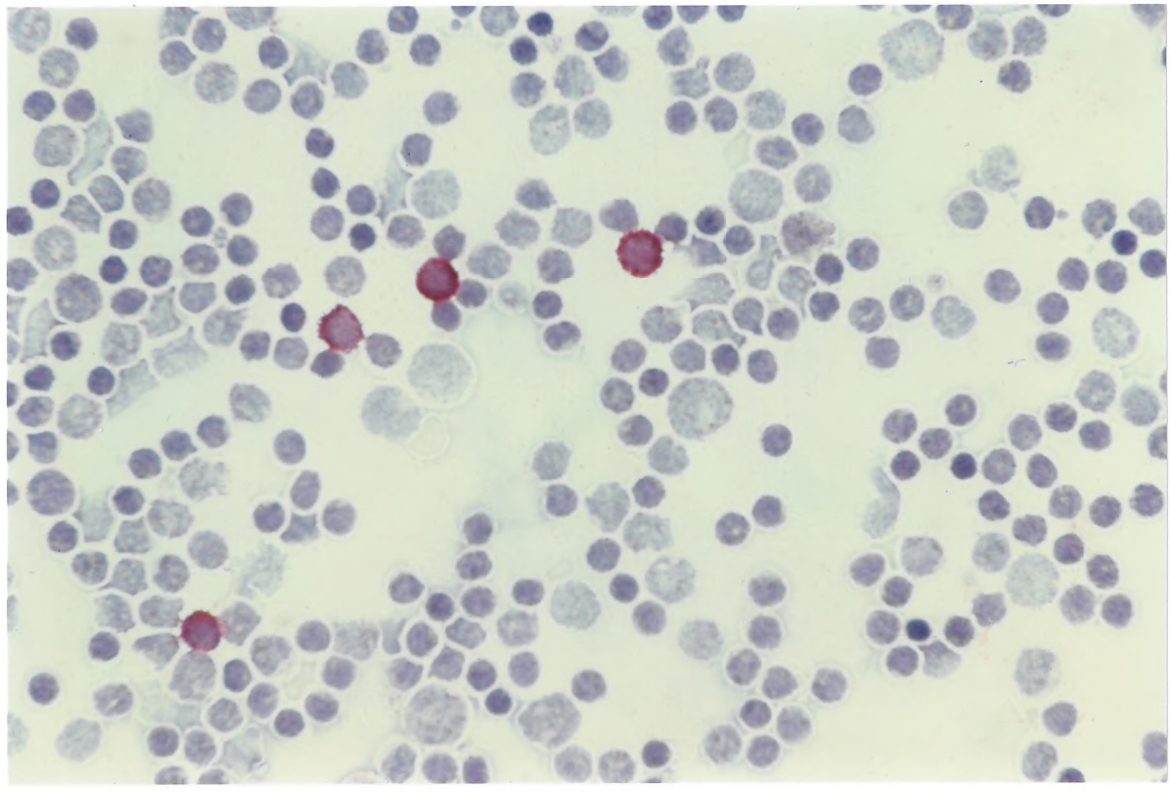


Fig 3.5

staining for IgG and IgM, as the 9G4+IgM+ cells when added to the 9G4+IgG+ cells total >100%. This is probably due to errors inherent in counting large numbers of cells and the fact that IgM+ and IgG+ cells were counted in different although sequential tonsil sections. In view of these ambiguities the only meaningful conclusion that can be drawn from this study is that both 9G4+IgM+ and 9G4+IgG+ cell are present in normal tonsil tissue albeit in very low numbers. Morphologically the 9G4+/IgM+ cells were small lymphocytes or lymphoplasmacytoid cells although an occasional larger reactive cell showed this phenotype. The 9G4+/IgG+ cells were small to mature plasma cells by morphology.

Table 3.3 The incidence of cytoplasmic 9G4 positive cells in tonsil tissue

	Cytoplasmic staining		
Tonsil No	9G4 positive*	%9G4 also IgM positive	%9G4 also IgG positive
1	0.55	32.5	79.0
2	0.33	32.2	83.3

* - Percentages are given as percentages of total lymphoid cells

3.5 **9G4 expression in cord blood**

Mononuclear cell cytospin preparations were made from seventeen cord blood samples and examined for 9G4 expression by the APAAP technique. As in the normal adult blood samples 9G4 was strongly expressed, being positive in 4.4% (±2.7%) of B cells, with B cells accounting for 21.6% (±8.9%) of the

cord blood lymphocytes. The percentage of 9G4 positive B cells in cord blood is similar to that found in other normal lymphoid tissues (see Table 3.2).

3.6 **9G4 expression in fetal spleen**

Fetal tissue was obtained from prostaglandin induced terminations, the age being determined by crown to rump measurement (188). Samples of fetal spleen were examined at 15, 17 and 20 weeks gestation. Cryostat sections were stained by the APAAP technique with 9G4, a monoclonal anti IgM and with an irrelevant rat IgG2a monoclonal (5H7), which gave negative results throughout. Surface IgM staining was used to indicate the total B cell numbers present in the tissue. To give an estimate of the percentage of IgM or 9G4 positive cells, the number of positively stained cells in fifty random microscope fields (600x magnification) were counted and related to the total numbers of lymphocytes counted in the same 50 fields.

Strongly staining IgM cells were scattered throughout the spleen at 15 weeks gestation, comprising 3% of the total lymphoid population. By 17 weeks the percentage was 6.4%, as small aggregates of B cells started to form around prominent blood vessels. At 20 weeks, the spleen had started to develop obvious follicular structures. IgM positive cells were found predominantly in the follicles and scattered throughout the tissue, accounting for 37.3% of the lymphocytes (see Fig 3.6).

At 15 weeks gestation the 9G4 positive cells made up only 0.088% of the lymphoid cells or 2.9% of the B cell population and by 17 weeks the percentages were 0.47% and 7.3% respectively. At 20 weeks although there was a marked increase in total 9G4 positive cells to 2.4%, the percentage as a portion of the B cell population remained at 6.5% simply reflecting the overall expansion in the total number of B cells (see Fig 3.7). Results are shown in Table 3.4. The distribution of the 9G4 positive cells in the tissue mirrored that of the IgM positive cells at each gestation age.

Fig 3.6. Frozen section of fetal spleen (20 weeks gestation) stained with monoclonal anti-μ, by the APAAP technique. Magnification x 310.

Fig 3.7. Frozen section of fetal spleen (20 weeks gestation) stained with anti-idiotype, by the APAAP technique. Magnification x 310.

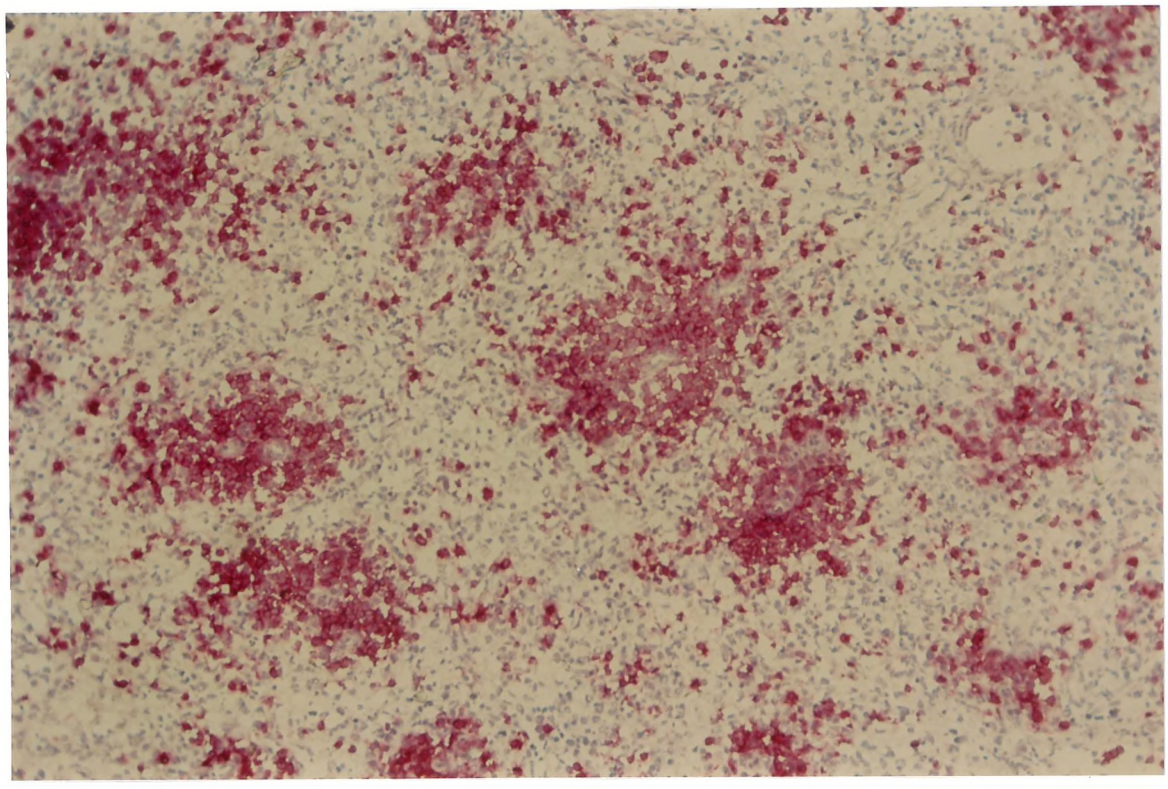


Fig 3.6

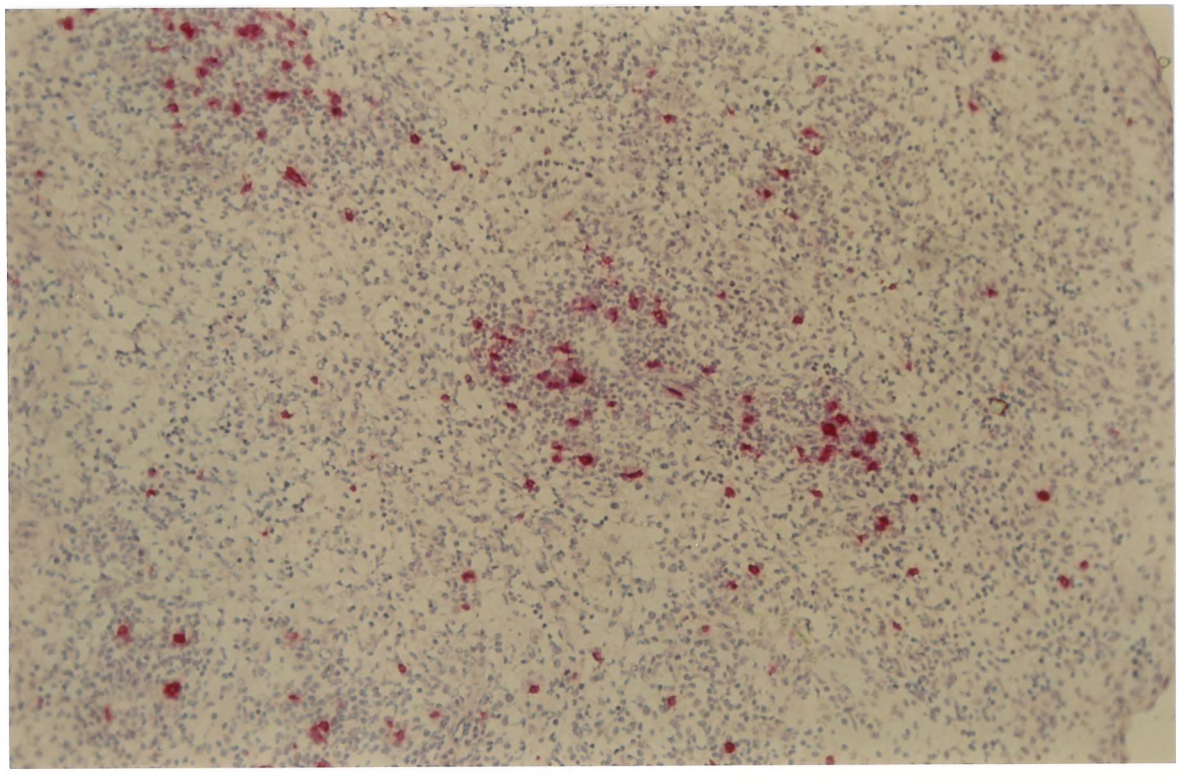


Fig 3.7

The 9G4 positive cells were small lymphocytes of normal morphology showing strong staining by the APAAP technique. Because of the primary fixation step used in this method it is not possible to say whether this staining is cytoplasmic or strong surface expression of 9G4. The numbers of 9G4 positive cells expressed as a percentage of B cells was consistent with that found in normal adult spleen tissue (see Table 3.2).

Table 3.4. Incidence of 9G4 positive cells in fetal spleen

	Cell populations		
Weeks gestation	B cells*	9G4 positive*	9G4 positive (% B cells)
15	3.0	0.088	2.9
17	6.4	0.47	7.3
20	37.3	2.4	6.5

* - percentages are given as percentages of total lymphoid cells.

3.7 Isolation of idiotope positive cells from normal tonsil tissue

The 9G4 antibody reacts strongly with the surface of a small population of lymphoid cells but shows no non specific binding to other cell types commonly found in lymphoid tissue. These features make it an ideal antibody with which to isolate idiotope positive cells. Magnetic particles, although a recent innovation, were used in preference to more established cell isolation techniques e.g, panning, FACS or rosetting, because they offered a rapid isolation with good cell yield and purity from a simple tonsil cell suspension. Cells attached to magnetic beads can be further

stained by immunofluorescence (187) or APAAP techniques and can be transformed with EB virus (189), as the cells maintain excellent viability.

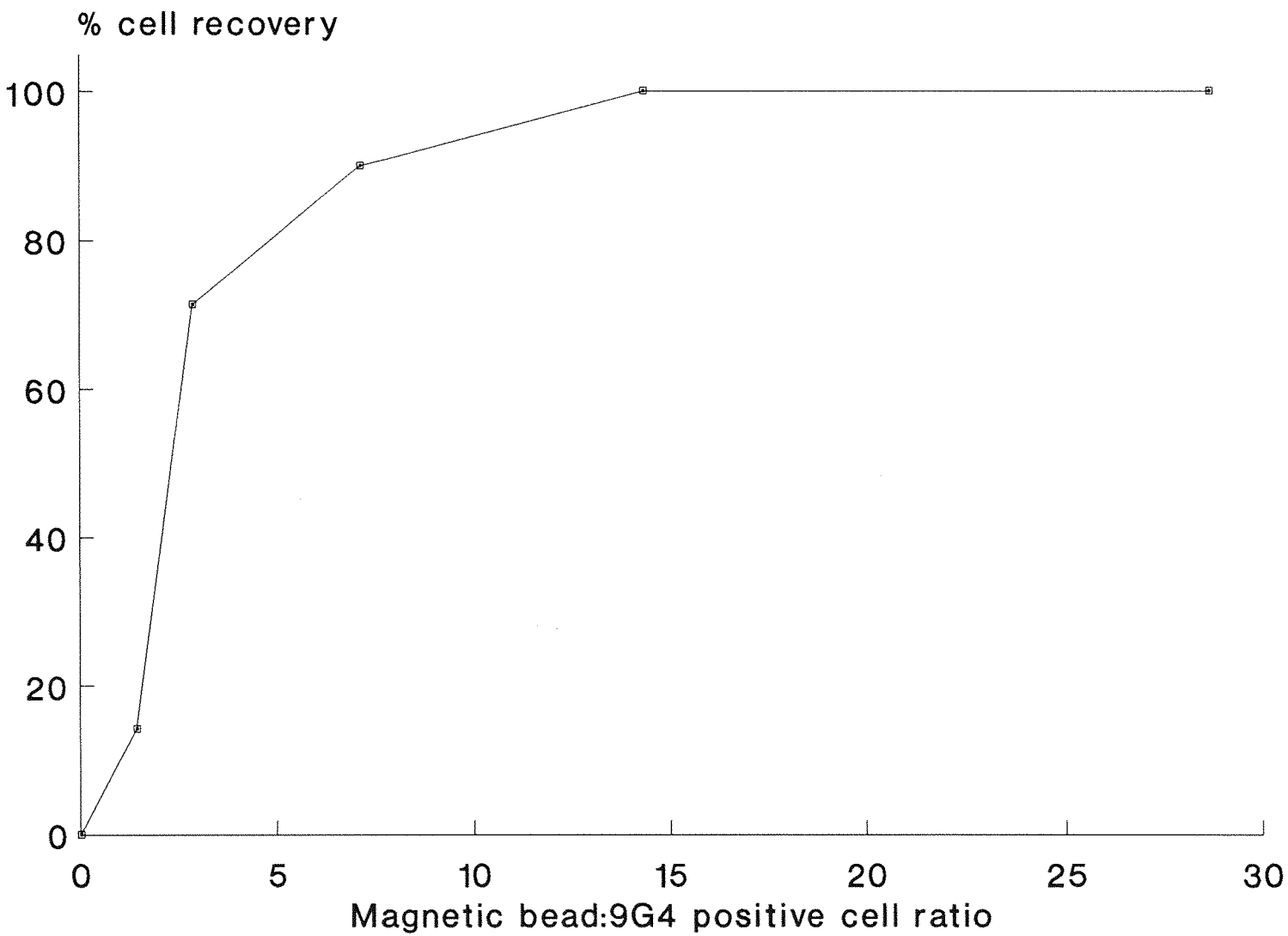
Bilobed tonsilectomy samples were used for the isolation of idiotope positive cells and provided 0.55-3.5x10^9 mononuclear cells (mean 2.2x10^9) with >95% cell viability as assessed by trypan blue dye exclusion. Samples showing microscopic bacterial contamination were discarded.

A preliminary experiment showed that an indirect separation procedure where the tonsil cells where first incubated with 9G4 antibody and subsequently rosetted with anti-rat IgG coated beads was preferable to a direct method. The direct technique involved binding 9G4 (at 2μg/ml) to magnetic beads coated with sheep anti rat IgG (Dynal Uk Ltd), the beads where washed and incubated with the tonsil cells to form rosettes with the idiotope positive cells. The indirect method gave a 76% recovery of 9G4 positive cells, assessed by counting the number of idiotope positive cells in the original tonsil cell suspension and the number of positive cells remaining in the cell suspension not bound to the magnetic beads. An APAAP technique was used to determine the numbers of 9G4 positive cells. The direct technique also using a bead:cell ratio of 3:1 gave only a 11.3% cell recovery.

To further assess the effect of the magnetic bead:cell ratio on the percentage cell recovery, the described indirect cell isolation technique was used and the ratio beads:cells varied from 1.43:1 to 28.6:1. The results are shown in Fig.3.8. Although a 2:1 ratio gave a 50% idiotope positive cell recovery, a ratio of ≈13:1 was required for 100% recovery. This ratio is impractical for routine separations, firstly because of the high cost and secondly because as suggested by the manufacturers highly rosetted cells with more than 4-5 particles per isolated cell can prove difficult to detach from the magnetic particles. A ratio of 1:1 or 2:1 is recommended for giving the best detachment of cells. The 3:1 ratio which gives ≈60% cell

Fig 3.8. The effect of varying the ratio of sheep anti rat IgG coated magnetic particles to the number of 9G4 positive cells, on the percentage recovery of idiotope positive cells. The percentage recovery was assessed by counting the number of 9G4 positive cells in the original tonsil tissue and the number of positive cells not bound to the magnetic beads during the separation.

Fig.3.8


% cell recovery
100
80
60
40
20
0
0
5
10
15
20
25
30
Magnetic bead:9G4 positive cell ratio

recovery was chosen to obtain adequate cell numbers for further analysis while potentially allowing the cells to be detached from the magnetic particles.

3.8 Immunophenotyping of rosetted cells

The cells attached to the beads were small/large lymphocytes of normal morphology, and the only contaminating cells were occasional macrophages (see Fig 3.9). To demonstrate that the rosetted cells were coated with 9G4, cytospin preparations of the cell rosettes were stained by the APAAP technique using no primary antibody, relying on the sheep anti mouse bridging antibody to detect the 9G4 antibody bound to the cells during the isolation procedure. By this method >99% of the rosetted cells where positive, with only rare macrophages showing a negative reaction (see Fig 3.10).

As the rosetted cell had been shown to be 9G4 positive further dual labelling studies could be performed. Cytospin preparations were stained using FITC labelled anti-human Ig reagents and indicated no cells expressing cytoplasmic IgA, IgG or IgD. Only very occasional cells (<0.1%) showed cytoplasmic expression of IgM or kappa/lambda light chains, and these cells were morphologically small lymphoplasmacytoid cells (which had been separated by virtue of their surface immunoglobulin expression).

Cell surface staining was performed using the rosetted cells in suspension and indirect immunofluorescence. To detect the mouse monoclonal antibodies a fluorescein conjugated $F(ab')_2$ fragment, rat anti mouse IgG (Jackson Immunoresearch Laboratories, INC.) was used to minimise binding to the rat 9G4 antibody already shown to be present on the surface of the rosetted cells. The conjugate had also been immunoabsorbed to remove antibodies cross reacting with human serum proteins. The results were read microscopically and are shown in Table.3.5. When used alone or with the rat monoclonal antibody 5H7 as a control the conjugate showed no staining reaction with the rosetted cells. The cells showed

Fig 3.9. Idiotope positive cells labelled with 9G4 antibody, rosetted by anti-rat Ig coated magnetic beads.
The magnetic beads appear out of focus because they are not compressed by the cytocentrifugation technique used in the samples preparation.
Wrights stain, magnification x 1550.

Fig 3.10. Idiotope positive cells labelled with 9G4 antibody, rosetted by anti-rat Ig coated magnetic beads. Cytocentrifuge preparations were stained by the APAAP technique to reveal 9G4 bound to the idiotope positive cells. No primary monoclonal antibody was used, the 9G4 being detected by the sheep anti-mouse bridging antibody.
Insert: Rosetted cells stained only with APAAP complex and Mayers Haemotoxylin as a negative control.
Magnification x 1550.

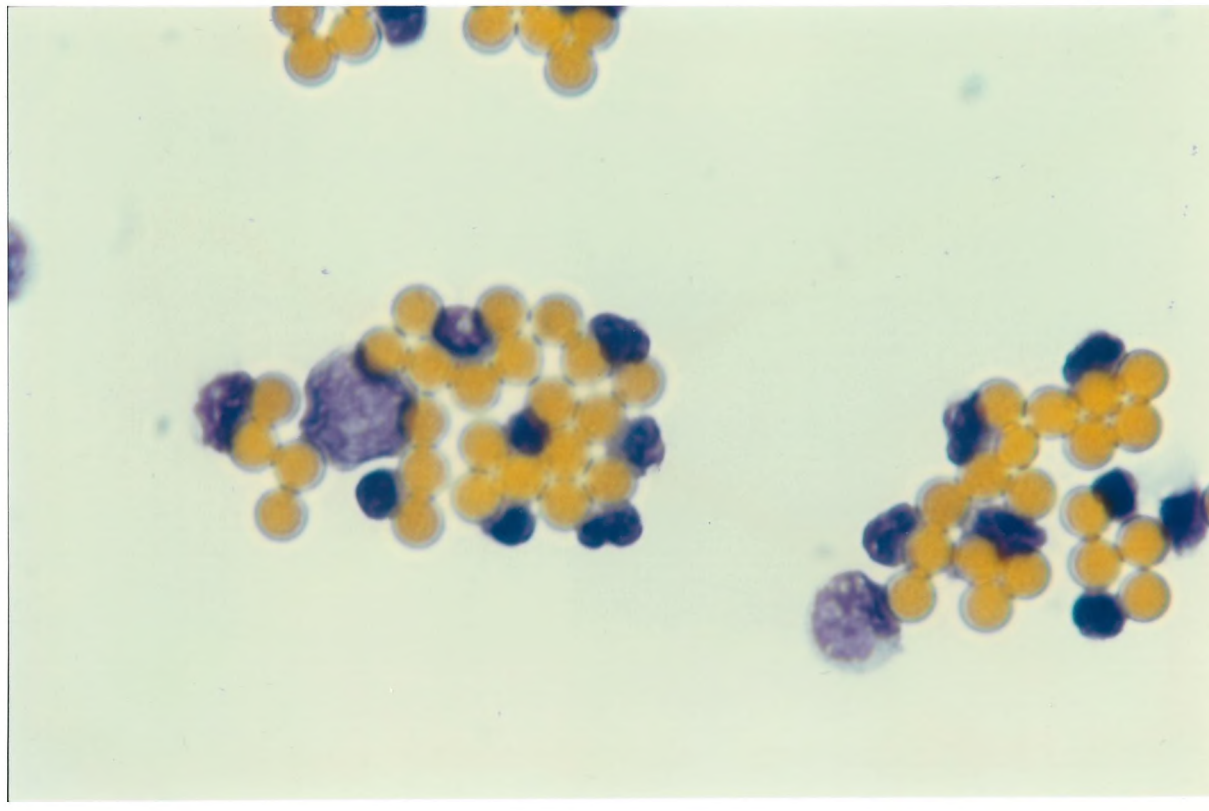


Fig 3.9

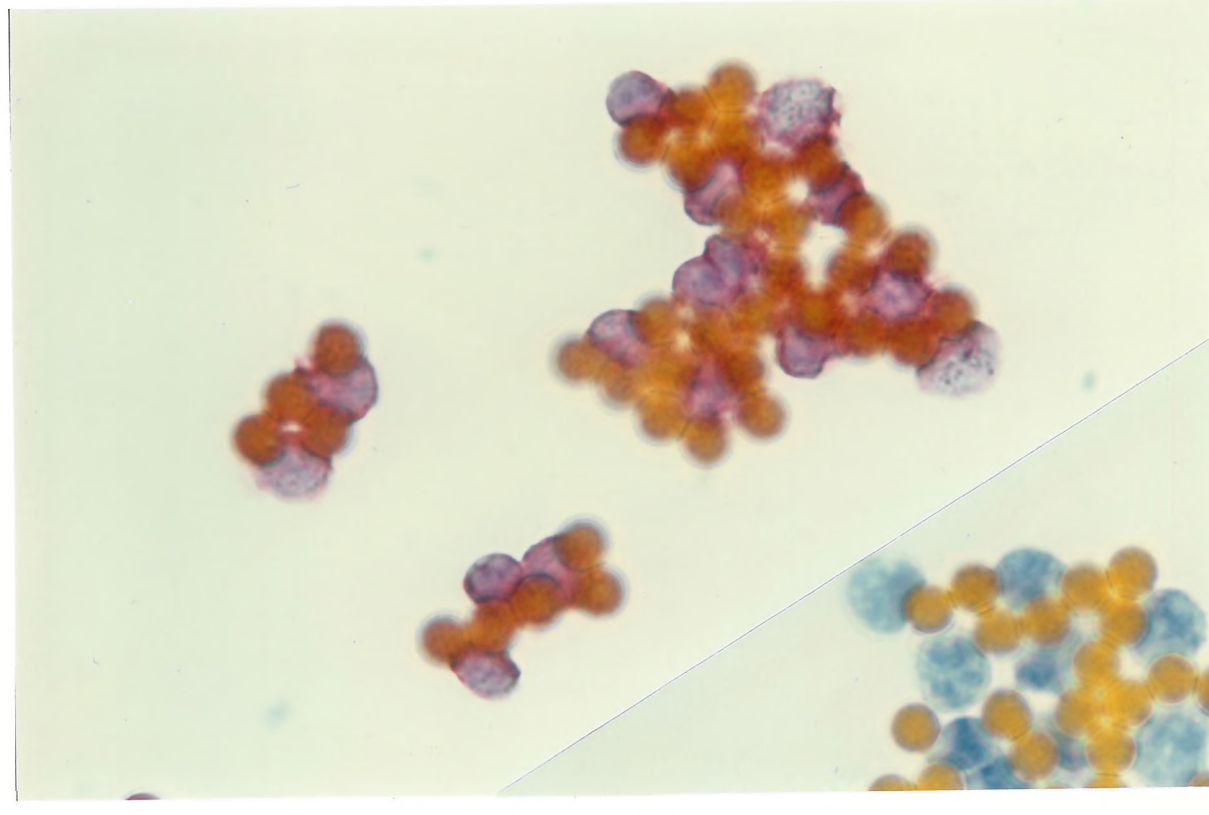


Fig 3.10

surface expression of CD19, CD37 and HLA class I and II molecules. No cells expressing the CD5, CD10, CD23 or CD71 antigens were demonstrated.

To answer the question as to whether or not the isolated cells expressed the CD5 antigen, a theoretically more sensitive technique was employed in addition to immunofluorescence. A biotinylated CD5 specific monoclonal antibody was allowed to react with the rosettes and after adequate washing, this was detected by an avidin biotin complex containing biotinylated phycoerythrin (ABC-PE)(Vector Laboratories Ltd). The increased sensitivity is a result of at least three molecules of phycoerythrin

Table 3.5 Immunophenotyping of idiotope positive rosetted cells

Primary antibody	Secondary antibody	Percentage rosetted cells positive	Staining strength*
PBS	RaM$^{\phi}$	0	
5H7	RaM	0	
9G4	RaM	92	± - +
CD19	RaM	96	+
CD37	RaM	98	+ - ++
CD5	RaM	0	
CD71	RaM	0	
CD23	RaM	0	
CD10	RaM	0	
HLA ClassI	RaM	98	++
HLA ClassII	RaM	98	+
PBS	ABC-PE	0	
CD5-Biotin	ABC-PE	0	

* - Staining strength is assessed on an arbitary scale from negative to bright staining - +++.

ϕ - Rat anti mouse IgG/FITC

(PE), being bound to each molecule of monoclonal antibody, as opposed to one molecule of fluorescein. PE when excited at a wavelength of 488nm is also ≈20 times brighter than fluorescein on a molecule to molecule basis.

By this method again no cells were found to express the CD5 antigen.

3.9 Release of cells from magnetic beads

To determine the optimum incubation time for removal of cells from the magnetic particles, tonsil cell rosettes were collected as previously described and divided into ten equal aliquots. These were incubated at 37°C in an atmosphere of 5% CO_2 and at timed intervals the eluted cells were recovered. The percentage recovery over time was assessed by counting the number of 9G4 positive cells in each original aliquot and the number eluted from the beads. The cell viability was determined by trypan blue dye exclusion at each time interval. The results of two experiments are shown in Fig.3.11. The optimum cell recovery occurred after 2-3 hours incubation and produced an 81-87% cell recovery. The cell viability was in the range of 85-95% in both experiments throughout eight hours incubation. In the one experiment where the incubation was extended to fifteen hours the viability fell to 75%. Using a three hour incubation period we recovered 1.5 - 9.1×10^7 cells from a bilobed tonsilectomy sample (mean = 5.3×10^7, N=10) with >85% cell viability in each case.

3.10 Immunophenotyping of cells released from magnetic beads

Released cells were stained by the APAAP technique for cytoplasmic immunoglobulin and idiotope on cytospin preparations and by indirect immunofluorescence in suspension for surface antigens. The surface fluorescence was read on the FACS analyser. Results from three tonsil separations are shown in Table.3.6. It is clear that the majority of cells obtained from the three tonsils were idiotope-positive although there were some T cells (≈10%)

Fig 3.11 The effect of incubation time on the release of idiotope positive cells from magnetic beads. The percentage recovery is determined by counting the number of 9G4 positive cells in the incubated aliquot of rosetted cells and the number of cells released from the beads.

Fig 3.11

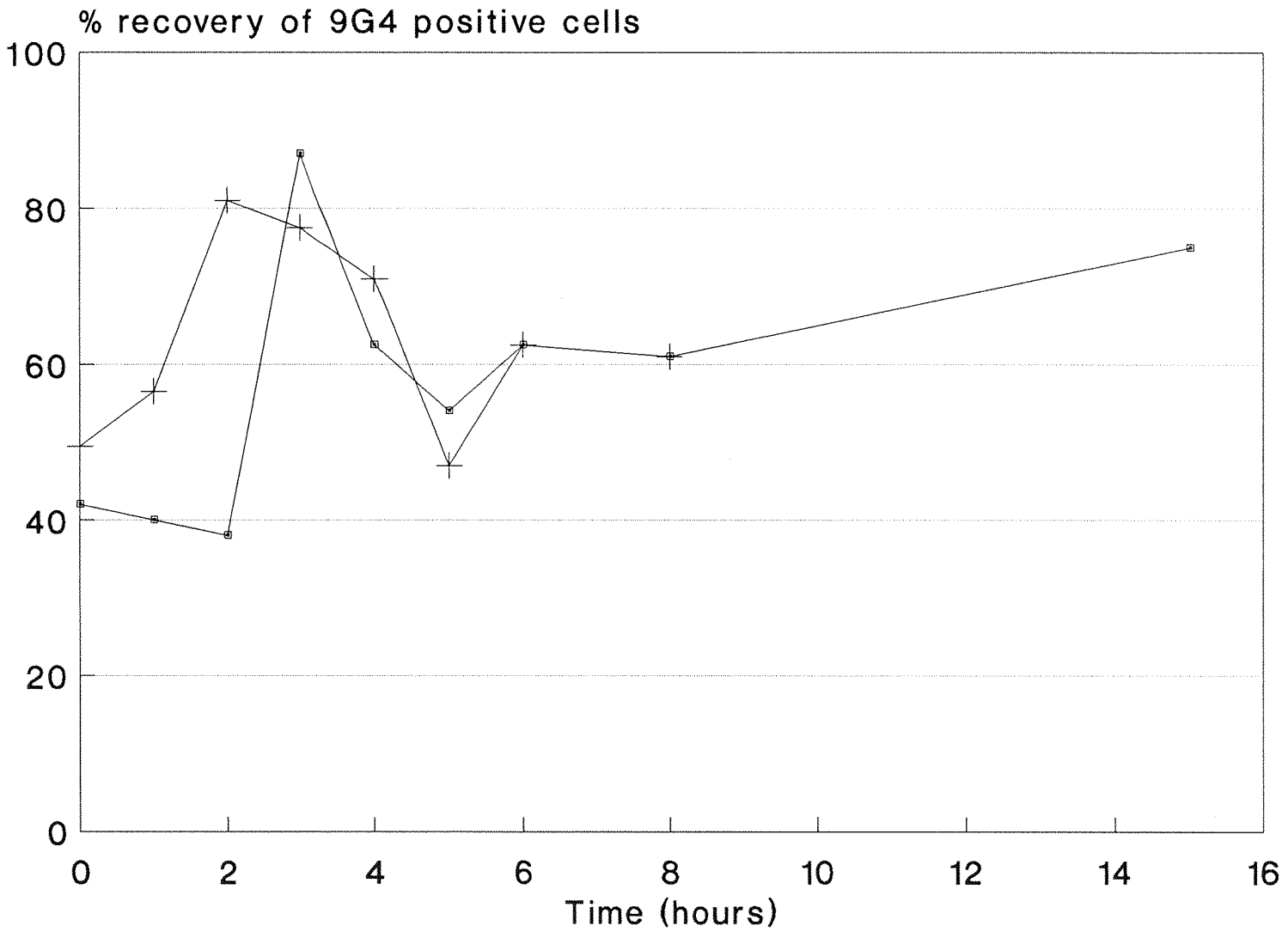

% recovery of 9G4 positive cells
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Time (hours)

Table 3.6 Immunophenotyping of idiotope positive cells released from magnetic bead rosettes*

Surface antigens	Cells from beads 1	2	3
Idiotope	34	60	67
IgM	68	80	62
IgG	22	-	-
CD3	5	-	12
CD5	6	28	27
CD19	81	51	75
CD22	88	-	84
CD23	17	26	-
CD37	94	97	91
CD45	96	-	92
CD38	12	-	74
Intracellular antigens			
Idiotope	62	62	92
IgM	85	78	87
IgG	2	-	-

* - Figures are given as percent positive cells.
- Not detected

carried over during separation on the beads. In tonsils 1 and 2 there were also minor populations of normal B cells which were idiotope-negative but IgM-positive. This reflects the sensitivity of the method to detect idiotype which was usually found to be below that to detect IgM. The FACS profile indicates that most of the separated tonsil cells expressed immunoglobulin but the surface immunoglobulin signal was usually below the APAAP figure suggesting that some idiotope-positive cells had only intracellular Ig. This

assessment is complicated by the fact that antibody to the surface Ig idiotope had been used in the separation procedure and that resynthesis of modulated Ig may not be complete. The predominant immunoglobulin class found in the isolated tonsil cells was IgM although IgG positive cells were present in some preparations. The B lineage antigens recognised by the CD19, CD22 and CD37 monoclonal antibodies were present on the cells, although in the case of CD19 the expression was weak and variable compared to that of CD22 and CD37. The small numbers of contaminating CD3 positive T cells carried over during the separation on beads, probably account for the finding of CD5 positive cells in the preparations. The CD23 and CD38 antigens found on certain activated B cells were variably expressed on the idiotope positive cells.

3.11 Epstein-Barr virus immortalisation of idiotope positive cells

3.11.1 Morphology and immunophenotyping of the cell lines

EBV immortalized cell lines were established from idiotope positive cells separated from normal tonsil tissue and from the idiotope positive bone marrow cells of a patient (R.H) with cold agglutinin disease. In the latter case the bone marrow cells were purified only by density gradient sedimentation and contained predominantly lymphoplasmacytoid cells (80%), a minor plasma cell population (10%) and occasional normal bone marrow elements. All the tumour cells stained for IgM-kappa and with 9G4. Transformation of both cell populations was rapid and efficient with 30-40 colonies visible per well of a 96 well tissue culture plate, 7 days post viral infection. Morphologically the transformed cells from the tonsil were predominantly lymphoplasmacytoid cells and plasma cells, although large multi nucleolated basophilic blast cells, plasmablasts and proplasma cells were present in small numbers. The transformed cells from the patient showed a similar range of cell types, the predominant cell being a lymphoplasmacytoid cell.

The immunophenotyping results are shown in Table 3.7.

Table 3.7 Immunophenotyping of idiotope positive EBV cell lines from normal tonsil and patient (R.H) tumour cells.

Surface antigens	Tonsil EBV line	Tumour (R.H) EBV line
Idiotope	60	83
IgG	28	0
IgM	41	73
kappa	39	79
lambda	36	2
CD3	2	0
CD5	3	1
CD19	73	93
CD22	63	82
CD23	60	92
CD45	89	95
CD38	-	17
Intracellular antigens		
Idiotope*	83	95
IgM	74	95
IgG	28	0
kappa	15	95
lambda	12	0

* - APAAP technique.

The EBV line from tonsil showed only 60% surface idiotope positivity by FACS analysis, the remainder of the population expressing cytoplasmic idiotope. The surface idiotope positive cells expressed IgG or IgM with no predominance of kappa or lambda light chain types. The cytoplasmic idiotope positive cells were mainly IgM positive lymphoplasmacytoid

cells, with a minor population of IgG expressing plasma cells, again there was no kappa or lambda light chain predominance showing we had isolated a polyclonal idiotope expressing EBV cell line. The cell line established from the patient (R.H) with cold agglutinin disease showed 83% surface idiotope staining and 95% of the cells expressed cytoplasmic IgM and kappa light chain. These results reflect the nature of the patients original tumour cells i.e monoclonal lymphoplasmacytoid cells and show we have isolated essentially a monoclonal idiotope expressing EBV cell line. As expected both cell lines strongly expressed the CD23 antigen and neither expressed the CD5 antigen.

3.11.2 Immunoglobulin secretion by the EBV cell lines.

Spinner culture supernatant from the tonsil derived EBV line contained IgM at 4.4μg/ml and IgG at 8.0μg/ml as measured by ELISA assay. A parallel supernatant from the patient derived EBV line contained IgM at 2.9μg/ml. Both supernatants were concentrated for testing in the cold agglutination assay.

The results of testing the ability of various IgM preparations to agglutinate I antigen positive red cells at 4°C are shown in Fig 3.12.

No agglutination was observed with sera from three randomly selected patients with macroglobulinaemia but no cold agglutinin disease. The serum from patient R.H (monoclonal IgM-kappa) shows efficient agglutination down to an IgM concentration of 1.8μg/ml.

After EBV immortalization, the IgM secreted by the cells from patient R.H showed a similar agglutinating ability. Culture supernatant from an EBV line established from normal B cells which were idiotope negative was also assayed for IgM and tested for cold agglutination, but was completely negative.

The next step was to investigate whether the IgM secreted by the idiotope positive cell line derived from normal tonsil could cause agglutination in the cold. The assay showed that the supernatant was as effective as that from the patient derived EBV line causing agglutination at

Fig 3.12 Cold agglutination activity of idiotypic IgM secreted by EBV immortalized normal or neoplastic cells. The ability of various IgM preparations to agglutinate I antigen positive group O red blood cells at 4°C was assessed.

—+——+— , serum idiotypic IgM (patient R.H).

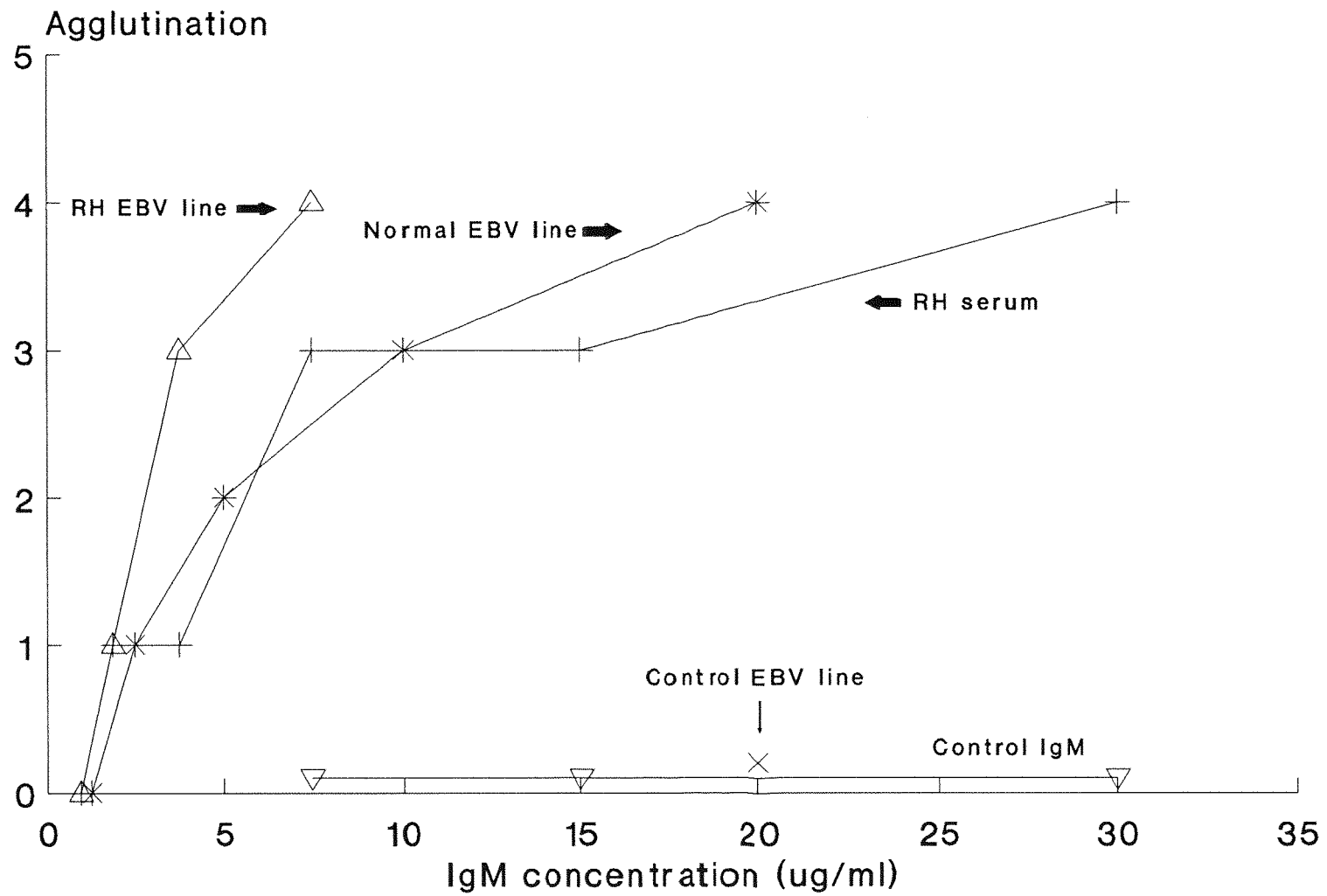
—∇——∇— , control serum IgM from a patient with macroglobulinaemia but no cold agglutination.

—*——*— , idiotypic IgM secreted by an EBV line derived from normal tonsil.

—Δ——Δ— , idiotypic IgM secreted by an EBV line derived from neoplastic cells, patient R.H.

X , culture supernatant from an idiotope negative EBV line derived from normal B cells.

Fig 3.12

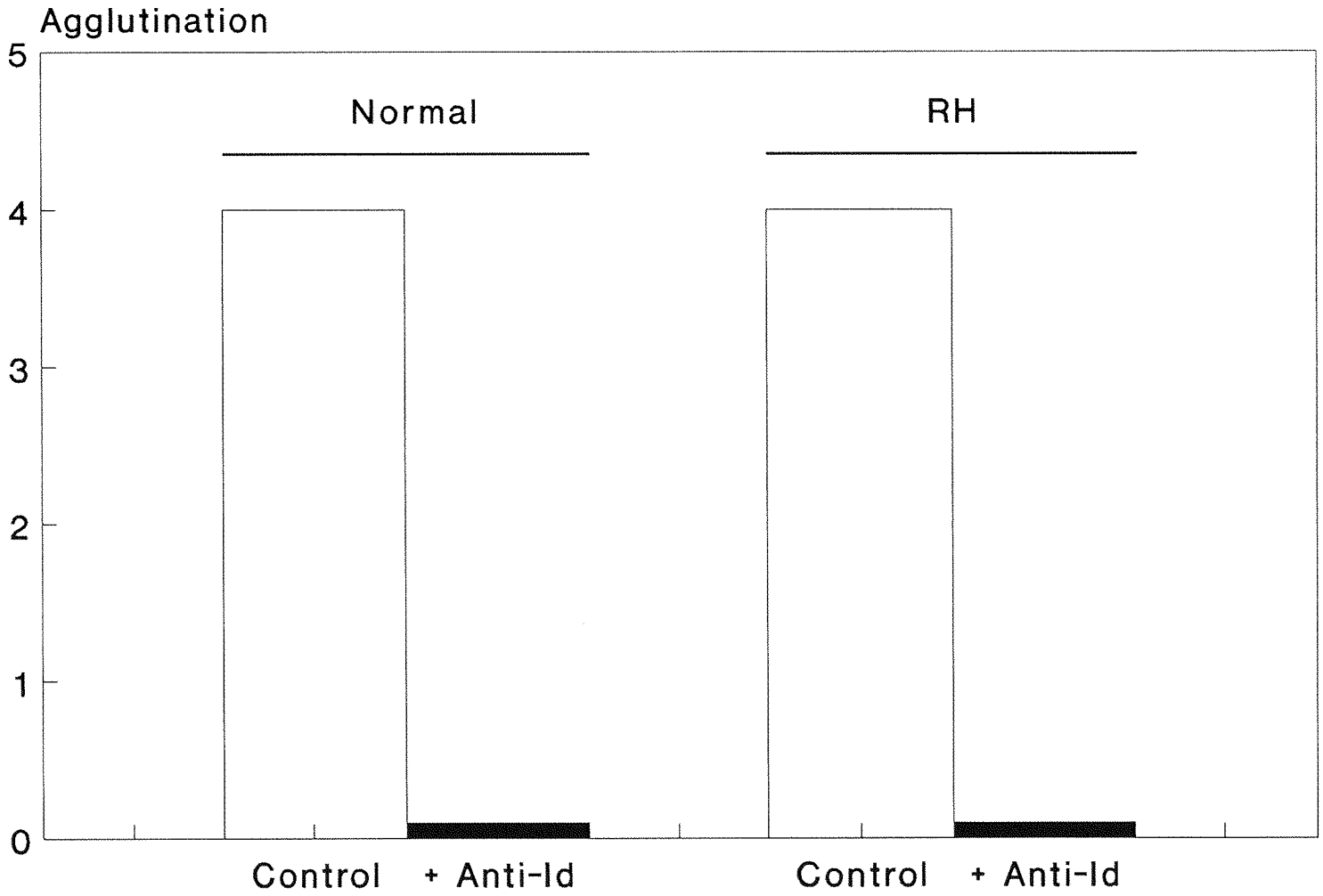

Agglutination
5
4
3
2
1
0
RH EBV line
Normal EBV line
RH serum
Control EBV line
Control IgM
0
5
10
15
20
25
30
35
IgM concentration (ug/ml)

2.5μg/ml of IgM. Agglutination by both EBV line supernatants was shown to be temperature dependent, no agglutination occurring at 37°C. The strength of agglutination caused by IgM from the tonsil derived EBV cell line was suprising because the line was not cloned and was secreting IgG and IgM. The IgG if it bound to the RBCs might have been expected to block the ability of the IgM to agglutinate. In order to investigate this, the IgM and IgG was purified by sizing chromatography and the IgM was run in the agglutination assay for comparison with the original culture supernatant. No difference in agglutinating ability could be discerned, indicating that the IgG either does not bind to the RBCs or binds in such a way as not to interfere with agglutination by IgM antibody. Both the IgG and IgM fractions were shown to be idiotope positive using an ELISA technique.

The effects of the anti-idiotypic antibody (9G4) on the ability of the IgMs secreted by the EBV cell lines to cause cold agglutination of red cells was investigated. The IgMs were tested at 7.5μg/ml (Patient R.H EBV line) and 11.9μg/ml (Tonsil EBV line) with the anti-idiotypic antibody at 64μg/ml. The results are shown in Fig 3.13 and it is clear that in both cases that the agglutination is totally inhibited by the anti-idiotypic antibody. A control rat IgG2a antibody at a similar concentration to the 9G4, caused no inhibition of agglutination.

Fig 3.13 Inhibition of cold agglutination by anti-idiotypic antibody. IgM secreted by EBV lines from idiotope positive normal tonsil cells, or from tumour cells (patient R.H) was assayed for cold agglutination in the presence of anti-idiotypic antibody (anti-Id) or a control rat antibody (control).

Fig 3.13


Agglutination
5
4
3
2
1
0
Normal
RH
Control
+ Anti-Id
Control
+ Anti-Id

DISCUSSION

Idiopathic cold agglutinin disease is an autoimmune disease characterised by the production of antibodies which bind to autologous red cells, particularly at low temperatures. These autoantibodies are termed cold agglutinins and they can lead to red cell agglutinination with consequent acrocyanosis, and occasionally give rise to intravascular haemolysis. Idiopathic CAD is usually associated with a benign monoclonal gammopathy. Transiently elevated CA titres, generally oligoclonal in nature, frequently occur in association with infections such as Mycoplasma pneumoniae or Epstein Barr virus, and these CAs can cause mild episodes of haemolytic anaemia.

The CAs most commonly recognize the Ii carbohydrate antigens which are expressed on the red cell surface. These antigens are composed of repeating N-acetyl lactosamine units, with the I antigen having a branched structure and the i antigen a linear chain structure. Antibodies specific for these antigens express cross-reactive idiotypic determinants (32). Similar CRIs have been described for other autoantibodies. For example, human monoclonal rheumatoid factors (RF), autoantibodies with specificity for epitopes on the Fc of IgG, express two major idiotypic systems, Wa and Po which have been delineated using polyclonal rabbit antibodies (178,195). Similarly, three dominant CRI determinants have been described on monoclonal anti-DNA antibodies (199,200,204). Curiously, in the diseases RA and SLE, idiotopes found commonly on tumour derived monoclonal rheumatoid factors or anti-DNA antibodies respectively may form only a minor component of the autoimmune patient's serum (194).

The expression of cross reacting idiotopes by CAs was first studied with polyclonal antisera and although H and L chains in combination were usually required for full expression of the idiotypic antigens (193), evidence suggested that CRIs were predominantly H-chain determined (174,181). In a study in which free heavy chains from 2 cold

agglutinins were used to raise polyclonal antibodies against CRI determinants, Feizi et al (206) showed that these determinants, in this case known as the V_H Mar antigen, were present on the majority of CAs of anti-Ii specificity. Using the available sequenced proteins of V_HI, II and III subgroups, the V_H Mar antigen was tentatively assigned to the V_HI subgroup.

The monoclonal anti-idiotypic antibody used throughout this study was produced while raising a panel of rat anti-idiotypic antibodies against the IgM expressed on the cells of a patient with B-NHL. The antibody (9G4) recognises a conformation dependent V_H determinant on IgM CAs of anti-I and anti-i type (184), but it is highly specific for CAs and does not react with any of the proteins in a panel of 56 myeloma proteins and 16 macroglobulins (184). The reactivity was similar to that of a monoclonal antibody reported previously which was raised against a CA (185), in that it reacted with 7/8 IgM CAs but failed to react with IgM Ma, which is known to lack the majority of idiotypic determinants shared by this group of proteins (186).

Agglutination inhibition studies using a monoclonal IgMκ CA from patient R.H. at a concentration of 22μg/ml, showed that 9G4 caused complete inhibition at 50μg/ml and was still causing partial inhibition at <2μg/ml. Similar results were found with two further CA proteins. In contrast, anti-μ and anti-κ antibodies were totally ineffective in inhibiting agglutinination at concentrations up to and including 300μg/ml. These data suggest that the idiotope lies close to the antibody binding site.

Although Ig heavy chains were originally assigned to three subgroups based on sequence homologies (V_HI - V_HIII), recent work (198) suggests that at least seven different V_H gene families exist. Of particular interest to the study of cold agglutinins is the V_HIV family which was defined (209) from two nucleotide sequences described by Kodaira et al (210) that did not seem to relate in any significant way to V_HI-III. Originally seven V_HIV gene segments were defined (209) and recently this number has been revised to fourteen

(211). The V_HIV family is most closely related to V_HII and in retrospect some protein sequences described as V_HII could be redefined as V_HIV (212).

Human monoclonal autoantibodies show restricted usage of both V_H and V_L families. The Wa idiotype expressed in human rheumatoid factors derived from patients with mixed cryoglobulinaemia was shown, using polyclonal antisera, to be derived from the minor V_KIIIb subgroup of light chains (195), and the sequences involved have now been elucidated with monoclonal anti-idiotypic antibodies against peptides with amino acid sequences corresponding to the CDRs of the RF molecule (194). Rheumatoid factors similarly show restricted use of V_HI genes (194). Cold agglutinins interestingly also show a similar preferential use of V_KIII V-region subgroups (201) and may use the same human kv325 derived germline genes as the rheumatoid factors. Work on the structure of the binding site of CAs was initially confined to the light chains (201) as the heavy chains are usually blocked at the N-termini making it difficult to obtain information on the protein sequences. However, by using polyclonal antibodies specific for peptides characteristic of the V_H families it has been found that CAs commonly arise from the minor V_HIV family (202).

In a recent collaborative study between this laboratory and that of Don Capra in Dallas, it has been possible to obtain sequences of the V_H regions of 2 IgM CAs, and both were derived from the V_HIV gene family. In fact, both sequences utilized the V_H4.21 gene segment and sequences associated with this gene segment appear to be responsible for the expression of the 9G4 defined cross-reactive idiotope (214).

Using the 9G4 antibody we have been able to trace the utilization of the idiotope in B-cell malignancies, in normal lymphoid tissues and in immature fetal tissue. Also we have examined the ability of idiotypic Ig produced by normal cells to cause the cold agglutination of RBCs. Clearly the expression of the idiotope does not indicate

that an Ig has CA activity but it is likely that cells producing the CAs found at low levels in normal serum are among this population.

A preliminary study using ELISA demonstrated that 9G4 reacted with 0.2% and 0.6% of normal human IgM and IgG respectively and detected a minor population of cells in a lymph node (184). In this project the analysis has been extended and small numbers of idiotope positive cells have been demonstrated throughout all the normal adult lymphoid tissues examined where they account for 3.2-10.8% of the B cell population. The finding of idiotope positive cells in fetal tissue suggests that the V_H gene used by the antibody expressing the idiotope is germ line encoded and evolutionary pressure appears to be acting to preserve the relevant VH gene sequences.

The total population of idiotope positive cells from the tonsil was isolated and analyzed for the presence of CA synthesising cells. These cells had been separated by virtue of their surface idiotypic Ig, and cells expressing only cytoplasmic idiotypic Ig would not be isolated by the method employed. The isolated cells expressed mainly surface IgM although small numbers of IgG positive cells were present; in addition the cells expressed the CD19, CD22 and CD37 antigens. The CD23 antigen which appears following activation of resting B-cells (216,217) was demonstrated on only a small proportion of cells. The separated cells do not express the CD5 antigen which is thought to mark a subset of B cells involved in autoantibody production. Evidence for this association in the human system comes from the finding of increased numbers of CD5 positive cells in rheumatoid arthritis (123) and an increase in immunoglobulins with rheumatoid factor or anti-DNA activity in cases of CD5 positive CLL (203). Idiotope positive cells do not appear to arise from this CD5 positive B cell subset, although patients with cold agglutinin disease associated with with a B cell tumour variably express the CD5 antigen (154).

The idiotope-positive cells have been immortalized with EBV to give a polyclonal cell line containing cells which synthesize IgM or IgG. Immortalization with EBV did not

markedly affect the phenotype of the cells isolated from tonsil except to induce expression of the CD23 antigen which is usually found on EBV cell lines (218). IgM secreted by the cell line caused agglutination of adult red cells preferentially in the cold, therefore at least a proportion of the cells seems capable of producing antibody with cold agglutinin activity. The IgM has been shown to be as efficient at agglutination as a IgM from a monoclonal EBV cell line derived from a patient (R.H) with cold agglutinin disease. This suggests that a majority of the idiotope positive cells can secrete antibody with cold agglutinin reactivity.

The tonsil derived EBV cell line also secreted idiotope-positve IgG which appeared not to hinder the cold agglutination of red cells by the IgM, and it is not yet clear whether the IgG fraction has specificity for the RBC. Although IgG CAs are not usually looked for clinically, since agglutination is less likely to occur than with IgM, examples of IgG antibodies binding with anti-I specificity to RBCs in the cold have been described (196,197,213). The IgG CAs can be of more clinical interest if accompanied by RF which can convert the IgG antibody to a strong agglutinator (215).

Our finding that idiotype positive cells isolated from tonsil tissue express surface IgM and on a smaller proportion of cells IgG, together with the presence of idiotope on normal serum IgG (184) strongly suggests isotype class switching occurs for this idiotope and therefore possibly for the $V_{H4.21}$ gene segment, although further work is required to assess the antibody activity of the IgG.

Using the separation technique outlined it should be possible to isolate idiotope positive cells from the blood of normal and post infective individuals and after EBV immortalization and subsequent cloning a comparison could be made of the V_H and V_L gene usage with cell lines established from cold agglutinin patients. It will be interesting to see if the same genes are used in normal and malignant cells and if the same level of somatic mutations occurs in both populations.

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APPENDIX 1

Monoclonal antibodies used in this study

CD Number/ specificity	Clone	Source
CD3	OKT3	American Tissue Culture Collection
CD5	OKT1	American Tissue Culture Collection
CD19	HD37	Gift from Dr.M Glennie, Tenovus.
CD22	HD39	Gift from Dr.M Glennie, Tenovus.
CD23	MHM6	Gift from Dr.M Glennie, Tenovus.
CD45	WR6	Wessex Regional Immunology Service
CD38	OKT10	American Tissue Culture Collection
CD37	WR17	Wessex Regional Immunology Service
CD71	OKT9	American Tissue Culture Collection
CD34	WR18	Wessex Regional Immunology Service
HLA Class I	HB95	American Tissue Culture Collection
anti IgG	HB60	American Tissue Culture Collection
anti IgA	HB63	American Tissue Culture Collection
anti IgM	HB57	American Tissue Culture Collection
anti IgD	MOBD4	Gift from Dr.N.Ling, Birmingham.
anti Kappa	HB61	American Tissue Culture Collection
anti lambda	M152	Tenovus Research Laboratory

Appendix 2

Scoring of results in red cell agglutination tests

++++ or 4+ = C (complete)	One complete mass of agglutinates, easily visible on the slide before microscopic examination.
+++ or 3+ = V (visual)	Large separate masses of agglutinates, easily visible on the slide before microscopic examination. Very few unagglutinated cells.
++ or 2+	Small agglutinates, still easily visible on the slide before microscopic examination.
+ or 1+	A granular appearance just visible on the slide. The microscope reveals big clumps of more than 20 cells.
(+)	Smaller clumps (12-20) cells only visible microscopically.
good weak	Clumps of 8-12 cells, only visible microscopically.
weak	Small clumps of 4-6 cells uniformly distributed.
negative	All cells free and evenly distributed.

Taken from Practical Haematology, 6th Ed, Sir John V. Dacie and S.M.Lewis (eds), 1984.