PRODUCTION OF ANTIBODIES FOR CELL SURFACE ANTIGENS

submitted by

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ABSTACT

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The aim of the project was to devise a general method for raising monoclonal antibodies to a particular cell-surface antigen, given at least one pre-existing antibody (monoclonal or polyclonal) reactive with that antigen. The motive for this approach arose from the fact that many available antibodies to tumour-cell surfaces are unsuitable for therapeutic use, for reasons which can include an inappropriate antibody isotype, an inappropriate epitope on the antigen, or an inadequate association constant. It was hoped that the available antibodies could be used to isolate the antigen and present it in an immunogenic form for monoclonal technology.

Two surface antigens on human neoplastic B lymphocytes were chosen for investigation: immunoglobulin (Ig) and CD37. Standard methods were used for solubilizing and detecting the IgM from the Namalwa B cell line. Antigenically intact CD37 could not be recovered after the cell surface was treated with papain, but it was recoverable after cell lysis using the non-ionic detergent NP40. The solubilized IgM and CD37 were isolated onto paramagnetic polymer beads (Dynabeads M-280, Dynal U.K Ltd) coated with a given anti-human IgM and anti-CD37 monoclonal antibody respectively. A rapid and easily implemented method was set up to detect the antigen uptake by the beads. The principle was to gauge the inhibition of rosetting of the antibody-coated beads with cells expressing the antigen at their surface. Then, those beads coated with antigen were directly used to immunize mice, which both avoided purification steps and optimized the presentation of the antigen to the immune system. Intra-peritoneal immunisation led to a high titer of serum antibody. In order to recover blast cells from the spleen for monoclonal antibody work, an intrasplenic boost was given 2 days before removing the organ. Finally, to derive antibody-secreting hybridomas, standard protocols were used for fusion, screening and cloning of antibody-producing cells.

ACKNOWLEDGEMENTS

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I wish to thank Profesor G.T stevenson for his time and precious guidance through this project.

I am very grateful for all menbers of Tenovus helping me to carry out this research.

To my parents,

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INTRODUCTION

MONOCLONAL ANTIBODIES

History

Discovery in 1890 of antibody by von Behring and Kitasato and the subsequent elucidation of antibody function initiated the modern era of scientific immunology. The exquisite specificity of antibody has also proved valuable in other areas, as in diagnostic medicine, and the detection and purification of macromolecules in biochemistry. In 1975, the usefulness of antibody was greatly enhanced by the discovery of monoclonal antibodies by Milstein and Köhler, who were awarded the Nobel Prize in 1984. They established a method for "a routine production of large amounts of homogeneous antibody of a predefined specificity" (Köhler and Milstein, 1975). Within 5 years the technique had spread to such a wide number of fields that it became difficult to construct an exhaustive list of all its uses (Kennett et al, 1980).

Definition

Köhler and Milstein achieved the growth in culture of hybridoma cells generated by the fusion of myeloma B cells with immunized splenocytes (Köhler and Milstein, 1975). The resulting hybridoma exhibits characteristics of both parents in being both immortal and in secreting Ig of a predefined specificity. This principle of monoclonal antibody

represented a remarkable feat considering the new properties conferred on antibody technology at one stroke (Goding, 1980). The antibody specificity was greatly enhanced owing to the clonality of the B cell line secreting antibody that recognizes a single epitope. Also, the immortal hybridoma could provide an unlimited amount of antibody that could be tapped at will.

Principle of fusion

Different approaches had been tried for immortalizing immunized B cells (Baumal et al, 1971), and the principle of fusing the B cells with a myeloma cell line to form a " hybridoma " proved to be the most successful (Köhler and Milstein, 1975). This phenomenon of cell fusion occurs naturally on very restricted occasions (Goding, 1980), and could be induced at useful frequencies by using virus or at a better rate with polyethylene glycol (PEG) (Davidson and Gerald, 1976; Gefter et al, 1977). The mechanism of cell-cell fusion is not fully understood but some stages have been observed. The formation of a bridge by inactivated Sendai virus bound to its receptor on B cells makes them agglutinate and fuse without any change in membrane structure (Knutton and Pasternak, 1979). In the case of cell fusion with PEG, intramembrane particles become aggregated and membrane denuded of protein at juxtaposed cell surfaces merges to form a polykaryon, and a hybridoma at the next division (Knutton and Pasternak, 1979).

Hybridoma characteristics

After the fusion, a strategy was adopted for isolating hybridoma from unfused cells and particularly myeloma cells that tend to overgrow hybrid cells. The technique was based on the existence of a salvage pathway for nucleotide synthesis (Littlefield, 1964) in which free nucleotides resulting from high RNA turnover are salvaged using the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). The principle was to fuse a myeloma mutant cell line lacking HGPRT with immunized splenocytes, and use a selective medium (HAT medium: hypoxanthine, thymidine and aminopterine). The folic acid antagonist aminopterine would inhibit the enzyme thymidine kinase and block the pathway for de novo DNA synthesis, while an exogenous contribution of hypoxanthine and thymidine would make the use of the salvage pathway available. Unfused cells cannot survive and only hybridoma cells are selected (Fig.1).

The hybridoma genome comprises almost 4n chromosomes which represents a challenge for cellular organisation. Indeed, a progressive loss of chromosomes was observed (Köhler and Milstein, 1975), as well as mutations or suppressions that could generate variants of Ig (Rothstein et al, 1983; Dildrop et al, 1982). Hybridomas were found to be most stable when they were fused with cells of the closest similarity, e.g: B cell with B cell or T cell with T cell (Kennett et al, 1980). It was established that the Ig produced by the fused cell showed unchanged association of V and C regions within single chains (Cotton and Milstein, 1973). B cell hybridomas

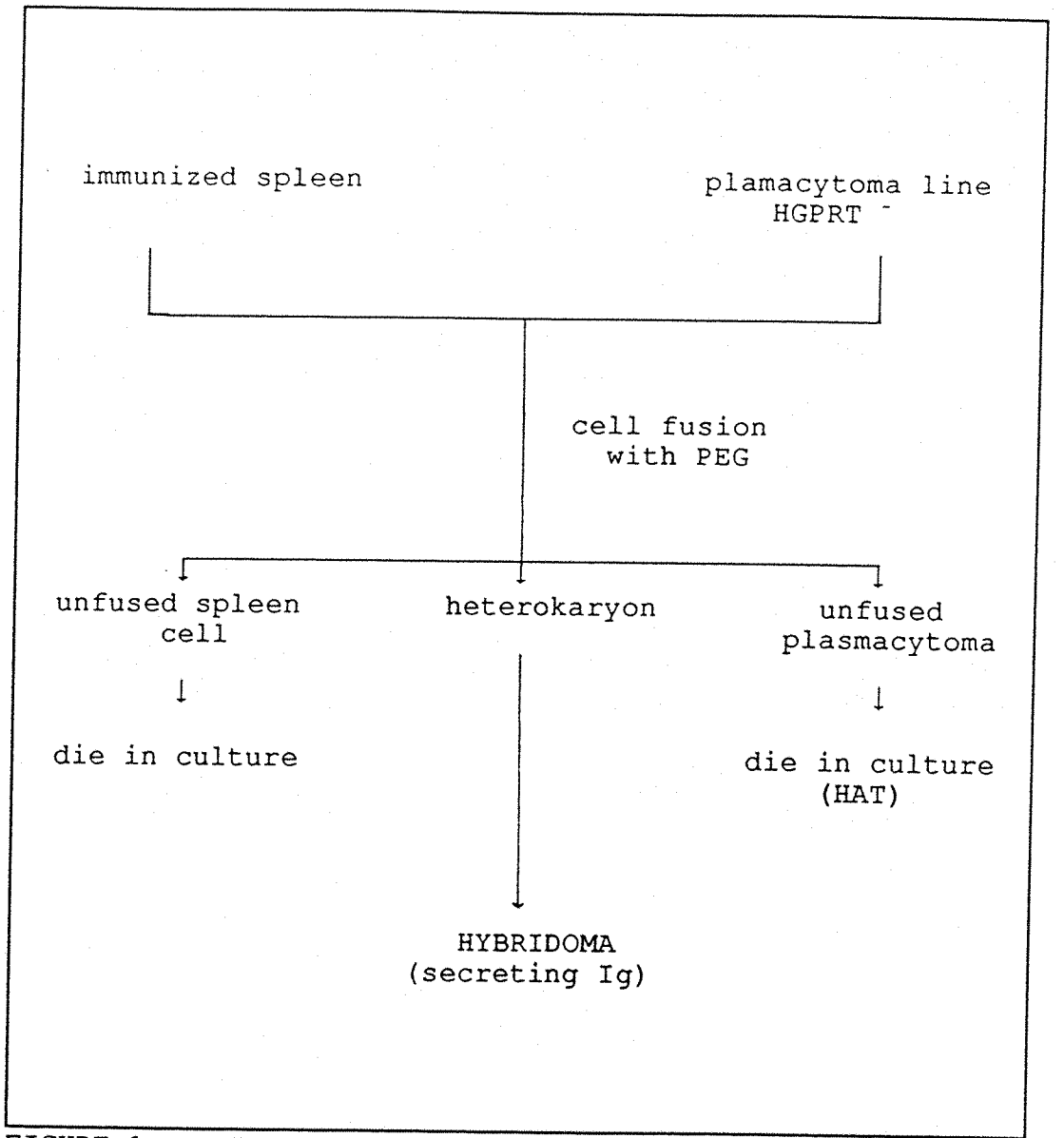


FIGURE 1 : PRINCIPLE OF HYBRIDOMA TECHNOLOGY

expressed in codominance antibody of both parent cells (Cotton and Milstein, 1973; Milstein et al, 1977) and could generate a new antibody due to random associations of heavy and light chains (Kennett et al, 1980). However, Ig production is not extinguished when one parent line lacks Ig expression (Kölher et al, 1976). Hence, the proportion of specific antibody was increased by fusing splenocytes with a myeloma line missing heavy or light chain synthesis (Coffino et al, 1972; Cotton and Milstein, 1973).

Comparing monoclonal and polyclonal antibodies

The preparation of monoclonal antibody presents the researcher with a variety of obstacles to overcome. For instance, equipment has to be adapted to work under sterile conditions. Moreover, screening and recloning hybridoma lines involve considerable time and work. Throughout the monoclonal production, a risk of loss of these hybridoma lines may occur either by contamination or chromosome loss and a special care must be taken. Thus preparation of monoclonal antibody is initially expensive and time-consuming, but finally rewarding by being easier to purify than an antiserum containing polyclonal antibody. Monoclonal antibody could happen to be euglobin, easily purified by precipitation from a saline solution. On the other hand, production of polyclonal reagents is far simpler but presents the major drawback of using a great number of animals while a hybridoma can now be grown in vitro at a high yield with techniques such as hollow fibre bioreactors (Evans, 1988).

For some purposes polyclonal antibodies remain more useful than monoclonal. Recognizing several epitopes on a protein, polyclonal can precipitate more easily in precipitin tests. Monoclonal antibody of high specificity can sometimes be a double edged sword. When looking for a protein among different species, the epitope could differ from one species to another due to divergent phylogeny and some proteins can escape detection (Campbell, 1984). On the other hand, a functional domain highly conserved could be shared by different types of proteins which could lead to contamination (e.g: NAD anchorage domain of dehydrogenase). In these particular situations, polyclonal antibodies would be more advisable. Also, sometimes monoclonal antibody presents weaknesses by having too low an affinity, a cryoglobin property that handicaps uses at room temperature, or an undue sensitivity to pH or salt concentration (Herrmann and Mescher, 1979; Masson and Williams, 1980).

Monoclonal antibody present major advantages in being reliable and having a high precision which limits cross reactions and permits fine study of functional domains of the target antigen (Bonnies, 1990). Unlike highly specific antisera that are non-reproducible and arduous to obtain, monoclonal antibody can be prepared for a wide range of antigens as the immunogen does not need to be extensively purified (Kennett et al, 1980). Moreover, monoclonal antibodies of a low affinity were found to be very useful in purifications of protein which could be eluted under mild dissociative conditions (Bonnies, 1990). Also, a better

precipitation of protein could be obtained with a cocktail of monoclonal antibodies specific for the same antigen. A summary of the main differences between monoclonal and polyclonal is set out in table I (Bonnies, 1990; Stevenson and Stevenson, 1983).

Monoclonal antibodies and cancer

Monoclonal antibodies have proved to be a valuable aid in the diagnosis of cancer, and offer promise for therapy (Glennie and Stevenson, 1982; Levy and Miller, 1983). Moreover, they have permitted the identification and purification of previously unrecognized cell surface antigens (Williams et al, 1977). Monoclonal antibodies reactive with leucocytes have been "clustered" with groups which display the same reactivities on leucocytic surfaces (Nadler et al, 1986).

ANTIBODY

Structure

Antibody function is embodied in a symmetrical monomeric molecule whose overall structure is in equilibrium between T and Y conformations. Variations in amino acid sequence over the entire population of molecules endow antibodies with an outstanding variety of combining sites which can accommodate virtually any antigen.

Antibody structure is made up of an assembly of four polypeptide chains joined together by non-covalent and disulphide bonds (Fig. 2). After reduction and exposure to strong dissociative conditions, immunoglobulins can be

Table 1: COMPARISON OF POLYCLONAL AND MONOCLONAL ANTIBODY.

major advantages of monoclonal antibody	major disadvantages of monoclonal antidody
single homogeneous antibody of a defined specificity	preparation slower and more expensive
in theory available in indefinite amount over an indefinite period	apt to have lower association constant
obtained as pure antibody	not apt for precipitin assay
multiple antibodies can be obtained and compared	apt to be non-cytotoxic
useful for study of functional domain of molecules	hybridoma line occured to be instable

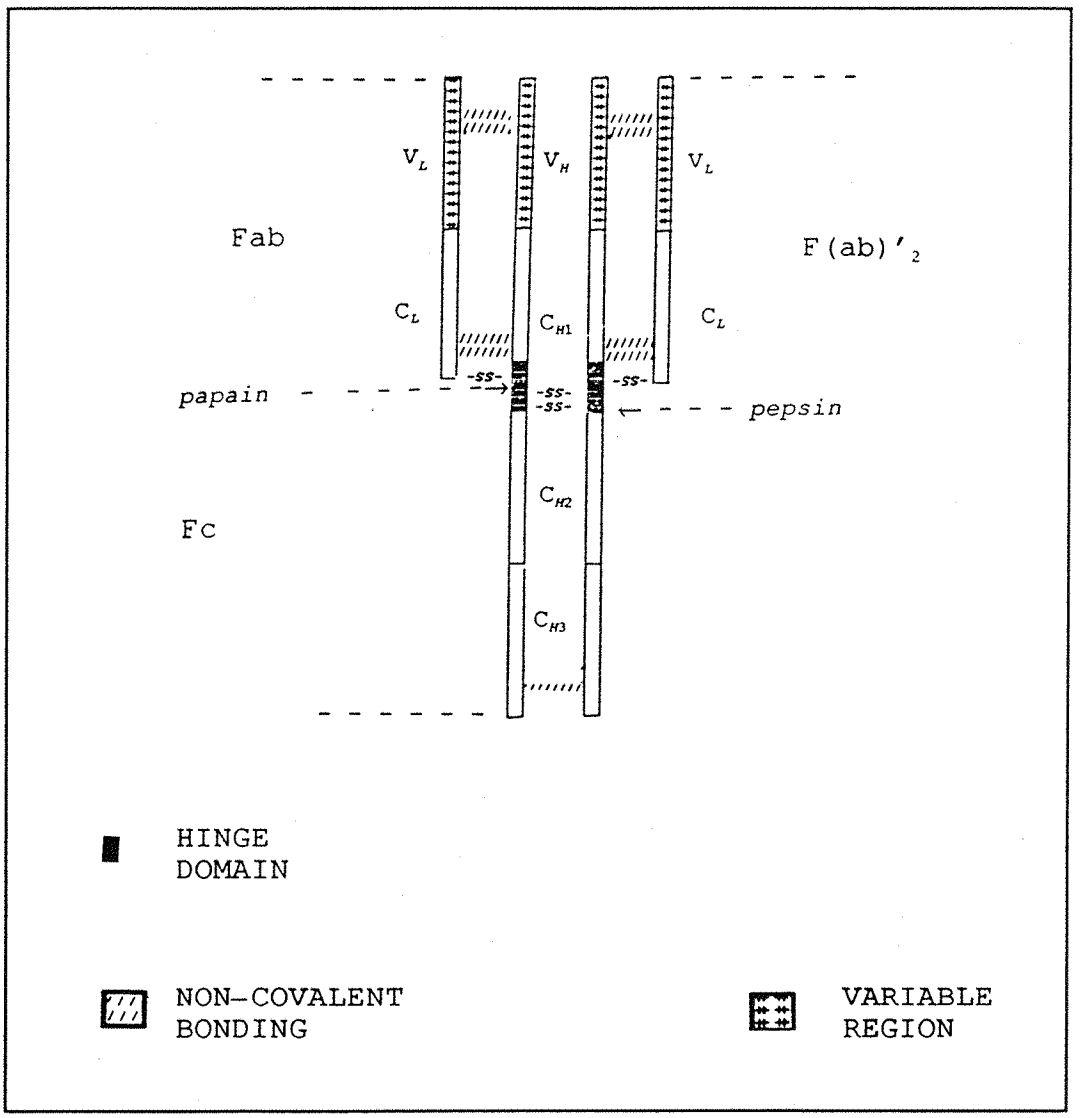


FIGURE 2 : ANTIBODY STRUCTURE

denatured to yield two pairs of chains with typical molecular weights of 23,000 and 50,000 Da that are named light and heavy chains respectively (Edelman, 1973). The light chain exists in two forms κ and λ, and each immunoglobulin molecule contains a pair of one or the other (Korngold and Lipari, 1956). The heavy chains exist in 5 classes (μ, δ, γ, ε and α), and the whole Ig molecule is named according to the class of heavy chain it contains: Ig M, D, G, E and A. Despite the classification into five classes the heavy chains display considerable homology, each possessing a variable domain at the N terminus, and then three (δ, γ, α) or four (μ, ε) constant domains. Light chains display one variable and one constant domain (Seidman, 1978; Kehry et al, 1982). Structural homologies between all these domains suggest a repeated duplication of a single primordial gene (Edelman, 1969). Each domain contains a primary sequence with a large (60- to 70-residue) disulphide-bonded loop, and is folded into 2 anti-parallel β pleated sheets joined by the intrachain disulphide bond (Poljak et al, 1972). Besides interchain disulphide bonds, immunoglobulin light chains are bound to the heavy chains by non-covalent interactions (Hubert et al, 1976). The variable domains of heavy and light chains come in close contact and construct the antigen-binding site with a contribution of three hypervariable loops from each chain (Inbar et al, 1972; Wu et al 1970; Schiffer et al, 1973). The globular domains prove to be very resistant to proteolytic actions while the hinge region, between the first and second constant domains of the heavy chains, is more open to

enzymatic cleavage. The most common enzymatic digestions at the hinge region are with papain (Porter, 1973) and pepsin (Nisonoff, 1975). Papain generates two fragments, Fab (Fragment, antigen-binding) and Fc (Fragment, crystallisable). The digestion with pepsin leads only to the Fab'_2 fragment, due to fragmentation of the rest of the molecule.

Functional organization

The structure of antibodies reflects the bifunctional activity of these molecules. The variable domains confer the potential ability to bind to a large array of antigens. This recognition of a foreign element in the organism is translated into protective action by the immune system via the constant domains in the Fc region. The 5 different immunoglobulin classes recruit appropriate effectors for dealing with the antigen.

Biosynthesis

At the initial stage of B-cell differentiation, the V region is determined by a DNA rearrangement which brings together 2 coding segments (V and J) for each light chain, and 3 segments (V, D and J) for each heavy chain (Weigert et al, 1978; Schilling et al, 1980; Sakano et al, 1981; Fig.3). Separate V-region clusters exist for κ and λ light chains, while a single cluster serves all the heavy chain classes. These genes have many copies, e.g : in the case of human heavy chains, between one and two hundred V (Variable), about 30 D (Diversity) and 6 J (Junctional) gene segments (Strominger,

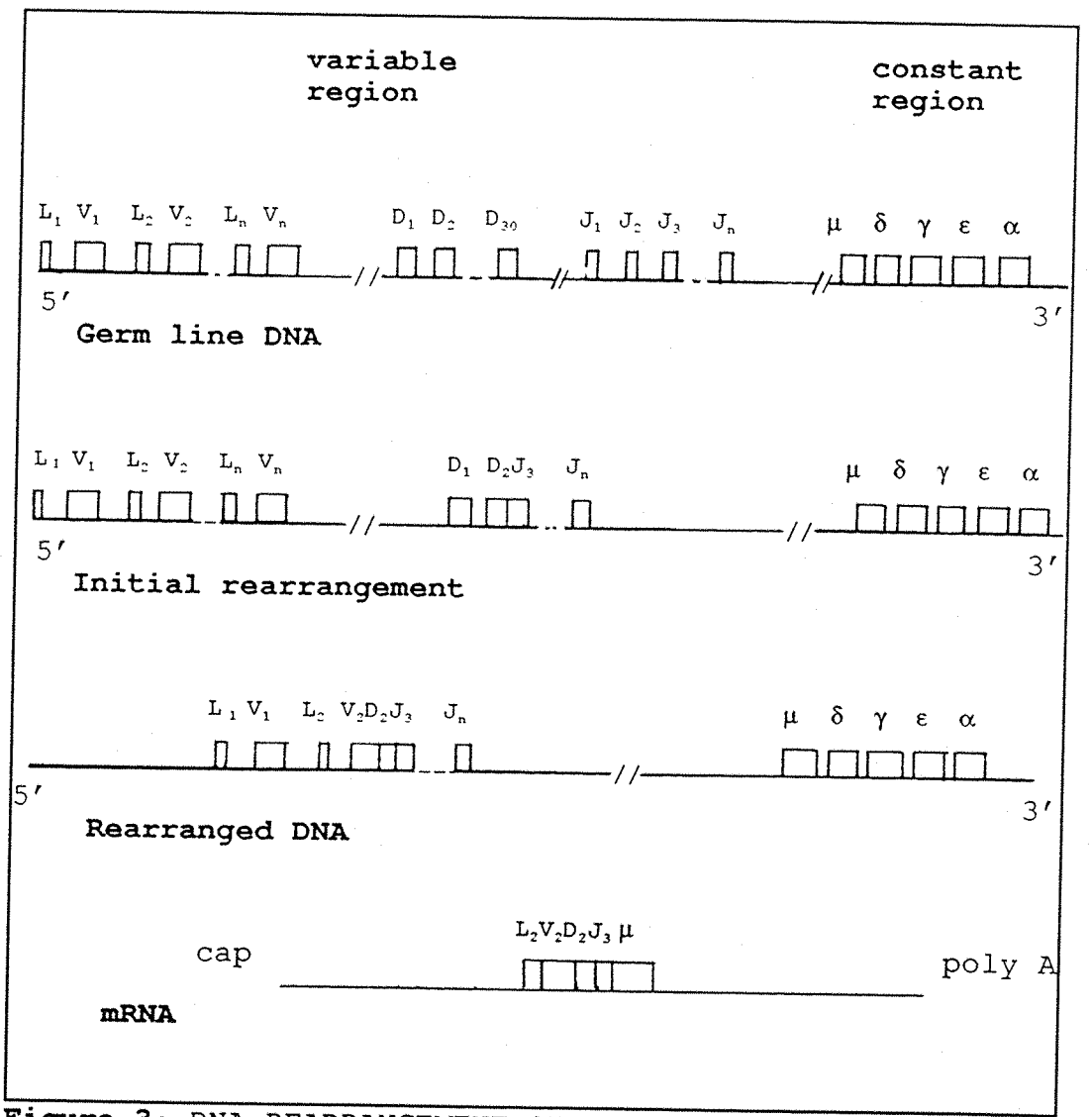


Figure 3: DNA REARRANGEMENT OF ANTIBODY SYNTHESIS.

1989). Misalignments at the junctions of these genes add a further element of diversity. The variability of antibodies is greatly enhanced by subsequent somatic mutations mainly localized at 3 hot spots for each chain (CDR for complementary determining regions; Wu and Kabat, 1970). Variation in the association of heavy and light chains further enhances the antibody diversity so as to serve a vast repertoire of reponses. Downstream of the DNA segments encoding the variable domains of the heavy chains, the genes responsible for the constant regions of the 5 classes can undergo a switch of classes due to an DNA excision. During an immunisation, a switch from IgM to IgG occurs after the primary immunization and accompanies somatic mutations that can increase the antibody affinity (Griffiths et al, 1984).

ANTIGEN

An antigen is an entity specifically recognized by an antibody. In principle , any foreign substance introduced into an animal and capable under favourable circumstances of eliciting an immune response is defined as an antigen. Three functional types of antigen can be considered. Haptens are usually small chemical groups that need to be conjugated to a larger carrier in order to be immunogenic. A second type of antigen is the T-independent antigen (generally a polymeric molecule with repeating units) which can induce a B-cell response without T-cell cooperation. The third and the most common type of antigen is the T-dependent antigen (a complex molecule bearing multiple determinants) which needs the

involvement of T helper cells in order to induce a significant antibody response, with the IgG response being particularly T-dependent.

Structural aspects of the antigen-antibody complex have been studied by X-ray diffraction, which has revealed that the surface of contact between antibody and a protein antigen extends beyond the antigen-combining site made of 6 hypervariable loops (Amit et al, 1985). In the case of the complex Fab-lysozyme the surface of contact was about 20 x 25 Å and involved 19 residues (Janin and chotia, 1990; Padlan et al, 1989). A close contact between the Fab and the antigen was observed, with 111 Van der Waals bonds and 14 hydrogen bonds (Padlan et al, 1989). The site of recognition on the antigen could be either an assembly of discrete segments from the primary sequence (a "conformational" determinant) or a continuous sequence of the polypeptide chain (Lando et al, 1982). In the case of a conformational determinant, a denatured protein might not be recognized by an antibody specific for the native protein (Brown et al, 1959).

Factors that determine immunogenicity and the structure of the determinant have been the subject of numerous investigations. With the advent of monoclonal antibodies, it became possible to analyze very precisely the topology of the antigen recognition site (Benjamin et al, 1984). Studies carried out on proteins like myoglobin or lysozyme converged to define virtually the entire protein surface as being potentially immunogenic with respect to the host responder (Benjamin et al, 1984; Amit et al, 1985). Evidence derives

chiefly from obtaining a panel of monoclonal antibodies that entirely covers, with some overlapping, the protein surface (Benjamin et al, 1984; Smith-gill et al, 1984; Metzger et al, 1983). It has been shown that the greater the phylogenetic distance between antigen and recipient the more enhanced is the immune response (Urbanski et al, 1977). Also, stuctural and chemical aspects of the protein have been claimed to correlate with immunogenicity. Aromatic residues are preferentially recognized by antibodies (Padlan, 1990); chemical structures of epitopes are on average 55% non-polar, 25% polar and 20% charged (Miller et al, 1987) and it has also been claimed that the mobility of the site favours immunodominance (Tainer et al, 1985; Westhof et al, 1984). Likewise, some molecular aspects favour immunogenicity, e.g. massive, aggregated or repeated antigenic determinant molecules are better immunogens. Some immunogenic factors come from physicochemical characteristics : for example particulate antigens tend to trigger better immune responses than water-soluble antigens. Also, non-specific substances, such as adjuvant, can magnify an antigenic stimulus.

CD37 ANTIGEN

In the present work CD37 was used as a target protein of a lymphocyte surface membrane. CD37 is known to be widely expressed during B-cell ontogeny but,as is discussed later, is not entirely restricted to the B lineage. In 1987, monoclonal antibodies anti-CD37 were clustered at the third international workshop in a pan B-cell goup (Ling, 1987). CD37 antigen was

classiied in a new protein family (Table 2, Gaugitsch et al, 1991; Fig. 4, Angelisova et al, 1990; Wright, 1990), whose stuctural aspects were regarded as suggesting transporter (Amiot et al, 1990; Angelisova et al, 1990; Chen et al, 1986; Classon et al,1989; Gaugitsch et al, 1991), secretory (Amiot et al, 1990) or growth-regulatory protein (Bellacosa et al, 1991; Wright, 1990). We shall consider structure, antibody clustering and the therapeutic potential of CD37.

CD37 has a highly glycosylated structure with a wide electrophoretic pattern which was rather unusual until the discovery of other proteins possessing similar characteristics, for instance rat OX44 and CD37(Classon et al, 1989), and human CD53 (Amiot et al, 1990; Angelisova et al, 1990) and ME491 proteins (Wright, 1990). The molecular weight of human CD37 has been found to lie between 40 and 52 kDa due to a microheterogeneity of the carbohydrate moiety (Schwartz-Albiez et al, 1988). CD37 from a Burkitt's lymphoma cell line was analyzed by SDS gel electrophoresis and endoglycosidase F digestion, and revealed a single chain structure, without any intrachain disulphide bonds (Schwartz-Albiez et al, 1988). Putative N glycosylation sites were located in the C-terminal region on at least CD37 (Classon et al, 1989) and CD53 (Angelisova et al, 1990). Glycosylation of CD37 in its extacellular regions accounts for up to the half of the total molecular weight (Schwartz-Albiez et al, 1988) and thus plays a major role in CD37 antigenicity (Schwartz et al, 1987). It was demonstrated that antibodies of the CD37 cluster all bind to the same sugar epitope or at least neighbouring epitopes

Table 2 : CHARACTERISTICS OF THE THE NEW FAMILY
(Gaugitsh et al, 1991)

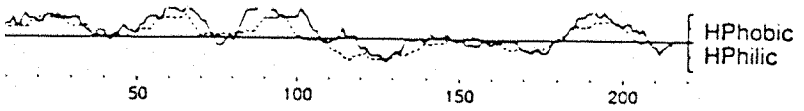
protein	identity to R2 %	similarity to R2 %	amino acid lengh	possible N-glycosilation
R2	-	-	267	3
CD37	33	52	281	3
CD53	38	64	219	2
ME491	26	56	238	3
Sm23	29	60	218	1

FIGURE 4

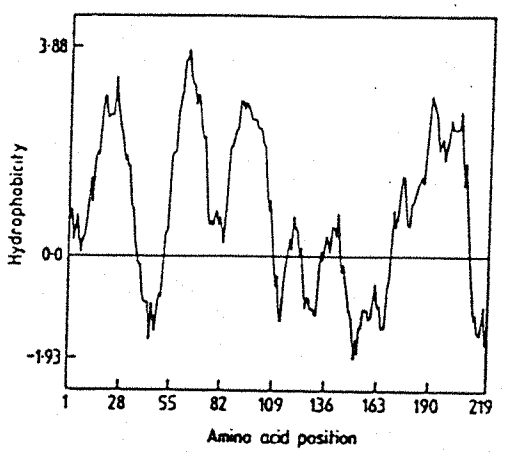
HYDROPHOBICITY PROFILES OF :

MRC OX-44, Sm23, ME491 AND CD53

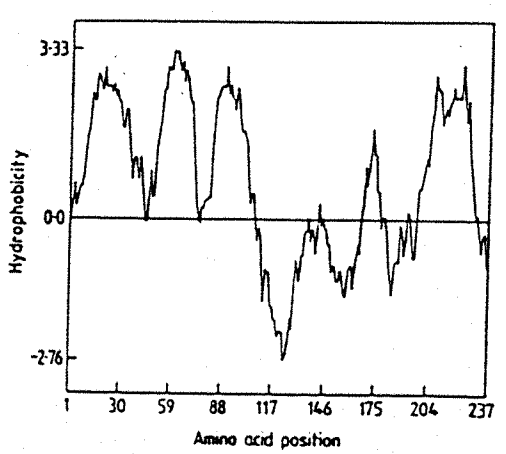
copied from: Bellacosa et al, 1991; Wrigh et al, 1990; Angelisova, 1990.



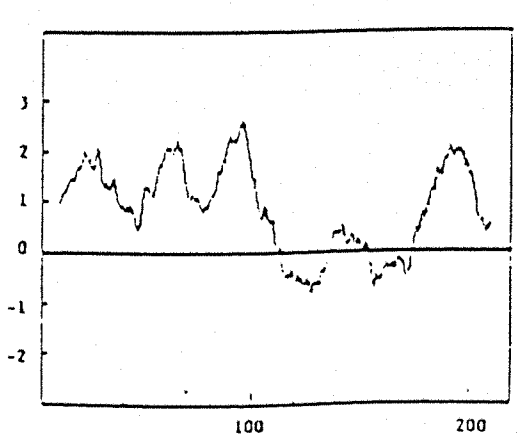
Rat MRC OX-44



Sm23



ME491



CD53

(Clark et al, 1986; Moldenhauer et al, 1987; Schwartz et al, 1987), although the core protein was necessary for the binding (Schwartz et al, 1987). The sugar framework contains a high level of mannose in a main N-linked oligosaccaharide chain. Schwartz suggested that glycosylation could participate in CD37 function, particularly during the process of ligand binding (Schwartz et al, 1987).

The second part of the molecule consists of a core protein of about 25 kDa (Schwartz-Albiez et al, 1988). Human CD37 was sequenced as were the two closely related proteins rat OX44, rat CD37 (Classon et al, 1989; Classon et al, 1990) and more recently human CD53 (Angelisova et al, 1990). This demonstrated that human CD53 and human CD37 were close protein homologues of rat OX44 and rat CD37 respectively (Fig.5: Angelisova et al, 1990; Classon et al, 1990). The overall structure of these proteins is composed of an intracytoplasmic N terminus owing to a lack of conventional leader sequence (Schwartz-Albiez et al, 1988), a triple transmembrane region followed by an extracellular hydrophilic region and finally by a fourth transmembrane sequence ending in an intracellular C terminus. The latter is highly conserved, suggesting a biological activity for this domain (Amiot et al, 1990; Angelisova et al, 1990; Classon et al, 1990; Fig.6). The function of this new family protein remains unknown at the present time, but the structural arrangement as well as the biocellular distribution is reminiscent of membrane protein transporter (Angelisova et al, 1990; Schwartz et al, 1988;

FIGURE 5

CYTOPLASMIC CD37

Detecting the presence of CD37 on Daudi cells before and after treatments with papain to remove surface antigen, and paraformaldehyde to render the surface permeable to indicator antibody. Fluorescent profiles of target cells labelled with FITC-conjugated anti-CD37 monoclonal antibody (WR17*).

FIGURE 5

THE CD37/CD53 FAMILY

(a) Aligned amino-acid sequences of human (upper) and rat (lower) CD37 antigens. Each protein contains 281 residues, with four putative transmembrane sequences. Differences are in bold type. Adapted from Classon et al (1990).

(b) Aligned N-terminal sequences of rat OX-44, human CD53 and human CD37 antigens. Again diferences are in bold type. OX-44 and CD53 may be truly homologous, with CD37 anothe member of the same highly conserved family. Adapted from Angelisova et al (1990).

MSAQESCLIKYFLFVFNLFFFVLG **S** LIFCFG **I** WILIDKTSFVSFVSFVGL **A** FVPLQ
MSAQESCLIKYFLFVFNLFFFVLG **G** LIFCFG **T** WILIDKTSFVSFVSFVGL **S** FVPLQ

I WSKVL **AI** SG **IF** TM **GI** ALLGCVGALKELRCLLGLYFGMLLLLFATQITLGIL
T WSKVL **SV** SG **VL** TM **AL** ALLGCVGALKELRCLLGLYFGMLLLLFATQITLGIL

ISTQR **AQ** LER **SLRDV** V **EK** TIQ **K** Y **G** TNP **E** ETAAEESWDY **V**
ISTQR **VR** LER **RVQEL** V **LR** TIQ **S** Y **R** TNP **D** ETAAEESWDY **A**

QFQLRCCGW **HY** P **Q** DW **FQVLI** L **RG** NGSE **AHR** VPCSCYN **LS**
QFQLRCCGW **QS** P **R** DW **NKAQM** L **KA** NGSE **ELF** VPCSCYN **ST**

ATNDS **TIL** DK **VI** L **P** QLSRLG **HL** A **RS** R **HS** ADICA **V** PA **ES**
ATNDS **SGF** DK **LF** L **S** QLSRLG **PR** A **KL** R **QT** ADICA **L** PA **KA**

HIYREGCA **QG** LQKWLHNN **L** ISIVGICLGVGLLELGFMTLSIELCRNLDHVY **N**
HIYREGCA **RS** LQKWLHNN **I** ISIVGICLGVGLLELGFMTLSIELCRNLDHVY **D**

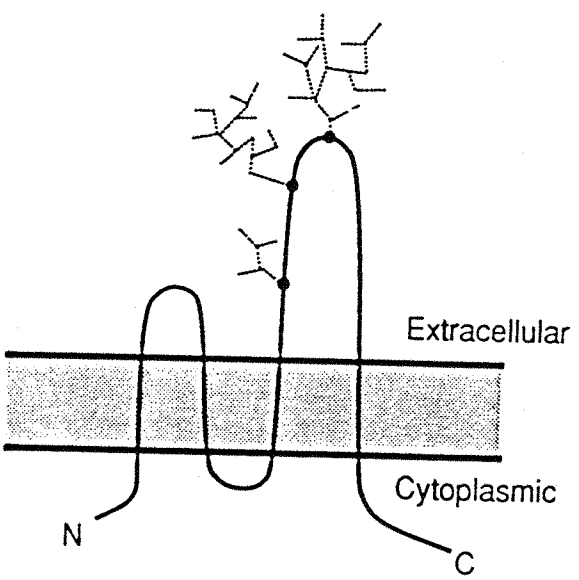
RLARYR Human CD37
RLARYR Rat CD37

GMSSLKLLKYVLFFFN**F**LFW**VSGEE**ILGF Rat OX-44
MGMSSLKLLKYVLFFFN**L**LFW**ICGCC**ILGFGI**YL**LI**HNNFG**VLF Hu CD53
MSAQESSLKLLKYVLFFFN**L**LFW**VLGSL**IFCFGI**WI**LI**DKTSF**VSF Hu CD37

FIGURE 6

RAT MRC OX-44 AT THE CELL SURFACE

Copied from : Bellacosa et al, 1991.



Wright et al, 1990) or growth-regulatory protein (Bellacosa et al, 1991; Wright et al, 1990).

In addition to its high concentration on B cells, CD37 has been found in lesser amounts on a broad variety of leucocytes : neutrophils, monocytes, macrophages and some T cell subclasses (Moore et al, 1987; Schwartz-Albiez et al, 1988). Nevertheless, it appears clear that the highest level of CD37 is found on the cell surfaces and intracytoplasmic membranes of B cells, which express about 10^5 molecules per cell surface (Ling and Ledbetter, 1987; Poncelet et al, 1987; Schwartz-Albiez et al, 1988). CD37 is expressed during a wide span of B cell ontogeny, from the pre-B stage (appearing after CD19, CD20 and CD22) up until plasmacytic differentiation (Schwartz-ALbier et al, 1988. Fig.7: Dörken et al, 1986).

The CD37 antibody cluster comprises 6 monoclonal antibodies, and, despite recognizing the same sugar epitope (Ling, 1987; Modenhauer et al, 1987; Schwartz-Albier et al, 1988), they display some differing characteristics, such as binding affinity and metabolic effects on the target cells. Immunological staining shows the highest intensity with WR17 and G28-1, a moderate intensity with HD28,HH1 and F973G6, while BL14 stained weakly (Pallesen and Hager, 1987). Affinity was found at 5×10^8 M^{-1} for HD28 (Schwartz-Albiez et al, 1988) and 2×10^9 M^{-1} for MB1 (Link et al, 1986). Binding of some of these antibodies to the cell surface affects cellular metabolism. It was observed that G28-1 enhances or suppresses B-cell proliferation depending on the primary

FIGURE 7

CD37 ASSOCIATED TO B CELL ONTOGENY

(Dörken et al, 1986)

pro B pre B μ^- $\mu^- \delta^-$ act B B blast PC

CD37 EXPRESSION AT THE CELL SURFACE

signal (Ledbetter et al, 1987) while HH-1, a weak antibody, does not interfere with B-cell activation or proliferation (Smeland et al, 1987).

With regard to the prospect of treating lymphoma using CD37 as a target, MB-1 linked to ^{131}I was used in radiotherapy on ten patients with non-Hodgkin's lymphoma (Bernstein et al, 1990; Press et al, 1989). This combination resulted in four total and one partial remission of the tumor, but was acompanied by high toxicity. It was found that ^{131}I MB-1 had a high tumor site retention but, when tumor exeeded 1 kg, antibodies penetrated the tumor poorly. Another therapy, using CD37 as a target on tumor cells, aimed to use T cell cytotoxicity to kill proliferating B cells (Tutt et al, 1991). In this approach, a tri-specific antibody was synthetised capable of recognizing both B and T cells, and aimed to link cytotoxic T effectors to B-cell targets; two arms of the antibody attached to T cells, thereby enhancing their activation.

CELLULAR BASIS OF THE IMMUNE RESPONSE

The immune system involves a complex variety of cells, each element fulfilling a defined task. In this section, we shall present the characteristics and function of lymphocytes and mononuclear phagocytes.

A homogeneous morphology is observed among lymphocytes and at their cell surface some proteins which are restricted to a specific type of lymphocyte are used as markers, e.g: CD3 is a T-cell associated peptide complex and CD4 and CD8 permit

a further classification of T cells into helper and cytotoxic subsets respectively. Antigenic receptors on B and T cells belong to the same immunoglobulin protein superfamily and the diversity of the receptors is generated through basically similar genetic rearrangement processes. Lymphocytic surfaces also bear other distinguishing markers such as receptors for Fcγ, complement C_3 and growth factors.

The maturation of lymphocytes takes place in the "bursa equivalent" (bone marow) for B cells and in the thymus for T cells. Selection of T cells gives rise to lymphocytes specific for antigens presented in association with molecules coded by the Major Histocompatibility Complex (MHC). The MHC I and II protein products are polymorphic proteins whose variability comes from a multiallelic and multigenic genome. Antigens associated with MHC class I are recognized by cytotoxic T cells (CD8 positive), antigens associated with class II by helper T cells (CD4 positive). Only antigen-presenting cells (APC) exhibit MHC class II, while all nucleated cells express MHC class I.

The activation of lymphocytes into lymphoblasts occurs after an antigenic stimulation. After antigen processing, parts of the lymphocytic population will proliferate and mount humoral responses: antibodies secreted by B cells and lymphokines by T cells (chemotactic, mitogenic, growth-regulatory and lytic- activating factors). This reaction triggers a cascade of events which aim to eliminate the intruder. A second element of the lymphocytic population

comprises memory cells. Memory B cells appear to undergo a competitive selection in the presence of a low antigen concentration, so that only those with high-affinity immunoglobulin proliferate. The switch from IgM to high-affinity IgG production requires the cooperation of helper T cells.

Mononuclear phagocytes are widely scattered over the body: bone marrow, blood, liver, lymphoid aggregates such as spleen, connective tissues and serous cavities. The cells start their maturation in the bone marrow as promonocytes, then migrate through the circulatory system as monocytes and finally differentiate as macrophages in tissues. The macrophage can be free or fixed in tissues, and exhibits a high polymorphism dictated by the degree of development or stimulation of the cells. The size could range from that of an average lymphocyte to a volume ten times larger (Van Furth, 1970). Macrophages in tissues have a long life span and can turn over at a very slow rate, in the order of a month in the peritoneal cavity (Van Furth, 1970).

MATERIALS AND METHODS

1 - TISSUE CULTURE

A - GROWTH MEDIA

Preparation of media and cultured cells involved working under sterile conditions: outfits were sterilized and the work was carried out under a laminar flow hood.

Cultured cells were grown in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 (Gibco, Scotland) with additional Hepes for buffering at the desired pH. The medium was supplemented with glutamine and pyruvate (1 mM final concentration), streptomycin and penicillin, fungizone and 10-15% foetal calf serum (FCS, Myoclone). The active components of the FCS which support the growth of cells are poorly understood (reviewed by Barnes and Sato, 1980), but are established as necessary for the cell culture.

Hybridoma cells were grown in medium complemented with HAT (hypoxanthine, aminopterine and thymidine) (Gibco, Scotland) for the first ten days after the fusion and thereafter with HT (Gibco, Scotland). Hybridoma cells were cloned on a feeder layer medium which was prepared with thymocytes extracted from Balb/c mice. Thymectomy was performed on Balb/c mice under sterile conditions, the thymus was squashed in a petri dish and the thymocytes were added to 100 ml of medium.

B - B CELL LINES (DAUDI, NAMALWA)

Cultured cells were grown at 37°C in a CO_2 incubator (7-10 %) under a moist atmosphere.

Cells stored in liquid nitrogen (-196°C) were thawed, washed, resuspended in RPMI with FCS, and grown in flasks (Labortechnik, Greiner). An exponential growth was maintained during culture.

C - HYBRIDOMAS

Fusion protocol

The immunized mice were sacrificed in a CO_2 chamber, and a sample of blood was withdrawn by cardiac puncture. The serum was recovered from the clotted blood by centrifugation at 2000 g for 10 minutes. The animal was swabbed with 70% ethanol and the spleen was removed under sterile conditions. The spleen was excised with fine scissors and squashed in a petri dish. The splenocytes were harvested, washed twice in warm DMEM medium and a small sample was erythrocyte-depleted and counted with the Coulter counter (Coulter Electronics Ltd, England). The NS-1 myeloma cells were grown in standard medium and the growth rate was maintained in log phase. They were washed twice in warm DMEM and a sample was counted. Splenocytes and NS-1 were then mixed at a ratio 1-2 : 1 (spleen : NS-1), centrifuged at 800 g for 5 minutes, and the supernatant was carefully decanted. Under sterile conditions, the centrifuge tube containing the pellet was placed in a beaker filled with distilled water at 37°C. Then 0.5 ml of 48% PEG (m.w. 4000, BDH) was added dropwise over 60 seconds

with continuous agitation. The pellet was stirred for a further 90 seconds and 10 ml of warm DMEM was added with stirring over 5 minutes. The initial 2 ml was added slowly over 2 minutes and then the rest of the medium in the remaining 5 minutes. The PEG was quickly removed by centrifugation at 400 g for 5 minutes and the pellet was broken up into small clumps in warm HAT medium . It was then placed in an incubator for 30 minutes. The cells were then fully resuspended and a drop of cells was added to each well of 96-well plates (Nunclon, Nunc, Denmark) containing feeder cells. The HAT medium bathing the cells was changed three times before screening.

Ref: Köhler and Milstein (1976).

Cloning by limiting dilution

The positive wells were cloned onto 96-well culture plates filled with feeder medium complemented with HT. A drop of cells was added into each well of the first row and diluted out with a multichannel pipette. The positive wells containing a single colony were then selected and recloned twice as described to ensure monoclonality. Hybridomas were transferred to 24-well culture plates and gradually to flasks of increasing sizes. Cells were then injected intraperitoneally into mice (Balb/c x CBA), and antibody-containing ascitic fluid was tapped when sufficient accumulation was judged to have occurred.

Cryopreservation

As soon as the hybridomas had grown to about 10^7 cells they were washed, resuspended in DMEM with 15% FCS and 10% dimethyl sulphoxide (DMSO), aliquoted in vials, placed immediately in a -75°C freezer for 3-10 days, and then stored permanently in liquid nitrogen at -135°C.

2 - PREPARATION OF CONJUGATED ANTIBODY

A - DIALYSIS OF PROTEINS

Dialysis tubing of Visking size 2 (18/32"; Medicell International Ltd) was boiled in distilled water with di-sodium ethylenediamietetraacetic acid (EDTA, Sigma) and rinsed twice with distilled water before use. Protein solutions were dialysed into appropriate buffer overnight with at least two changes of buffer.

B - FITC-CONJUGATED ANTIBODY

Fluorescence is the emission of photons associated with the reversion of a molecule from an excitated state to its stable level. In the case of the FITC, the aromatic π bonding which absorbs at a wavelength (λ) of 488 nm emits light of λ>500 nm ($500 < \lambda < 550$). The intensity of fluorescence can be measured using a filter for the emitted light (dichroic mirror or beam-splitter).

A fluorochrome can be coupled to an antibody without interfering with its antigen-binding capability and therefore

can be used as a probe for detecting antigen. Fluorescein isothiocyanate (FITC) is widely used because it is very stable (Riggs et al, 1958). The coupling reaction involves a nucleophilic attack of unprotonated ε-amino groups of lysine residues on the isothiocyanate group of the fluorochrome. A ratio of fluorochrome : protein from 0.6 to 0.8 should be achieved. A lower ratio would give a lower sensitivity during detection of antigen due to a competition with unlabelled antibody. A higher ratio would alter the biochemical property of the antibody and increase the non-specific binding of the conjugated antibody to the cells.

The protein concentration can be measured spectrophotometrically. The absorbance of the aromatic residues of the protein at 280 nm correlates with the protein concentration by the Beer-Lambert formula:
$OD = \varepsilon \times c \times 1$ (ε, molar extinction coefficient; c, concentration; 1, length of light path; OD, optical density). Likewise, the absorbance of the π bonding of the fluorochrome at 495 nm correlates with the fluorochrome concentration. A correction is required for the absorbance of the fluorochrome at 280 nm. The F/P ratio as well as the conjugated antibody concentration can be calculated according to the following formulae (The and Feltkampt, 1970):

$$F/P = (2.87 \times O.D_{495}) / (O.D_{280} - 0.35 \times O.D_{495})$$

$$[Ig^*] = (O.D_{280} - 0.35 \times O.D_{495}) / 1.4 \quad \text{(in mg/ml)}$$

A solution of purified antibody was mixed with FITC solution (Isomer 1, BDH Limited Poole, England) at a fluorescein : protein ratio of 1:50. The reaction was carried out in a carbonate buffer (pH 9.5) for 45 minutes at 25°C. The conjugate was purified by two sequential chromatographic procedures. Unreacted FITC can be removed by a Sephadex G-25 fine column (Pharmacia LKB, Sweden), and the conjugated protein can be fractionated with a diethylaminoethyl (DEAE)-cellulose anion-exchange column (Whatman BioSystems Ltd, England) according to the degree of FITC incorporation. Absorbance of collected fractions were measured at 495 and 278 nm. Antibodies with unduly low and high fluorescein contents were discarded.

C - RADIOACTIVE LABELLING OF ANTIBODY

Radioactivity is the property of some molecules of disintegrating with emission of radioactive particles (α, β, β^+, or γ). Disintegration of ^{125}I is accompanied by the emission of an γ wave which can be measured with a gamma-counter (1282 Compugamma, Pharmacia-LKB). The kinetics of disintegration follow an exponential decay rule: $N/N_0 = e^{-\lambda t}$, where N and N_0 are the number of nuclei at times t and t_0 respectively, and λ is a constant of disintegration for the isotope concerned. The γ emission of a ^{125}I-conjugated antibody can be correlated with the protein concentration with due allowance for the isotopic decay.

The coupling of ^{125}I to antibodies was performed according to the Iodo-beads iodination technique (Pierce Chemical Co.) Iodine was oxidised to I^+ which can react with tyrosine residues by electrophilic susbtitution. A monoclonal antibody (M15/8) at 1 mg/ml in PBS was mixed for 10 minutes with 2 µl of radioactive [125] iodine. The mixture was extensively dialyzed in PBS until >96% of radioactivity was precipitable , with the protein, by 8% trichloroacetic acid. Radioactivity achieved was 2 x 10^8 cpm/mg protein.

3 - ANTIGEN PREPARATION

A - IgM (NAMALWA)

The IgM secreted into the culture medium by Namalwa cells was harvested after removing the cells by centrifugation at 2000 rpm for 5 minutes. The culture supernatant was concentrated 10-fold using an Amicon ultrafiltration chamber (Amicon Ltd, High Wycombe, Bucks) with Amicon PM10 Diaflo membranes.

An ACA 22 chomatography column (LKB) was prepared. The fine powder was soaked in appropriate buffer and allowed to swell. The gel was packed into a column and equilibrated with 0.2M Tris EDTA buffer, pH 8.0. After standardizing the column, the concentrated IgM sample was applied to it at 10 ml/min and the fractions were analyzed by ELISA.

B - CD37 ANTIGEN

Cell preparation

Cells from culture were centrifuged at 2000 rpm for 5 minutes, resuspended in PBS and the procedure repeated. For staining studies, cells were resuspended in PBS/BSA/azide, the azide serving to reduce endocytosis of antibody into the cells (Taylor et al, 1971). In the rosetting assay, cells were washed and resuspended in RPMI, with 10% FCS to help preserve the cellular viability.

Cell digestion with papain

A cell surface digestion for CD37 was carried out with papain, which catalyzes the hydrolysis of peptide bonds with the requirement of an active enzyme sulfhydryl group in concert with an aldehyde nearby (Morihara, 1967). Hydrolysis *in vitro* takes place at 37°C after activating the papain with a reducing agent (Smith, 1958; Finkle and Smith, 1958). The catalytic activity of papain can be inhibited with reagents such as iodoacetamide which inactivate sulfhydryl groups (Morihara, 1967).

Papain solution, 2x crystallized suspension in 0.05M sodium acetate (Sigma), was pre-incubated for 15 minutes at 37°C in a buffer containing 100 mM dithiothreitol (DTT, BDH Chemicals Ltd, Poole, England). Daudi cells were washed twice in PBS and resuspended at 10^7 cell/ml. The papain digestion was carried out in a water bath at 37°C with adequate agitation at a final enzyme concentration of 50 µg/ml, and was

stopped by chilling on ice for 10 minutes before centrifugation at 2000 rpm for 10 minutes.

In the attempt to isolate CD37 from the digest, an additional micrococcal nuclease solution (from *Staphylococcus aureus* (Sigma)), at a final concentration of 50 µg/ml, was added during the digestion. This is necessary in order to fragment the released DNA which otherwise can raise the viscosity of the digest to the point of gelling. The digestion was stopped by adding a solution of iodoacetamide (BDH Ltd, Poole, England) to a final concentration of 20mM in the supernatant.

Cellular permeabilization.

Daudi cells were washed twice in PBS and resuspended at 10^7/ml in a PBS solution with 2% paraformaldehyde (BDH) for 10 minutes at room temperature. The fixative reagent was removed by washing the cells with PBS. The cells were further permeabilized by incubating with n-octyl β-D-glucopyranoside solution (Sigma) for 5 minutes at room temperature (150 µg for 10^6 cells). The cells were then washed twice with PBS before labelling.

Ref. Hallden et al (1989).

Lysis of cells

The non-ionic surfactant NP40 was used to extract the membrane protein CD37. This detergent, at 0.5% concentration, solubilizes the intra- and extra-cytoplasmic membrane proteins without disrupting the nuclear envelope. The protein

solubilization is largely independent of temperature, and the lysis was performed at 0°C to inhibit proteolysis of the solubilized protein. Soya-bean trypsin inhibitor (BDH) and iodoacetamide were added as inhibitors of proteolytic enzymes, the iodoacetamide serving also to prevent untoward disulophide cross-linking by free sulphydryl groups.

Cells were washed and resuspended in PBS containing iodoacetamide (50 mM) and soya inhibitor (.04 mg/ml). The cell suspension was mixed with an equal volume of NP40 solution and incubated at 0°C for half an hour. The lysate was centrifuged at 2000 g for 20 minutes and the supernatant recovered. Lysis of Namalwa cells produced an IgM concentration of about 20 µg/ml (estimation from ELISA analysis).

Ref. Stevenson et al (1981).

4 - PREPARATION OF BEADS

A - ANTIBODY-COATED BEADS

The binding of antibody onto beads was carried out according to the manufacturer's recommendations. Tosyl-activated Dynabeads M-280 (Dynal A.S, Norway) were concentrated to 20 mg/ml in a borate buffer (PH 9.5). The antibody was prepared in that buffer to give a concentration of 450 µg/ml. Beads and the protein solution were mixed in equal volumes and incubated for 24 hours at 37°C with slow tilting and rotating. The beads were collected with the Dynal magnet concentrator and washed by slow rotation at room

temperature three times for 10 minutes, once for half an hour, and once at 4°C overnight. Beads were kept in PBS/BSA/azide and appeared stable for more than 6 months when stored at 4°C.

B - ANTIGEN LOADING OF BEADS

The antibody-coated beads were washed in PBS/BSA twice before incubating with the lysate supernatant at 4°C with slow rotation overnight. The beads were shown to bind antigen efficiently at room temperature during a two-hour period without agitation. They were then washed 4 times for half an hour at room temperature in PBS/BSA with slow rotation. Beads were collected with the magnetic particle concentrator (Dynal A.S, Norway) and kept in PBS/BSA/azide at 4°C.

5 - ASSAYS FOR DETECTING PROTEIN

A - ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The commonly used double-determinant immunometric version of this assay permits detection of antigen or antibody in solution at concentrations as low as picograms/ml as quoted by Monroe (1984). The general steps involved are (1) coating the plastic assay plate with a "capture" antibody; (2) allowing this antibody to react with test antigen in solution; (3) allowing captured antigen to react via another determinant with "indicator" antibody. The presence of indicator antibody is detected by virtue of an enzyme label, either on the antibody itself or – in the indirect method – on a further

layer of anti-antibody. Commonly the enzyme is horseradish peroxidase which hydrolyzes its substrate with production of colour measured spectrophotometrically at 490 nm.

Application of ELISA can be illustrated by an assay for screening antibody secreted by hybridoma. One hundred microliters per well of rabbit anti-mouse Ig polyclonal antibody in a coating buffer (pH 9.6) was incubated overnight at 4°C in 96-well micro-ELISA plates (Nunc Immunoplates, Dynatech, Denmark). The liquid was discarded and replaced by 100 µl of blocking buffer containing 1 % BSA-PBS and incubated for one hour at 37°C. The plates were washed 3 times with PBS/Tween using Immuno Washer NK-350 (Teknunc), and 100 µl per well of test samples (fusion supernatant) were added and incubated for 90 minutes at 37°C. The plates were washed 5 times in PBS/Tween and 100 µl per well of peroxidase labelled rabbit anti-mouse Ig polyclonal antibody were incubated for 1 hour at 37°C. The plates were washed 5 times and 100 µl of the peroxidase substrate was added and left in the dark for half an hour. The reaction was stopped by adding 50 µl of 2.5 M sulfuric acid and the optical density was read at 490 nm using Dynatech MR 5000 (ELISA plate reader).

B - FLOW CYTOMETRY

This technique permits an analysis of cells labelled with an immunoreagent linked to a fluorochrome. The FACS has a very powerful readout and can analyze up to 5000 cells per second.

Cell-surface antigen can be detected either by direct or indirect fluorescence. In direct fluorescence the FITC-conjugated antibody is specific for the given cell surface antigen. Cells prepared in PBS/BSA/azide were resuspended at 10^7/ml and 50 µl was incubated at 4°C for 20 minutes with an equal volume of FITC-conjugated antibody solution. Cells were washed twice in PBS/BSA/azide, resuspended in 400 µl of PBS/BSA/azide and analyzed by a fluorescence-activated cell sorter (FACS; Facscan, Becton Dickinson U.K. Ltd). In indirect fluorescence, a rabbit FITC-conjugated anti-mouse antibody was incubated at 4°C for 20 minutes with cells coated with mouse monoclonal antibody specific for a given cell surface antigen (e.g. anti-CD37, anti-human µ).

Screening for secretion of monoclonal anti-CD37 was carried out with indirect immunofluorescence analysed by FACS. Target cells were suspended at 2 x 10^6/ml in PBS/BSA/azide and incubated with supernatant from the fusion in Falcon "U" 96-well plates (Becton Dickinson U.K. Ltd). Incubation of 50 µl of target cells with 100 µl from the fusion was carried out at 4°C for 30 minutes. The cells were then prepared for FACS analysis according to the indirect fluorescence method.

Dyna beads carrying antibody ± antigen were labelled with FITC-conjugated antibody and analyzed by flow cytometry as described above. Beforehand, beads were diluted 100-fold in PBS from the initial pot to produce a concentration of 6 x 10^8 beads/ml.

Negative controls were set up for each assay with irrelevant FITC-conjugated antibody to monitor any non-

specific binding on the cells due either to cross-reaction of FITC-conjugated rabbit anti-mouse with human Ig, or to non-specific binding via Fc receptors on the target cell suface.

Adsorption analysis was performed by pre-incubating, at equal volumes, the antibody with a test solution of antigen for 15 minutes at 4°C before labelling the target cells.

C - ROSETTING ASSAY

Target cells were resuspended at 1.2 x 10^6/ml in RPMI with 10 % FCS. Antibody-coated beads were diluted 100-fold in the same medium to produce a concentration of 6 x 10^6/ml. A ratio of 5 : 1 (beads : cells) was obtained by mixing cells and beads at equal volume. The mixture was left for half an hour at room temperature and 400 µl of medium was added before analyzing the rosetting by flow cytometry. The percentages of rosetted cells yielded by antibody-coated beads, and by antibody-coated beads which had been incubated with a test solution of antigen, were compared to deduce the percentage of rosette inhibition.

6 - SCHEDULE OF IMMUNISATION

Six Balb/c mice at six months old were immunised with CD37- and Namalwa IgM-coated beads. The beads were suspended in PBS and 250 µl at 6 x 10^8/ml was concentrated to 150 µl using the magnet particle concentrator and was then administered to each mouse intra-peritoneally using a 21 gauge needle. The mice were boosted twice at three-week intervals and the serum titer was monitored by ELISA for anti-(Namalwa

IgM) and by FACS for anti-CD37. The immunized mice having the highest titer were then given an intrasplenic injection (Spitz et al, 1984; Spitz, 1986) by the chief animal technician, Mr Richard Reid. They were anaesthetized with metofane and an incision was made with sterilized entomological scissors and curved forceps. The injection into the spleen was carried out with a 30-gauge needle. Two days later, the mice were sacrificed in a CO_2 chamber and the spleen was removed for fusion.

RESULTS

The present work arose from a need to obtain anti-CD37 monoclonal antibodies of higher affinity than those already available, which were obtained after immunizing mice with whole cells obtained from B-cell lymphomas. The B-cell surface exhibits a vast variety of antigens capable of triggering an immune response. Our project aimed to generate a range of CD37-specific monoclonal antibodies made available by the use of a much more selective immunogen, while avoiding the difficulty of rigorously purifying the CD37 antigen.

An attempt was made also to raise a monoclonal anti-idiotypic monoclonal antibody specific for surface IgM on the B-cell line Namalwa. This approach was used as a model of investigation for several reasons. First, IgM is a protein more intensively studied than CD37, with methods available for obtaining it in pure solution. Second, a range of monoclonal and polyclonal antibodies reactive with the constant regions of human IgM was already available in the laboratory. Third, an anti-idiotype (Namalwa) was considered highly suitable for cellular killing studies.

The main feature of our approach was to harness the available anti-CD37 and anti-IgM antibodies (1) to detect solubilized antigen in cellular extracts; (2) to isolate the antigen on beads and measure it on the beads; and (3) to act as a carrier in further immunisations of mice.

1 - DETECTING THE ANTIGEN

Initial work was carried out to detect the presence of antigen at the cellular level, and in solution in culture supernatant, cell-surface digest or cell lysate.

Studies at the cellular level

Namalwa surface IgM was readily detectable with FITC-conjugated anti-human μ monoclonal antibody (M15/8*).

The level of IgM secretion by Namalwa cells was monitored using ELISA. IgM concentration was measured by setting up a standard curve O.D. = F[IgM] (data not shown). Figure 1 shows the secreted IgM concentration in the medium over a period of 48 hours. The secretion increased from 0 to 30 hours and then levelled off to form a plateau. From this experiment it can be concluded that IgM is secreted by Namalwa cells in amounts which are small but nevertheless useful as immunogen. As shown in the graph IgM secretion was slow for the first 8 hours, but after this initial period the secretion rate increased and possibly reflected optimum activity of the cells before depletion of growth constituents stopped secretion after 30 hours.

The molecular weight of the secreted IgM was measured to verify its pentameric structure. An ELISA was performed on aliquots of culture supernatant passed through an ACA 22 column (Figure 2). The chromatographic column had been standardized beforehand using a standard IgM (19S) and IgG (7S). It can be seen that the major antigenic activity in the column effluent was in the 19S region, indicating that IgM in

FIGURE 1

IgM SECRETION BY NAMALWA CELLS

Concentration of IgM secreted into the culture medium over a period of 48 hours. Aliquots were analyzed by ELISA.

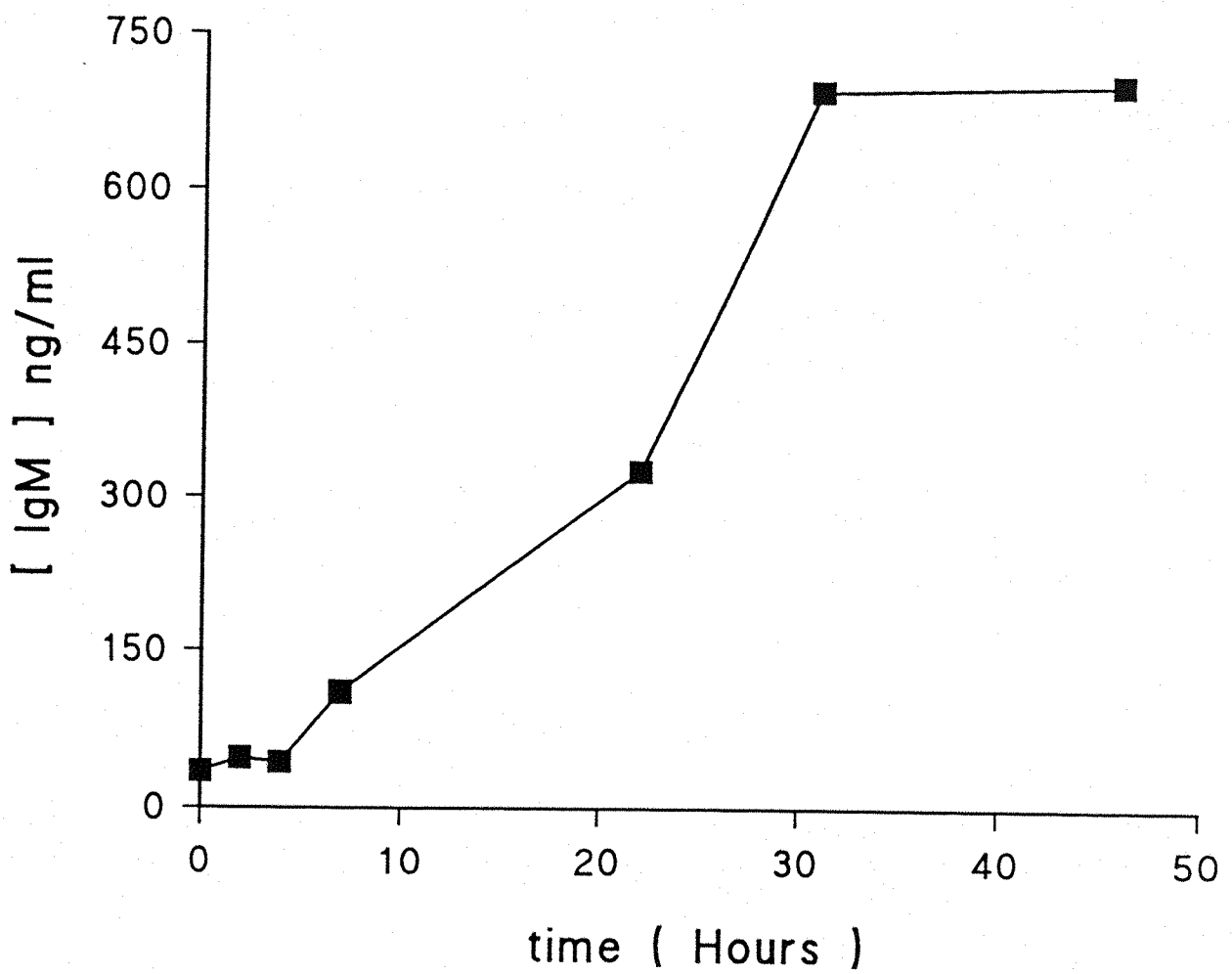
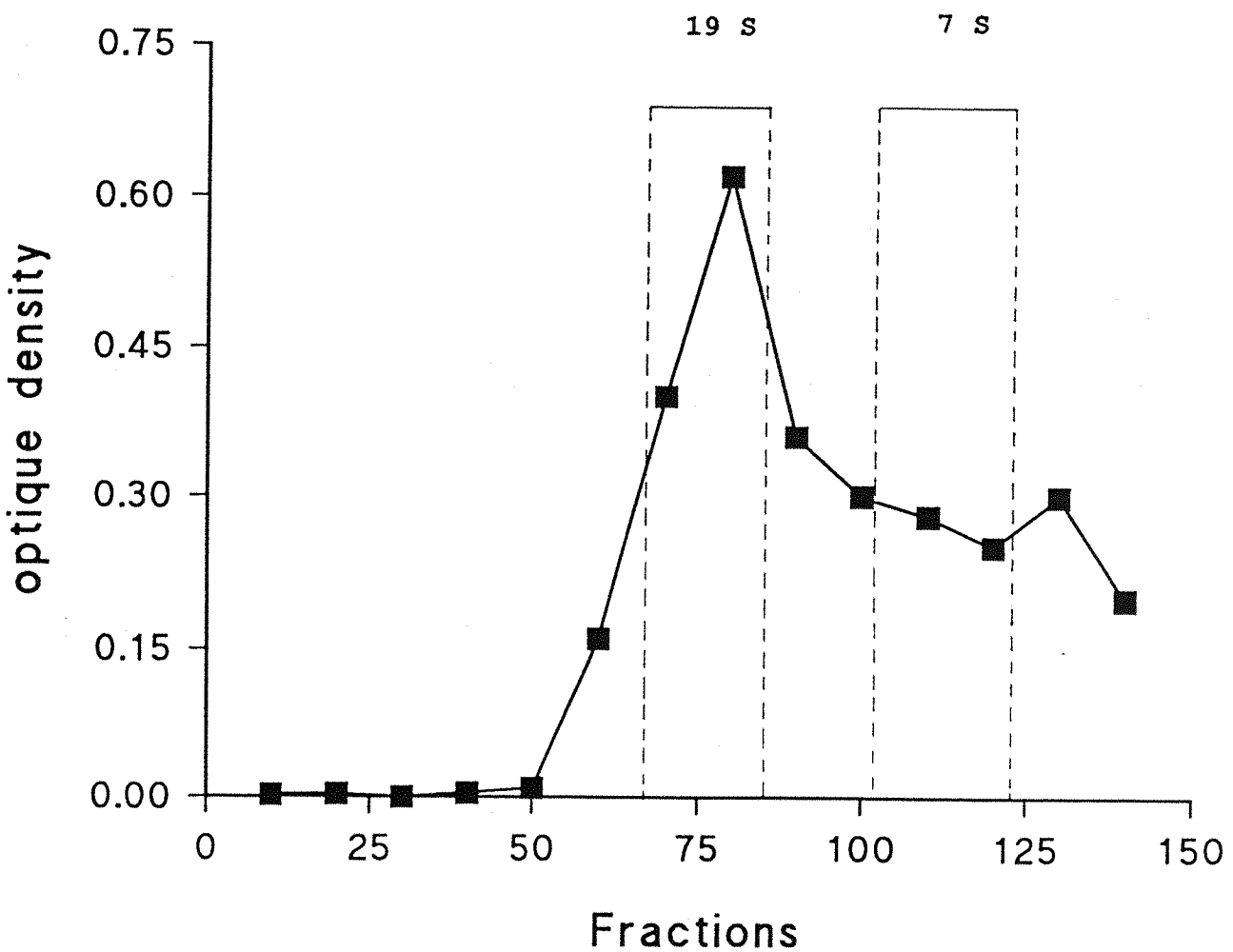


FIGURE 2

DETERMINATION OF THE MOLECULAR WEIGHT OF SECRETED IgM

ELISA for IgM performed on fractions eluted from an ACA 22 chromatography column.



the culture supernatant was predominantly pentameric.

The presence of CD37 at the cell surface was assessed using WR17*. Figure 3 shows the fluorescence intensity of Daudi and Namalwa cells. A comparison of the fluorescence level between these two cell lines reveals a higher expression of surface CD37 on Daudi than Namalwa. The negative control of this experiment was set up with FITC-conjugated anti-CRBC (chicken red blood cell) monoclonal antibody which binds to the Daudi cell surface to a small extent, possibly through a combination of Fc-receptor binding and non-specific interactions.

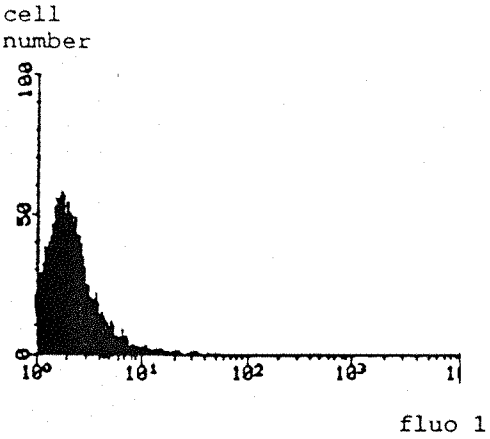
Investigations were also conducted to determine the approximate level of intracellular expression of CD37 in Daudi cells. The cells were fixed, permeabilized and labeled with WR17*. Results showed that the brightness of the cells did not increase appreciably above the high expression of CD37 at the cell surface (data not shown). Hence, a cell surface digestion for CD37 was carried out to reduce the background. Figure 4 shows a time course for a cell surface digestion with papain, monitored with WR17*. This graph exhibits a decrease of fluorescence in the initial 30 min period for cells labeled with WR17* (■) while the negative control remains at the same level (▲). The differential between test and control staining was disappointing in this experiment. Nevertheless we proceeded to investigate CD37 in papain-treated cells. In these experiments, cell surface membrane was papain digested, fixed and permeabilized. The presence of CD37 was monitored throughout the process by comparing the brightness of the

FIGURE 3

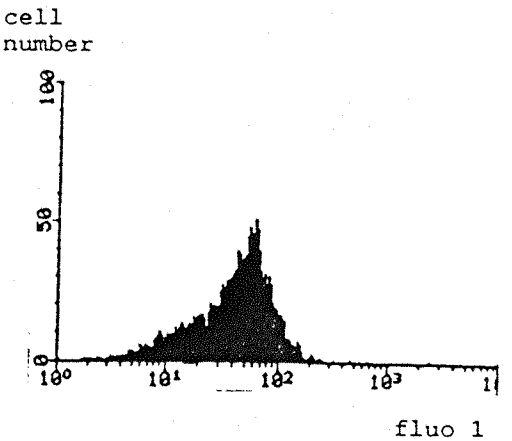
CD37 EXPRESSION AT THE CELL SURFACE

Fluorescent profiles of cells from the B-lymphoblastoid lines Daudi and Namalwa, labelled with FITC-conjugated anti-CD37 monoclonal antibody (WR17*).

DAUDI

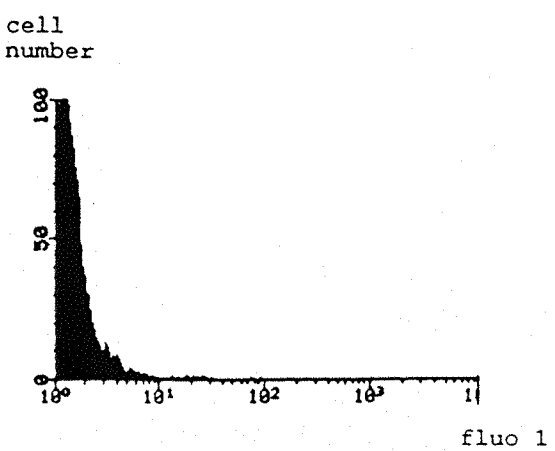


Control
Mean : 19.66

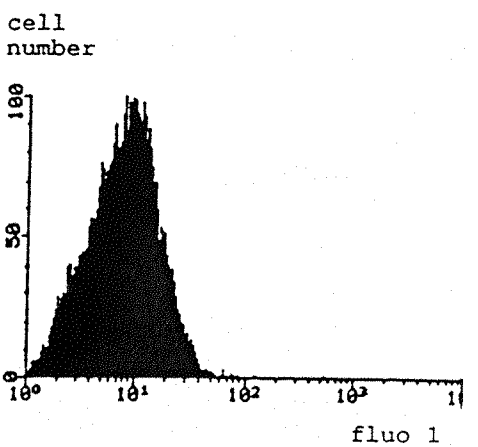


WR17*
Mean : 101.32

NAMALWA



Control
Mean : 7.74



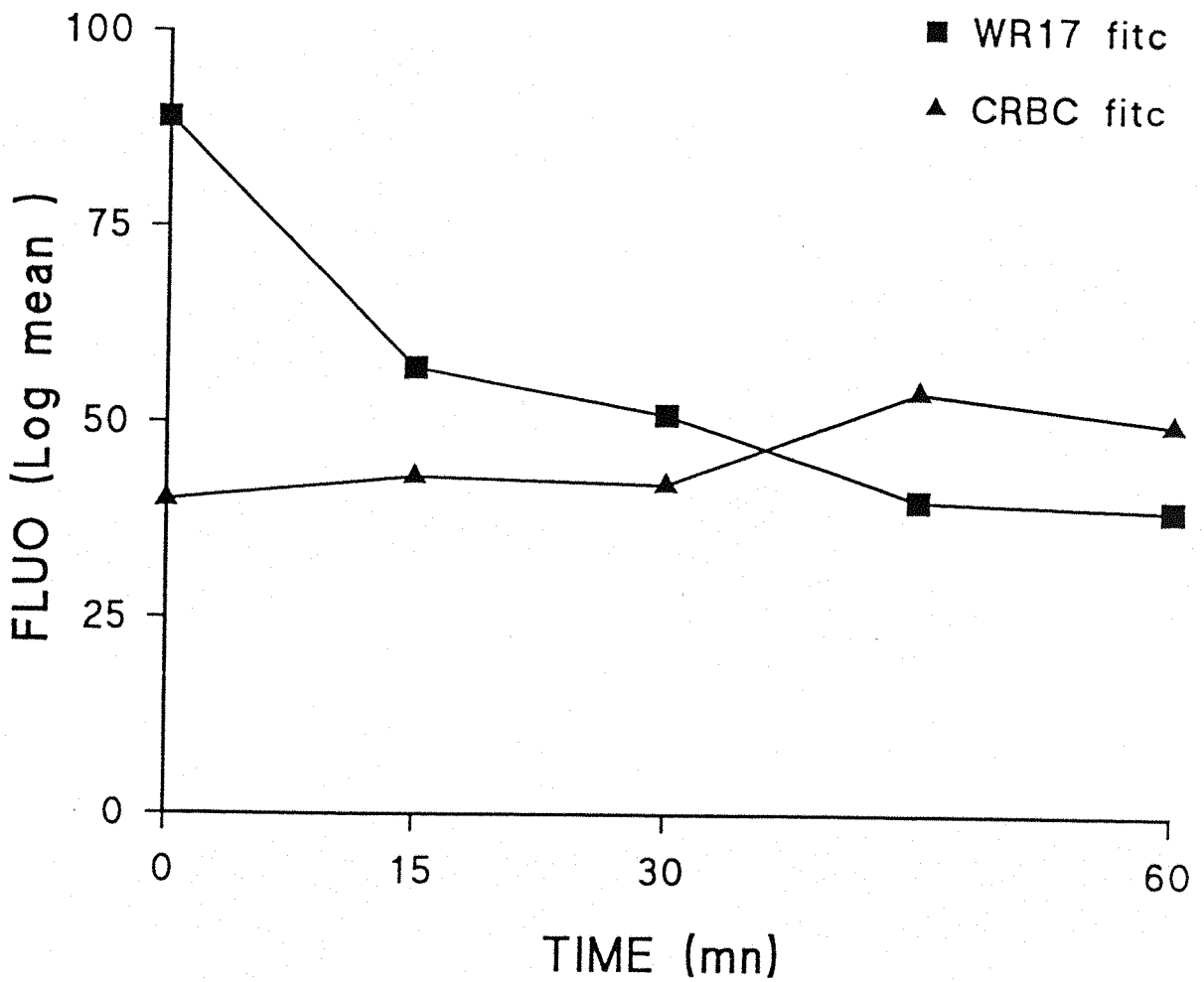
WR17*
Mean : 56.17

Control :
FITC conjugated monoclonal antibody;
anti- chicken red blood cell (anti-RCBC*)

FIGURE 4

ENZYMIC REMOVAL OF SURFACE CD37

Time course of papain digestion of Daudi cell surfaces, for removal of cell-surface CD37. Monitoring was undertaken by staining with a FITC-conjugated anti-CD37 monoclonal antibody (WR17*).



WR17*-labelled cells with the negative control (labelled with an anti-RCBC* (Figure 5)). The fluorescence profiles showed that papain digestion removed a majority of CD37 on the cell surface and, once permeabilized, the cells revealed a substantial quantity of CD37 located intracellularly. This result suggested that a useful amount of CD37 could be found in lysates of Daudi cells.

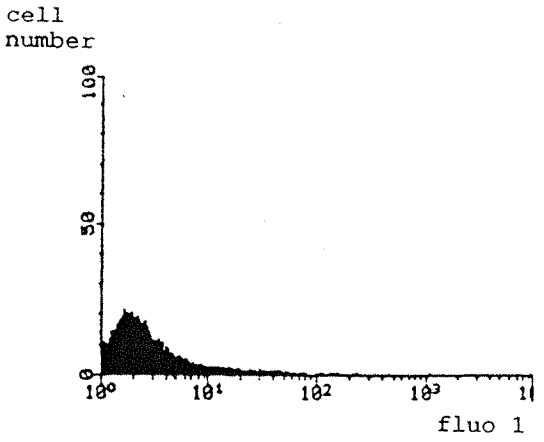
Detection of solubilized CD37 antigen

Our first approach was to attempt the detection of CD37 in surface digests or cell lysates by a standard double-determinant ("sandwich") immunometric ELISA. Unfortunately the three anti-CD37 monoclonal antibodies available in the laboratory proved unsuitable for this purpose. MB1 and WR17 showed mutual blocking activity, indicating that they recognized the same or closely neighbouring epitopes. In Figure 6 cells pre-coated with MB1 or WR17 at varying concentrations were labelled with WR17* at a saturating concentration. The two curves fell steadily and almost in parallel. The somewhat greater blocking by MB1 suggests that it has a higher affinity than WR17. The antibody HD28 was found to have too low an affinity for use in either ELISA or blocking experiments.

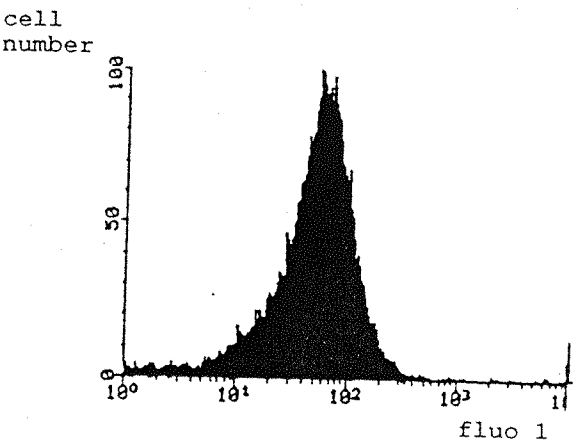
The lack of availability of a suitable ELISA led to a decision to demonstrate CD37 antigen in cell-surface digests or cell lysates by blocking of immunofluorescence.

Although it was shown that CD37 could be removed from the cell surface by papain, its survival in the digest supernatant

DAUDI CELL SURFACE

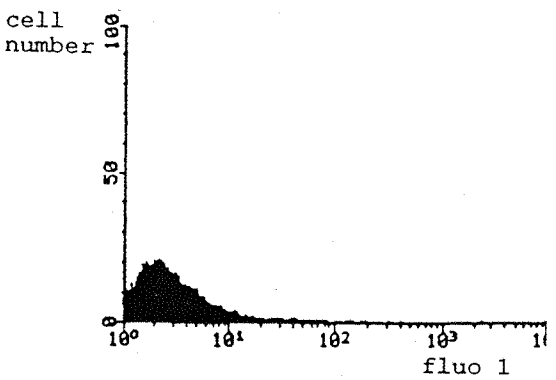


Control, Mean: 25.94

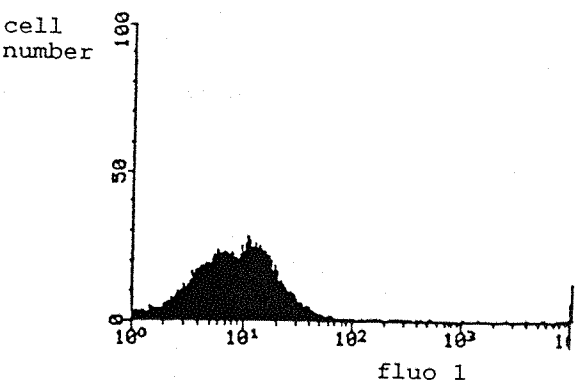


WR17*, Mean : 106.56

DIGESTED DAUDI CELL SURFACE

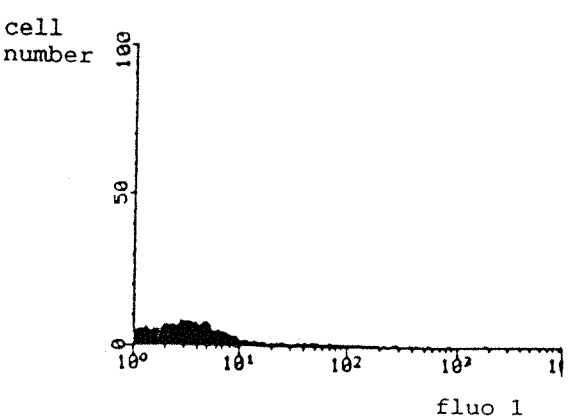


Control, Mean : 27.54

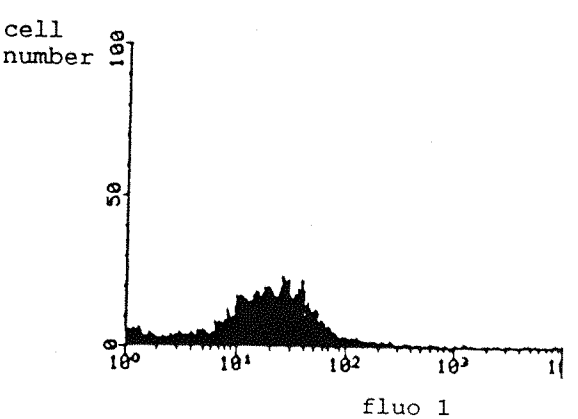


WR17*, Mean : 57.27

DIGESTED, FIXED AND PERMEABILIZED DAUDI CELL



Control, Mean : 31.01



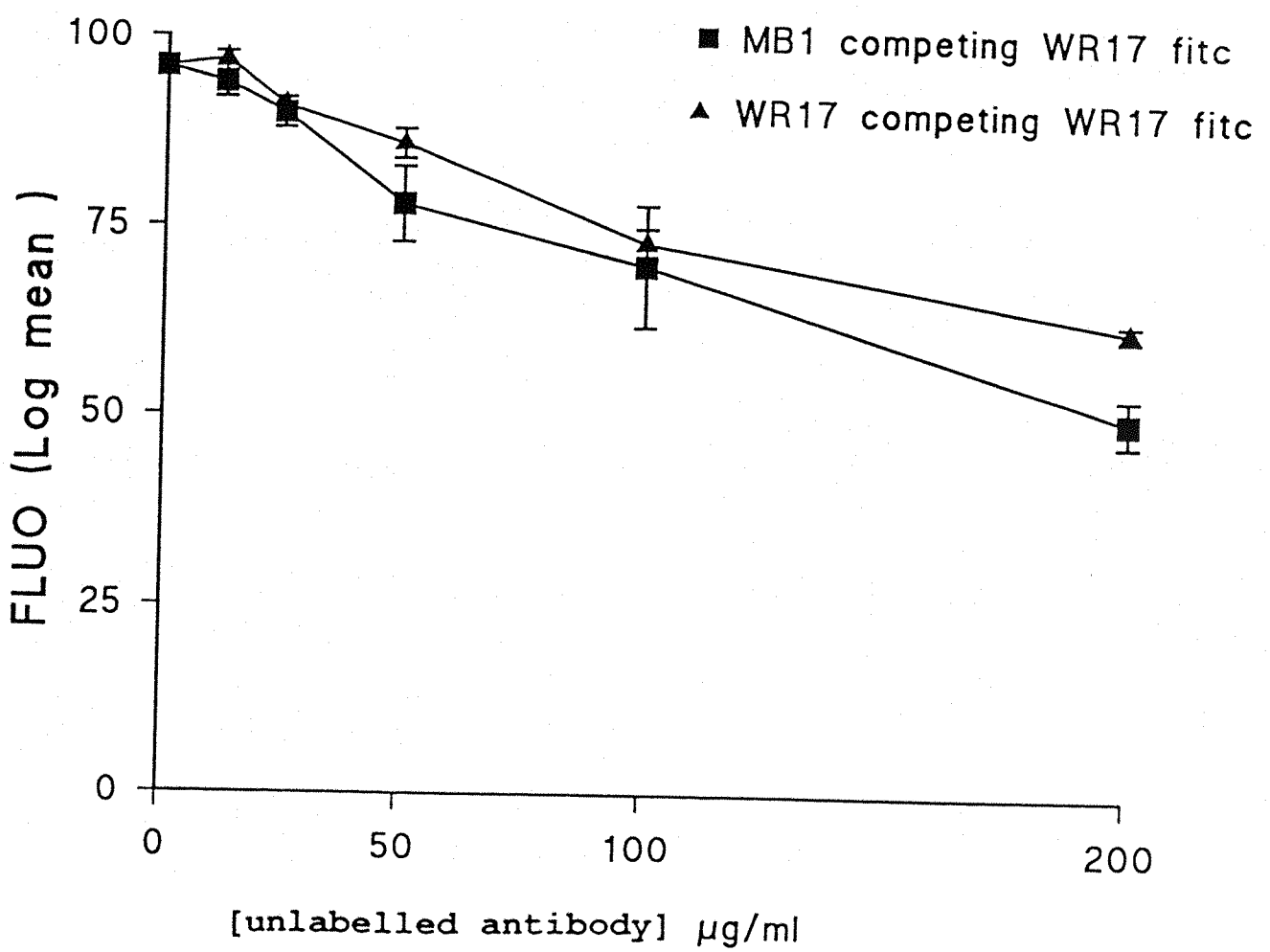
WR17*, Mean : 80.66

Control: anti-RCBC*

FIGURE 6

COMPETITION BETWEEN TWO ANTI-CD37 ANTIBODIES

Cells were allowed to react with an unlabelled anti-CD37 antibody (MB1 or WR17) at varying concentrations, washed, and then allowed to react with FITC-conjugated WR17.



could not be detected regularly by inhibition of immunofluorescent staining with anti-CD37, suggesting that the protein is unstable in the presence of the enzyme.

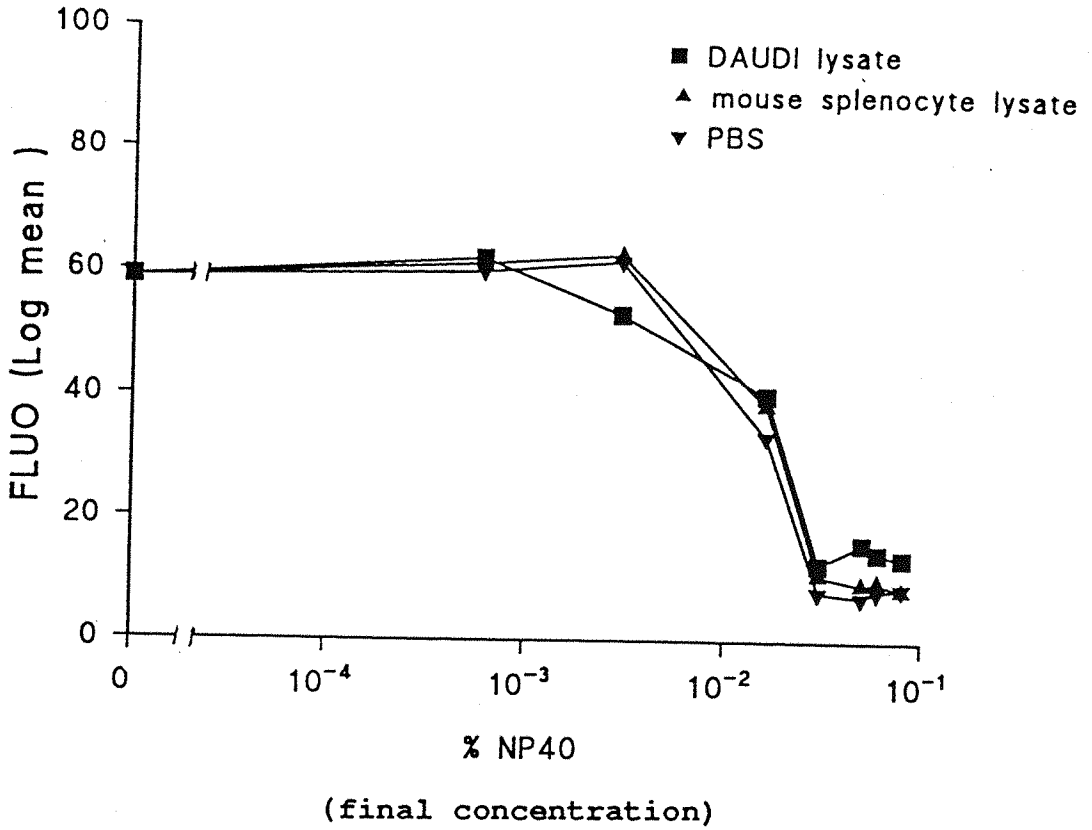
We were led by the above results to seek CD37 antigen in cell lysates by the blocking of immunofluorescence. The amounts of antigen anticipated limited the dilution of lysate which could be allowed in the detection system. Figure 7 shows the problems encountered when a lysate from cells at 5×10^7/ml was diluted 3-fold in an indicator system of Daudi cells and WR17*. In (A) it is seen that the antigen present was insufficient to yield significantly greater inhibition of staining than was seen with NP40-containing controls. In (B) it is apparent that a final concentration of NP40 above .03% will seriously compromise the viability of the indicator cells in the time taken for the assay. Nevertheless it was possible, by maximizing the cell density at lysis (up to 1.4×10^8/ml) and minimizing the concentration of NP40 (down to 0.1%), to obtain lysates in which CD37 could regularly be demonstrated. Figure 8 show that lysates from high cell densities show a clear difference in the level of inhibition between test and control. A WR17* solution at half saturating level on the indicator cells was used in order to enhance the sensitivity of the assay. Lysates obtained from cells at densities $>1.4 \times 10^8$ gave irregular results. The figure also illustrates another difficulty: it is seen from the upward slope of the control samples, and from the zero immunofluorescence occurring in the control lacking any cells (the point at the origin), that NP40 is actually consumed by

FIGURE 7

PROBLEMS POSED BY NP40 IN DETECTING INHIBITION OF FLUORESCENCE

A - Effect of varying concentrations of NP40, in simple buffer or cell lysate, on the binding of FITC-antibody (WR17*) to Daudi target cells.

B - Effect of NP40 on the viability of Daudi target cells. (Cells excluding trypan blue were counted with a haemocytometer)


DAUDI lysate
mouse splenocyte lysate
PBS
FLUO (Log mean)
100
80
60
40
20
0
0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
% NP40
(final concentration)

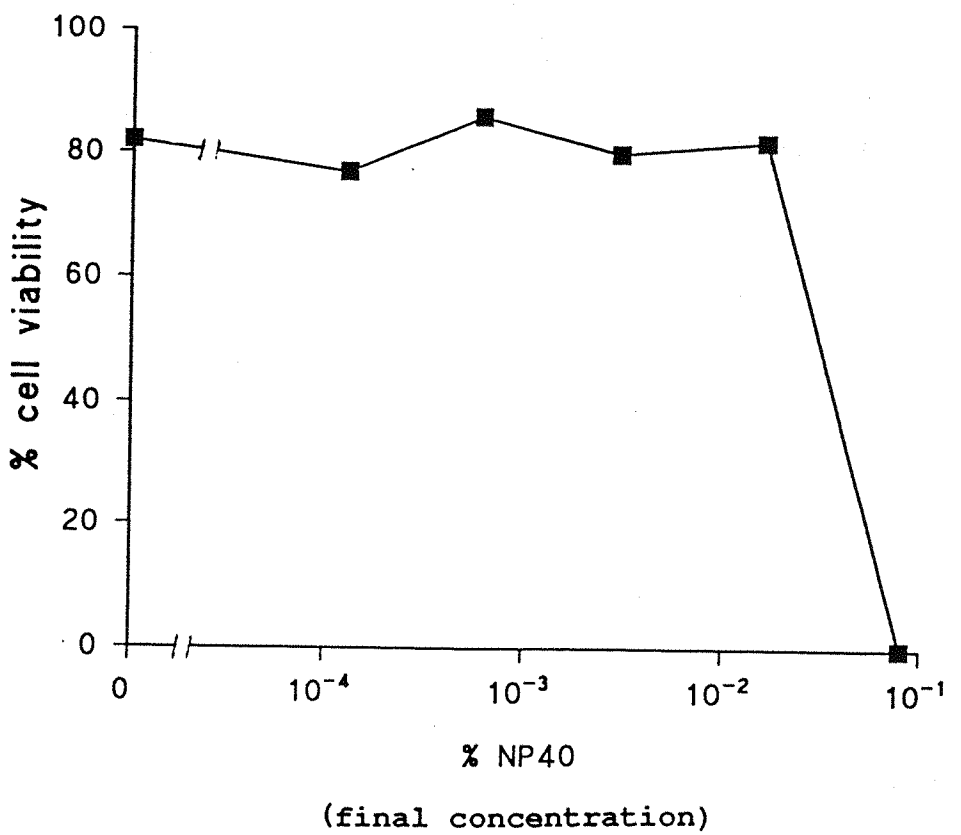
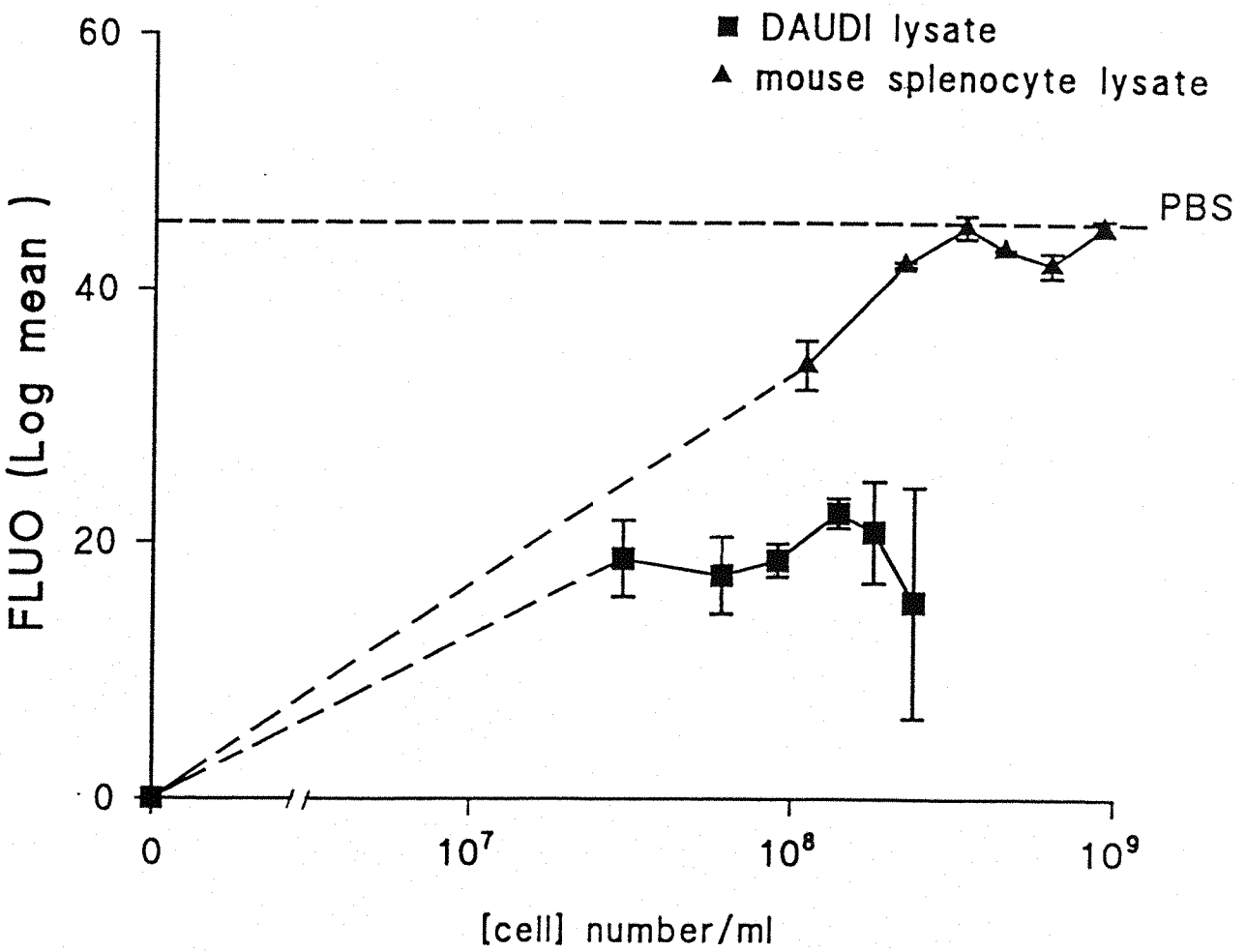

% cell viability
100
80
60
40
20
0
0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
% NP40
(final concentration)

FIGURE 8

DETECTING CD37 BY INHIBITION OF FLUORESCENCE

The presence of CD37 in lysate supernatant was assessed by the ability of the supernatant to inhibit specifically the immunofluorescent staining of Daudi cells with FITC-conjugated anti-CD37 (WR17*).



lysed cells so that its final concentration in the indicator system, and the degree of inhibition which it causes, are uncertain. It is likely that CD37 antigen being detected in the lysate is in mixed micelles with NP40.

To help validate this result a positive control was set up. In this experiment, it was found that a purified IgM solution blocked the binding of a conjugated FITC-anti-human μ monoclonal antibody (M15/8*) to Daudi cells (Figure 9).

2 - ISOLATING THE ANTIGEN

The solubilized antigen was isolated using immuno-Dynal beads (2.8 μm diameter). The main characteristics of these beads lie in their aptitudes of being both easily coupled to a given antibody as well as being rapidly collected with a magnet concentrator. The outline of the process was to link the monoclonal antibody covalently to the beads, incubate them with the antigen in a solution made of a vast variety of proteins (lysate supernatant), and isolate the antigen-laden beads using the magnet concentrator.

Beads were calibrated for their coupling capacity using a radio-iodine-conjugated anti-human Fcμ (^{125}I-M15/8) monoclonal antibody. Figure 10 represents the ^{125}I-M15/8 uptake per bead as a function of antibody concentration. The curve increases sharply until a concentration of 2 μg/ml and then levels off at a plateau. A satisfactory uptake was observed at a ^{125}I-M15/8 concentration of 20 μg/ml which gives rise to 2.6 x 10^5 ^{125}I-M15/8/bead. From this graph, it can be concluded that beads pick up the radio-iodinated antibody avidly.

FIGURE 9

BLOCKING ANTIBODY BINDING TO TARGET CELLS.

Adsorption analysis of a FITC-conjugated anti-human μ monoclonal antibody using a pure IgM solution to compete with surface IgM of target cells.

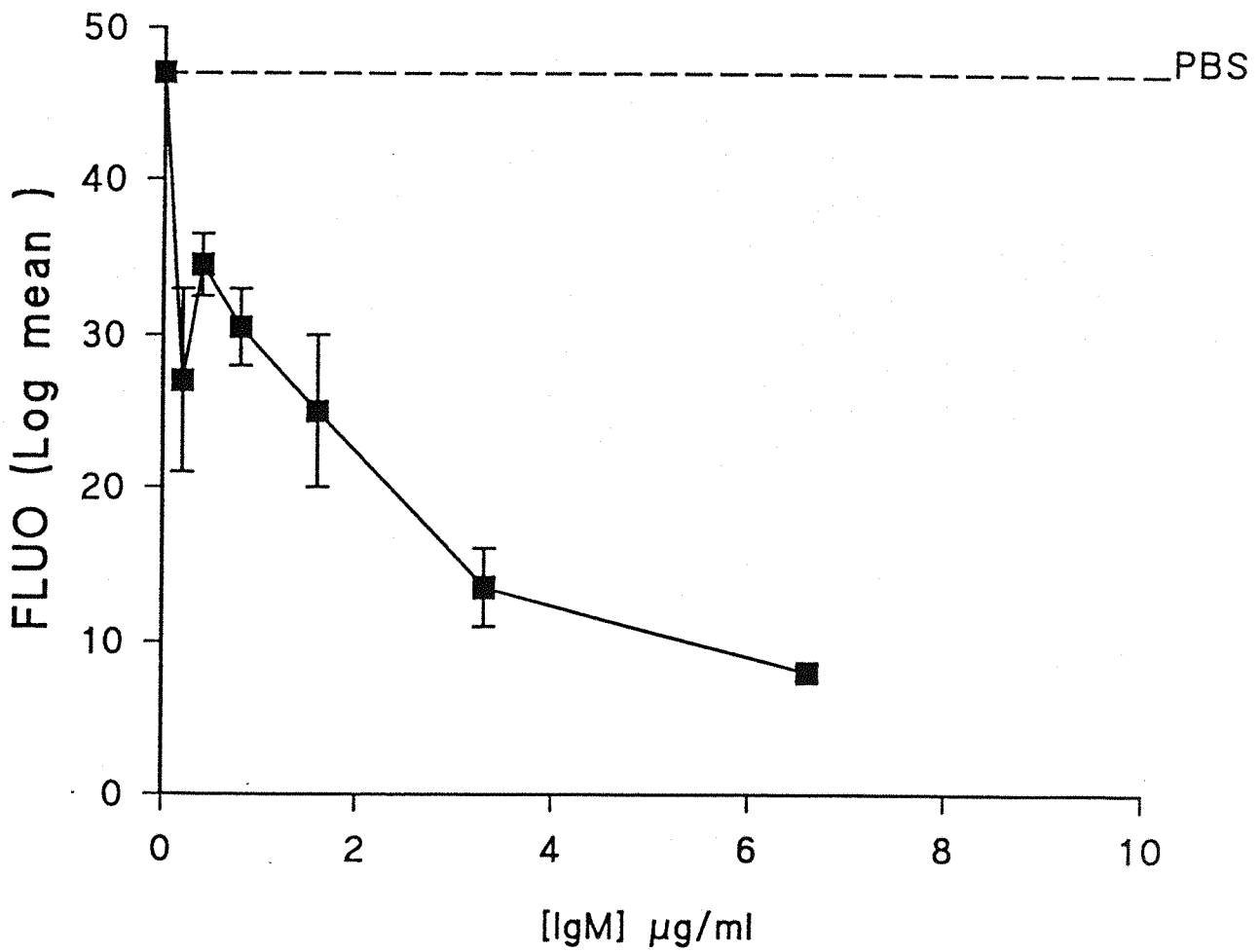
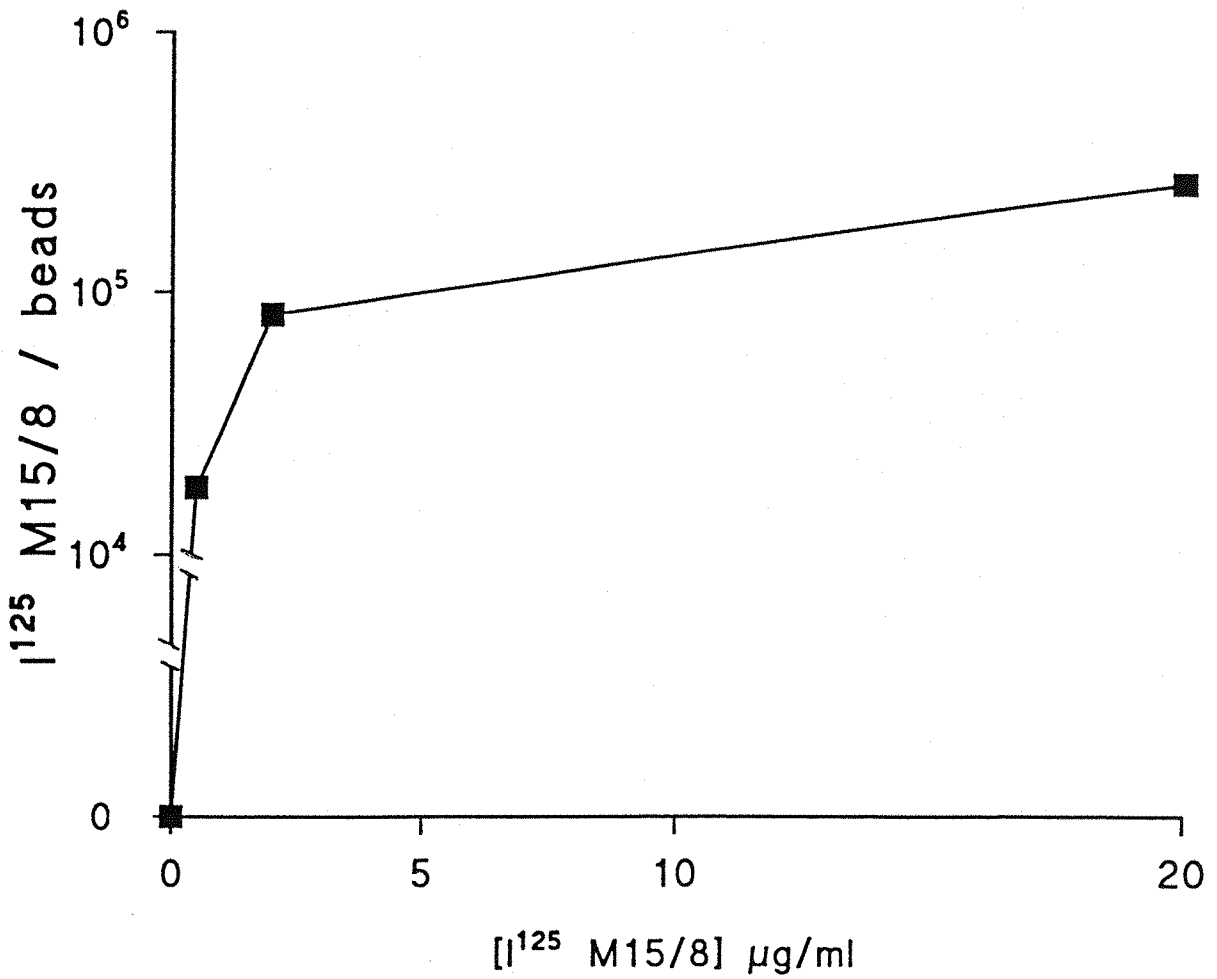


FIGURE 10

CALIBRATION OF M15/8 PER BEAD

Uptake of ^{125}M15/8 by the beads with increasing antibody concentration measured with the gamma counter.



Binding of antigen (IgM) to beads coated with M15/8 was monitored by labelling these beads with a FITC-conjugated polyclonal anti-Fdμ antibody (Figure 11-A). Excellent uptake is seen to have occurred from Namalwa culture fluid and lysate as well as from a control IgM solution. Provided the beads present represented ≤1% of the suspension volume, the uptake after 2 hours at room temperature without stirring was just as good as under conditions regarded as optimal: an overnight incubation at 4°C with slow rotation. The specificity of uptake was checked by labelling the same antigen-laden beads with FITC-conjugated anti-CD37 (WR17*): the beads remained entirely negative (Figure 11-B). A further control entailed incubating beads coated with anti-CD37 (b-WR17) with IgM and then anti-Fdμ* (Figure 12): again the beads remained negative, apart from some fluorescence exhibited by a minor population among those incubated with the Namalwa lysate. It should be noted that the modal fluorescence among the lysate beads remained unaltered.

It has been demonstrated previously that NP40 could hamper antigen-antibody complex formation. Accordingly the binding of IgM to b-M15/8 in the presence of NP40 at 0.1% was studied. Figure 13 shows the titration curve of IgM on b-M15/8. Controls were set up with a range of IgM concentrations in PBS (■) compared with the IgM in NP40 at 0.1% (▲). This graph displays two overlapping curves indicating that NP40 at 0.1% has not interfered with antigen uptake by the beads the beads.

Finally, calibration of the antigen-binding capacity

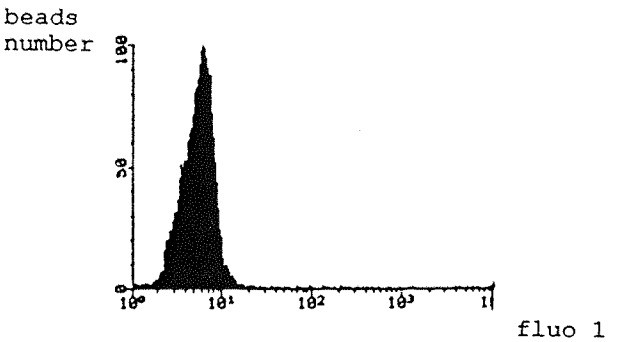
FIGURE 11

SPECIFICITY OF BEADS-M15/8

A - POSITIVE CONTROLS

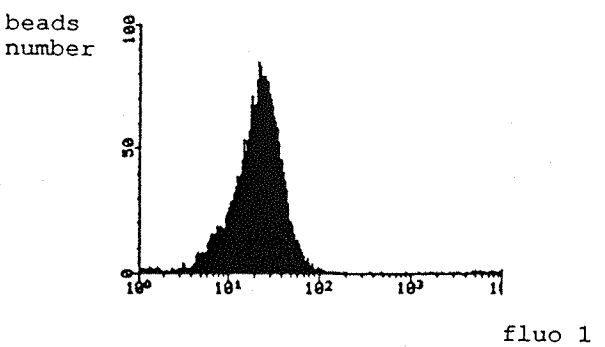
Fluorescent profile of b-M15/8 (anti-human μ monoclonal antibody coated beads) before and after incubation with IgM solutions and lysate supernatant. A FITC-conjugated goat anti-human Fd μ was used as indicator.

UNINCUBATED BEADS :



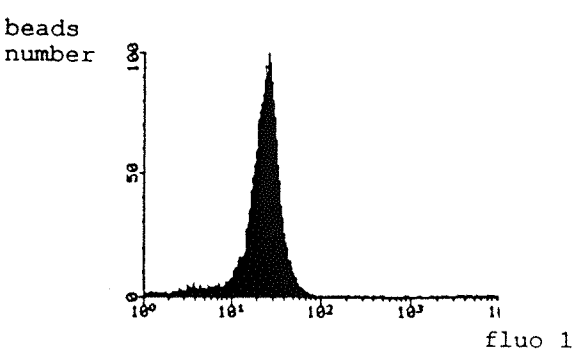
mean : 45.44

BEADS INCUBATED WITH :



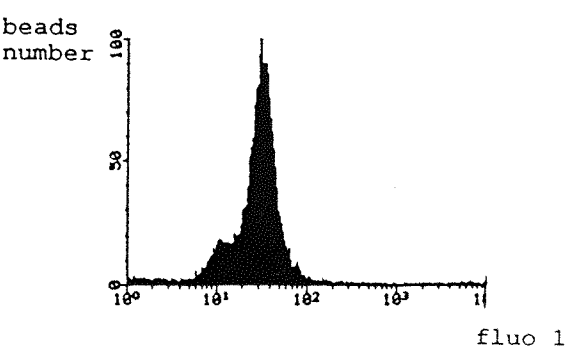
mean : 81.56

IgM AT 10 µg/ml



mean : 82.97

NAMALWA
CULTURE SUPERNATANT



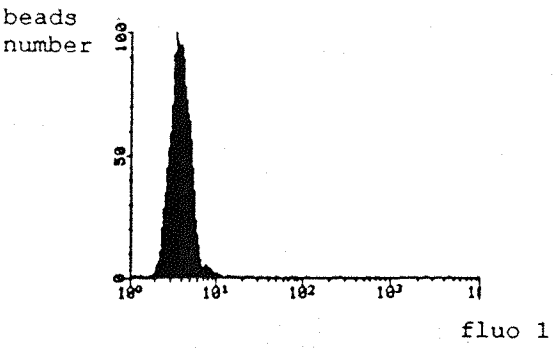
mean : 89.61

NAMALWA LYSATE

B - NEGATIVE CONTROLS

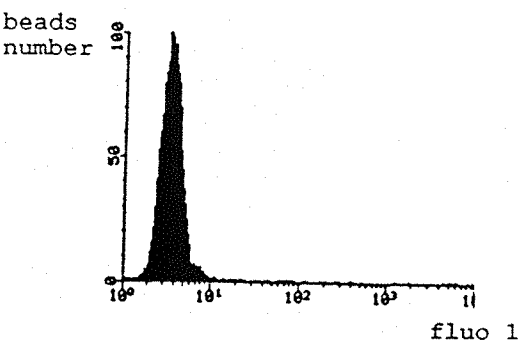
Fluorescent profile of b-M15/8 (anti-human μ monoclonal antibody coated beads) before and after incubation with IgM solution and lysate supernatant. A FITC-conjugated anti-CD37 (WR17*) was used as indicator.

UNINCUBATED BEADS :



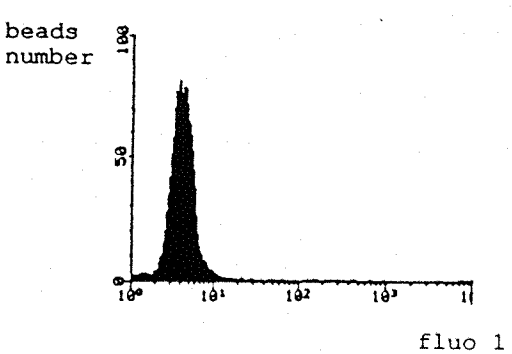
mean : 36.31

BEADS INCUBATED WITH :



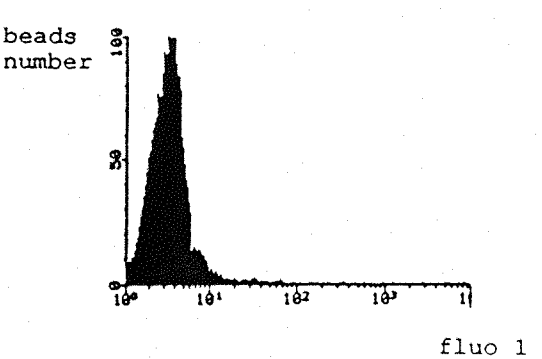
mean : 34.47

IgM AT 10 µg/ml



mean : 37.63

NAMALWA
CULTURE SUPERNATANT



mean : 32.31

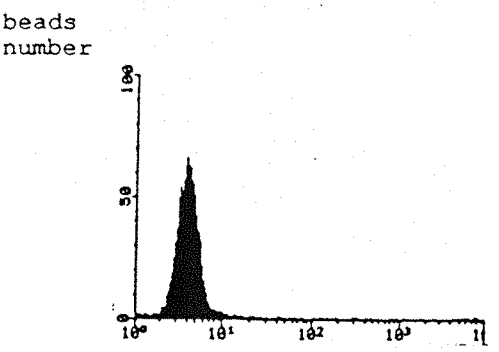
NAMALWA LYSATE

FIGURE 12

SPECIFICITY OF B-WR17

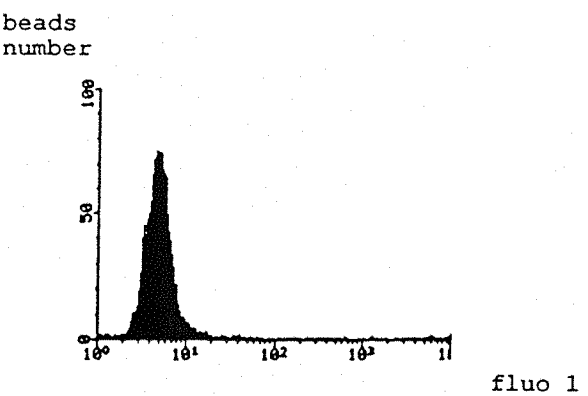
Fluorescent profile of b-WR17 (anti-CD37 monoclonal antibody coated beads) before and after incubation with lysate supernatant. A FITC conjugated goat anti-human Fd μ was used as indicator.

UNINCUBATED BEADS :



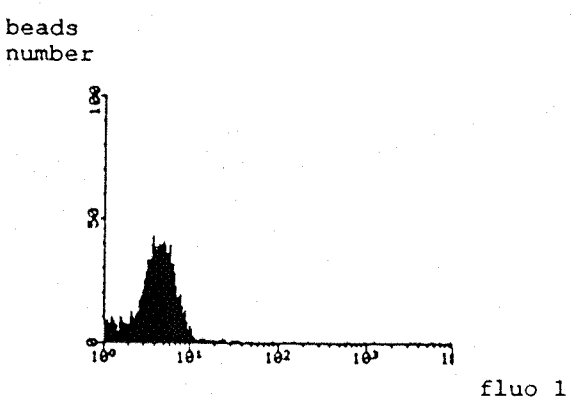
mean : 37.20

BEADS INCUBATED WITH :



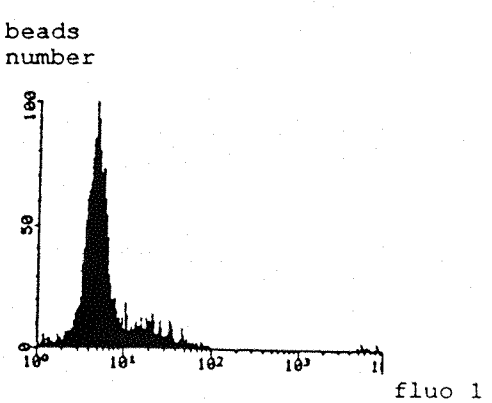
mean : 46.42

IgM AT 10 µg/ml



mean : 37.57

NAMALWA
CULTURE SUPERNATANT



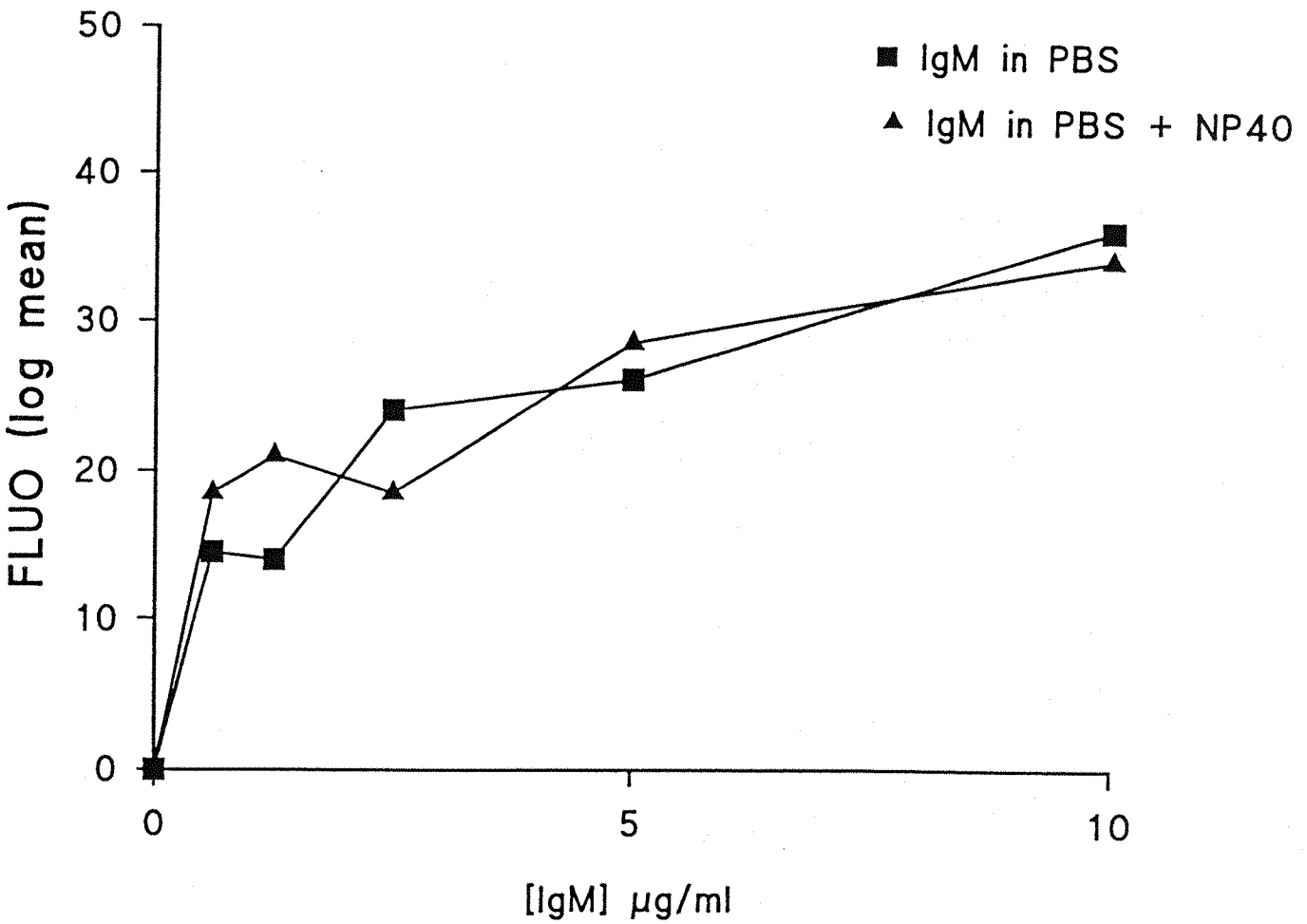
mean : 50.22

NAMALWA LYSATE

FIGURE 13

EFFECT OF NP40 ON BINDING OF ANTIGEN TO ANTIBODY-COATED BEADS

Fluorescent profile of b-M15/8 (anti-human μ monoclonal antibody-coated beads) incubated with IgM either in PBS solution or in 0.1% NP40. A FITC-conjugated anti-human Fd μ polyclonal antibody was used as indicator.



of b-M15/8 was carried out. Attempts to quantify the uptake of ^{125}I-IgM proved difficult owing to too low a number of molecules bound. Hence, investigation relied on ELISA as a very sensitive technique. The procedure involved incubating a large number of b-M15/8 with a solution of Namalwa IgM (cell culture or lysate supernatant). Repeated incubations revealed the attainment of saturation. Depletion of IgM was monitored by ELISA throughout the process. A first batch of 0.25 ml of b-M15/8 at 6 x 10^8 beads/ml was incubated twice in 0.5 ml of Namalwa lysate. Initial IgM concentration was 20 µg/ml, and the successive incubations led to respectively 15 and 18 µg/ml. The total uptake was then 3.5 µg of IgM for 0.25 ml of b-M15/8 resulting to an average of 2.0 x 10^4 IgM molecules per bead. Figure 14 shows the fluorescence intensity of the b-M15/8 incubated twice with Namalwa lysate supernatant at four degres overnight with slow rotation. These profiles shows higher intensity compare to the fluorescence intensity of the beads after a single incubation at room temperature for two hours (fig.11).

3 - DETECTING CD37 ANTIGEN ON ANTIBODY-COATED BEADS

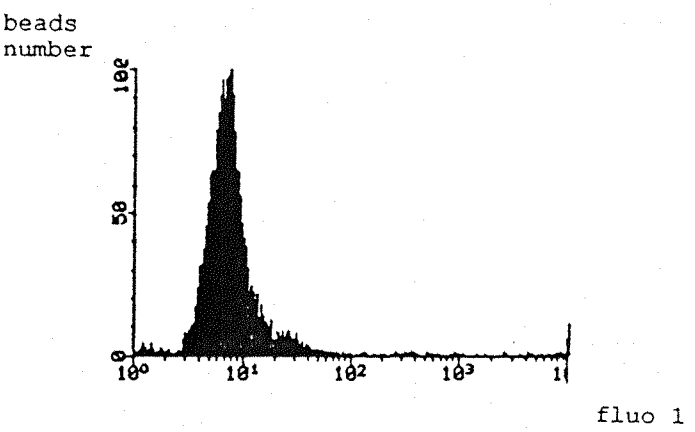
Because no two available monoclonal antibodies could be induced to bind simultaneously to CD37, a particular problem existed in quantifying its uptake on beads: indicator antibody would not find available epitopes on bead antigen (the epitopes being occupied by the bead antibody), and an ELISA was not available for monitoring antigen depletion in absorbed solutions. Accordingly a rosetting-inhibition assay was set

FIGURE 14

FLUORESCENCE OF BEADS-M15/8

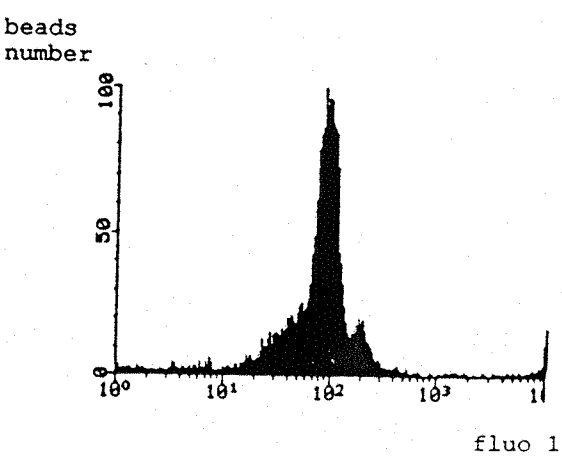
Fluorescent profile of b-M15/8 (anti-human μ monoclonal antibody coated beads) using a FITC-conjugated polyclonal anti-human Fdμ as indicator.

UNINCUBATED BEADS :

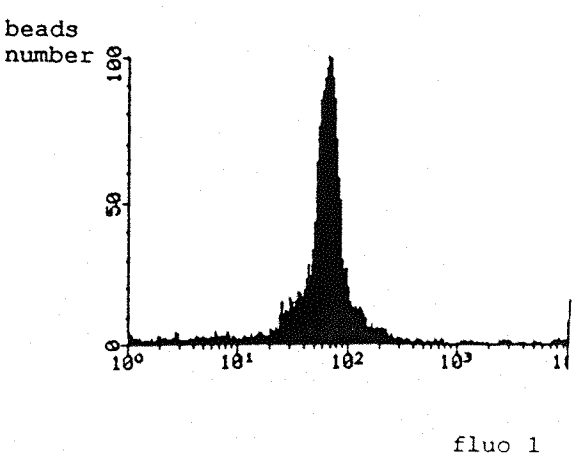


mean : 57.56

IgM SATURATED BEADS :



mean : 119.19



mean : 111.14

up, to compare the rosetting of antibody-coated beads with antigen-bearing cells before and after incubating the beads with solubilized antigen.

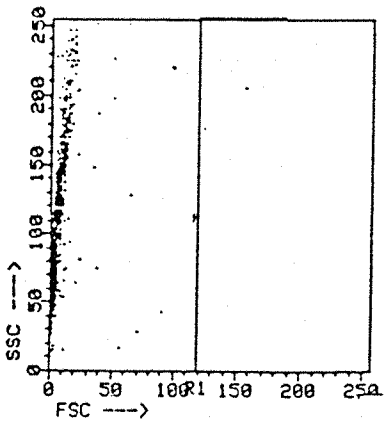
Beads forming a rosette around a cell, which could be observed under the microscope, change the cell scatter profile observed on flow cytometry (Figure 15). From the cytometry profiles distinct localisations of the bead, cell and rosette populations can be observed. Beads present a very low forward scatter, but variable side scatter, and are tightly bunched near the y-axis. Cells have a much higher forward but predominantly low side scatter. It can be seen that the rosetting of cells confers on them a considerable increase in side scatter, so that a semi-quantitative indication of the rosetting of cells can be obtained by (1) gating the forward scatter (above 120) so as to exclude non-rosetting beads; (2) quantifying the proportion of gated cells which lie above the normal side scatter boundary (70).

Negative controls were worked out to establish the extent of rosetting not mediated by antigen-antibody interaction. Figure 16-A compares the rosetting of Daudi cells with b-BSA and b-M15/8, where a small proportion of rosettes is observed for target cells incubated with b-BSA. Also, possible interactions between the Fc of mouse IgG_1 M15/8 and the Fc receptors on human target cells were investigated. Figure 17 shows that the scatter profile of mouse splenocytes incubated with b-M15/8 reveals no significant rosette formation. Figure 18, which exhibits the fluorescence intensity of Daudi cells labelled with a mouse FITC-conjugated

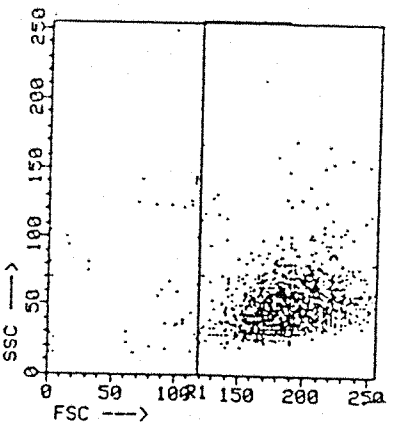
FIGURE 15

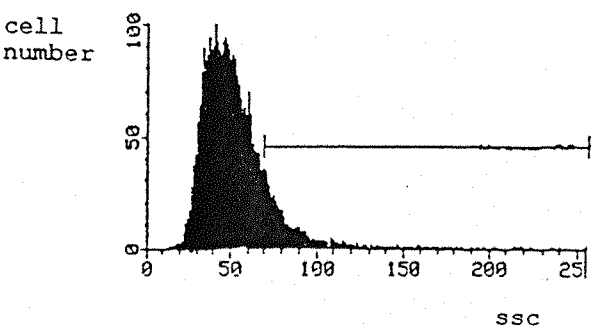
SCATTER PROFILES OF DAUDI CELLS, BEADS AND ROSETTES

BEADS



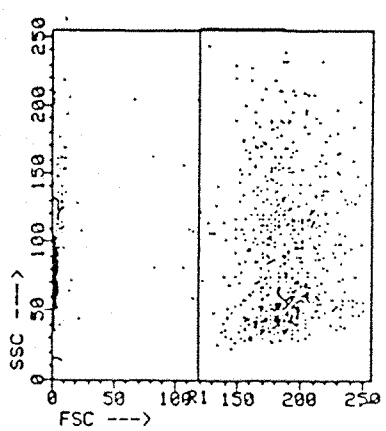
CELLS

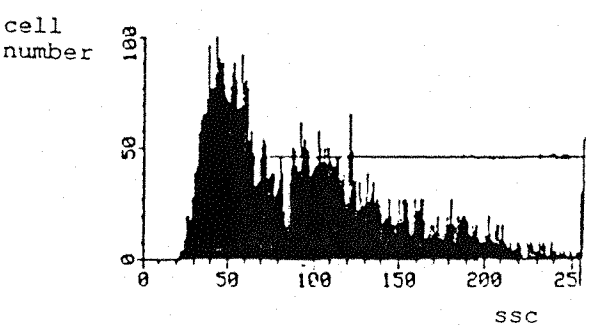




population above
70 (ssc): 13.38 %

BEADS-M15/8 + CELLS





population above
70 (ssc) : 56.88 %

FIGURE 16

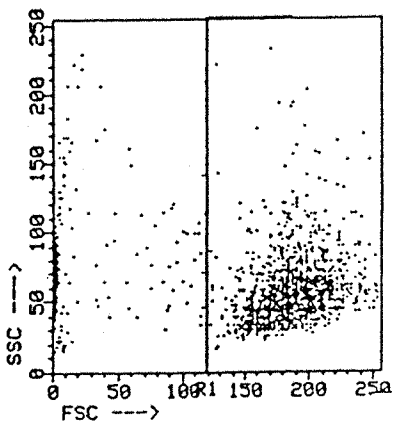
INHIBITION OF ROSETTING

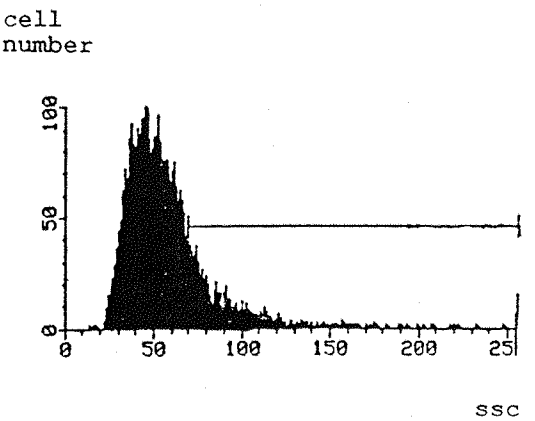
Beads coated with BSA or the antibody M15/8 (anti-human μ) were incubated with Daudi cells.

A - POSITIVE AND NEGATIVE CONTROLS

Scatter profile of cells incubated for half an hour with beads coated with BSA and M15/8 (anti-human μ monoclonal antibody).

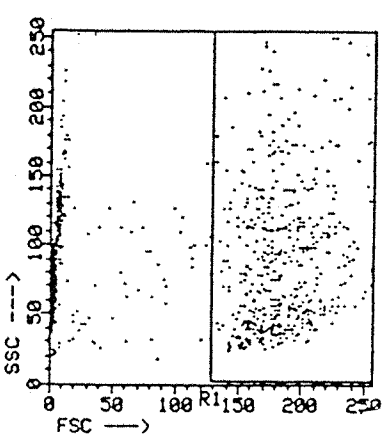
BEADS-BSA + CELLS

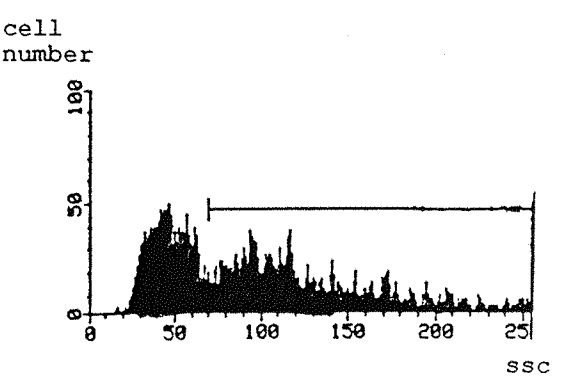




population above 70 (ssc) : 20.02 %

BEADS-M15/8 + CELLS



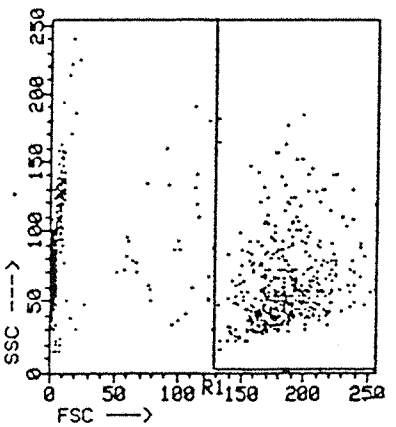


population above 70 (ssc) : 58.51 %

B - INHIBITION OF ROSETTING OF B-M15/8 WITH CELLS (5:1 RATIO)

Scatter profile of cells incubated for half an hour with b-M15/8 (anti-human μ monoclonal antibody coated beads) presaturated with Namalwa IgM. Considering an optimal rosetting at 58.5% and a minimal rosetting at 20%, a 27.2% of rosetting coincides by interpolation with an inhibition of rosetting of 81.5%.

CELLS AND IgM SATURATED BEADS-M15/8



POPULATION ABOVE 70 (ssc) : 27.15 %

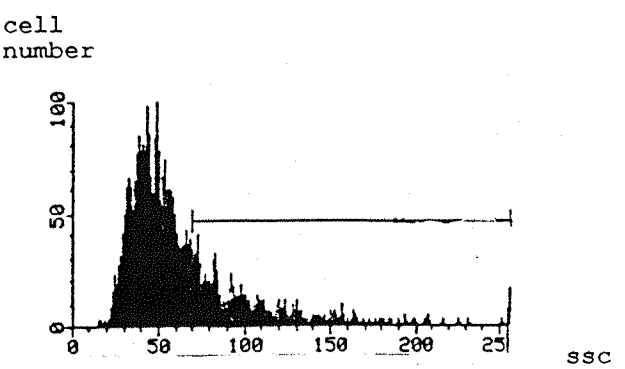
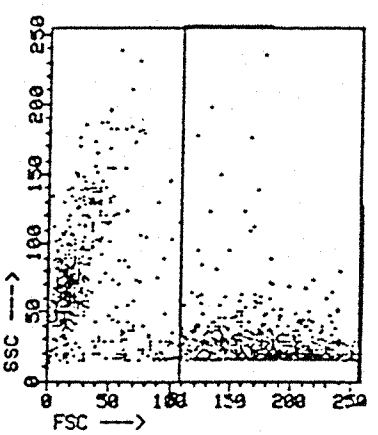
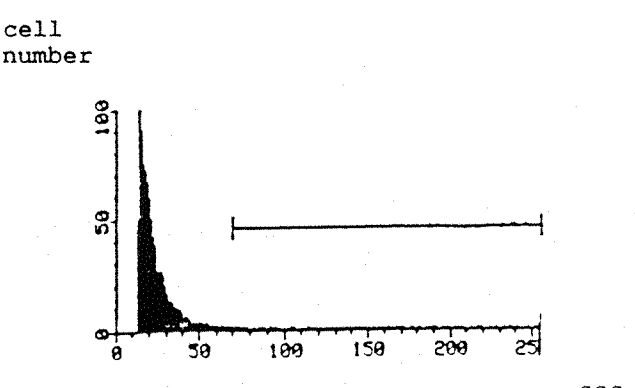


FIGURE 17

SCATTER PROFILE OF MOUSE SPLENOCYTES AND BEADS

Mouse splenocytes were incubated for half an hour at room temperature with beads coated with M15/8 (a mouse IgG_1 anti-human μ monoclonal antibody).



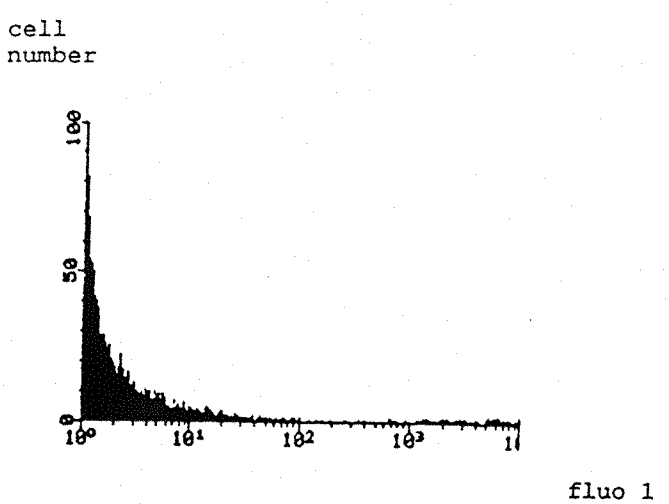


Population above 70 ssc : 6.37%.

FIGURE 18

FLUORESCENCE OF MOUSE SPLENOCYTES

Mouse splenocytes were incubated for 20 minutes on ice with a FITC-conjugated anti-human CD3 monoclonal antibody (OKT3, a mouse IgG1).



mean: 8.55

IgG_1 (OKT3*), shows that the antibody does not bind to the cells. Hence it can be concluded from these two experiments that b-M15/8 is unlikely to cross-react with the human Fc receptor of the target cells and lead to non-specific rosetting.

A rosette titration is shown in Figure 19, in which the rosette population is measured according to the bead:cell ratio. The rosetting of cells incubated with b-M15/8 (■) increases rapidly and levels off at a 50:1 ratio. Cells incubated with b-BSA (▲) form only a small number of rosettes. From this graph, it can be concluded that b-M15/8 bind avidly to target cells.

To examine inhibition of rosetting a bead:cell ratio of 5:1 was chosen to maximize sensitivity. The calculation of inhibition of rosetting was achieved according to the maximal and minimal rosetting at a 5:1 ratio (beads:cells)(fig.16-B). Figure 20 shows the inhibition of rosetting obtained with b-M15/8 exposed to increasing concentrations of IgM, washed, and incubated with Namalwa cells. This graph shows that the percentage of inhibition correlates with the IgM concentration, with 50 % inhibition occurring at an IgM concentration of 3 µg/ml. The sensitivity of the assay therefore permits the detection of antigen on the beads even at a low concentration.

Rosette formation was used to monitor the isolation of CD37 and IgM antigens. Figure 21 shows that the rosetting of b-WR17 with Namalwa cells is inhibited after the beads had been incubated with a Daudi lysate, indicating uptake of CD37

FIGURE 19

ROSETTING OF B-M15/8 WITH CELLS

Beads b-M15/8 were incubated for half an hour at room temperature with Daudi cells, at varying bead:cell ratios, and the percentage of cells rosetted was assessed by gated flow cytometry.

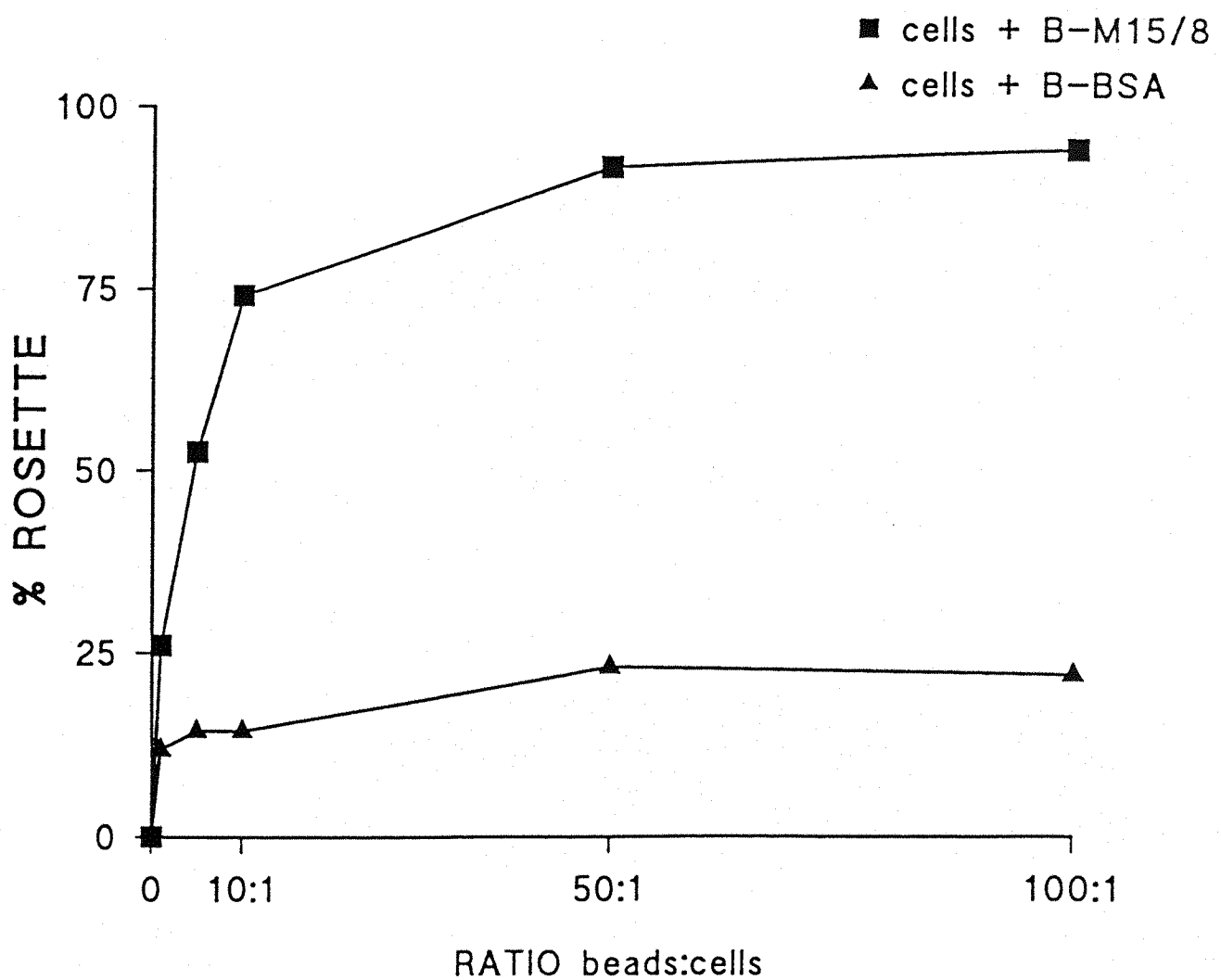


FIGURE 20

INHIBITION OF ROSETTING

Comparing the rosetting of b-M15/8 (anti human μ monoclonal antibody coated beads) with Namalwa cells (at a 5:1 ratio) before and after pre-incubating for 2 hours b-M15/8 with a pure solution of IgM.

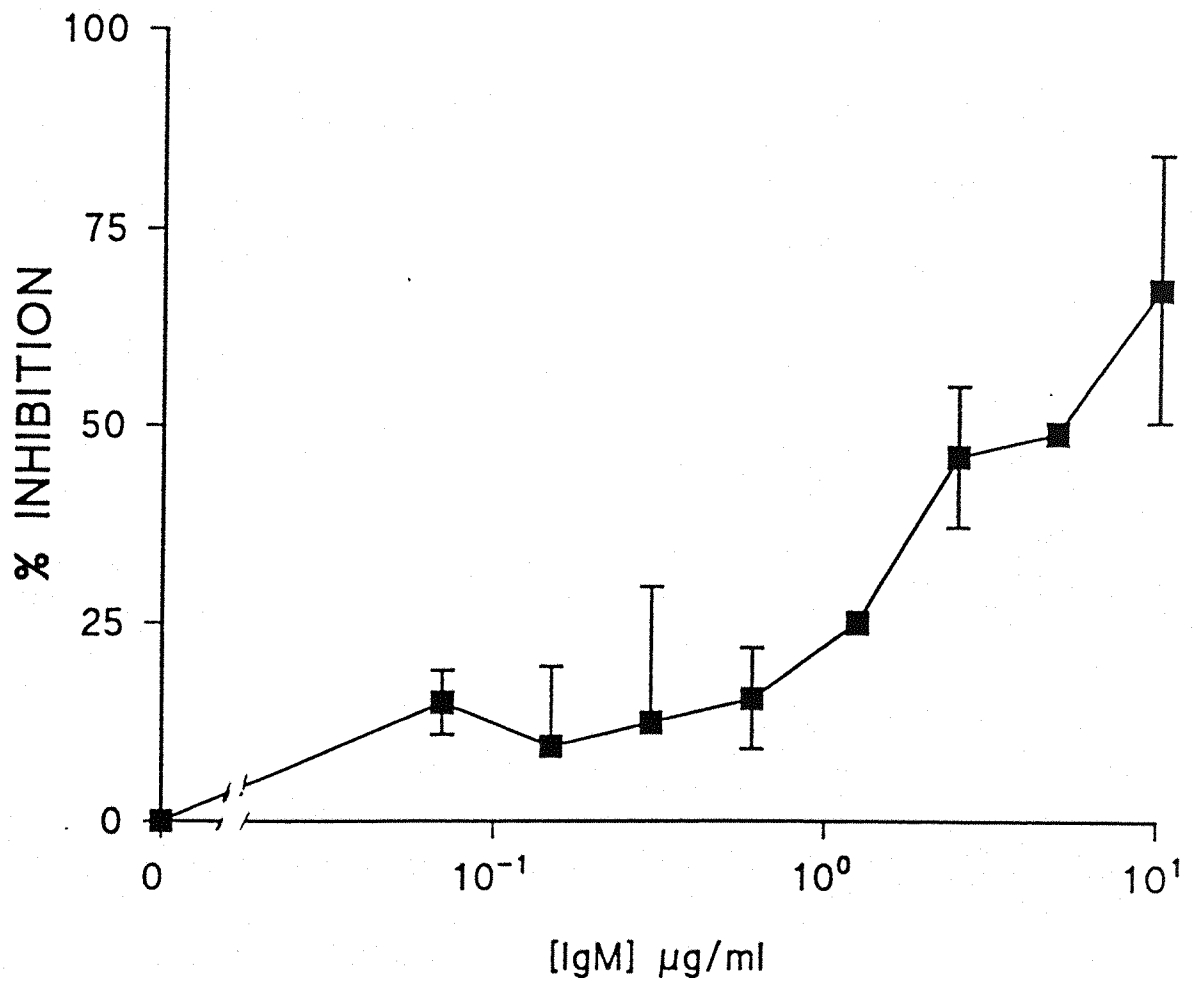


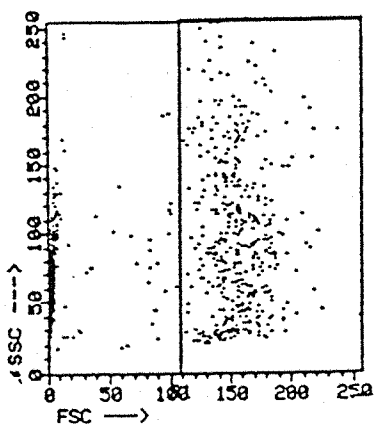
FIGURE 21

INHIBITION OF ROSETTING

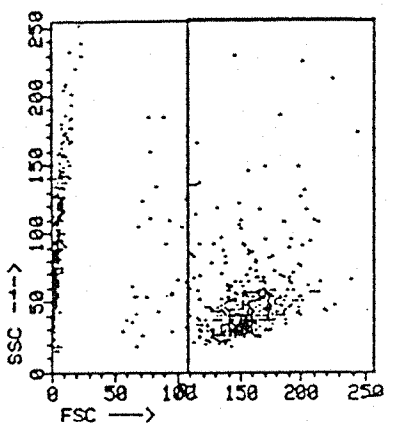
Beads b-WR17 were incubated with buffer or lysate supernatant, and then exposed to Namalwa cells.

NAMALWA CELLS + BEADS-WR17

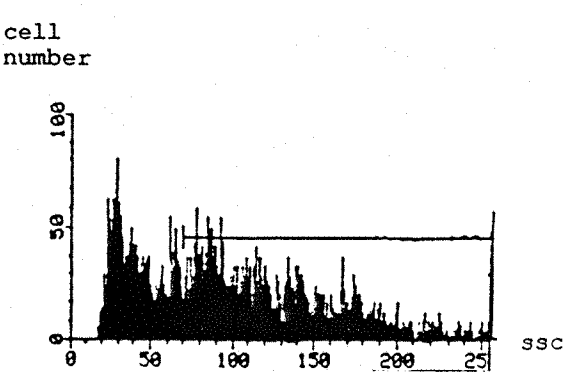
T_0 :
Before incubation with
Daudi lysate supernatant



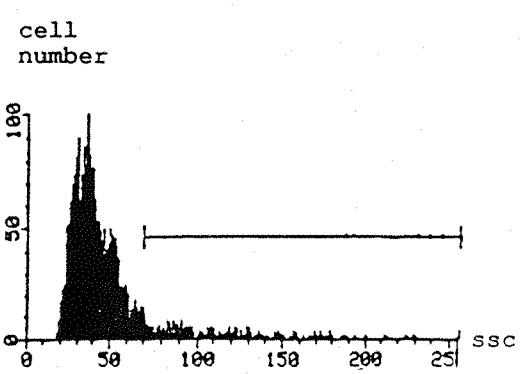
T_t :
After incubation with
Daudi lysate superntant



populaton above 70 (ssc) :



65.22 %



10.73 %

antigen. Similarly, incubation of b-M15/8 with IgM resulted in an average of rosetting inhibition of 81.5% (fig.16-B).

4 - SCHEDULE OF IMMUNISATION

The antigen-coated beads were used as immunogen, using initially an intraperitoneal (i.p) route of injection throughout the schedule of immunisation. On the basis of 2.0 x 10^4 IgM molecules/bead, an estimated amount of 3.5 µg IgM and 0.5 µg of CD37 were given per injection. The immune response was monitored and high serum titers (judged by immunofluorescence) were obtained after a single boost. Mice were boosted once more after a three-week interval via the same route. The final boost by i.p injection lead to immune spleens yielding an average of 1.7 x 10^8 splenocytes, but hybridomas derived from the fusion were not stable. The final boost was therefore performed with an intrasplenic injection, following which the yield of antibody-secreting cells from the spleen vastly increased. Several anti-CD37 monoclonal antibodies were then produced from a single immune spleen.

DISCUSSION

The production of monoclonal antibody against a given cell-surface antigen was shown to be optimized by immunizing with semi-purified antigen attached to beads. However prior to immunization some preliminary studies, that we shall discuss, were carried out both on the preparation of the immunogen and on the route of immunization.

Early work was carried out to detect the presence of CD37 at the cellular level. A comparative study of CD37 expression, with FITC-conjugated anti-CD37 monoclonal antibody, revealed a higher staining on Daudi than on Namalwa cells. The B-cell associated CD37 antigen presents a polydispersity on sieving electrophoresis, and the pattern has differed among B cell lines. The polydispersity is believed to be due to variable glycosylation. In addition to variable oligosaccharide chain lengths, one non-Hodgkin's lymphoma B line has been reported to have an additional oligosaccharide chain (Schwartz, 1988). In the present typing study, both Daudi and Namalwa cell lines derive from Burkitt's lymphoma (Minowada et al, 1981, Nyormoi et al, 1973). The difference in expression of CD37 on their surfaces is presumably due to a difference in the number of expressed molecules, rather than a difference in the number of recognized epitopes per molecule.

Daudi cells were therefore chosen as a source for the preparation of soluble CD37 in bulk.

Further studies at the cellular level were carried out to detect the presence of cytoplasmic CD37. The molecule is widely expressed in association with intracellular membranes, including presumably, the Golgi apparatus (Schwartz, 1988). In spite of this it appears difficult to quantify the amount of intracellular CD37, which may be lowered during fixation and permeabilization processes (Schwartz, 1988).

The antigenic activity of CD37 solubilized by papain was checked initially by inhibition of immunofluorescence, which led to non-reproducible results. Sometimes there was a detectable increase of fluorescence of the indicator cells (data not shown), possibly due to CD37 being aggregated on the cell surface with involvement of Fc-receptors. Further studies using Fab from FITC-conjugated anti-CD37 still failed to detect CD37 satisfactorily. However it was shown that CD37 was solubilized using the surfactant NP40, with retention of antigenic activity. NP40, a non-ionic detergent, creates a shielded hydrophobic microenvironment and replaces the lipid bilayer of the membrane with soluble micelles (Parish et al, 1986). This surfactant extracts proteins by interacting only with the lipophilic region of the molecule without denaturing the remainder (Helenius, 1975). Despite the fact that Daudi

lysate led to an appreciable inhibiton of immunofluorescence using WR17* (FITC conjugated anti-CD37 monoclonal antibody), it might be worthwhile emphasising that this adsorption analysis technique was fairly insensitive. It became necessary to optimize the assay by using a half-saturating level of indicator antibody. It appeared that antibodies would bind better to the cell surface than to soluble antigen, which could be due both to a cooperative binding through a Fc interaction (Greenspan et al, 1989) and to a higher affinity of antibody for cell surface antigen due to the lower dissociation constant of antibody bivalently bound to the antigen through both Fab (Mason and Williams, 1980). The positive control for this assay was set up with FITC-conjugated anti-human IgM monoclonal antibody (M15/8*) and soluble IgM. As judged by its ability to yield immunofluorecence on indicator cells, a polyclonal antibody could not be fully adsorbed at a high IgM concentration (data not shown). Likewise, an adsorption of M15/8* which reduced immunofluorescence by 50% required a higher concentration of soluble antigen than at the cell surface, assuming an average of 10^5 surface antigen molecules per cell. On the other hand, using a monoclonal antibody for detecting the antigen from a lysate was hampered by the interfering action of NP40 on the antigen-antibody complex formation and an appropriate NP40 concentration should be investigated. This interaction of NP40 was not detectable when using a polyclonal antibody (Crumpton, 1972).

The strategy of isolating antigen on beads does not

represent a rigorous purification. After extraction of a lysate the beads presumably have bound to their surfaces micelles which could incorporate molecules additional to the antigen sought. However the antigen has at least been semi-purified. Furthermore it is now in an immunogenic form, so one is offered the convenience of simply injecting unwashed beads into an animal which is to be immunized (Stevenson, 1974).

Initially the route of injection was intra-peritoneal throughout the schedule of immunisation. The cell population of this serous cavity is composed mainly of macrophages and lymphocytes, which thereby can supply an appreciable immune response (Van furth, 1970). Macrophages acting as APC (antigen presenting cells) would presumably enhance greatly the inflamation. It has been shown that agarose beads coated with the complement factor C_{3b} were engulfed by macrophages through their C_{3b} receptors (Pettersen et al, 1990). In the present situation, beads would presumably be phagocytosed by macrophages due to opsonization by Fc regions of antibodies, and possibly also by host complement activated by the antibody. The immune response following the intraperitoneal schedule showed a substantial antibody response as judged both by a high titre and a large immune spleen (average size 2.6×10^8 cells/spleen). However, the local immune response was not sufficiently strong to induce B cells into the appropriate stage of maturation for fusing. Optimal conditions for production of hybridoma require the maturating B cells to reach the early blast stage (highest dividing rate) before

secretory stage (Galfre and Milstein, 1981). An intrasplenic injection was thus performed to provoke the B-cell maturation at the stage prior to fusing (Spitz et al, 1984, Tosic et al, 1989).

Preparation of monoclonal antibody against cell-surface antigens can be performed by immunizing with whole cells without any separation of the antigen (Schwartz et al, 1988, Link et al, 1986). This requires the use of an adjuvant or multiple intra-venous injections of the antigen to enhance the immune response (Schwartz et al, 1988). However, the response to the desired antigen is somehow weakened due to the diverse responses to an immense number of antigenic sites on the cell surface. In contrast immunisation with antigen immunosorbed onto particles focusses and enhances the immune response (Stevenson, 1974). As shown in this study, several monoclonal anti-CD37 antibodies were obtained as a result of using the adsorbed immunogen. The amount of CD37 antigen per boost probably lay between 0.1 and 0.3 μg, which would encourage selection of clones producing antibody antibody of high affinity (Jerne, 1955, Burnet, 1957).

The present work focussed on the preparation of a B-cell surface antigen in order to optimize its presentation during immunisation for subsequent production of monoclonal antibodies. Investigations carried out on CD37 showed that this protein can be prepared in bulk, with antigenic activity intact, from lysates of Daudi cells yielded by NP40. Moreover, the detection of this antigen in the fluid phase required setting up a rosette-inhibition assay that might be

of general use in the detection of antigens when only a single monoclonal antibody is available. By using this assay, the amount of antigen bound to the beads can be gauged approximately before immunizing mice with the whole complex of antigen-laden beads. The overall strategy succeeded in focusing the immune response towards the antigen and led to the raising of a set of monoclonal antibodies from a single immune spleen.

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APPENDIX

Standard PBS

NaCl	7.0 g/l
Na_2HPO_4	3.44 g/l
KH_2PO_4	0.79 g/l

PBS/BSA/Azide

Standard PBS +	BSA	10 g/l
	Azide	1.08 g/l

PBS/TWEEN

0.5 ml tween per liter of PBS

0.2 M TE.8

tris	24.2 g/l
5 M HCl	20 ml/l
EDTA Na_2	3.72 g/l

BORATE BUFFER

0.05 M borate solution pH 9.5 (H_3BO_3, NaOH)

ELISA BUFFER

Citric acid	19.2 g/l
Na_2HPO_4	28.4 g/l

COATING BUFFER

NA_2CO_3	1.59 g/l
$NAHCO_3$	2.93 g/l

O.P.D (orto-phenylene diamine)

O.P.D	0.02 g/100 ml
H_2O	50 ml
Citrate buffer	24 ml
phosphate buffer	26 ml
H_2O_2	20 µl/100 ml

CO_3^{2-}/HCO_3 BUFFER (pH: 9.5)

$NaHCO_3$	3.7 g/100 ml
Na_2CO_3	1.03 g/100 ml

Adjust to pH 9.5 with NaOH