CELL SEPARATIONS BY IMMUNOAFFINITY PARTITION

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ABSTRACT

The partition of cells in an aqueous polymer two-phase system composed of dextran T500, polyethylene glycol 8000 and buffer was studied. The effect of various immunoaffinity ligands on erythrocytes and lymphocytes was examined. Separations of rabbit and human erythrocytes were achieved using a combination of monoclonal mouse anti-NN glycophorin IgG with trypan blue-derivatized sheep anti-mouse F_c fragment IgG as well as with a polyacrylamide-derivatized rabbit anti-human erythrocyte IgG (PAA-rαhrbc). The PAA-rαhrbc was able to completely resolve the two erythrocyte species in a countercurrent distribution (CCD) of 20 transfers. The lymphocyte separation problem was of two sub-lines of a transformed mouse lymphocyte, MBL-2(4.1) and MBL-2(2.6), which differ in the surface densities of an antigen recognized by a rat monoclonal IgG, YE1.48.10. Binding studies showed that at saturation MBL-2(4.1) bound 2.4×10^6 molecules of YE1.48.10. per cell whereas MBL-2(2.6) bound 8×10^5 molecules of YE1.48.10. per cell. This represented an extremely stringent separation problem compared to previous immunoaffinity erythrocyte separations. Attempts to separate the cells by immunoaffinity partition using the following combinations of ligands were not sufficiently successful to achieve useful separations: (a.) PEG 1900-derivatized YE1.48.10. (b.) PEG 1900-derivatized RG7/11.1, a mouse monoclonal IgG specific for rat F_c fragment, and YE1.48.10. (c.) biotin-derivatized YE1.48.10. and PEG-derivatized avidin. However polyacrylamide grafted onto YE1.48.10. produced an effective immunoaffinity ligand, separating MBL-2(4.1) and MBL-2(2.6) on the basis of their antigenic differences. A separation was achieved in 60 CCD transfers.

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CHAPTER 1

METHODS OF CELL SEPARATION

The present need for methods to separate, identify and characterize different types of cells is recognized amongst immunologists, hematologists, cell biologists, clinical pathologists and biomedical researchers. Unless cells exhibiting different functions or at different stages of differentiation are separable it will be difficult to study some of the mechanisms involved in cell recognition, specialization, cytotoxicity and transformation. Some of the immediate practical benefits obtained by applying cell separation techniques are the clinical diagnosis of disease and the use of isolated cells for therapeutic (*e.g.* immunotherapy) or survival (*e.g.* transfusion) purposes. A number of techniques for cell separation exist based on the physical or biological properties of the cells and a brief discussion of these methods follows:

1. Sedimentation. The technique of velocity sedimentation was first described by Lindahl (1948) who termed it counter streaming centrifugation. However little interest was shown in the method until the late 1960's when several adaptations of the technique such as sedimentation at unit gravity (Peterson and Evans, 1967; Miller and Phillips, 1969), elutriation (McEwen *et al*, 1968; Meistrich *et al*, 1977; 1981), sedimentation in an isokinetic gradient (Pretlow, 1971; Pretlow *et al*, 1975) and centrifugation using a reorienting gradient zonal rotor (Wells *et al*, 1977a, b) increased the efficiency and popularity of sedimentation techniques.

Velocity sedimentation can be carried out using a continuous or discontinuous density gradient. In the case of the discontinuous gradient the chamber is filled with successively less dense solutions resulting in interfaces at which the solution density changes abruptly. This has been largely superseded by the use of continuous gradients, partially to avoid the problem of the cells collecting at the interfaces where they tend to aggregate. However one frequently used discontinuous gradient method employs a Ficoll/Hypaque (sodium diatrizoate) density

gradient to remove erythrocytes from blood (Auiti *et al*, 1974). The more common gradient forming polymers include Ficoll, albumin and Percoll (colloidal silica). The sedimentation of cells in a centrifugal field is described by (Pretlow and Pretlow, 1982):

$$\frac{dr}{dt} = \frac{a^2(D_c - D_m)\omega^2 r}{k\eta} \qquad (1)$$

where r is the distance of the cell from the center of revolution, t is time, a is the diameter or radius (depending upon the value of k) of the cell, D_c is the density of the cell, D_m is the density of the gradient at the location of the cell, ω is the angular velocity (the speed of the centrifugation), η is the viscosity of the gradient at the location of the cell, and k is a constant.

Velocity sedimentation uses both cell density and diameter as the criteria for separation since insufficient centrifugation time is used for the cells to reach their respective buoyant densities. For this reason it is generally considered superior to isopycnic sedimentation where sufficient force is applied for sufficient time to allow the cells to arrive at their respective buoyant densities therefore making cell density the only criteria for separation (Pretlow and Pretlow, 1982).

Differential sedimentation at unit gravity or differential centrifugation does not make use of a density gradient. The less rapidly sedimenting cells remain in suspension (Anderson, 1966). This generally gives poor resolution and may need to be repeated several times resulting in the loss of some of the desired cells. Sedimentation at unit gravity is sometimes combined with electrophoresis where the applied field is across the density gradient to give a 2-dimensional separation (Platsoucas, 1983).

Centrifugal elutriation balances the outwardly directed inertial (centrifugal) forces acting on a cell against an inwardly directed hydrodynamic force created by pumping fluid through a chamber in the centrifugal rotor. This is usually arranged such that the hydrodynamic force is slightly dominant causing the cells to move inward, *i.e.* those with low sedimentation rates move inwards faster.

The main disadvantages of velocity sedimentation techniques are

that the basis for separation is not directly involved with cell function and care must be taken to maintain an isosmotic gradient to avoid selective shrinking of the cells. The capacity is small, usually in the order of $1\text{-}5\times10^{7}$ cells although large rotors have been used for up to 10^{10} cells (Wells and James, 1972). If the capacity is exceeded in any one band the cells in that particular band will tend to sink lower in the gradient. This is a result of the concentration of sedimented substances sufficiently altering the density of the part of the gradient where they are located to exceed the density of the centrifugally located gradient. The denser solution then sediments rapidly as a large bolus to a denser part of the gradient. There are problems with aggregation especially at the wall of the tube where cells tend to collect since the force vectors radiate from the center of the rotor. Swirling during acceleration and deceleration can be a problem as well as the streaming of cells left standing in the gradient for extended time periods.

2. Single cell sorting and multiparameter analysis. This technique sorts cells individually according to the signal generated when fluorescently labelled or optically characterized cells interact with a light beam as they pass by in suspension in single file. The development of high speed flow systems for single cell sorting combined with automated analysis has made it possible to resolve homogeneous cell populations from heterogeneous populations. Sorting is rapid, usually based on electrostatically charging drops containing cells of interest. Typically 10^{5} cells per minute may be analyzed and the data displayed as frequency histograms. Since many cells may be examined the statistical precision is high and small sub-populations can be detected and separated with confidence *e.g.* 1-5% of the total population.

The beginnings of flow cytometry occurred in 1934 when Moldaven reported a bright-field photometric method for counting individual cells in liquid suspension flowing through a capillary tube located on a microscope stage (Moldaven, 1934). With the development of the Coulter counter and size analyzer in the mid 1950's contributing significantly, the first system capable of automatically measuring multiple parameters was described by Kamentsky *et al* (1965), who sorted cells on the basis

of uv light absorption and visible light scatter as they flowed through a narrow channel under a microscope. Modern multiparameter cell sorters combine both optical and electrical sensing techniques to discriminate according to preselected parameters relating to cell volume and surface area, total or two color fluorescence from stains bound to specific biochemical features and light scatter related to internal and external cellular characteristics. Other parameters measured include axial light extinction, time of flight and membrane polarization.

The main disadvantage of single cell sorting is that the apparatus is very expensive, large cell numbers cannot be handled and problems may be encountered in disaggregating the cells and keeping them monodisperse during fixing, staining and analysis. In addition only two or three isolated sub-populations result from the process, limiting its capacity for separation in comparison to the analytical capability of the method. For further information see Steinkamp *et al* (1974); Mullaney *et al* (1976) and Preffer and Colvin (1987).

3. Magnetic separation. In this method cells are made weakly magnetic either on the basis of intrinsic magnetization, *e.g.* erythrocytes, or by specific labelling to make them magnetizable for the purposes of the separation, *e.g.* by attaching paramagnetic materials to lectins or antibodies. The separation of magnetic from non-magnetic cells in a suspension is carried out by a flow system through permanent magnets (low gradient magnetic filtration) or by high gradient magnetic filtration. In the latter case a column is loosely packed with fine magnetic stainless steel wire and placed between the poles of an electro- or super-conducting magnet. As the cell suspension is passed through the column the magnetizable cells become bound to the wire. The small diameter of the wire ensures that the distance over which the induced magnetic field vanishes is small. This means that a large field gradient is generated providing the necessary attractive force. The column must be sufficiently long to ensure that each cell will come close enough to the wire to be attracted if magnetizable. Loose packing of the column means that few problems due to clogging are encountered (Oder, 1976; Owen and Liberti, 1987). Some methods which are used to generate paramagnetic cells include:

a. Phagocytosis. Cells which ingest magnetic compounds may be removed from those incapable of rapid phagocytosis *e.g.* the incubation of spleen cells with carbonyl iron is used to deplete macrophages from cell suspension (Hudson and Hay, 1976). Several trivalent lanthanide ions such as erbium and dysprosium have been incorporated into cells rendering them magnetic but no selective uptake has been demonstrated (Graham and Selvin, 1982).

b. Pre-existing magnetic compounds. Erythrocytes are intrinsically non-magnetic but the iron present in the hemoglobin may be rendered paramagnetic by the removal of reversibly bound oxygen. This may be done by the addition of sodium dithionite or the use of an artificial lung machine. The low efficiency of erythrocyte removal (60-70%) makes this method more appropriate for the removal of erythrocytes from blood prior to a more rigorous separation procedure (Paul *et al*, 1978). Malarial parasites are known to cause intracellular degradation of hemoglobin. Some of the breakdown products are paramagnetic which may prove useful in malaria diagnosis (Paul *et al*, 1981).

Another method of rendering erythrocytes paramagnetic is the formation of methemoglobin by treatment of oxy-hemoglobin with sodium nitrite (15-30 mM). The loss of one electron from each heme-iron results in a stable paramagnetic erythrocyte.

Due to the relative inefficiency of the procedures discussed in a and b magnetic separation techniques are not used extensively to debulk red blood cells from whole blood, but targeted cells have been studied in greater detail.

c. Rosetting. When an excess of anti-immunoglobulin or protein-A coated sheep erythrocytes are centrifuged with immunoglobulin-coated lymphocytes, the erythrocytes will adhere to the surface of the lymphocytes forming rosettes (Goding, 1983). If sheep red blood cells are treated with nitrite to form methemoglobin then used to rosette T-lymphocytes, the rosetted T-lymphocytes may be magnetically removed from the suspension (Owen, 1982).

d. Magnetic particles. Coating magnetic spheres with targeting agents such as antibodies has produced a variety of particles which can bind to specific types of cells, thus rendering them magnetic. The first magnetic particles were agarose-polyacrylamide beads (Guesdon and Aurameas, 1977) but since then microspheres of starch (Mosbach and Schröder, 1979), dextran and agarose (Molday and MacKenzie, 1982; Schröder and Mosbach, 1983), cellulose (Forrest and Landon, 1978), polystyrene (Ugelstad *et al*, 1983; Treleaven *et al*, 1984), styrene-divinyl benzene (Ugelstad *et al*, 1984), methacrylate (Molday *et al*, 1977) and heat-denatured albumin (Widder *et al*, 1981) containing Fe_3O_4 have been used. The most monodisperse and uniform microspheres are those made of styrene-divinyl benzene (Ugelstad *et al*, 1983).

Magnetic microspheres coated with antibody have been used to remove tumor cells from bone marrow (Treleaven *et al*, 1984). The use of sheep anti-mouse IgG coated spheres means that the same beads may be used for separations of different malignancies where the marrow is pretreated with monoclonal IgGs of differing specificities. These beads have also been used for the separation of T- and B-lymphocytes (Schröder *et al*, 1986). The main disadvantage of this method, as with any solid-phase affinity technique, is the problem of non-specific absorption discussed later in the text. In addition high magnetic fields can be required, making the method expensive, particularly when liquid helium-cooled magnets are employed.

4. Solid phase affinity fractionation. This technique involves the specific binding of cells directly to a solid phase support. Often the support is modified by the attachment of ligands. Various ligands have been used; antibodies provided the first cell separation by this method (Wigzell and Anderson, 1969) but avidin (Basch *et al*, 1983), protein A (Langone, 1982) and several lectins (Sharon, 1984) have also been used. There are two serious drawbacks to this technique. First a substantial fraction of the cells often become non-specifically bound to the matrix. Supports of hydrophilic polymer gels such as dextran (Schlossman and Hudson, 1973), agarose (Carton and Nurden, 1979), polyacrylamide (Baran *et al*, 1982), poly(2-hydroxyethyl methacrylate) (Tlaskalová-Hogenová *et al*, 1981) and a copolymer of acrylamide and N-acryloyl-2-amino-

2-hydroxymethyl-1-3-propane diol (Bonnafous *et al*, 1983) have been reported to show less non-specific absorption than conventional matrices such as glass and poly(methyl methacrylate).

Another type of affinity cell selection uses nylon strings to which ligands are physically adsorbed. The fibers are incubated with the cell suspension and cells bound to the fibers are recovered by plucking each fiber with a needle (Edelman *et al*, 1971). Another "panning" method uses a plastic petri dish as the support (Wysocki and Sato, 1978).

A group of block and graft copolymers have been found to eliminate adsorption and subsequent contact-induced activation of platelets (Akemi *et al*, 1986). These polyamine graft copolymer matrices have been used to separate T- and B-lymphocytes (Kataoka *et al*, 1988).

5. Electrophoresis. Electrophoretic methods of cell separation are based on differences in the density and distribution of the surface charges on a cell. The electrophoretic mobility is a complex function of the number of charge-bearing lipid, protein and glycoprotein species on the surface, the depth and charge distribution of this surface-bound material and the ionic strength of the suspending medium (Levine *et al*, 1983; Sharp and Brooks, 1985). The more common electrophoretic methods of cell separation are:

a. Free-flow electrophoresis. This may be done in a vertical or horizontal, rotating column. The cells are introduced into a flowing buffer curtain and deflected to varying degrees within the buffer curtain. They are collected at the end of the column into tubes depending on their position within the buffer (Hannig, 1972). Problems with this method include sedimentation of cells due to gravity and the creation of thermal convection currents.

b. Dielectrophoresis. In this case an alternating current is applied which causes the charged cells to oscillate around a mean position whereas the polarization of neutral particles means that they will migrate to regions of high field intensity (Pohl, 1977).

c. Density gradient electrophoresis. This is done in a vertical column

containing a density gradient, usually formed with ficoll/sucrose (Platsoucas, 1983). The density gradient helps to avoid sedimentation problems but the very low ionic strength buffers that must be employed in order to minimize electric heating, as this results in mixing of the cells by convection currents, may cause cell damage (Seaman, 1975).

6. Biological Separations. These methods are applicable to cells which may be maintained in culture for limited or extended time periods. The techniques include differential outgrowth from tissue fragments attached to a culture dish (Lechner *et al*, 1981) or differential attachment which is frequently used to separate fibroblasts from epithelial cells (Kasten, 1973). Differential detachment using enzymes and/or chelating agents is commonly used (Rheinwald, 1980) as well as differential digestion with trypsin or collagenase (Owens *et al*, 1976). Perfusion has been used to isolate rat liver parenchymal cells (Seglen, 1973). Cloning is the most rigorous separation procedure available but a considerable fraction of the culture life span can be used up in the isolation procedure (Ham, 1972).

The use of selective reagents exploits the differences between dividing and non-dividing cells, normal and neoplastic cells and specific cell types, *e.g.* nucleic acid components and analogs may be used to selectively block metabolic pathways of unwanted cell types as in HAT selection for hybridoma cells (Littlefield, 1964). Deficiencies in particular amino acids may be used to suppress growth of certain cell types (Leffert and Paul, 1972) or substitution of some amino acids may be selective, *e.g.* the replacement of L-valine by D-valine will select out epithelial cells (Gilbert and Migeon, 1975).

In some cases selective media may be used. This usually involves the absence of serum with or without the addition of hormones and growth factors (Sato, 1975). Optimization of the basal medium may also give some degree of selectivity (Jennings and Ham, 1983; Lechner *et al*, 1982; Kaighn *et al*, 1983).

7. Partitioning in aqueous polymer two-phase systems. Despite this variety of cell separation methods with varying advantages and disadvantages, most cell separations are based on properties not

directly associated with cell function, *i.e.*, size, density, shape or charge of the cell. Cell sorting and solid phase affinity techniques are more selective because of their use of specific ligands. These methods, based on cell surface biochemistry, are likely to be associated with cell function or dysfunction and to be more discriminating. However individual cell sorting is expensive and impractical for bulk separations and the use of solid phase supports generally poses problems in cell removal from the solid support and non-specific adsorption.

One method of cell separation which depends primarily on cell surface properties is partitioning in aqueous polymer two-phase systems (APTS) and it is with this technique that this thesis is concerned. Partitioning in an APTS involves the unequal distribution of cells between the interface and one of the phases of a two phase system composed of two incompatible polymers, usually polyethylene glycol (PEG) and dextran (dx), and an aqueous buffer. A typical procedure, Fig 1., involves adding the cells to the APTS, mixing the phases then allowing them to settle, followed by sampling and analysis. It is analogous to a traditional solvent extraction. As the separation is performed in solution there is no problem with non-specific adsorption and the method has been scaled up almost 40,000 times with no loss in resolution when used for enzyme purification (Kroner *et al*, 1982).

It is a well established fact that a two phase system can be formed from two incompatible neutral polymers in a common solvent (Flory, 1953) and while many other types of two and multiphase systems exist, the former is the only type used to any degree for cell separations. Since the common solvent is generally water, these two phase systems may be buffered and made isotonic, making them compatible with biological materials and living cells. Partitioning in aqueous polymer two phase systems has proven to be a most versatile separation technique, having been used to separate amino acids, nucleic acids, membrane fragments, organelles, micro-organisms and cells, amongst others. The partition coefficient, K, is defined as the ratio of the concentration of solute in the upper phase to the concentration in the lower phase (*i.e.* $K = C_T/C_B$) and in the case of cells or particles as the ratio of the number of cells or particles in the upper phase to total number at the interface and in the lower phase (*i.e.* $K = n_T/(n_I + n_B)$. If the single

step partition process is repeated by exposing each phase to fresh complementary phases in a continuous manner, a multistep procedure of increased resolution can be developed known as a countercurrent distribution (CCD). This is the discrete analog of, and precursor to, chromatography.

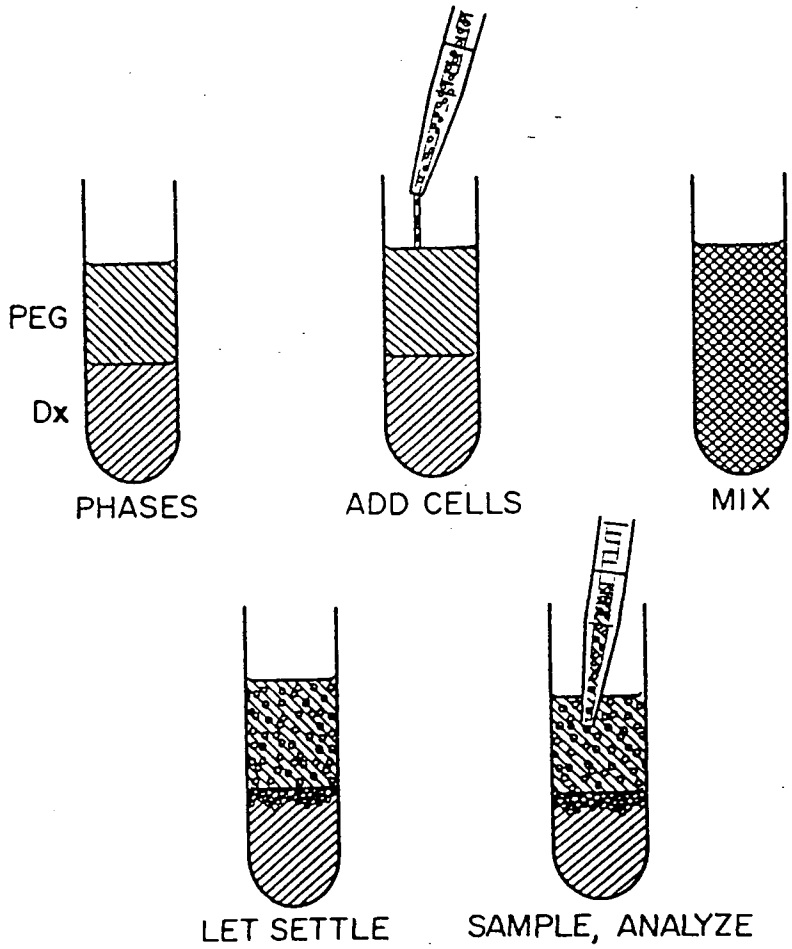


Fig. 1. A typical cell partitioning experiment is illustrated. The phase system is composed of polyethylene glycol (PEG), dextran (dx) and an aqueous buffer. From Walter (1969).

a. History of the development of partitioning in aqueous polymer two-phase systems. The use of APTS for the separation of biological material was developed by Albertsson (1956) who used a two-phase system formed by mixing PEG and phosphate to isolate chloroplasts from green algae. He went on to pioneer the use of two incompatible hydrophilic polymers as the APTS forming agents which enabled separations at physiological pH and tonicity. By the use of CCD he demonstrated that the partition coefficient observed in an APTS composed of two polymers was a reversible, thermodynamic partition ratio rather than an adsorption process for both particles and cells (Albertsson and Nyns, 1959; 1961; Baird *et al*, 1961; Albertsson and Baird, 1962). Albertsson also studied the relationship between the molecular weight or surface area of proteins and viruses and their partition coefficient (Albertsson, 1958, 1959), isolated ribosomes from rat brain microsomes (Albertsson *et al*, 1959) and concentrated and purified viruses (Frick and Albertsson, 1959). Antibody-antigen binding was also found to affect the partition coefficient (Albertsson and Philipson, 1960). All this information was published as a book (Albertsson, 1960) which is now in it's third edition (Albertsson, 1986). The development of a thin layer CCD apparatus which decreased settling time compared to the traditional Craig apparatus increased the resolution over a single partition step (Albertsson *et al*, 1965) and enzyme purifications by partition using salt-PEG systems were accomplished (Okazaki and Kornberg, 1964). Salt-PEG systems are now used extensively for industrial scale enzyme separations (Hustedt *et al*, 1985).

It was known that small changes in the ionic composition of the APTS had a large effect on the partition of proteins, nucleic acids or cells (Albertsson, 1986). Studies by Johansson (1970a) showed that salts partitioned unequally in an APTS and the electrical potential created by this unequal partition was estimated using electrodes (Reitherman *et al*, 1973; Johansson, 1974). A linear relationship between the log of the partition coefficient and protein net charge was observed in some cases (Johansson, 1970b; 1971) which was used to determine isoelectric points by cross-partition (Albertsson, 1970) and estimate net charge by partition (Blomquist, 1976). Cross partition is a technique which makes use of the fact that at the isoelectric point (pI) of the protein or

particle the partition is not determined by electrostatic effects such as the potential difference, ionic strength or electrostatically induced conformation changes. Thus a plot of K as a function of pH or net charge in two systems with different potential differences due to their salt compositions should cross at the pI.

b.i. More recent uses of partitioning in APTS (proteins, nucleic acids and sub-cellular particles). Over the past twenty years partitioning in APTS has been applied to the study and separation of a wide range of solutes and particles. The partition coefficients for a variety of proteins have been measured (Albertsson, 1958, 1960) and several general rules describing the partition behavior of proteins were established. Theoretical descriptions of partitioning described later in the text agree with these observations:

1. The higher the concentration of phase forming polymer, the more extreme the partition of the protein (providing no precipitation occurs).
2. With constant PEG/dx concentrations (or constant tie line length as defined later in the text) lowering the molecular weight of one of the polymers increases the partition of the protein into the phase in which that polymer predominates.
3. Protein dissociation may alter the partition of a protein as demonstrated by the pH dependence of the partition coefficient.
4. The partition may be influenced by the addition of salt, the effect depending on the pH, ionic composition and concentration of the salt. For a negatively charged protein the partition coefficient is often successively decreased in the series: $PO_4^{3-} > SO_4^{2-} > F^- > CH_3COO^- > Cl^- > Br^- > I^-$ and $Li^+ > NH_4^+ > Na^+ > K^+$.
5. The partition coefficient is independent of the concentration of the partitioned material up to 50g/L.

One of the more useful applications of partitioning in APTS has been the separation of proteins from nucleic acids *e.g.* the purification of RNA polymerase α peptide from *E. Coli*/bacteriophage (Goff, 1974). For a complete list of protein/nucleic acid separations see Johansson (1985). The incorporation of charged PEGs such as trimethylamino-PEG (TMA-PEG) and PEG-sulfonate (PEG-S) into the APTS causes the partition

of proteins to become extremely pH sensitive. These charged systems have been applied to the fractionation of baker's yeast (Johansson, 1985). Many other proteins have been partitioned including milk proteins (Igarashi *et al*, 1974), histones (Axelsson and Shanbhag, 1976; Bidney and Reeck, 1977), cellulases (Tjerneld, 1985), various albumins, ovalbumins and lactalbumins, hemoglobulins, serum globulins, interferon and various enzymes (For details see Johansson, 1985).

A more specific effect on protein partition may be obtained by including a PEG-bound ligand in the APTS. Trypsin was extracted into the upper phase of an APTS by the addition of diaminodiphenylcarbamoyl-PEG to the system (Takerkart *et al*, 1974). Similarly S-23 myeloma protein was extracted with dinitrophenyl-PEG (Flanagan and Barondes, 1975). Fatty acid derivatized PEGs and APTS have been used for assessing the degree of exposure of hydrophobic surfaces on proteins (Axelsson, 1978). PEG and dx derivatized procion dyes are now used industrially to purify glycolytic yeast enzymes (Johansson and Andersson, 1984; Johansson and Joelsson, 1985; Johansson, 1984). A novel use of an APTS was the separation of free and bound labelled ligand in a binding assay which investigated interactions between concanavalin A and various glycoproteins and carbohydrates (Mattiasson and Ling, 1980).

Partitioning has also been applied to the purification of nucleic acids. As with proteins the partition coefficient depends on the electrolytes present in the APTS, the structure and size of the nucleic acid and the presence of ligands. Separations include native and denatured DNA (Alberts, 1967), ribosomal genes from sea urchin sperm (Patterson and Stafford, 1970), plasmid DNA from bacterial lysates (Ohlsson *et al*, 1978a), DNA from rat tumors (Furnica, 1975) and the fractionation of chromosomal deoxyribonucleoproteins (Turner and Hancock, 1974). The development of chromatographic techniques whereby one of the phases is bound to a support has proven useful for nucleic acid separations (Müller, 1985).

The partitioning of sub-cellular particles has also received attention and is reportedly the only practical means for biomedical characterization of organelles and plasma membranes (Flanagan, 1985). Membranes have been isolated by partitioning for the past 20 years (Brunette and Till, 1971; Johansson, 1986). One example is the

separation of plasmalemma from human mammary carcinoma (Leung and Edgington, 1980). Three phase systems were used to partition hydrophobic membrane components (Albertsson, 1973) and partitioning has also been used for the separation of post-synaptic density structures (Gurd *et al*, 1982). The separation of right-side-out and inside-out plasma membrane vesicles by partitioning is used analytically and preparatively (Walter and Krob, 1976a). Affinity partitioning using a TMA-PEG ligand has proven useful for the isolation of nicotinic cholinergic receptors (Flanagan, 1976). Many organelles including peroxisomes (Suga *et al*, 1979) and microsomes (Ohlsson *et al*, 1978b) have been partitioned (Flanagan, 1985). Affinity partitioning of organelles includes the separation of calf brain synaptosomes with a PEG-procion dye affinity ligand (Muino Blanco *et al*, 1986).

Other uses of APTS include phase transfer catalysis (Harris *et al*, 1982), the separation of actinides (widely occurring terpene alkaloids, Sotobayashi, 1977) and the immunological quantification of bacterial cells (Ling *et al*, 1982).

ii. The partitioning of mammalian cells. One increasingly used application of partitioning in APTS is the separation and study of mammalian cells. It is with this aspect of partitioning that this thesis is most concerned. The partition of intact cells, as for all the previously mentioned solutes and particles, depends on the composition of the APTS, *i.e.*, on the pH and salt and polymer type and concentration as well as on the surface properties of the cell (Walter and Krob, 1976b). Separations may be on the basis of the native or ligand exposed cell partition.

The majority of work done to examine the determinants of cell partition to date has been carried out on erythrocytes, probably due to their availability as single cells in large quantities. Erythrocyte partition coefficients have been shown to correlate with electrophoretic mobility (Brooks *et al*, 1971) and separations of species-specific erythrocytes have been accomplished by CCD (Walter *et al*, 1967). Removal of sialic acid by neuraminidase was found to either lower or raise the partition coefficient, depending on the buffer (Walter and Coyle, 1968; Walter, 1985). The age of the individual erythrocyte in the same species

has an effect on the partition (Walter *et al*, 1981) as does the age of the donor (Seaman *et al*, 1980). The inclusion of PEG-fatty acid esters in the APTS has a large effect on erythrocyte partition. The fatty acid part of the ligand is presumed to insert into the erythrocyte membrane thereby coating the erythrocyte with PEG and causing it to partition into the PEG-rich upper phase of the APTS (Raymond and Fisher, 1980; Van Alstine and Brooks, 1984). It has been observed that ghosts and membrane vesicles have highly different partition coefficients to the erythrocytes from which they were prepared (Walter and Krob, 1976c).

An effort to correlate erythrocyte partitioning behavior with diseases associated with erythrocyte surface changes has been made. Rats treated to become highly anemic show a large increase in numbers of circulating reticulocytes. These "stress" reticulocytes have distinctly lower partition coefficients than mature erythrocytes (Walter *et al*, 1972). This observation was also made in patients suffering from reticulocytosis. Deposition of complement protein 3Cb on abnormal erythrocytes was used to separate sub-populations of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH, Pangburn and Walter, 1987). Small but significant differences in partition behavior between normal erythrocytes and those from multiple sclerosis patients have been observed in the presence of PEG-fatty acid esters (Van Alstine and Brooks, 1984). Erythrocytes from rats bearing subcutaneous Leydig cell tumor F344 have a lower partition coefficient than normal rat erythrocytes (Gascoine *et al*, 1983). However no differential partition behavior has been observed in the erythrocytes of sickle cell anemia patients (Walter, 1985) or erythrocytes of chronic alcoholics despite increased membrane cholesterol levels in these erythrocytes (Walter *et al*, 1979a).

Partitioning of mammalian cells other than erythrocytes has also been studied in systems containing no ligands. These include the separation of lymphocytes and polymorphonuclear cells by CCD (Walter *et al*, 1969) and observations of differences between mouse leukemic cells during lag and exponential growth phases illustrated by CCD profiles (Gersten and Bosmann, 1974). In fact changes associated with cell differentiation may be traced by partitioning (Stendahl *et al*, 1982). Walter *et al* (1979b) were able to separate T-lymphocytes, B-lymphocytes

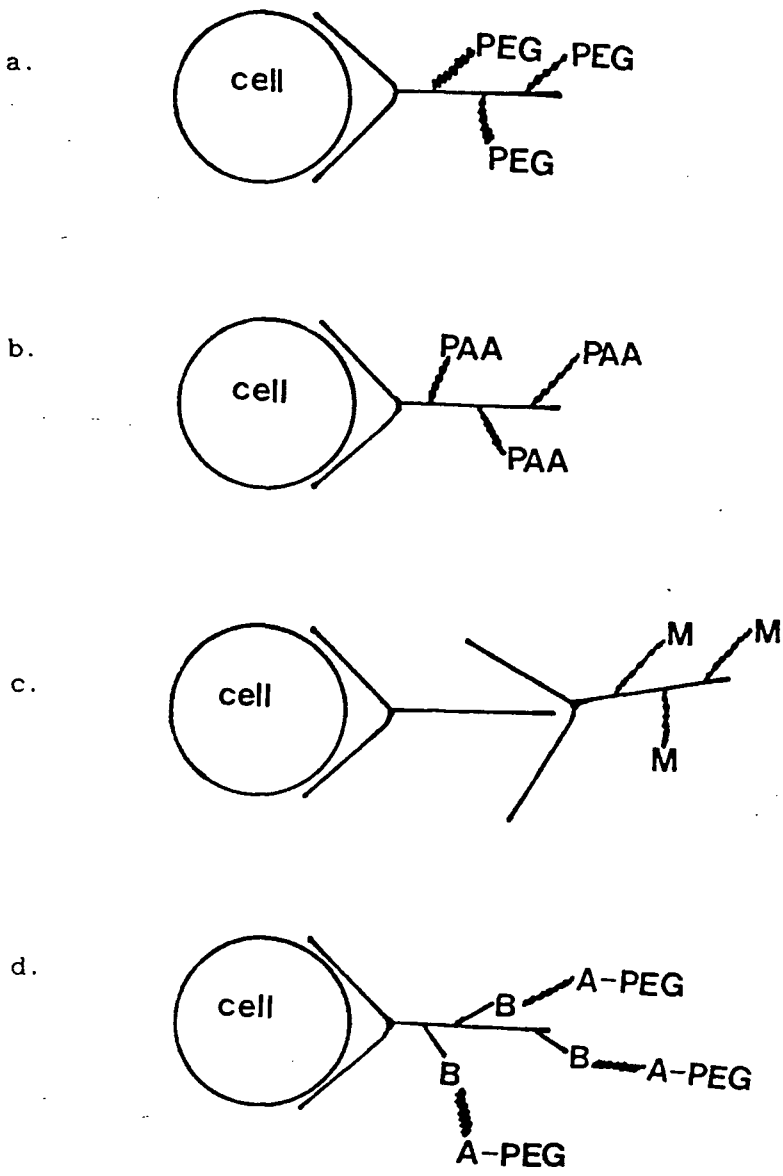
and F_c receptor bearing cells by CCD and Malmström (1980a,b) showed that natural killer (NK) cells were located in fractions with higher G values than the T- and B-lymphocytes and NK cells. Differences in the partition of normal and transformed fibroblasts have been observed (Sherbet and Lakshmi, 1981) and monocytes with high phagocytosing capacity have been found to partition differently to those with lesser phagocytic properties (Walter *et al*, 1980). There has been some success at distinguishing cells with high metastatic potential from those with low metastatic potential (Bosmann *et al*, 1973; Miner *et al*, 1981; Van Alstine *et al*, 1986) and the effects of some drugs on cell surface hydrophobicity has been investigated by partitioning (Kessel and McElhinney, 1978). Other cells studied by partitioning include bone marrow cells (discussed later in the text), rat liver cells (Walter *et al*, 1973a), intestinal epithelial cells (Weiser, 1973; Walter and Krob, 1975) and irradiated cells which appear to show only small, if any, change in partition behavior even after extremely high levels of irradiation (Gersten and Bosmann, 1975; Niepokojczycka *et al*, 1982).

Although partitioning of cells in the absence of ligands may achieve the desired separation in some cases, it is often necessary to separate cells on the basis of more discriminating factors than the overall surface properties of the cell. The incorporation of a suitable affinity ligand into the system may increase the resolution of the separation. The most specific type of affinity ligand is an antibody. These provide a powerful class of ligands for use in partitioning, provided they have been modified such that they partition predominantly into one of the phases

c. Immunoaffinity partition. Immunospecific separations of high resolution can be obtained using antibodies in a variety of separation techniques, for instance affinity chromatography. By applying these same principles to partitioning in a technique known as immunoaffinity partition, the separation of populations of viable intact cells on the basis of their surface antigens is possible. An immunoaffinity ligand is generally an antibody which has been modified such that it partitions into one of the phases of an APTS, *e.g.* a PEG-derivatized antibody will tend to partition into the upper phase of a PEG/dx system. The ligand

simultaneously binds to the solute or particle to be separated while partitioning into one of the phases, thereby increasing the partition of that solute or particle into that phase. The approaches taken in cell immunoaffinity partition have utilized a primary antibody affinity ligand (Sharp *et al*, 1986; Karr *et al*, 1986) and a second antibody affinity ligand (Stocks and Brooks, 1988). In the primary antibody affinity ligand case, a PEG-modified antibody recognizing a cell surface antigen has been incorporated, along with the cell mixture to be separated, into an aqueous two phase system containing PEG and dx. The PEG-antibody on binding to the cell surface effectively coats it immunospecifically with PEG, thereby increasing the partition of that cell into the upper, PEG-rich, phase (Fig. 2.a).

The first studies of cell separations by immunoaffinity partition were of species specific erythrocytes (Sharp *et al*, 1986; Karr *et al*, 1986). Although primary affinity ligands clearly demonstrate immunospecific effects on cell partition, there are disadvantages to the use of a directly-modified primary antibody as an affinity ligand, the most significant being the need for a different modified-antibody ligand for each cell separation problem. This entails time consuming optimization of the modification chemistry as well as separate analysis and characterization of the ligand for each cell separation. This disadvantage may be overcome by using a second antibody or other modified reagent as the affinity partitioning ligand in combination with a native primary antibody. One approach is to raise a monoclonal antibody (MAb) which specifically binds to the F_c region of a primary antibody. The F_c region of an antibody of any one class is constant irrespective of antibody specificity (Goding, 1983). This antibody may be modified such that it partitions into one of the two phases *e.g.* by attachment of PEG or trypan blue dye (Fig. 2c). Trypan blue partitions strongly into the upper phase (see later in discussion). Thus the same second ligand may be applied to any separation problem for which a primary antibody is available (Stocks and Brooks, 1988). Another advantage of selecting the second antibody to bind to the F_c region of the primary antibody is that the attached PEG is remote from the site of cell-primary antibody binding. Since some steric interference by PEG in the cell-antibody binding is thought to occur when using the primary



PEG polyethylene glycol B biotin

PAA polyacrylamide A avidin

M trypan blue or PEG

Fig. 2. Schematic diagrams of approaches to immunoaffinity partition of cells. a. PEG-derivatized primary ligand (Sharp *et al*, 1986; Karr *et al*, 1986) b. Polyacrylamide (PAA)-derivatized primary antibody (this work). c. PEG- or trypan blue-derivatized second antibody (Stocks and Brooks, 1988; this work). d. Biotin-derivatized primary antibody and PEG-derivatized avidin (this work).

antibody technique (Sharp *et al*, 1986), this is a distinct advantage. Monoclonal antibodies are of particular use in this context since they can be isolated as a pure molecular species, an infinite constant supply is potentially available and they may be chemically modified in a reproducible way. The use of a MAb as a primary ligand is the only way in which the resolution of immunoaffinity partition can approach that required for the more stringent cell separation problems discussed later.

Another approach to the second ligand technique is the use of PEG-derivatized Staphylococcal protein A in combination with a primary IgG ligand (Karr *et al*, 1987). Staphylococcal protein A binds to the F_c region of IgG with a high affinity and specificity (Goding, 1983). However there is considerable variation in the strength of binding among the different IgG sub-classes in each species, some of which are not bound at all. In some cases IgA and IgM will also be bound and multivalent protein A is likely to cause greater aggregation than a divalent antibody.

Another method is to derivatize the primary antibody with biotin and use PEG-derivatized avidin or native streptavidin as the second ligand (Fig. 2.d.). Streptavidin partitions into the upper phase (Flanagan, 1985). This has similar disadvantages to the directly modified primary antibody but has advantages in that it uses less labile reagents and an exceptionally strong binding reaction (Stocks and Brooks, 1987).

There are few reports of cell separations by immunoaffinity partition and for the most part these separations have been of species specific erythrocytes using the primary antibody technique. For the technique to be of clinical use it must be capable of separating closely related cell types with only small differences in surface antigens. The main object of this study is to apply immunoaffinity partition to a more stringent and potentially useful cell separation, while simultaneously examining novel affinity ligands and optimizing partition conditions for an efficient separation of viable cells. Two particularly interesting practical problems to which immunoaffinity partition could be applied are the purging of bone marrow prior to reinfusion during autologous bone marrow transplants as leukemia therapy and the separation of fetal

islet of Langerhans cells for implantation in patients with insulin-dependent diabetes mellitus.

i. Immunoaffinity partition - An approach to bone marrow purging. Numerous studies have demonstrated that bone marrow transplants from histocompatible donors in conjunction with ablative therapy can be an effective treatment for acute leukemia and lymphoma (Zwann *et al*, 1982; Tutschka *et al*, 1980). Although considerable controversy remains regarding the relative merits of bone marrow transplant and intensive chemotherapy as treatment for acute leukemia (Jehn and Grunewald, 1988) most reports favor bone marrow transplants for other types of cancer (Williams *et al*, 1989). Only 40% of patients have histocompatible donors, however, and the relevance of HLA compatibility to sustained marrow engraftment is well known. A far more immunosupressive regime is required in cases where the donor is not an HLA-identical sibling (Anasetti *et al*, 1989). Autologous bone marrow transplant, in combination with purging of the tumor cells, has been successfully used and does not require a bone marrow donor (Ritz *et al*, 1982; Wells *et al*, 1979). A similar treatment for severe combined immunodeficiency disease has also been applied; this involved the removal of immunocompetent T lymphocytes by MAb (Reinhertz *et al*, 1982). Other diseases which have been treated by bone marrow transplants include Hodgkin's disease (Gribben *et al*, 1989), thalassemia and other inherited metabolic diseases (Levinsky, 1989). Bone marrow transplants have also been used to obtain donor-specific unresponsiveness in kidney allograft recipients. A course of anti-lymphocyte serum at the time of transplant is followed by transfusion of donor bone marrow (Barber *et al*, 1989).

A typical procedure for autologous bone marrow transplant in the treatment of leukemia or lymphoma commences with the induction of remission by chemotherapy, followed by harvesting of the bone marrow. The bone marrow is processed in some way to remove tumor cells, then cryopreserved. Meanwhile the patient undergoes ablative chemotherapy and finally total body irradiation (TBI). Approximately 12 hours after TBI, the cleansed bone marrow is reinfused into the patient.

The purging of the bone marrow is a crucial step in the treatment. As a result, considerable research is currently underway in the areas of

long term storage and culture of bone marrow cells and methods of enriching the bone marrow white cells while eliminating the tumor cells from the bone marrow *in vitro*. Several methods have been applied to the removal of tumor cells from normal cells while maintaining the latter in a viable condition, including:

1. Treatment with MAbs and complement (Linker-Israeli *et al*, 1981; Wells *et al*, 1979; Economu *et al*, 1978; Netzel *et al*, 1980).
2. Long term marrow cultures which favor the production of multipotential stem cells while disfavoring the maintenance of tumor cells (Chang *et al*, 1986).
3. Incubation with MAbs, followed by exposure to anti-mouse antibody bound to magnetite-containing microspheres. Exposure of the cells to a series of magnets results in the removal of the tumor cells recognized by the primary antibody (Treleaven *et al*, 1984). A more recent method employs polystyrene magnetic beads (Morecki and Slavin, 1988).
4. Short incubation with 4-hydroperoxycyclophosphamide (Sharkis *et al*, 1980). Although cyclophosphamide derivatives are the most commonly used drugs others such as Ditercalinium (a pyridocarbazole derivative) and cis-platinum have shown potential, the latter in particular since it exhibits low myelotoxicity (Benard *et al*, 1988).
5. Fractionation on discontinuous albumin gradients (Dicke *et al*, 1979). This method has been semi-automated using a ficoll-hypaque gradient and transfused cells prepared by this method engrafted within 20 days (English *et al*, 1989).

All of the above approaches suffer from drawbacks of varying severity. Complement lysis is not 100% efficient due to the presence on most cell surfaces of enzymes which inactivate the complement complex before cell lysis occurs (Ritz and Schlossman, 1982). The selectivity of long term marrow cultures for the normal population will vary depending on the type of tumor and condition of the donor. The use of solid phase supports in the magnetic bead-based separation suffers from the same problem as occurs in immunoaffinity chromatographic separations of cells, namely the non-specific adsorption of non-target cells to the beads. Similarly, separations based on size and density differences are never specific since the relevant properties vary so widely through the normal and tumor cell populations and change depending on the stage in

the cell cycle in which the members of each population occur. Hence, it seems clear that alternate techniques for bone marrow purging ought to be explored.

A converse approach is the positive selection of stem cells rather than the removal of tumor cells. MAbs directed against a human progenitor cell antigen have been used for autologous stem cell selection in primates and these selected cells were able to fully reconstitute hemopoietic function following TBI (Levinsky, 1989).

Partitioning in APTS is one method with potential for the fractionation of populations of bone marrow cells. There have been a few studies of the CCD profiles of bone marrow cells but only the hemoglobin-containing cells have been studied in any detail. In erythroid-stressed rats (due to phenyl hydrazine injection) a large proportion of the bone marrow cells (94%) are reticulocytes which have a lower partition than normal erythrocytes located in the bone marrow in appropriate phase systems (Walter *et al*, 1973b). This was also observed in peripheral erythrocytes (Walter *et al*, 1972). X-ray irradiation *in vitro* (10 Gray) does not alter the CCD pattern of bone marrow cells (Walter *et al*, 1974). The shift in hemoglobin synthesis from embryonic to adult hemoglobins in the bone marrow cells of adult rats has been assigned to erythroid cells with different CCD profiles at different stages of their development (Weiser *et al*, 1976).

The development of MAbs specific for tumor-associated cell surface antigens (TAAs) is extensively documented (Hawkey *et al*, 1986; Robinson *et al*, 1986; Shipman *et al*, 1983; Springer, 1985; Linker-Israeli *et al*, 1981). Such monoclonal antibodies have been used for the identification (Dhokia *et al*, 1986a; b), separation (Treleaven *et al*, 1984; Kemshead and Ugelstad, 1985) and selective destruction of malignant cells (Nadler *et al*, 1980; Thierfelder *et al*, 1977). For example, a MAb which reacts with the cells of patients with acute nonlymphoblastic leukemia (ANLL) has been shown capable of indicating periods of remission and relapse up to three months prior to the event (Levy *et al*, 1985). The same MAb has been used to label and selectively kill ANLL-associated tumor cell lines using an antibody-hematoporphyrin conjugate and laser irradiation. (Mew *et al*, 1985).

To date the principal use of monoclonal antibodies against TAAs has

been in the identification of malignant cells, although the potential development of "magic bullets" to destroy target cells following antibody binding has received considerable attention. Less effort has gone into applying anti-TAA MAbs in the separation of malignant from normal cells, fluorescence-activated cell sorting (FACS, Herzenberg, 1977) and a recently developed technique using MAb-coated magnetic microspheres (Treleaven *et al*, 1984) being the principal approaches used.

The possibility of applying immunoaffinity partition to cell types relevant to the bone marrow purging problem is investigated in this thesis. The approach taken is the development of a second, MAb affinity ligand to be used in combination with one of the aforementioned panel of MAbs to various TAAs in an APTS to separate tumor cells recognized by the MAb. In order to model to a degree the bone marrow purging problem, a separation of two sub-lines of a transformed mouse lymphocyte was attempted. The problem was selected to test more stringently the limits of immunoaffinity partition since the lymphocyte sub-lines selected were qualitatively identical in surface characteristics, only the surface concentration of a membrane antigen differing in the two cell types.

ii. Immunoaffinity partition - An approach to transplantation of fetal islet tissue for Type II diabetes treatment. The ultimate goal of pancreatic islet transplantation is to reinstate glucose and insulin homeostasis in the insulin-dependent patient, thus preventing the long-term complications of the disease. The main problem with islet transplantation is not only immune rejection but the fact that the islets of Langerhans constitute only 1-2% of the mass of the pancreas. The fetal pancreas has many potential advantages as a donor organ for the treatment of insulin-dependent diabetes mellitus due to its capacity for growth and development (Clark and Rutter, 1972). Also, removal prior to exocrine development avoids problems due to immune responses to acinar cells (Brown *et al*, 1976), the small size of the organ means that it is easily cryopreserved (Mazur *et al*, 1976) and possibly unlimited supplies are available, subject to public opinion. However, although transplantation of fetal pancreas effectively reverses diabetes in rodents (Brown *et al*, 1984; Federlin and Bretzel, 1984) the transplant

of human fetal pancreas has had only marginal success (Farkas and Karacson, 1985; Groth *et al*, 1980).

The detrimental effect of transplanted acinar tissue on graft function and viability may be avoided if the islet cells can be positively selected. The selection procedure involves disruption by mechanical distension, collagenase perfusion or mechanical or enzymic digestion (Scharp, 1984) followed by purification by sedimentation with or without density gradient, hand-picking, elutriation, FACS, electrophoresis or affinity techniques using anti-islet antibodies. An auto-analyzer which employs cell culture to form aggregates of predominantly islet cells and elutriation has been used with some success for dog transplants (Scharp, 1984). A similar automated method employing a ficoll gradient is currently in clinical trials (Ricordi *et al*, 1989). Other relevant developments in islet cell isolation include the development of MAbs to human islet cells (Soon-Shiong *et al*, 1988), the discovery of lectins that bind islet cells (Peterson *et al*, 1986), the fact that addition of nicotinamide to islet cells in culture increases islet DNA replication (Sandler *et al*, 1989) and the encapsulation of islet cells in agarose resulting in a longer lifetime on transplantation (Iwata *et al*, 1989). The most commonly used method to enrich islet cells is long-term culture which forms aggregates of islet cells which can be separated by density gradients (Sandler *et al*, 1985).

Since MAbs to the islet cells exist it seems that this separation may be feasible using immunoaffinity partition. A model separation such as that outlined below of cells distinguished by low surface density antigens recognized by monoclonal antibodies is relevant to this problem as well as to the bone marrow purging problem.

8. The model separation problem. The separation problem chosen for this study was of two sub-sets of a Moloney-virus transformed T lymphocyte cell line, MBL-2 (2.6) and MBL-2 (4.1) (Takei, 1983; Chan and Takei, 1986). The only known difference between these two cell lines is the surface density of an antigen recognized by the rat monoclonal antibody, YE1.48.10. Prior to the development of this antibody these two sub-sets of MBL-2 were unknown and the only currently available method of separating these two sub-sets is by FACS. The MBL-2 cells and YE1.48.10.

hybridoma were gifts from Dr. F. Takei, Terry Fox Laboratory, Vancouver.

Thus the specific objective of this study is to examine a variety of novel immunoaffinity ligands and to apply them to separate populations of MBL-2 (2.6) and MBL-2 (4.1) cells by immunoaffinity partition using the rat monoclonal antibody YE1.48.10 as the primary affinity ligand. The dye trypan blue, a system utilizing biotin, avidin and streptavidin and polyacrylamide-MAb ligands are all examined. The trypan blue and polyacrylamide derivatized antibodies are first used on an erythrocyte separation problem and the polyacrylamide antibody is then applied to the separation of the MBL-2 lymphocytes. A schematic diagram of the approaches studied in this thesis is shown in Fig. 2.

CHAPTER 2

THEORETICAL ASPECTS OF PARTITIONING

1. Phase Separation. The statistical mechanical mean field theory of phase separation for any number of polymer species in a common solvent is based on the approach first taken independently by Flory (1941) and Huggins (1941). The method is to calculate the free energy of mixing polymer and solvent molecules, ΔG_m, from the sum of the enthalpy and entropy of mixing (ΔH_m and ΔS_m). The theory is based on a lattice in which sites can be occupied by a solvent molecule or a segment of a polymer molecule. In the case of two polymers and a solvent, the expression turns out to be (Flory, 1953):

$$\Delta G_m = kT\ [n_1 \ln\phi_1 + n_2 \ln\phi_2 + n_3 \ln\phi_3 + (n_1 + n_2P_2 + n_3P_3)(\phi_1\phi_2\chi_{12} + \phi_1\phi_3\chi_{13} + \phi_2\phi_3\chi_{23})] \qquad (2)$$

where subscript 1 denotes the solvent and 2 and 3 denote the two polymer species (characterized by their molecular volumes), ϕ_i is the volume fraction of component i ($\phi_i = n_iP_i/[n_1 + n_2P_2 + n_3P_3]$), n_i is the number of molecules of i on the lattice, P_i is the number of segments of volume equal to a solvent molecule per polymer molecule and χ_{ij} is the Flory interaction parameter between i and j. χ_{ij} represents the maximum interaction energy a segment of molecule i can possess in a mixture, *i.e.* when completely surrounded by segments of molecule j.

By differentiating ΔG_m with respect to n at constant temperature and pressure for any of the species the chemical potential of that species may be calculated, *i.e.* $(\mu_i - \mu_i^\circ) = N_A(\partial \Delta G_m / \partial n_i)_{n_j}$ where μ_i is the chemical potential of i when its volume fraction is ϕ_i, μ_i° is the standard state chemical potential of i when $\phi_i = 1$ and N_A is Avogadro's number. If two phases are to be present at equilibrium then the chemical potential, μ, must be identical in either phase. The critical conditions for the appearance of two phases are described by (Flory, 1953):

$$\frac{\partial \mu_1}{\partial \phi_2} = \frac{\partial^2 \mu_1}{\partial \phi_2^2} = 0 \qquad (3)$$

The character of the solution may be seen for a simplified case as follows. Solving this expression for critical values of ϕ_1 and χ_{1j}, i.e., those which will produce phase separation in a two polymer, single solvent system, assuming that $P_2 = P_3$, *i.e.* both polymers have the same number of segments per molecule, and that both polymers are equally soluble in the solvent (implying $\chi_{12} = \chi_{13}$) results in:

$$\phi_{2C} = \phi_{3C} = (1-\phi_{1C})/2 \quad (4)$$

$$\chi_{23C} = 1/P_2\phi_{2C} \quad (5)$$

Where c denotes critical values.

These expressions describe the general features of phase separation in an APTS. That is, phase separation will occur readily since P_2 is large for high molecular weight polymers. Hence, phase separation is the rule rather than the exception implying only small positive (unfavorable) value of χ_{23C} is necessary to produce separation in mixtures of polymers. The ease of separation will increase with increasing molecular weight, and since χ_{12} and χ_{13} are absent from these expressions, only polymer-polymer interactions are important in determining phase separation in systems of equally soluble polymers.

The range of concentrations above which a two phase system will arise as defined above may be represented by a phase diagram (Fig. 3). The curved line dividing the two areas of the phase diagram is termed the binodial and all compositions of, in this case, PEG and dx above the binodial will produce two-phase systems. Points along the binodial or nodes (*e.g.* B,B', C,C') can be joined to form tie lines (eg.B-C, B'-C'). Any point along the tie line will give rise to a two-phase system with the same phase composition but the phase volumes will differ. The tie line length (TLL) becomes zero at the critical point, K, and this defines the minimum polymer concentrations which will phase separate. The %w/w ratio of bottom to top phase is equal to the ratio between those parts of the tie line defined by the total system composition (eg.AC:AB).

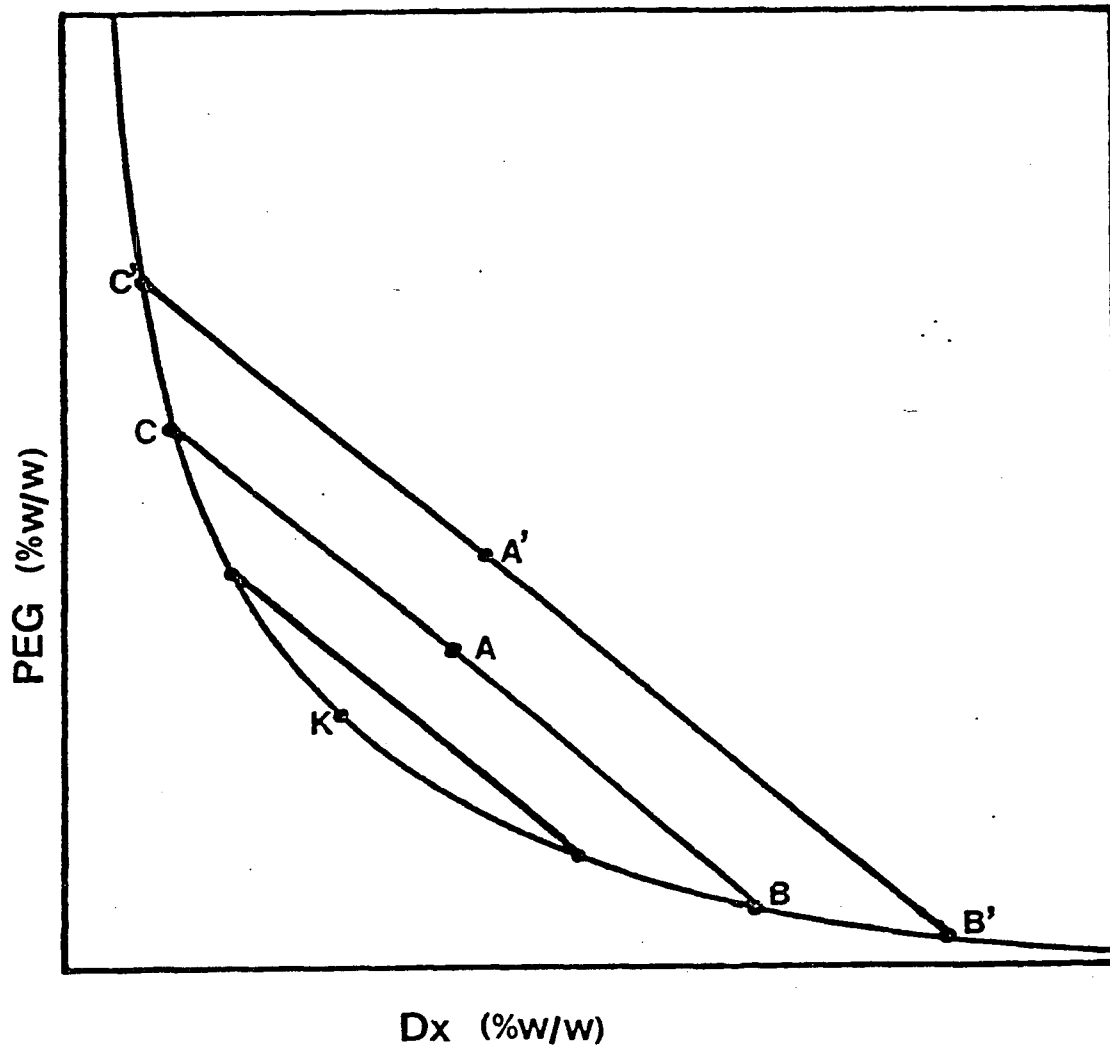


Fig. 3. General phase diagram for a PEG/dx/water phase system. The lines BC or B'C' connect the points representing the composition of the two phases at equilibrium *i.e.* B represents the bottom phase composition and C the top phase composition. The total composition is given by A and the ratio of AC:AB will give a weight ratio of bottom to top phase. K is the critical point.

The process of phase separation does not occur instantly but takes a significant time depending on density and viscosity differences between the phases and the time taken for droplets to coalesce into larger drops (Raymond and Fisher, 1981). It has been described in terms of the movement of complex microphases and is similar to the upward and downward creaming of emulsions (Becher, 1965). The rate of phase separation increases with TLL for moderate concentrations which seems to relate to density differences, interfacial tension and viscosity differences which in turn depend on the polymer composition. Systems close to the critical point (K) are slow to separate because of their small density difference.

2. Molecular Affinity Partition. If the Flory-Huggins expression (Eq.[2]) for the free energy of mixing is applied to a four component system, the fourth component considered to be the partitioned molecule and all the components are considered equally soluble, then the chemical potential, μ_4, may be calculated. At equilibrium μ_4 must be identical in both upper and lower phase so by equating μ for each phase and assuming ϕ_4 to be small so second order terms can be dropped, an expression for K_4 the partition coefficient of the fourth component, is obtained (Brooks *et al*, 1985):

$$K_4 = \exp P_4[(\phi_1^T-\phi_1^B)(1-\chi_{14}) + (\phi_2^T-\phi_2^B)(1/P_2-\chi_{24}) + (\phi_3^T-\phi_3^B)(1/P_3-\chi_{34})] \quad (6)$$

where $K_4 = \phi_4^T/\phi_4^B$ and T and B denote top and bottom phases.

This illustrates several features of partitioning which are experimentally observed. That is, the partition coefficient depends exponentially on the properties of the phase system and the partitioned material and will become more one-sided with increasing molecular weight of the partitioned material or increasing difference in polymer concentration between the phases. The partition coefficient will be determined by the balance of the interaction energy of the partitioned material with the phase polymers and the solvent. If the molecular weight of one of the phase polymers is decreased, then the partitioning into that phase will be increased.

It must be recognized, however, that the above concentrated solution Flory-Huggins theory is limited in that it assumes there is no volume change on mixing nor any entropy change (other than configurational) associated with segment-solvent contacts. There are also general, model-independent, thermodynamic expressions which can be used to describe partition behavior (Brooks *et al*, 1985). Consider the chemical potential of an uncharged solute molecule of species i, μ_i, in a single phase, treated as a continuum with the average properties of the solution:

$$\mu_i = \mu_i^0 + RT \ln a_i \qquad (7)$$

where μ_i^0 is the standard state chemical potential in that phase, R is the gas constant and a_i is the activity of i in that phase ($a_i = f_i c_i$ where f_i is the activity coefficient and c_i is the concentration of species i). At equilibrium μ_i must be the same in either phase, therefore:

$$\mu_i^{OT} + RT \ln a_i^T = \mu_i^{OB} + RT \ln a_i^B \qquad (8)$$

If sufficiently dilute solutions are considered then the activity coefficient approximates to one and the above expression may be considered in terms of concentration rather than activity. Solving for the partition coefficient, $K_i = c_i^T/c_i^B$, results in:

$$K_i = \exp \frac{-(\mu_i^{OT} - \mu_i^{OB})}{RT} \qquad (9)$$

By equating this with Eq.[6], the Flory Huggins expression for K_i, it is apparent that in this interpretation the standard state chemical potential difference of the partitioned molecule in the two phases contains contributions from the energies of interaction between the solute and all three components of the phase systems. If a ligand associates strongly with another molecule and if the ligand has a different partition coefficient from the molecule, the binding will change the partition coefficient of the complex. This is the basis of affinity partition (Flanagan and Barondes, 1975) and the use of phase

systems in studying association reactions (Müller and Gautier, 1975; Hustedt and Kula, 1977). It can be considered simply as a way of altering μ_i^{OT} and μ_i^{OB} thereby changing K_i.

A more detailed treatment of affinity partition can be provided, as follows (Brooks *et al*, 1985). If a macromolecule has n equivalent, independent binding sites for a ligand, then the binding of ligand to the macromolecule can be analyzed by considering the sequential addition of ligand up to saturation. If M_i denotes the concentration of molecules which have i of their sites occupied by ligand at a given equilibrium concentration, L, then the total concentration of macromolecule, M_{tot} is the sum of all the species of M_i, that is $M_{tot} = \sum_{i=0}^{n} M_i$. This may be evaluated by applying the binomial theorem (Cantor and Schimmel, 1980) resulting in:

$$\sum_{i=0}^{n} M_i = M_0(1 + k_a L)^n \qquad (10)$$

where k_a is the microscopic association constant for ligand binding to any site.

$$\text{Since } K_m = \sum_{i=0}^{n} M_i^T / \sum_{i=0}^{n} M_i^B, \quad \text{then } K_m = \frac{K_0(1 + k_a^T L^T)^n}{(1 + k_a^B L^B)^n} \qquad (11)$$

where K_0 is M_0^T/M_0^B, the partition coefficient of empty macromolecules.

k_a^T, k_a^B are the microscopic association constants in top/bottom phase.

L^T, L^B are the equilibrium concentrations of ligand in top/bottom phase.

If the ligand concentration is very high, all molecules will be saturated and Eq.[11] will simplify to (Flanagan and Barondes, 1975):

$$K_m = K_0 K_L^n \, (k_a^T/k_a^B)^n \qquad (12)$$

In the case where the ligand is covalently bound, as in a PEG-derivatized protein, the association constant becomes infinite in

both phases, therefore $K_m = K_0 K_L^n$. These expressions (11) and (12) predict a very strong effect of ligand binding on partition coefficients providing K_L is not equal to 1.

3. Particle partition. The above theory for molecular partition cannot be applied to cell and particle partition since the concept of chemical potential for a particle as large and slowly diffusing as a cell does not really apply. Moreover, it is generally observed that cells distribute between the interface and one of the phases rather than between the two bulk phases. Therefore, numbers of cells at the interface and in the phases rather than concentrations are the more appropriate measure of cell number. One possible starting point for developing a theory for cell and particle partition is the Boltzmann equation (Brooks *et al*, 1985).

$$K = n_1/n_2 = \exp(-\Delta G^\circ/kT) \qquad (13)$$

This relates the probabilities of a particle being in either of two compartments, designated 1 and 2, to the standard state change in free energy required to move the particle between the compartments, ΔG°. The use of kT in the expression assumes that the particle diffuses freely and is distributed by thermal energies. For large particles such as cells, however, a significant reduction in free energy can result from adsorption at the interface. If a particle is located at the liquid-liquid interface, the interfacial area is reduced by the cross-sectional area of the particle. The decrease in area produces a proportionate decrease in free energy equal to the interfacial tension times the area lost, thus stabilizing particles adsorbed at the interface. If a particle has equal affinity for both phases, then adsorption at the interface will be significant when the free energy associated with adsorption is of the order of the average thermal energy of a particle. For example, a typical phase system with an interfacial tension 5×10^{-3} erg/cm^2 would adsorb particles with a diameter over 320Å at the interface. The greater the surface tension, the smaller the particles that will adsorb at the interface.

The work done on moving a particle from the interface into the top

phase, ΔG_{TI}, is the sum of two components: (i) the transfer of part of the particle surface of area A_B from the bottom phase to the top phase, with a net energy change of $-A_B\Delta\gamma$; (ii) the change in surface area of the interface by A_{TB}, with a net energy change of $A_{TB}\gamma_{TB}$. Thus:

$$\Delta G_{TI} = A_{TB}\gamma_{TB} - A_B\Delta\gamma \qquad (14)$$

where γ_{TB} is the surface free energy at the interface, $\Delta\gamma$ is the particle cell surface free energy difference in top and bottom phase, $A_{TB}\gamma_{TB}$ is a contribution from the net energy change associated with the change in interfacial surface area, and $A_B\Delta\gamma$ is a contribution from the energy required to transfer a portion of the particle surface area, A_B, from bottom phase to top phase.

Equation [14] has been tested *via* the microscopic measurement of the contact angle, θ, formed between the interface and the tangent to the cell surface at the contact line (Adamson, 1976). In terms of the contact angle, the expression for particle partition is as follows (Brooks *et al*, 1985):

$$\ln K = -\Delta G_{TI}/kT \qquad (15)$$

$$\ln K = \frac{-\gamma_{TB}\pi a^2(1 - \cos\theta)^2}{kT} \qquad (16)$$

The contact angle is related to the free energies in the system by $\cos\theta = \Delta\gamma/\gamma_{TB}$, where $\Delta\gamma$ is the difference in particle surface free energy in top and bottom phase, γ_{TB} is the interfacial tension between top and bottom phase and a is the particle radius. When typical values are substituted into this equation, it is found that ΔG_{TI} is several orders of magnitude larger than kT, suggesting essentially no particles should partition into the upper phase. Experimentally this is not observed. However, it would still be expected that particle partition would depend on the surface properties of the particle, their size and area, the temperature and the interfacial tension between the phases. In fact an exponential dependence of K on ΔG_{TI} has been observed (Sharp, 1985).

4. Particle affinity partition. The following is taken from Sharp and Brooks (1989). An expression for the effect of an affinity ligand binding to a particle on the surface free energy difference, $\Delta\gamma$, of the particle may be obtained by integrating the Gibbs equation:

$$d\gamma = -\Sigma\ \Gamma_i\ d\mu_i \qquad (17)$$

where Γ_i is the surface excess of the ith component and $d\mu_i$ is the change in chemical potential of the ith component. As eqn [17] applies in both phases then the surface free energy difference between a particle in top and bottom phase may be written as:

$$d\Delta\gamma = \Sigma\Gamma_i^B\ d\mu_i^B - \Sigma\Gamma_i^T\ d\mu_i^T \qquad (18)$$

If it is assumed that the binding of ligand does not significantly alter the particle area and the ligand is the only significant contribution to the integral of eqn [18] then:

$$d\Delta\gamma = \Gamma_L^B\ d\mu_L^B - \Gamma_L^T\ d\mu_L^T \qquad (19)$$

where subscript L refers to the ligand. The chemical potential of the ligand in the top phase is $\mu^T = \mu^{oT} + kT\ \ln\ c^T$ and the differential is:

$$d\mu^T = kT\ \frac{dc^T}{c^T} \qquad (20)$$

The surface excess of ligand in the upper phase, Γ_L^T, is the amount of ligand bound per unit area. The simplest binding isotherm which may be used to describe this is the Langmuir isotherm for n identical, independent binding sites per unit area (Cantor and Schimmel, 1980):

$$n^T = \frac{nc^Tk^T}{1 + c^Tk^T} \qquad (21)$$

where k^T is the association constant for the binding reaction. The integral of the 2nd term in equation [19] is:

$$\int_o^{c^T} \Gamma_L^T\ d\mu_L^T = \int_o^{c^T} \frac{nkTk^T\ dc^T}{(1 + c^Tk^T)} \qquad (22)$$

$$\gamma_L^T - \gamma_o^T = nkT \ln (1 + c^T k^T) \quad (23)$$

Repeating this for the lower phase and substituting into eqn [19] gives:

$$\Delta\gamma_L - \Delta\gamma_o = nkT \ln \frac{(1 + c^B k^B)}{(1 + c^T k^T)} \quad (24)$$

Using eqn [21] to substitute for the terms in parentheses in eqn [24]:

$$\Delta\gamma_L - \Delta\gamma_o = nkT \ln \frac{(n^T k^B)}{(n^B k^T K_L)} \quad (25)$$

Experiments using PEG-palmitate and erythrocytes were consistent with this theory (Sharp, 1985).

5. Counter current distribution (CCD). CCD is a method for carrying out repeated partition steps and is useful for separations in which one partitioning step does not sufficiently resolve the components. It was originally designed for aqueous-organic or organic-organic two-phase systems (Craig, 1960) but was modified by Albertsson (1965) who developed a thin-layer CCD apparatus with faster settling times. This made the technique more appropriate for viscous, polymer-containing phases and biological separations. A CCD apparatus consists of multiple chambers in a circular arrangement. By rotation of the top half of the apparatus in a clockwise direction while the bottom half remains stationary, the upper phase is transferred to the chamber immediately to the right as shown in Fig. 4, while the upper phase in the chamber to the left replaces the upper phase that has been transferred.

The three parameters useful in predicting CCD curves are P, the fraction of the total amount of material in the upper phase, K, the partition coefficient, which is the ratio of the concentrations of the distributed material and G, the distribution ratio, which is the ratio of the masses of the material of interest in the phases. These are related as follows:

$$G = KV_t / V_b \quad (26)$$

$$P = G/ (G + 1) \quad (27)$$

A schematic diagram in Fig. 4 depicts stages in the generation of a five transfer CCD starting with 1000 units of a material with a partition coefficient (K) of 1. Materials with K other than 1 will produce asymmetric distribution curves. It is relatively straightforward to predict theoretical CCD curves providing the distribution ratio, G, of the solute or particle is known.

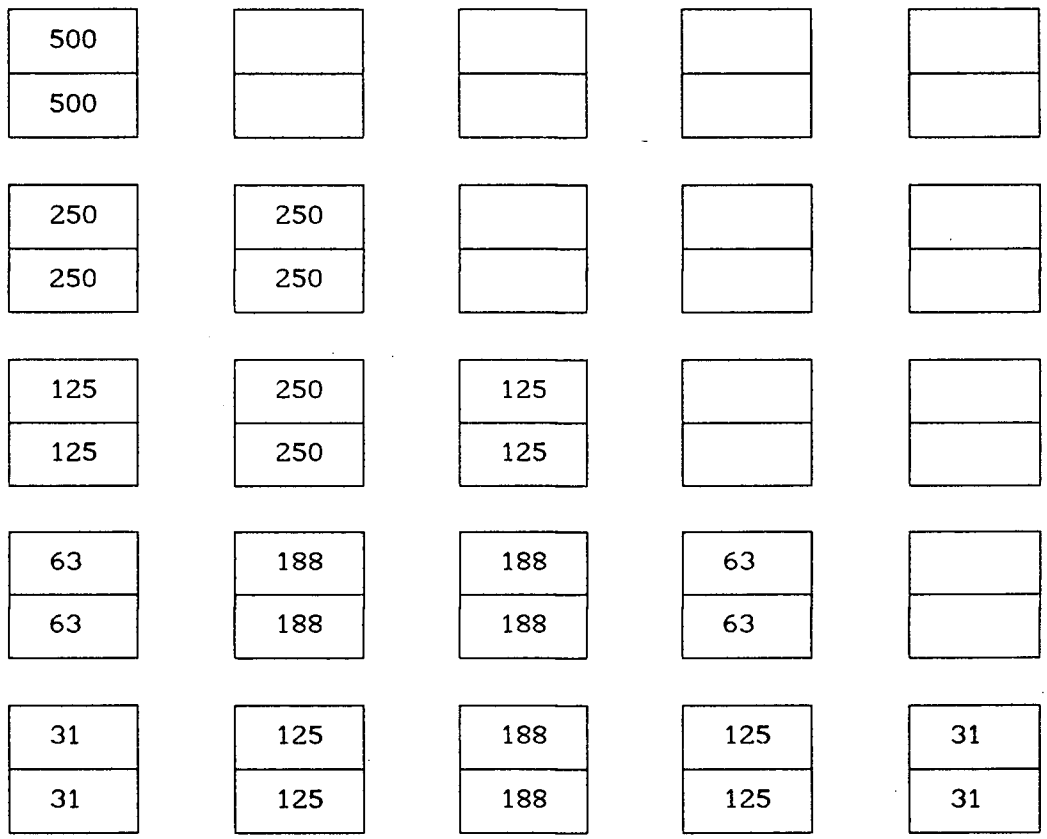


Fig. 4. A diagrammatic representation of 5 CCD transfer steps following the loading of 1000 units of a soluble sample (K=1, G=1, P=0.5). From Treffry *et al*, 1985).

The partitioning of solutes occurs between the two phases but the partition of particles is between the interface and one of the phases (Fig. 5). In order to apply these parameters as defined in (26) and (27)

to predict CCD curves, the physical separation of the phases must be at the interface. This is possible when solutes are being partitioned but not for particles when a small volume of, in the case of an upper phase partition, upper phase is left behind to ensure that no particles adsorbed at the interface are carried over. Thus the amount transferred is $C_t(V_t-v)$, *i.e.*, the number in the upper phase minus that which is not transferred, and the amount remaining stationary is $a + C_t v$, *i.e.*, the number adsorbed at the interface and the number in the upper phase that is not transferred. (see Fig. 5). Then the distribution coefficient, G, will be:

$$G = \frac{C_t(V_t-v)}{a + C_t v} \qquad (28)$$

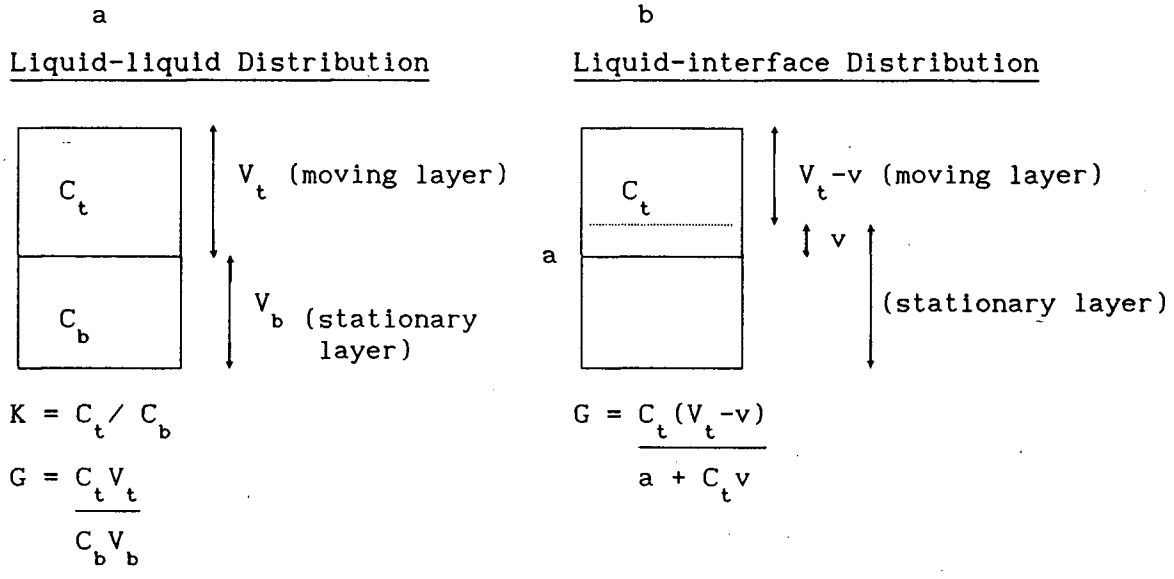
For n transfers the distribution of solute is described by $(1+G)^n$ or $(1-P)^{n-1}$ which may be expanded using the binomial distribution (Hecker, 1955). From this P may be calculated and the CCD curve predicted. If n distributions are carried out the fraction of the total population appearing in the rth cavity is given by:

$$F(r) = \frac{n!}{r!(n-r)!} P^r(1-P)^{n-r} \qquad (29)$$

The peak of the distribution will be defined as those locations in which two adjacent cavities contain equal amounts *i.e.* $F(r_m) = F(r_{m+1})$. Applying (29) to this equation and solving gives:

$$r_m = nP \qquad (30)$$

Hence, from eqn [27]: $$r_m = nG/(G+1). \qquad (31)$$



where a is the number of particles adsorbed at the interface, C_t is the concentration in the upper phase, V_t is the volume of the upper phase, v is the volume of upper phase not transferred in particle partition, G is the distribution ratio and K is the partition coefficient.

Fig. 5. Differences between the distribution type in liquid-liquid CCD (a) and liquid-interface CCD (b). To the left no significant interfacial adsorption takes place and the whole top phase is transferred at each step. To the right, the distribution takes place between the top phase and the interface. The bottom phase, the interface and a small layer above the interface together form the stationary layer which is not transferred. From Albertsson and Baird (1962).

CHAPTER 3

BACKGROUND TO THE SEPARATION PROBLEM.

1. History of the cell lines used for the separation. A variety of cultured cell lines and products thereof form the basis of this study. The rat monoclonal antibody specific for MBL-2 cells, YE1.48.10., used as the primary ligand throughout this study was raised against a mouse lymphocyte line resulting from the fusion of a Con-A activated normal mouse spleen cell and a transformed mouse T cell (EL-4BU). A "transformed" cell is one that has undergone a stable heritable change that usually enables it to grow into a tumor in an appropriate recipient animal. As the animal from which a cell is derived may not be available for tumorgenicity testing *in vivo* other tests have been developed although nude mice are still frequently used. One test is the ability of transformed cells to grow in the absence of substratum (*i.e.* in culture) or the rapid metabolism of glucose exhibited shortly after transformation. Some other changes commonly observed on transformation are growth to an unusually high cell density, lowered requirement for growth factors in serum and they are less anchorage dependence. There are also abnormalities in adherence and plasma membrane related properties. Transformed cells generally have a different morphologic appearance than normal cells in culture. They are often more rounded and are usually covered with microvilli and lamellopodia. It is possible to transform almost all the cells in a culture into cancer cells within a few days with RNA tumor viruses. Cells can also be transformed with chemical carcinogens.

Concanavalin A (con-A) is a mitogen commonly used to activate lymphocytes. When confronted with a stimulus such as con-A, the resting lymphocyte undergoes changes in virtually every aspect of cellular metabolism culminating in DNA synthesis and mitosis. *In vivo* this only occurs in cells having specific mechanisms for antigen recognition but *in vitro* a large number of cells can be stimulated in this way. It appears that mitogens mimic the action of antigens on lymphocytes. However, while an antigenic determinant stimulates only a very small proportion of all lymphocytes, mitogens stimulate a large number of

them. The mechanism of mitogenic stimulation is thought to involve aggregation of con-A receptors to form small clumps on the cell surface. These receptors are proposed to be transmembrane-linked to the cytoskeletal system which results in cytoplasmic changes in the cell leading to changes in metabolism (Golub, 1977).

The fusion of EL-4BU and the con A activated spleen cell resulted in ECA17.9.8, a doubly cloned hybrid. The technique for fusing cells with polyethylene glycol is also used to produce hybridoma lines secreting monoclonal antibody. Aqueous solutions of PEG at a sufficiently high concentration will fuse almost all cell types . The fusing concentration of PEG is usually above 30%w/w and the optimum concentration is determined by the balance between increasing fusion efficiency and minimizing cell damage. The mechanism by which PEG causes fusion is not known with certainty. The concentration dependence seems to be related to the ability of PEG to decrease the water activity of the solution since solutions inducing maximum fusion have virtually no water unassociated with the polymer. Many other polymers and viruses, in particular Sendai virus which was used in early hybridoma technology, will induce cell fusion.

The primary ligand YE1.48.10., was a rat MAb raised against ECA17.9.8. cells (Takei, 1983). The spleen cells of a rat immunized with ECA17.9.8. cells were fused with rat myeloma Y3 cells (Galfrè and Milstein, 1981). The hybrid culture supernatants were selected for ECA17.9.8. specific clones with an indirect binding assay using radio-iodinated $(F_{ab})_2$ fragment of anti-rat IgG. All positive hybrids were doubly cloned in soft agar. Cloning ensures that the hybridoma line is descended from a single cell. In this case dilute concentrations of cells were seeded into medium solidified by agar and cells forming colonies were transferred individually to culture dishes. The MAbs produced by the resulting hybridomas were tested for their reactivities with various lymphoid cells. One of the antibodies, YE1.48.10., an IgG2b, reacted strongly with two transformed T cell lines EL-4 and MBL-2. EL-4 is a chemically induced T-leukemia cell line of B6 origin (B6 refers to the mouse strain). A 5-bromo-2-deoxyuridine sub-line of EL-4, EL-4BU was one of the fusion partners in the hybrid cell line used to generate the YE1.48.10. hybridoma. MBL-2 is a Moloney

virus-induced T-leukemia cell line, also of B6 origin.

The antigen recognized by YE1.48.10. has been immunoprecipitated from EL-4 cells and is a dimer of 90kD (non-reduced). There are approximately 1.5-3 x 10^5 antigen molecules per EL-4 cell as measured by binding of radiolabelled YE1.48.10. The antigen is also expressed on normal T-cells but is hidden, not exposed as on EL-4 and MBL-2 cells. The antigen may be exposed on normal T-cells by non-ionic detergent solublization so it is likely to be hidden by by membrane-associated molecules rather than chemical modifications such as glycosylation (Chan and Takei, 1986).

The MBL-2 cells were resolved into two sub-populations, MBL-2(2.6) and MBL-2(4.1) by FACS on the basis of a higher surface antigen density on MBL-2(4.1) cells as defined by YE1.48.10. (Takei, 1986).

2. Structure of Immunoglobulin G (IgG). IgG (Fig. 6) is a symmetrical molecule made up of two identical glycosylated heavy chains (MW = 50 000-75 000) and two identical non-glycosylated light chains (MW ≈ 25 000). The heavy chains are joined by two disulfide bonds in the non-variable or F_c region and each light chain is joined to a heavy chain by a disulfide bond. Enzymic digestion by papain splits IgG into one F_c and two F_{ab} fragments. The F_{ab} fragment consists of one light and part of a heavy chain and includes the variable region (F_v) which binds antigen, thus varying in antibodies of different specificities (Goding, 1983). The F_c fragment consists of only heavy chain and is constant in any one class of IgG irrespective of antibody specificity as it is not involved in antigen binding. Papain digestion produces fragments with retention of biological activity making it preferable to chemical degradation.

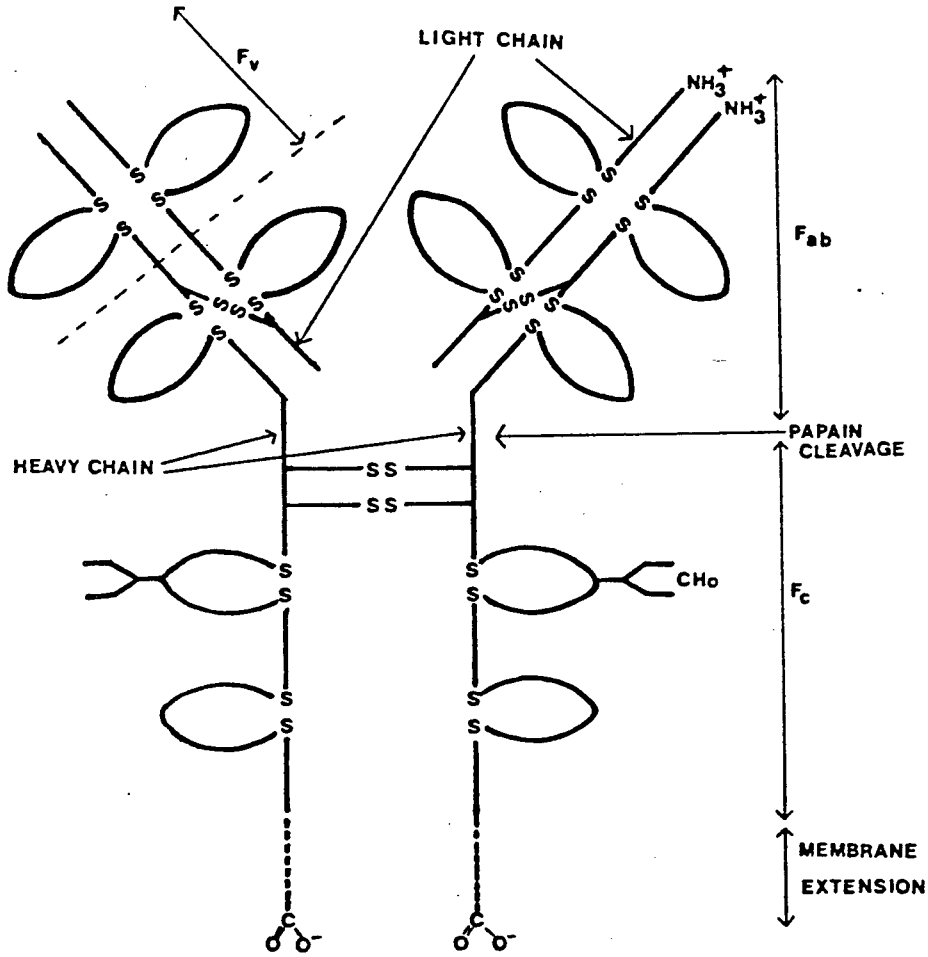


Fig. 6. Structure of the IgG molecule. Reduction of the disulfide cross links will split the molecule into heavy and light chains. The enzyme papain will cleave the molecule into F_{ab} and F_c fragments during limited digestion.

3. Avidin-Biotin reaction. One of the ligand combinations utilized in this study is a biotin-derivatized antibody and PEG-avidin (Fig. 2.d.) Biotin (Fig. 7, vitamin H) was first encountered as early as 1916 when it was observed that diets high in raw egg white were toxic to rats (Batemann, 1916). Later the symptoms of egg white injury in rats were described as muscle incoordination, dermatitis, hair loss and nervous problems (Boas, 1927). It was also noted that cooked egg white did not have any effect and that liver, yeast and other foods protected rats against raw egg white toxicity. The protective substance was called vitamin H (György *et al*, 1940). Previously a potent growth stimulant for yeast had been identified and named biotin (Kögl and Tonnis, 1936). In 1941 the structure of biotin was elucidated, synthesized and confirmed to be vitamin H (György *et al*, 1941). At the same time the presence of avidin in egg white, a basic glycoprotein with anti-vitamin H activity was confirmed.

Dietary deficiencies of biotin are rarely observed, however, due to its synthesis by intestinal bacteria (although the daily requirement is estimated at 0.01 to 0.2 mg). A deficiency of biotin results in reduced activity in urea synthesis, purine synthesis, carbamylation and tryptophan catabolism. This is not accounted for by any known biotin enzymes nor is the above effect observed on administration of avidin.

Biotin is covalently linked to the enzyme (*e.g.* a carboxylase) through the ε-amino group of a lysine residue resulting in a fairly long, flexible attachment (Fig. 7). The biotin-lysine peptide has been isolated by hydrolysis of biotin-containing enzymes and is known as biocytin. The most important function of biotin is as a coenzyme for a variety of carboxylating enzymes.

One of the most important enzymes for which biotin is a coenzyme is pyruvate carboxylase which interconverts pyruvate and oxaloacetate. The biotin becomes carboxylated at N_1 only when acetyl CoA is bound to the pyruvate carboxylase. This allosteric activation is an extensively studied physiological control mechanism.

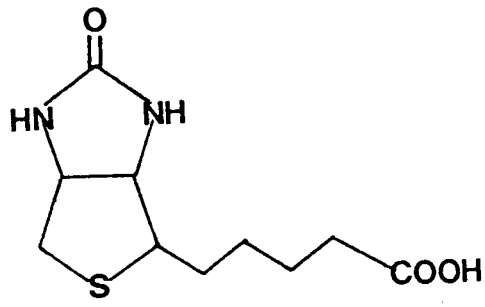


Fig. 7. The structure of biotin.

Other enzymes for which biotin is a cofactor are acetyl CoA carboxylase, which is involved in fatty acid biosynthesis, and propionyl CoA carboxylase which metabolizes propionyl CoA, a breakdown product of fatty acids with odd numbers of acyl carbons. The reactions involved are summarized below.

$$\underset{\text{Acetyl CoA}}{CH_3CO\text{-}SCoA} \xrightarrow[\text{biotin, Acetyl CoA carboxylase}]{ATP,\ CO_2 \rightarrow\ ADP + P_i} \underset{\text{Malonyl CoA}}{HO_2CCH_2CO\text{-}SCoA}$$

$$\underset{\text{Propionyl CoA}}{CH_3CH_2CO\text{-}SCoA} \xrightarrow[\text{biotin, propionyl CoA carboxylase}]{ATP,\ CO_2 \rightarrow\ ADP + P_i} \underset{\text{Methyl Malonyl CoA}}{HO_2CCH(CH_3)CO\text{-}SCoA}$$

Biotin contains fused imidazole and thiophene rings as illustrated in Fig. 7. and is bound tightly by avidin ($k=10^{21}\ M^{-1}$). This strong biotin-avidin interaction is used extensively in cell-labelling procedures and various immunoassays.

4. Polyacrylamide-protein graft copolymers as ligands. Polyacrylamide (PAA) is highly hydrophilic compared to most non-ionic water soluble polymers. For instance, it has a partition coefficient of 18 in favor of the water-rich phase in a water/phenol two phase system (Dobry, 1956). Before this work was carried out its behavior in dx/PEG systems was not known but it seemed likely that PAA would partition in favor of the

dx-rich phase. This supposition is based on the use of PAA-coated chromatographic supports to bind stationary dx-rich phase for use in liquid-liquid partition chromatography (Müller, 1986). Only small surface coverage of the bead with PAA was required to immobilize large volumes of stationary phase.

In the work to be described, polymerization of acrylamide was carried out *via* a free radical mechanism. It is known that some ceric salts such as nitrate and sulfate form effective redox systems in the presence of organic reducing agents such as alcohols, thiols, glycols, aldehydes and amines. This produces cerous ions and free radicals capable of initiating vinyl polymerization. The ceric salts form complexes with alcohols and glycols and the disproportionation of these complexes is the rate determining step (Duke and Forist, 1949; Duke and Bremer, 1951). The mechanism of the initiation reaction for alcohols can be written generally as:

$$Ce^{IV} + RCH_2OH \rightleftharpoons B \longrightarrow Ce^{III} + H^{+} + \overset{*}{R}CHOH$$

where Ce^{IV} represents the aqueous ceric complex, B the ceric-alcohol complex and $\overset{*}{R}CHOH$ a free radical which initiates polymerization in the presence of a vinyl monomer. If the reducing agent is a macromolecule and the reduction is carried out in the presence of a vinyl monomer, the free radical produced on the macromolecule initiates polymerization to produce a polymer graft. This yields predominantly grafted polymers since the free radicals are formed preferentially on the backbone. In the absence of a reducing agent, ceric salts will initiate acrylamide polymerization at a very slow rate. For instance, in the case where polyvinyl alcohol was the reducing agent the initial rate was 3.0%/minute compared to 0.04%/min in the absence of reducing agent (Mino and Kaizerman, 1958). This means that there will be a small amount of free polyacrylamide (PAA) in the PAA-macromolecule solution.

In the present work polyacrylamide was grafted onto the primary and secondary hydroxyls of IgG to form a lower phase partitioning immunoaffinity ligand.

CHAPTER 4.

METHODS AND MATERIALS.

1. Fragmentation of mouse IgG. This is an adaptation of the methods of Utsumi (1969) and Porter (1959). Mouse IgG (Sigma, 1 mg/mL, 1 mL) in Na_2HPO_4/ NaH_2PO_4 buffer (0.01 M, pH 8.0) containing ethylene-diaminotetraacetate (EDTA, 2 mM) and dithiothreitol (1 mM) was incubated with activated mercuripapain (BDH, 0.1 mg/mL, 0.1 mL, 15 minutes, 37°, buffer as above) for one hour. The reaction was stopped with iodoacetamide (final concentration 20 mM) and kept in the dark at 0° for one hour. The mixture was dialyzed against sodium acetate (NaAc, 10 mM, pH 5.5) at 4°, then fractionated on a mono Q anion exchange column coupled to a Pharmacia Fast Protein Liquid Chromatography (FPLC) apparatus with an elution gradient of 0.01-1 M NaAc (pH 5.5). The fractions were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2. Production of sheep anti-(mouse F_c fragment) IgG (s α mF_c). A sheep was injected subcutaneously with mouse F_c fragment (50 μg), obtained as described above, in complete Freunds adjuvant[1] (1:1 emulsion). Two subsequent injections at two week intervals were given in incomplete Freunds adjuvant.[1] After one week, blood was drawn and subsequent blood drawing was preceded by a booster injection one week beforehand. The blood was allowed to clot at room temperature and the serum obtained by centrifugation and decantation. A solution of saturated $(NH_4)_2SO_4$ was added over 10 minutes to the slowly stirred serum at room temperature. At about 40% v/v saturated solution, most of the immunoglobulins were precipitated (Goding, 1983). This was confirmed by SDS-PAGE. The

[1]An adjuvant is a substance which augments immune responses in a non-specific manner. Freund's complete adjuvant is a water in oil emulsion in which killed and dried *M. tuberculosis* bacteria are suspended in the oil phase, Freund's incomplete adjuvant omits the bacteria (Herbert, 1973).

suspension was stirred for a further 30 minutes. This was followed by centrifugation at 10 000 g for 10 minutes at 4° and the pellet washed three times with 40% saturated $(NH_4)_2SO_4$ solution. The pellet was dissolved in phosphate buffered saline (PBS, 16.7 mM Na_2HPO_4, 3.3 mM NaH_2PO_4, 130.4 mM NaCl, pH 7.2), dialyzed against water, the precipitate discarded and the solution redialyzed against Na_2HPO_4/ NaH_2PO_4 (10 mM, pH 8.0). The solution was chromatographed on a mono Q anion exchange column using FPLC with a 0-1 M NaCl ionic strength elution gradient. SDS-PAGE was used to identify the fractions.

3. Monoclonal antibody production via ascites fluid. This method (Goding, 1983) was used to produce mouse anti-NN glycophorin (mαNN glyc) from the hybridoma cell line NN-5 (American Tissue Culture Collection HB 8476). BALB/c mice were injected intraperitoneally (i/p) with 2,6,10,14-tetra-methylpentadecane (pristane, 0.5 mL per mouse) and 3-4 days later injected with 10^6 hybridoma cells i/p in 1 mL of serum-free medium. After 7-10 days, the ascites fluid was drained by inserting a needle i/p and this was repeated every 2-3 days until death of the mouse. Each mouse typically yielded 5-6 mL of fluid containing 5-15 mg/mL of antibody. The ascites was centrifuged and the supernatant stored at -20° until purification. The mαNN glyc was purified by $(NH_4)_2SO_4$ precipitation as described earlier followed by FPLC on a mono Q column with a 0.01-0.3 M Na_2HPO_4/ NaH_2PO_4, pH 8.0 ionic strength elution gradient. The peaks were identified by SDS-PAGE.

4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The method used for slab gels was an adaptation of the methods of Davis (1964) and Ornstein (1964). The stock solutions were made up as follows:
1. Acrylamide:bis (30:0.8). Acrylamide solution (60 g in 200 mL of water) was heated to 60°, then maintained at 56° for 30 minutes in the dark. The solution was filtered through Whatman #1 filter paper and N,N'-Methylene-bis-acrylamide (bis) (1.6 g per 200 mL) added. The solution was stored at 4° in the dark.
2. 1.875 M [Tris(hydroxymethyl)aminomethane, pH 8.8 (tris-base).
3. 1.0 M [Tris(hydroxymethyl)aminomethane hydrochloride], pH 6.8

(tris-HCl).

4. Sample preparation solution (10% SDS). The following were combined: SDS (0.41 g), tris-HCl (1.28 mL), water (12.3 mL) and bromophenol blue dye to color.

5. Running buffer. Glycine (57.6 g) and SDS (4 g) were added to 4 L of tris-base (0.05 M), pH 8.3.

6. Coomassie stain. Coomassie brilliant blue dye (0.4 g) was dissolved in isopropanol (30 mL), water (130 mL) and acetic acid (30 mL).

7. Destain solution. An aqueous solution of 35% v/v ethanol and 10% v/v acetic acid was used.

Acrylamide:bis (10 mL), tris-base (6 mL), EDTA (0.2 M, 0.3 mL) and water (13.4 mL) were mixed and degassed under vacuum. N,N,N',N'-tetramethylethylenediamine (TEMED, 0.015 mL), SDS (10%, 0.3 mL) and freshly made $(NH_4)_2S_2O_8$ (10%, 0.3 mL) were added and this, the resolving gel, poured into a Bio-Rad Mini-PROTEAN II gel electrophoresis apparatus. Water was layered over the surface and polymerization was complete within 30-40 minutes. This procedure was repeated using acrylamide:bis (2.5 mL), tris-HCl (1.88 mL), EDTA (0.2 M, 0.15 mL), water (10.3 mL), TEMED (0.0075mL) and $(NH_4)_2S_2O_8$ (10%, 0.15 mL) to prepare the stacking gel. The water was removed from the surface of the running gel, a sample comb inserted and the stacking gel poured. Polymerization was complete within 30-40 minutes. The quantities quoted are for a 10% resolving gel and 5% stacking gel.

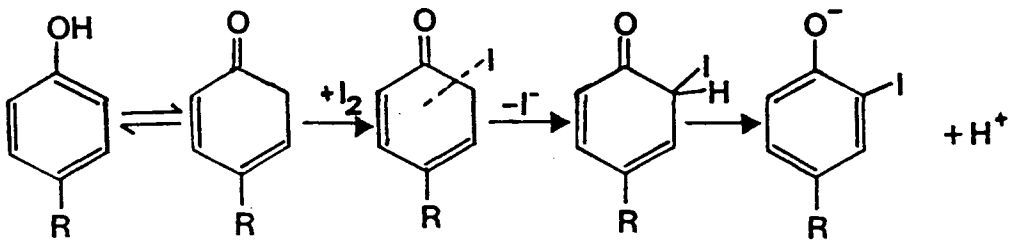
Each protein sample (10-20 μg) was mixed with an equal volume of sample preparation solution and reduced with mercaptoethanol (5%) at 100° for 3 minutes in the case of reduced samples. The samples were applied to the gel (10-20 μL), the apparatus was filled with a 4:1 dilution of running buffer, air bubbles removed, and a constant current of 130 mA applied until the tracking dye reached the end of the gel. The gel was stained for 2 hours, destained over several hours followed by overnight soaking in 3% glycerol then dried for 3 hours on a Bio-Rad 543 gel drier.

When rod gels were run the resolving gel was cast in 125 x 5 mm inside diameter tubes which were mounted in a Bio-Rad 150A electrophoresis chamber and run at an initial current of 0.5 mA per tube

until the sample entered the gel at which point the current was increased to 8 mA per tube. When the tracking dye reached the end of the gel it was marked with India ink and the gels fixed and stained as before.

5. **Hemagglutination assay.** Serial dilutions of antibody solution (50 μL) were made up in PBS in a microtiter plate (a PVC plate containing multiple wells). An equal volume of 1% hematocrit fresh cell suspension (usually erythrocytes) was added to each well. The suspensions were mixed and examined after four hours. In the wells containing sub-agglutinating concentrations of antibody the cells were rolled down the sides of the well and settled as a button at the bottom of the wells whereas in the wells containing agglutinating concentrations of antibody the cells were prevented from rolling by adhesion to their neighbors and uniformly distributed over the well.

6. **Radiolabelling of proteins with ^{125}I.** This was done using iodobeads which are N-Chloro-benzenesulfonamide (sodium salt)-derivatized, uniform, non-porous, polystyrene beads (Markwell, 1982). Of all the amino acids that are capable of reacting with iodine in a protein, tyrosine is of overriding importance because of the relative stability of the bond between the iodine and the protein (Krohn *et al*, 1977). This reaction is summarized below.



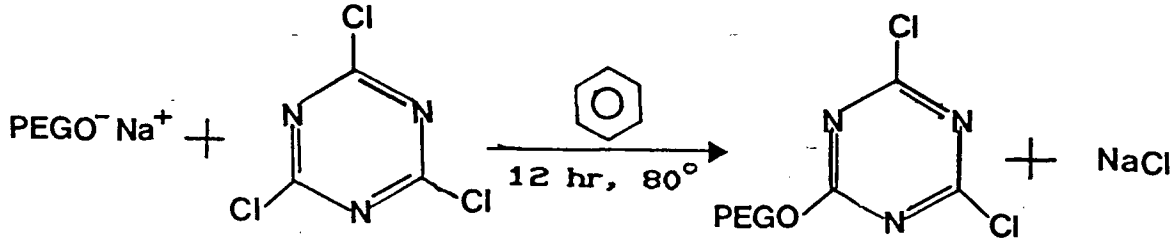
Protein solution (0.5 mL, 0.5 mg/mL in PBS) was added to 2-3 iodobeads (Pierce) and $Na^{125}I$ (2 μCi), then stirred end over end for 40 minutes. A small aliquot (≤ 1μL) of the reaction mixture was added to a mixture of trichloroacetic acid (20%, 1 mL) and bovine serum albumin (BSA, 0.5 mg/mL, 1 mL). The precipitate and supernatant were separated, counted

and the fraction of bound ^{125}I calculated. When the reaction was over 90% complete, usually within one hour, the free iodine was separated from the labelled protein with a G-150 Sephadex column using PBS as the elution buffer. The column fractions (1 mL) were sampled (1 μL) for counting and the first, labelled protein-containing peak was broken into aliquots and stored at -20°. The labelled stock protein solution was added to unlabelled protein to obtain the required specific activity for binding or partitioning studies. The specific activity of the stock solution was measured by absorbance at 280 nm ($A^{1\%}_{1-cm}$ = 13.5, Kirschenbaum, 1973) and γ counting on a LKB 1282 Compugamma γ counter. In all cases proteins were labelled prior to derivatization with PEG, trypan blue or biotin.

7. Trypan blue-derivatization of protein. Radiolabelled protein solution (5-10 mg/mL in 0.1M NaAc, pH 4.8, 4 mL) was mixed with trypan blue solution (50 mg/mL in 0.1M NaAc, pH 4.8, 1 mL). 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide-HCl (EDC, 200 mg/mL in 0.1M NaAc, pH 4.8, 0.5 mL) was added and the pH of the mixture adjusted to 4.8. The mixture was rotated end over end for 4 hours. Free dye was removed by dialysis against NaCl solution (1 M) and the amount of dye bound to the protein was estimated from the ratio of ^{125}I to dye absorbance at 610 nm. The validity of this estimate is discussed later.

8. Synthesis of 2(alkoxypolyethyleneglycoxy)-4,6 dichlorotriazine (PCC). The usual approach to preparing protein-PEG conjugates is to synthesize an "activated" PEG which will readily couple with a protein functional group. In this study PEG 1900 was activated using cyanuric chloride. The resulting product reacted mainly with protein primary amines (Abuchowski *et al*, 1977). The solvents were dried as follows: Benzene was distilled from CaH_2 onto 4Å molecular sieves, acetone was kept over 4Å molecular sieves and petroleum ether was predried over $MgSO_4$ then distilled from CaH_2 onto 4Å molecular sieves. To synthesize PCC, NaH (0.18g) was suspended in anhydrous benzene (50 mL) and the system purged with N_2. PEG-1900 (10g) in anhydrous benzene was added over fifteen minutes. The reaction mixture was stirred for one hour under N_2 flow and one hour

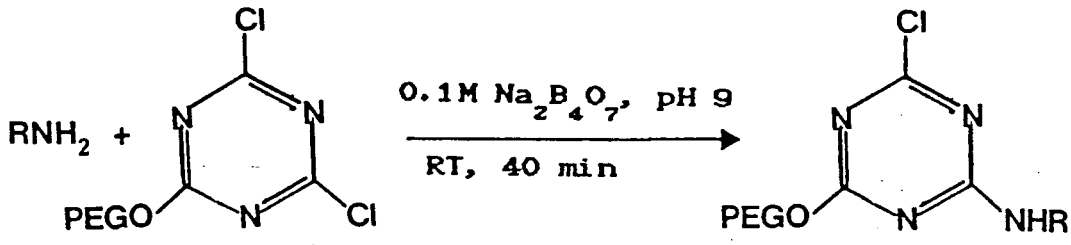
under a stationary N_2 atmosphere. A four-fold molar excess of cyanuric chloride (3.9 g) was added and the mixture refluxed (80°) for 12 hours. The reaction mixture was poured into petroleum ether (b.p. frac. 60-80°, 150 mL) at 0° and the precipitated product collected by filtration on Whatman #42 paper. The product was redissolved in a minimum of anhydrous acetone and reprecipitated with two volumes of petroleum ether at 0°. The reprecipitation was repeated three times. The PCC was vacuum dried and stored at -70° under N_2. It was stable for up to 6 months or over 2 years when stored under N_2. The reaction efficiency was determined by assaying the hydrolyzable chlorides of the PCC. In all cases PEG-1900 was used to modify proteins and PEG 8000 was used to form APTS.



9. Determination of the hydrolyzable chlorides of PCC. The Buchler Cotlove Chloride Titrator applies the coulometric principle of titration. Silver ions are generated at the anode which react with the chloride ions and precipitate. When all the free chloride has reacted, the increase in current is detected as the end point. Four samples of PCC (approx. 40 mg) were accurately weighed and two of them hydrolyzed in $Na_2B_4O_7$ (0.1M, pH 10) for at least 2 hours. The other two samples were dissolved in the same buffer, pH 9, immediately before titration. A solution of HNO_3 (0.1M) and CH_3COOH (10%, 4 mL) and gelatin (0.62%, 0.2 mL) was added to each sample, a blank and a NaCl standard. The free chlorides were titrated using the chloridometer.

10. Reaction of the PCC with protein. Protein solution (2-8 mg/mL in $Na_2B_4O_7$, 0.1M, pH 9) was mixed with PCC to give a PCC:lysine molar ratio of 1:1, 3:1 or 5:1, assuming a lysine content of 124 lysines per IgG molecule (Edelman *et al*, 1969). After 40 minutes at room temperature the mixture was either used immediately, stored at -20° or the unbound PCC

and PEG removed by ultrafiltration through an Amicon PM 10 membrane or dialysis. The degree of protein primary amine modification was estimated by reaction with 4-phenylspiro[furan-2H(3H),1'-phthalan]-3,3'dione (fluorescamine).



11. Degree of modification of the protein. The reagent 4-phenylspiro-[furan-2H(3H),1'-phalanx]-3,3' dione (fluorescamine) reacts with primary amines to form fluorophors. The resulting fluorescence is stable over several hours and excess reagent is hydrolyzed within a minute. Moreover, significant quantities of ethylene glycol-based detergents have no effect on the resulting fluorescence (Stocks *et al*, 1986). The amount of protein-bound PEG was estimated by assaying the protein primary amines with fluorescamine before and after reaction with PCC. Protein solutions were made up in sodium phosphate buffer (0.1M, pH 8), each test tube containing between 0 and 2.5 μg of protein made up to 1.5 mL. Fluorescamine (0.3 mg/mL in acetone, 3 mL) was added to each test tube while vortexing. After a minimum time of 7 minutes the fluorescence in the solution was measured on a Turner Model 430 spectrofluorometer with an excitation wavelength of 390 nm and emission detected at 475 nm. (For details see Stocks *et al*, 1986).

12. Enzyme-linked immunosorbent assay (ELISA). A 96 well PVC microELISA plate was coated by adsorption with antigen solution (1 μg/mL in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6, 200 μL per well) at 4° overnight. The plate was washed three times with PBS-Tween (0.5% Tween 80) manually or in a SLT EAW8/12 ELISA plate washer. An aliquot of a serial dilution of antibody solution was added (200 μL per well) and incubated for one hour

at 37°. The plate was washed three times with PBS-Tween. A solution of BSA (0.5%) in PBS-Tween was added (200 μL per well) as a blocking agent and incubated 30 minutes at 37°. The plate was washed three times and a solution of horseradish peroxidase (HRP) conjugated antibody (Sigma) specific for the primary antibody was added (1:8000 dilution of a 0.5 mg/mL stock solution), followed by incubation at 37° for 2 hours. In some cases where the primary antibody was derivatized, the HRP-conjugated antibody was specific for the antigen. The unbound conjugate was removed by washing three times and O-phenylenediamine solution (OPD, 0.04% in 25 mM citric acid, 51 mM Na_2HPO_4, pH 5.0) containing H_2O_2 (0.12%) added immediately before use was added to each well (200 μL per well). The color was allowed to develop in the dark until the reaction was stopped by the addition of H_2SO_4 (4 M, 50 μL per well) and the absorbance of each well measured at 492 nm with a 690 nm reference beam on a SLT EAR 400 AT ELISA plate reader interfaced to an Epson LX-800 printer.

13. Culture of hybridoma and transformed lymphocyte cells. Freezing and thawing of cells: The cells were frozen by resuspending approximately 5 x 10^6 cells in freezing medium (90% fetal bovine serum (FBS), 10% DMSO, 1 mL), followed by initial freezing at -70° for 24 hours. They were stored in liquid N_2 indefinitely. The cells were thawed over 5 minutes at room temperature, then 5 min at 37°, added to cold (4°) serum-free medium and mixed by inversion. An aliquot (100 μL) was stained with erythrosin B dye and the viability of the cells estimated by counting live and dead cells on a hemocytometer. Only dead cells are stained by erythrosin B. If the viability was over 80%, the cells were centrifuged (300 g, 2 minutes), the medium aspirated off and the cells resuspended in growth medium containing FBS (10-15%) to give a final concentration of 10^5-10^6 cells per mL. Incubation was at 37°, 100% humidity and 5% CO_2. The cells were passed at the appropriate cell density for each cell line. All cell lines were gradually transferred into 5% FBS-containing medium within several weeks. The NN-5, RG7/11.1 and MBL-2 cells were all cultured in RPMI-1640 (Cancer Control Agency, Vancouver, B.C.) medium but YE1.48.10. cells were started in DMEM (Gibco) then transferred into

RPMI-1640.

14. Supplemented serum-free medium (SSFM). RPMI-1640 medium (Gibco) containing L-glutamine (0.3 mg/mL, Gibco) and penicillin-streptomycin (50 units/mL and 50 μg/mL respectively, Gibco) was supplemented (Murakami *et al*, 1982) by the addition of bovine insulin (5 μg/mL, Sigma), human transferrin (35 μg/mL, Sigma), sodium selenite (2.5 mM, Sigma) and ethanolamine (20 μM, Sigma). Insulin stock solution (2 mg/mL) was prepared in distilled water adjusted to pH 2.5 by addition of HCl (1 M) and transferrin stock solution (2 mg/mL) was prepared in PBS. The protein stock solutions were stored at -20° and the other stock solutions at 4°. The SSFM was made immediately before use or stored at 4°.

15. Culture of YE1 48.10. and RG7 11.1 cells in SSFM. Rapidly growing cells (30 mL) adapted to growth in 5% FBS-containing medium were centrifuged (300 g, 2 minutes) and the serum-containing medium aspirated off. The cells were resuspended in SSFM (100 mL) in an 850 cm^2 roller bottle (Fisher) and incubated overnight, upright, in an atmosphere of 100% humidity and 5% CO_2 at 37°. The following day, the volume of SSFM was made up to 700 mL and the bottle allowed to re-equilibrate with the 5% CO_2, 100% humidity atmosphere. It was then sealed and rotated at 1 rpm at 37°. Each day the bottle was re-equilibrated with the 5% CO_2, 100% humidity atmosphere until the cells were confluent, usually approximately 10 days later. Cell numbers were estimated by counts using a hemocytometer and viabilities by erythrosin B dye uptake. (For details see Stocks and Brooks, 1989).

16. Harvesting and purifying the monoclonal antibodies. The contents of the roller bottles were centrifuged (300 g, 5 minutes, 4°) in a Sorvall RC-5 centrifuge equipped with a GSA rotor and the supernatant decanted and filtered through a 0.22 μm filter. The supernatant was then concentrated 50 fold by ultrafiltration using an Amicon XM100A or PM10 filter (Stocks and Brooks, 1989). The concentrated supernatant (2 mL) was injected onto a Pharmacia mono Q anion exchange column linked to a

FPLC apparatus. The elution buffer was 10mM sodium phosphate with a pH gradient from pH 8.5 to 7.0 and a 0-1 M sodium chloride ionic strength gradient (Gemski *et al*, 1985). The IgG peak, identified by SDS-PAGE was concentrated and stored at -70°.

17. Measurement of the binding isotherms of YE1 48. 10. and RG7 11.1 antibodies to MBL-2(4.1) and MBL-2(2.6) cells. All binding studies were performed at 4° in PBS containing NaN_3 (3 mM). The cells were washed three times in PBS, then resuspended at a measured hematocrit (H, 30-50%) and kept at 0° for a minimum of 30 minutes or until required. Eppendorf tubes were incubated with BSA solution at 4° overnight, washed three times with PBS, dried and weighed (w_1). Antibody of a known specific activity (S) and buffer to make the volume up to 1 mL were added and the eppendorf tube reweighed (w_2). The antibody solution was incubated for 1 hour at 4° with end over end stirring, followed by the removal of 100 μL for γ counting (γ_1) and reweighing of the eppendorf tube (w_3). Up to 0.3 mL of cell suspension of hematocrit H was added and the eppendorf tube reweighed (w_4), followed by a further 1 hour incubation with end over end stirring at 4°. The cells were centrifuged (300 g, 4 minutes) and an aliquot of supernatant (200μL) removed for counting (γ_2). The eppendorf was reweighed (w_5). If a wash analysis was required, the remaining supernatant was removed and the cells washed three times with PBS, each of the discarded washings being counted. The final pellet was resuspended, weighed, counted and the hematocrit of the resuspended pellet measured. In this case a comparison could be made between the equilibrium binding and that bound after washing the cells. The following equations were used to calculate the bound and free ligand concentrations:

$$\nu = \frac{[\gamma_1(w_3-w_1)/(w_2-w_3)] - [\gamma_2(w_4-w_1) - (w_4-w_3)H]/(w_4-w_5)}{(w_4-w_3).H.S}$$

$$\frac{\nu}{L} = \frac{\{[\gamma_1(w_3-w_1)/(w_2-w_3)] - [\gamma_2(w_4-w_1) - (w_4-w_3)H]/(w_4-w_5)\}(w_4-w_5)}{(w_4-w_3).H.\gamma_2}$$

where ν = surface concentration of bound ligand (molec./ g of packed cells).

L = free ligand concentration at equilibrium (molec./ g of supernatant).

w_1 = weight of empty eppendorf tube (g).

$w_2 = w_1$ + weight of radiolabelled antibody solution (g).

$w_3 = w_2$ - weight of sample γ_1 for counting (g).

$w_4 = w_3$ + weight of cell suspension added (g).

$w_5 = w_4$ - weight of sample γ_2 for counting (g).

γ_1 = γ counts of approx. 100 μL of supernatant before adding cells (cpm).

γ_2 = γ counts of approx. 100 μL of supernatant after adding cells (cpm).

H = mass fraction occupied by cells in added cell suspension, assumed equal to the hematocrit, or volume fraction of cells, in the added cell suspension. The mass of the packed cells was 10^9 cells per g.

S = specific activity of antibody solution (cpm/ molec.).

Error estimates for these measurements were made from the partial derivatives of the above expressions for ν and ν/L and are shown in Appendix I. The surface area per MBL-2 cell was estimated from a photomicrograph and this information used to calculate the number of ligands bound per cell.

18. Fluorescent staining of cells. Approximately 5 x 10^6 cells were washed 3 times in PBS containing NaN_3 (3.0 mM). The cells were incubated in YE1.48.10. culture supernatant at 4° for 30 minutes with end over end stirring, then centrifuged. The supernatant was aspirated and the cells washed three times in PBS containing NaN_3. Fluorescein isothiocyanate-conjugated goat anti-rat IgG (FITC-gαrIgG) was added (0.5 mL, 0.05 mg/mL) and the incubation repeated as before. The cells were centrifuged, washed three times then resuspended at approximately 50% hematocrit and placed on a slide. All cells except erythrocytes were

fixed in 95% ethanol and mounted under glycerol:PBS (90:10). Photofluorescence microscopy was carried out using a Zeiss Photomicroscope II equipped with a Zeiss Neofluor 40/0.75 Oel oil immersion lens and Kodachrome 400 ASA film with an approximately 30 second exposure. Excitation was at 450-500 nm with a 510 interference filter and a high pass filter of greater than 528 nm.

19. Trypsin digestion of MBL-2 cells. MBL-2 cells (approximately 10^{10}) were washed 3 times in PBS, resuspended in serum-free RPMI 1640 containing trypsin (10 mL, 0.25 mg/mL) and incubated 30 minutes at room temperature with end over end stirring. The cells were centrifuged, washed 3 times with PBS and used for fluorescent staining in some cases. Phenylmethyl sulfonyl fluoride (PMSF, 0.02 mM final concentration) was added to the supernatant which was stored at -70° and used to coat ELISA plates.

20. Preparation of the two phase systems. Dextran T500 (M_w=494 000, M_n = 181 200, lot 26066) was obtained from Pharmacia and PEG 8000 (M_w = 8000) was obtained from Union Carbide. The following stock solutions were prepared:

1. Dextran. Dextran T500 (22g) was made up to 100g with water and stirred until dissolved. The %w/v was determined with a Steeg and Reuter polarimeter on a 10 fold dilution of the stock solution using $[\alpha]_D^{25}$= +199° followed by conversion to %w/w using the partial specific density of dextran.
2. PEG. PEG 8000 (30g) was made up to 100g with water. The %w/v was determined by measuring the refractive index of a 10 fold dilution of the stock solution using a Bausch and Lomb "Serum Protein" Meter then converted to %w/w using the partial specific density of PEG.
3. NaCl (0.6M).
4. Na_2HPO_4 / NaH_2PO_4 buffer (214 mM / 67 mM, mixed to give pH 7.2).
5. Sorbitol (0.6 M).

The stock solutions and water were mixed to give the required composition, mixed well and allowed to separate either at room temperature or 4° or by centrifugation (200 g, 10 minutes). The phases

were stored separately at 4°or -20°. The systems used in the study were designated by the abbreviation X,Y,Z where X denotes the % w/w dx, Y denotes the % w/w PEG and Z is the buffer composition which may be I, II, III or S. I is 110 mM Na_2HPO_4/ NaH_2PO_4, II is 96 mM Na_2HPO_4/ NaH_2PO_4 and 50 mM NaCl, III is 10 mM Na_2HPO_4/ NaH_2PO_4 and 130 mM NaCl and S is 10 mM Na_2HPO_4/ NaH_2PO_4 and 100 mM sorbitol. An example of a phase composition description would be 5,4,II which would be 5% dx, 4% PEG, 96 mM Na_2HPO_4/ NaH_2PO_4 and 50 mM NaCl. The phase systems used in this study were 5,4,Z where Z is I, II, III or S; 5,3.4,Z where Z is I, II, III; 5,3.5,I; 5,3.6,I; 5,3.7,I; 5,3.8,I; 5,3.9,I; 5,5,I; 5,4,S and 7,4,S.

21. Partitioning of cells and proteins. Protein solution (up to 100 μL) was added to 2 mL of phase system. If the resulting dilution of the phase system formed one phase, then stock PEG 8000 (up to 10 μL, approx. 30% w/w) was added to reform the phases. The phases were mixed by 20 inversions and allowed to settle before sampling and assay of the phases for protein concentration with Coomassie blue dye or radioactive counts or in the case of biotin or avidin with 4-hydroxyazobenzene-2'-carboxylic acid (HABA). For cell partitioning, erythrocytes were collected into 0.38% sodium citrate (final concentration) by venipuncture and cultured cells were removed from the medium by centrifugation. All cells were washed three times with PBS and resuspended in upper phase. The load mix concentration was determined by impedance cell counting on a Particle Data Inc. Electrozone Celloscope, usually approximately 2 x 10^8 cells/mL. Approximately 2 x 10^6 cells were added to 2 mL of phase system, the system mixed by inversion 20 times and upper, lower and total phases sampled. The cells were counted by impedance counting or radioactive counts.

22. Biotin-derivatization of protein. N-hydroxysuccinimidobiotin (BNHS, 25 mg/mL in dimethylformamide, Sigma) was added to protein (1-50 mg in 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0) to give molar reaction ratios ranging from 1:10 lysine:BNHS. The mixture was stirred end over end for 4 hours then dialyzed overnight against the appropriate buffer, usually 10 mM Na_2HPO_4/NaH_2PO_4, pH 8.0. The biotin content of the conjugate was assayed

with HABA as defined and described below.

23. Determination of avidin, biotin and degree of protein derivatization with biotin. The assay of avidin and biotin is based on the use of the dye 4-hydroxyazobenzene-2'-carboxylic acid (HABA) which binds to avidin. The binding of HABA by avidin is accompanied by spectral changes summarized in Table 1.

Table 1. Extinction coefficients of 4-hydroxyazobenzene-2'-carboxylic acid and its complexes with avidin. From Green (1970).

	λ (nm)	ε_{282}	ε_{350}	ε_{500}
Avidin	282	25 000	0	0
HABA	350	2 800	20 500	480
Avidin-HABA complex	500	-	2 500	34 500

HABA is not bound by the avidin biotin complex and since the dissociation constant of the latter is so low (10^{-21} M) the dye is stoichiometrically displaced by the biotin. HABA was recrystallized from aqueous methanol and dissolved in one equivalent of sodium hydroxide.

a. Assay for avidin. HABA (10 mM, 50 μL) was added to avidin solution (0.1 - 1 mg, 2mL) in sodium phosphate buffer (0.1, pH 8) in a 1 cm cuvette. The absorbance at 500 nm (A_{500}) was measured then biotin added (2 mM, 50 μL) and the A_{500} remeasured. The number of binding sites and the concentration of the original solution were calculated using the following expressions.

$$[\text{binding sites}] = \frac{\Delta A_{500}}{34} \text{ mM}$$

$$\text{avidin (mg/mL original solution)} = \frac{2.05 \text{ mL}}{2 \text{ mL}} \times \frac{16.2}{34} \Delta A_{500}$$

$$= 0.49 \, \Delta A_{500}$$

b. Assay for biotin. A solution of avidin-HABA complex (0.2 - 0.4 mg avidin/mL, 0.25 mM HABA in 0.1M sodium phosphate buffer, pH 8, 2mL) was pipetted into a 1 cm cuvette and the absorbance at 500 nm was measured (A_1). A known volume of biotin solution (v mL) was added and the absorbance remeasured (A_2). The biotin concentration was calculated as follows.

$$[\text{biotin}] = A_1 - A_2 \; \frac{(v + 2)/2}{34} \; \text{mM}$$

c. Assay of degree of protein modification by biotin. When solutions of biotinyl proteins are added to avidin-HABA complex, the dye is displaced more slowly than by the free biotin. When about 30% of the dye has been displaced the reaction becomes very slow and does not go to completion (Green, 1970). For this reason the protein-biotin complex was digested prior to assay for biotin. The modified protein was dialyzed against 2x4L of sodium phosphate buffer (0.1M, pH 8), then digested with pronase (Sigma, 0.2 mg/mL, 2 hours, 37°) and assayed for biotin as described above.

24. Polyacrylamide-derivatization of proteins. Protein solution in water (25 mL, approximately 2 mg/mL), in a three neck 250 mL flask equipped with a condenser, a dropping funnel and a N_2 inlet through an empty gas washing bottle, was degassed by bubbling N_2 through the solution for three hours under reflux at room temperature. The N_2 exited the apparatus through a Dudley bubbling tube. $(NH_4)_2[Ce(NO_3)_6]$ (54 mg/mL in 1 M HNO_3, 0.1 mL) in the dropping funnel was degassed by means of a Y junction in the N_2 line, then added to the degassed protein mixture. The reaction mixture was stirred minimally in order to mix the reagents but no further stirring was required throughout the reaction. It has been shown that a higher degree of polymerization is obtained in the absence of stirring (Müller, 1986). The reaction continued for three hours with constant N_2 bubbling through. The relative extent of reaction was estimated by the increase in solution viscosity. A Cannon-Manning Semi-Micro viscometer was filled by suction and allowed to equilibrate at 25°. The liquid was pumped up to the upper mark, allowed to flow to

the lower mark and the time taken measured. The mean of three measurements was taken. The density of the solution was measured using a 2 mL pycnometer which was filled and weighed accurately twice. The viscosity was calculated using the following expression.

$$\eta_s = \eta_{H_2O} \times \frac{\text{time (s)}}{\text{time } (H_2O)} \times \frac{m(H_2O)}{m(s)}$$

where η_s = sample viscosity (cp).

η_{H_2O} = viscosity of water (0.8904 at 25°).

time (s) and time (H_2O) are the measured times for the sample and water.

$m(H_2O)$ and m(s) are the densities for water and the sample ($m(H_2O)$ = 1.0015 g/mL at 25°).

The derivatives were concentrated three fold and free acrylamide was removed by washing with 10 volumes of water and ultrafiltration through an Amicon XM100A filter.

25. Radiolabelling of MBL-2 cells. Approximately 10^7 cells (10 mL) close to 100% viability at approximately 10^6 cells/ mL (*i.e.* underconfluent) were incubated overnight with 6 μCi of 5-[^{125}I] Iodo-2'-deoxyuridine (Amersham) or ^{14}C amino acid mixture (Amersham high specific activity mixture CFB.104). The cells were washed three times in PBS and the specific activity determined by cell impedance counting and radioactivity counting in a LKB Compugamma γ counter or a Phillips PW 4700 liquid scintillation counter . Typical specific activities were 10^7 cpm/mL or 10^7 dpm/mL.

26. ^{51}Cr labelling of erythrocytes. Erythrocytes (0.5 mL packed cells) were washed three times in PBS and incubated with [^{51}Cr] $NaCr_2O_7$ (0.5 mL, 10^7 Bq/mL, Amersham) for 30 minutes. Free label was removed by washing four times in 10 volumes of PBS. Typical specific activities were 10^7- 10^8 cpm/mL of cells.

27. Counter current distribution (CCD) of cells. CCDs were run on a 60 cavity thin-layer CCD rotor (CCD 1240C, Buchler Instruments, Fort Lee, N.J.). Cavities were loaded with 0.6 mL of lower phase and 0.7 mL of upper phase when a lower phase partition was expected and vice versa for an upper phase partition. In the case of polyacrylamide (PAA)-derivatized antibodies a lower phase partition was anticipated and the concentrations of derivatized-antibody were 0.08 mg per cavity for polyacrylamide-derivatized rabbit anti-human erythrocyte IgG (PAA-rαhrbc) and 0.2 mg per cavity for polyacrylamide-derivatized YE1.48.10. (PAA-YE1.48.10.). The appropriate number of cavities were loaded with phase system containing ligand or control phase systems without ligand except for the first cavity to which the load mix of cells was added (approx. 2×10^7 total cells). The required number of transfers was performed, with the systems being mixed for 30 seconds and allowed to settle for 5 minutes. Erythrocyte CCDs were performed at room temperature and MBL-2 cell CCDs at 4°. After the run was finished, 1 mL of PBS was added to each cavity to dilute the phase system and the contents collected in tubes. The cell concentrations were determined by impedance counting or radiolabel counts.

CHAPTER 5

RESULTS AND DISCUSSION.

1. Separation of Erythrocytes using a Trypan Blue-Derivatized Second Ligand. Trypan blue (3,3'-[(3,3'-Dimethyl[1,1'-biphenyl]-4,4'-diyl) bis (azo)bis[5-amino-4-hydroxy-2,7-napthalenedisulfonic acid] tetrasodium salt) is a blue dye (MW = 961) which partitions strongly into the upper phase of a PEG/dx two phase system (Fig. 8). The maximum partition coefficient measured for trypan blue was 10 at 0.5 μg/mL which decreased to 7.5 at 5 μg/mL. The partition coefficient decrease at higher concentrations was probably due to aggregation. As trypan blue is approximately half the molecular weight of PEG 1900 it seemed possible that antibodies modified by covalent attachment of trypan blue may be less deactivated than those modified by covalent attachment of PEG 1900.

Trypan blue was linked to bovine serum albumin (BSA) using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) at various trypan blue concentrations. It was confirmed that the trypan blue and BSA were linked by a red spectral shift of 6 nm in the visible spectrum of trypan blue. This is shown for IgG in Fig. 9. PEG 1900 was also linked to BSA using cyanuric chloride activated PEG 1900 to make a comparison of the two affinity modifying agents. The degree of modification of BSA with trypan blue was estimated from the ratio of absorbance at λ_{596} to γ counts of radiolabelled BSA of a known specific activity. However since the trypan blue was bound to the protein as indicated by the spectral shift, it would be anticipated that the extinction coefficient of the dye-protein conjugate would be different to that of the free dye. In order to estimate the number of trypan blue molecules bound to each protein molecule the value of the extinction coefficient for the free dye was used to estimate the concentration of trypan blue in the dye-protein conjugate. There is no basis for assuming the extinction coefficient of the free dye is the same as that of the bound dye. In fact changes in extinction coefficient on dye-protein binding are used in this study to assay avidin and biotin concentrations. However measurements of the absorbance of trypan blue at λ_{max} before and after the dye was coupled to the protein were similar at the same dye

concentration. As this was the only simple method available for estimating the amount of dye bound to the protein it was used in this study. Although the absolute values of dye molecules bound to the protein may not be correct, it gives an indication of the relative amount of dye bound in the conjugate. The amount of PEG 1900 bound per BSA molecule was estimated with fluorescamine (Stocks *et al*, 1986).

A comparison of the partition of trypan blue and PEG 1900-derivatized BSA in various phase systems is made in Table 2. It must be realized, however, that these are only estimates of bound trypan blue as discussed above. Trypan blue derivatized BSA requires a higher degree of modification than PEG 1900-derivatized BSA to obtain an equivalent partition coefficient. As it is possible to obtain an equally high partition coefficient using trypan blue as the modifying agent as with PEG and because of the possibility that trypan blue modification would have a less deactivating effect on the antibody than PEG 1900 modification due to its lower molecular weight, the use of trypan blue as an affinity ligand modifying agent was investigated. Other studies suggest that the molecular weight of the modifying agent is more

Table 2. A comparison of the effect of PEG 1900 and trypan blue on bovine serum albumin as affinity ligand modifying agents.

Ligand	DOM[1]	% Upper Phase Partition in the System Specified					
		5,3.4,I	5,3.4,II	5,3.4,III	5,4,I	5,4,II	5,4,III
native BSA	0	52	52	53	53	49	42
PEG-BSA	16	100	100	100	100	100	100
PEG-BSA	34	100	100	100	100	100	100
PEG-BSA	46	100	100	100	100	100	100
BSA + TB	0			40	43		
TB-BSA	1			42	45		
TB-BSA	12			56	58		
TB-BSA	36			83	85		
TB-BSA	60			100	100		

DOM[1] degree of modification (molec. of PEG 1900 or TB per BSA molec.).

TB - trypan blue.

BSA - bovine serum albumin.

significant with respect to antibody activity decreases than the coupling chemistry employed to modify the protein (Sharp *et al*, 1986). A model erythrocyte separation was examined to investigate the potential of trypan blue-modified antibodies as second affinity ligands.

The problem used to test this approach was to separate human and rabbit erythrocytes using a monoclonal mouse anti-NN glycophorin IgG (mαNN glyc) as the primary ligand and a trypan blue-derivatized polyclonal sheep anti-mouse F_c fragment IgG (TB-sαmF_c) as the second ligand. This is depicted schematically in Fig. 10.

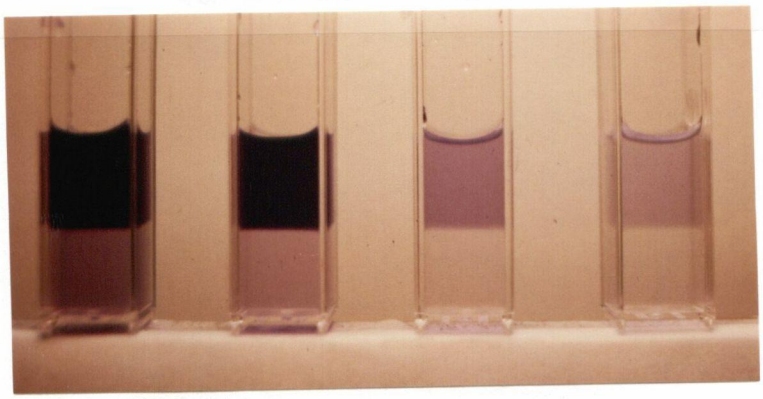


Fig. 8. Trypan blue dye in a 5,4,III phase system at total concentrations from left to right of 5, 2.5, 1.25, 0.75 μg/mL. The corresponding partition coefficients are from left to right 7.5, 9.0, 9.8 and 10.5.

Mouse F_c fragment was prepared from mouse IgG by papain digestion followed by FPLC. The fractions were identified and the purity assessed by SDS-PAGE. The F_c fragment was the second peak eluted by FPLC (Fig. 11). The SDS-PAGE gel of the IgG digestion products is shown in Fig. 12. In lane 2, Fig. 12, the crude IgG digest, there are three major bands. The reduced F_c fragment has a MW of less than 30 000 and any IgG light chain or F_{ab} fragment, which would also have a molecular weight less than 30 000, would also be included in this band. The other two major bands are probably partially digested F_c fragments defined as $1F_c$ and sF_c (MW = 48 000 and 40 000 respectively, Utsumi, 1969). A small amount of heavy chain at over 70 000 suggests that the digestion was close to completion and that only small amounts of light chain are present in

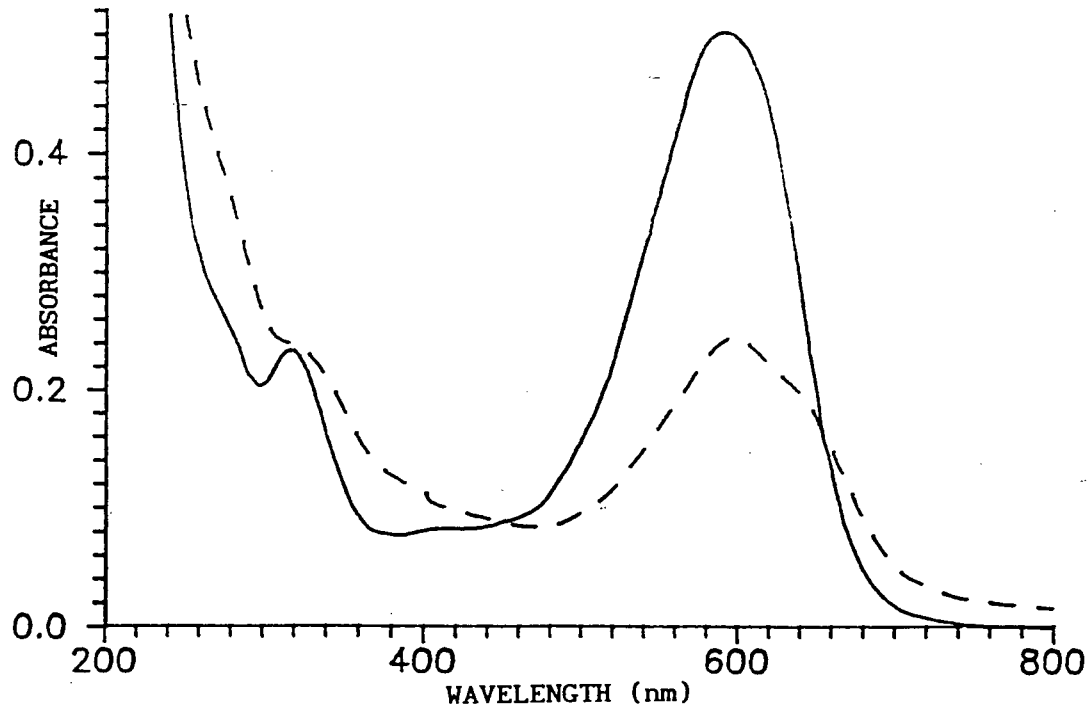


Fig. 9. Visible spectrum of trypan blue and sheep anti-mouse F_c (sαmF_c, ——) and trypan blue-derivatized sαmF_c (– – –). The conjugate shows a 6 nm red spectral shift.

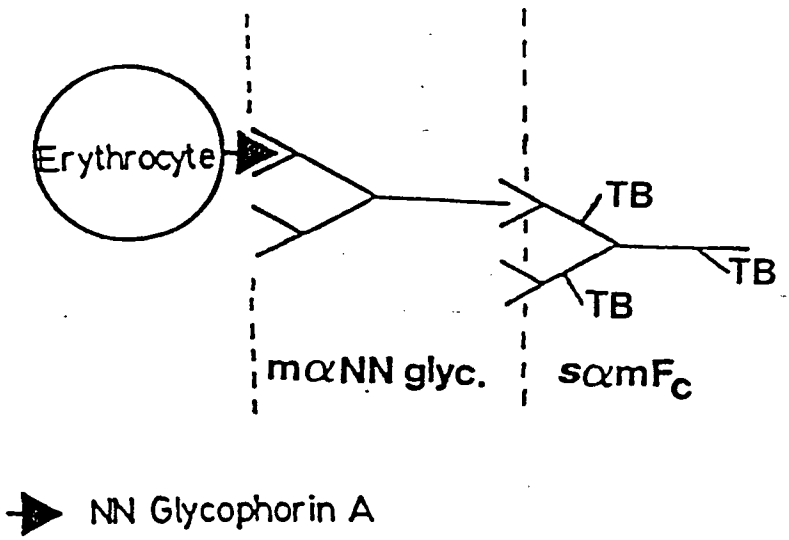


Fig. 10. A schematic diagram of the binding of trypan blue-derivatized sheep anti-mouse F_c ($s\alpha mF_c$) to mouse anti-NN glycophorin ($m\alpha$NN glyc) which is bound to an erythrocyte, thus immunospecifically coating the erythrocyte with trypan blue.

the digest.These partially digested products, $1F_c$ and sF_c, have also been observed in the papain digest of rabbit IgG (Stocks and Brooks, 1988) and in other mouse F_c fragment preparations (Clezardin *et al*, 1985). The FPLC purified F_c fragment, shown in Fig. 12, lane 3, is slightly contaminated with a band at approximately 50 000 but this preparation is considerably purer than the commercial F_c fragment preparation, also shown in Fig. 12, lane 4, which has contaminating bands at 40 000 and 48 000 corresponding to the putative partially digested F_c fragments, $1F_c$ and sF_c. The absence of F_{ab} fragment in the F_c preparation was confirmed by ELISA using an anti-F_{ab} enzyme-conjugated antibody.

The F_c preparation was used to raise $s\alpha mF_c$ by standard techniques and purified from sheep serum by ammonium sulfate precipitation and FPLC (Fig. 13). The first two peaks eluted are both IgG; it is likely that these represent different classes of IgG. SDS-PAGE gel of the pure $s\alpha mF_c$ is shown in Fig. 12, lanes 7 & 9, which shows the $s\alpha mF_c$ to be

homogeneous. The second antibody, mαNN glyc MAb, was purified from ascites by ammonium sulfate precipitation and FPLC (Fig. 14). The IgG was eluted in the first and only IgG-containing peak reflecting the monoclonal origins of the antibody. This also produced a homogeneous IgG preparation as shown by SDS-PAGE (Fig. 12, lanes 6 & 8). It was shown by hemagglutination assays that the MAb, mαNN glyc had no specificity for NN erythrocyte agglutination over that for MM or MN erythrocyte agglutination. In this case since the antibody was required to distinguish between human and rabbit erythrocytes, this was not important.

Table 3. Results of partitioning experiments using trypan blue-modified sheep anti-mouse F_c IgG as a second affinity ligand.

Cell Type	1° Ligand[1]	2° Ligand[1]	PC	%UP	Control[2] PC	%UP
	-	TB-sαmF$_c$	5	83	0.30	23
	mIgG	TB-sαmF$_c$	3.4	77	0.60	37
human rbc	mαNN glyc	TB-sαmF$_c$	2.6	70	0.10	18
rabbit rbc	mαNN glyc	TB-sαmF$_c$	0.25	20	0.09	8
human rbc	TB[3]	-	-	21	-	18
human rbc	TB[4]	-	-	12	-	18

[1]All antibody concentrations were 0.25 mg/mL and at a modification ratio of trypan blue:sαmF$_c$ of 28:1.

[2]The control partition is measured in the presence of primary ligand only. In the case of native TB the control is the partition in phase system only.

[3]At a TB concentration of 1 μg/mL and [4]10 μg/mL.

PC = partition coefficient, TB = trypan blue.

%UP = the % of total cells located in the upper phase.

mIgG = non-specific mouse IgG.

The results of partitioning experiments in the model system outlined are summarized in Table 3. All experiments using this ligand combination

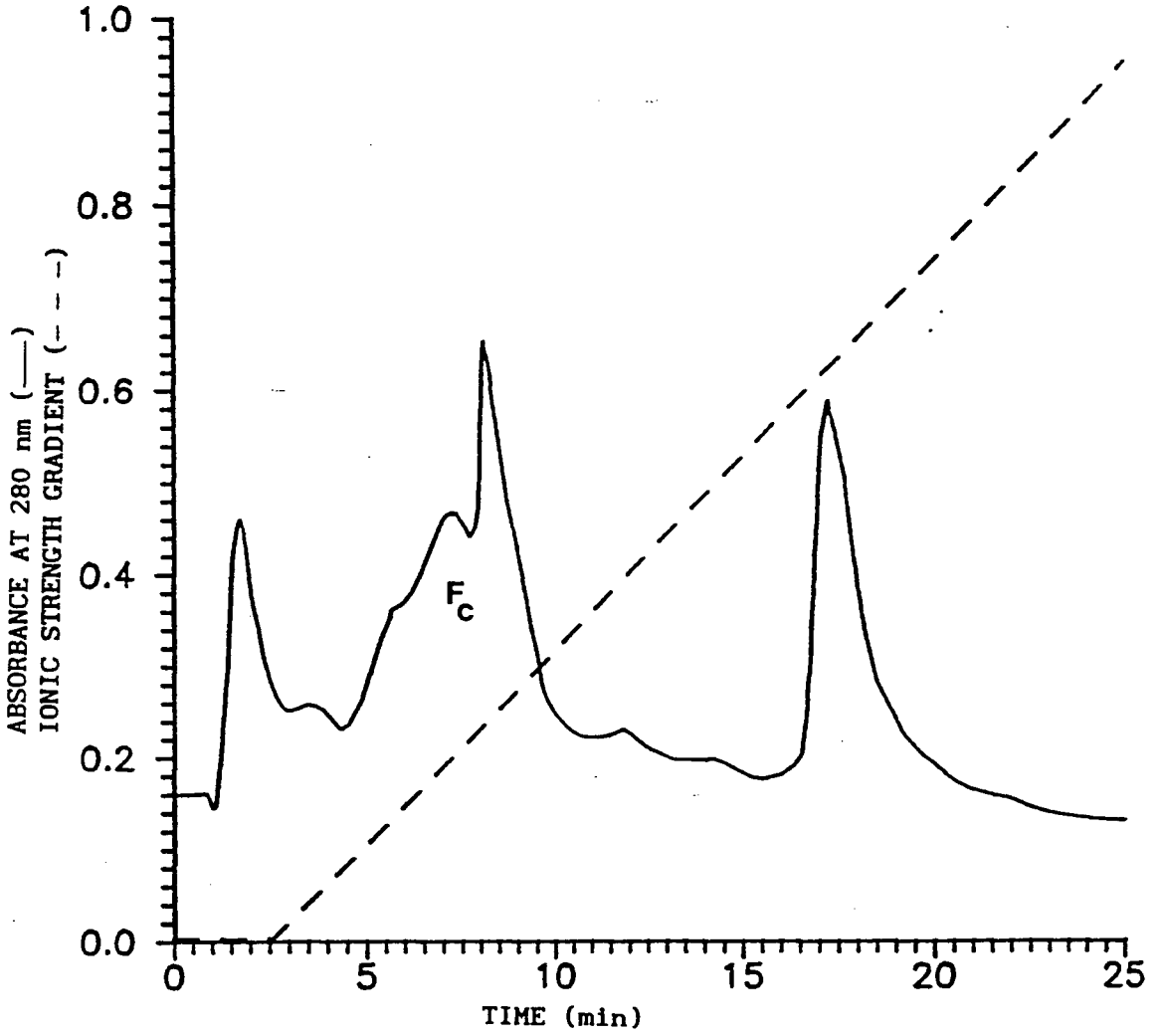


Fig. 11. Fast protein liquid chromatography (FPLC) profile of the papain digest of mouse IgG on a Mono Q column. The ionic strength elution gradient (- - -) was 0.01-1M NaAc, pH 5.5. The second peak was identified as F_c fragment by SDS-PAGE (see Fig. 12).

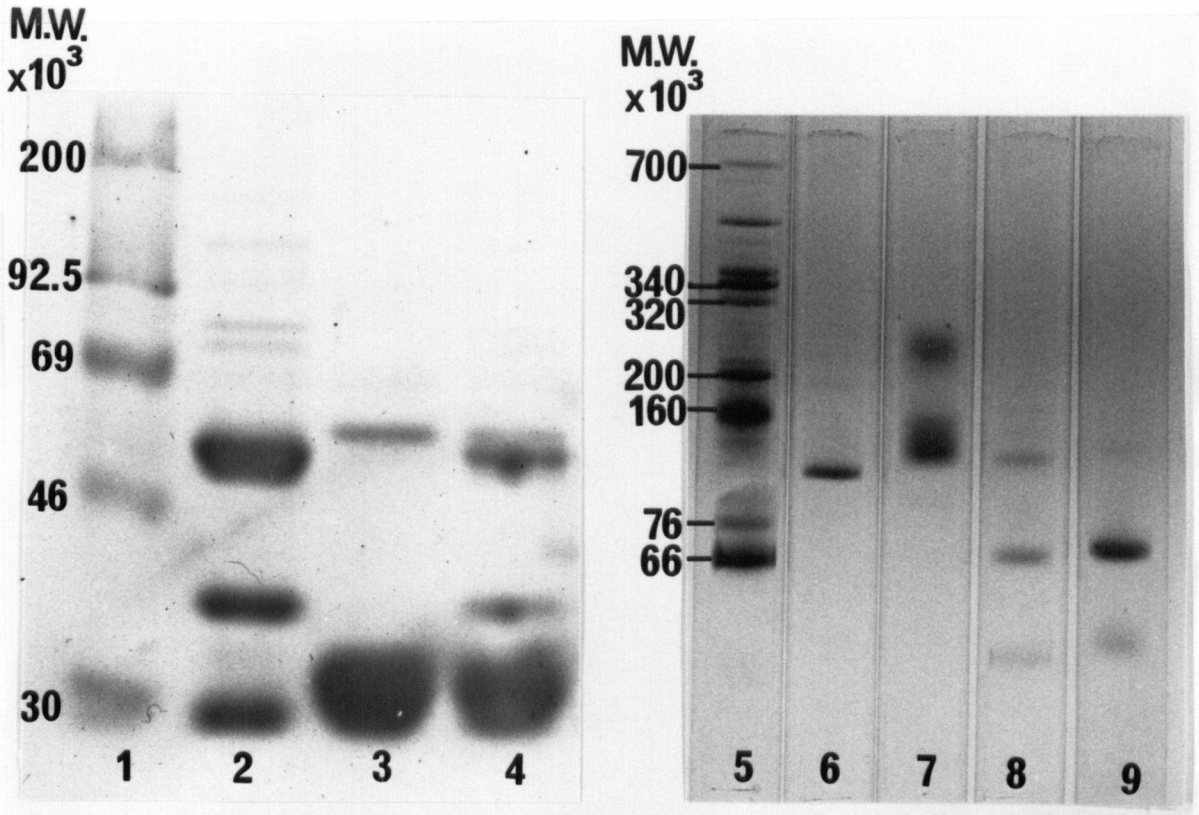


Fig. 12. 10% (lanes 1-4) and 3% (lanes 5-9) SDS-PAGE gels of IgGs and F_c fragment used in studies of the effect of trypan blue-derivatized second affinity ligand on erythrocyte partition. Lanes 2-5 and 8-9 are reduced, the others are native.

1. Molecular weight markers.
2. Reduced papain digest of mouse F_c fragment. The reduced F_c fragment has a MW of less than 30 000. The two other major bands represent partially digested IgG (see text).
3. Reduced FPLC-purified F_c fragment (MW ≤ 30 000) from mouse IgG digest in lane 2.
4. Reduced commercial mouse F_c fragment (Sigma).
5. Molecular weight markers.
6. Native monoclonal mouse anti-NN glycophorin IgG.
7. Native polyclonal sheep anti-mouse F_c fragment IgG. The band at approximately 300 000 is likely a dimer of IgG.
8. Reduced monoclonal mouse anti-NN glycophorin IgG as in 6.
9. Reduced polyclonal sheep anti-mouse F_c fragment IgG as in 7. The putative dimer in 7 is reduced and no longer observed.

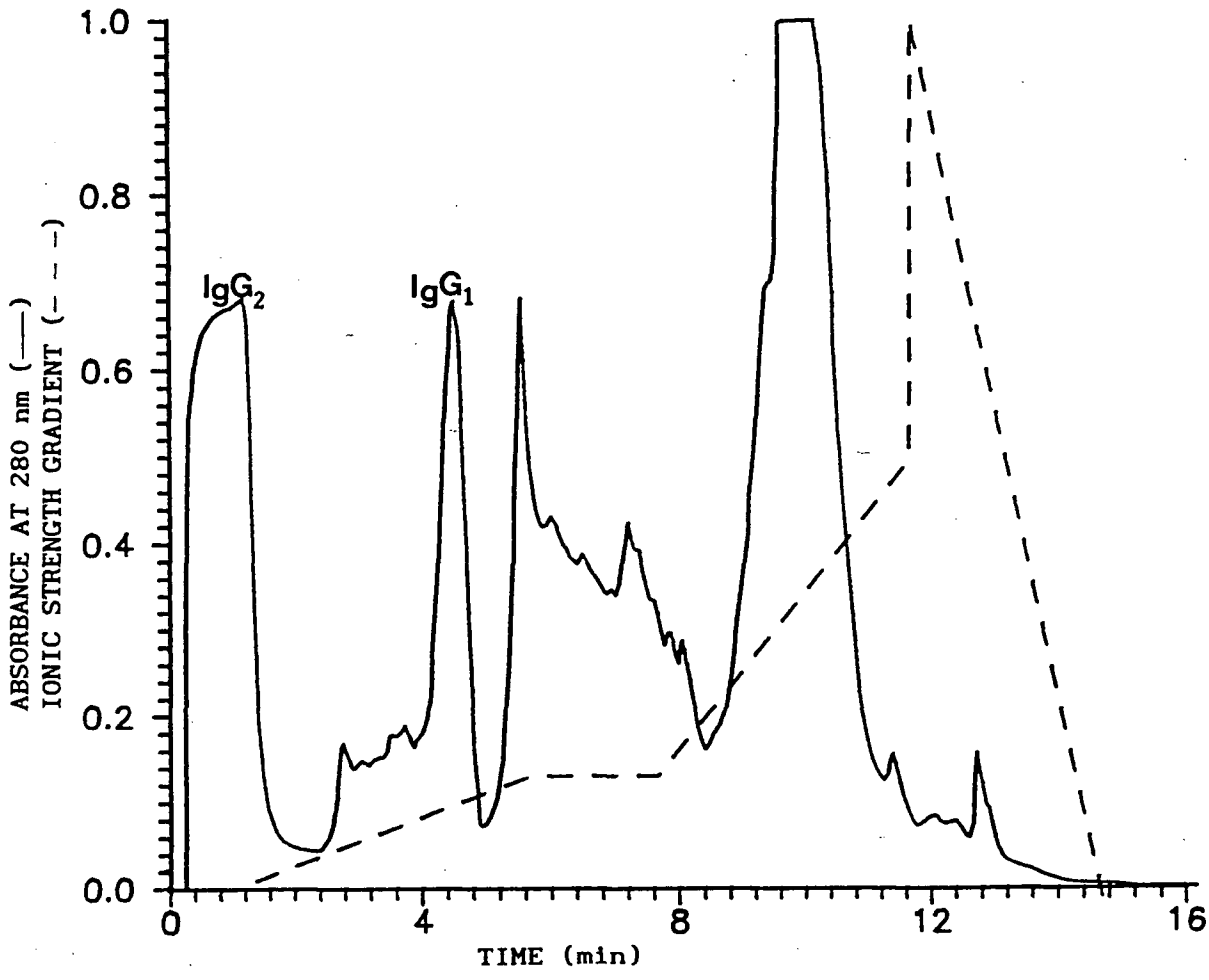


Fig. 13. FPLC profile of sheep anti-mouse F_c fragment (sαmF_c) preparation. The elution gradient (- - -) was 0-1M NaCl in 0.01M NaH_2PO_4/ Na_2HPO_4 buffer, pH 8. Both IgG peaks were identified by SDS-PAGE and ELISA. (Fig. 12, lanes 7 & 9). IgG_2 is more basic than IgG_1 and is therefore eluted first (Goding, 1983). The two IgG peaks, representing different classes of IgG, were combined and were indistinguishable by SDS-PAGE.

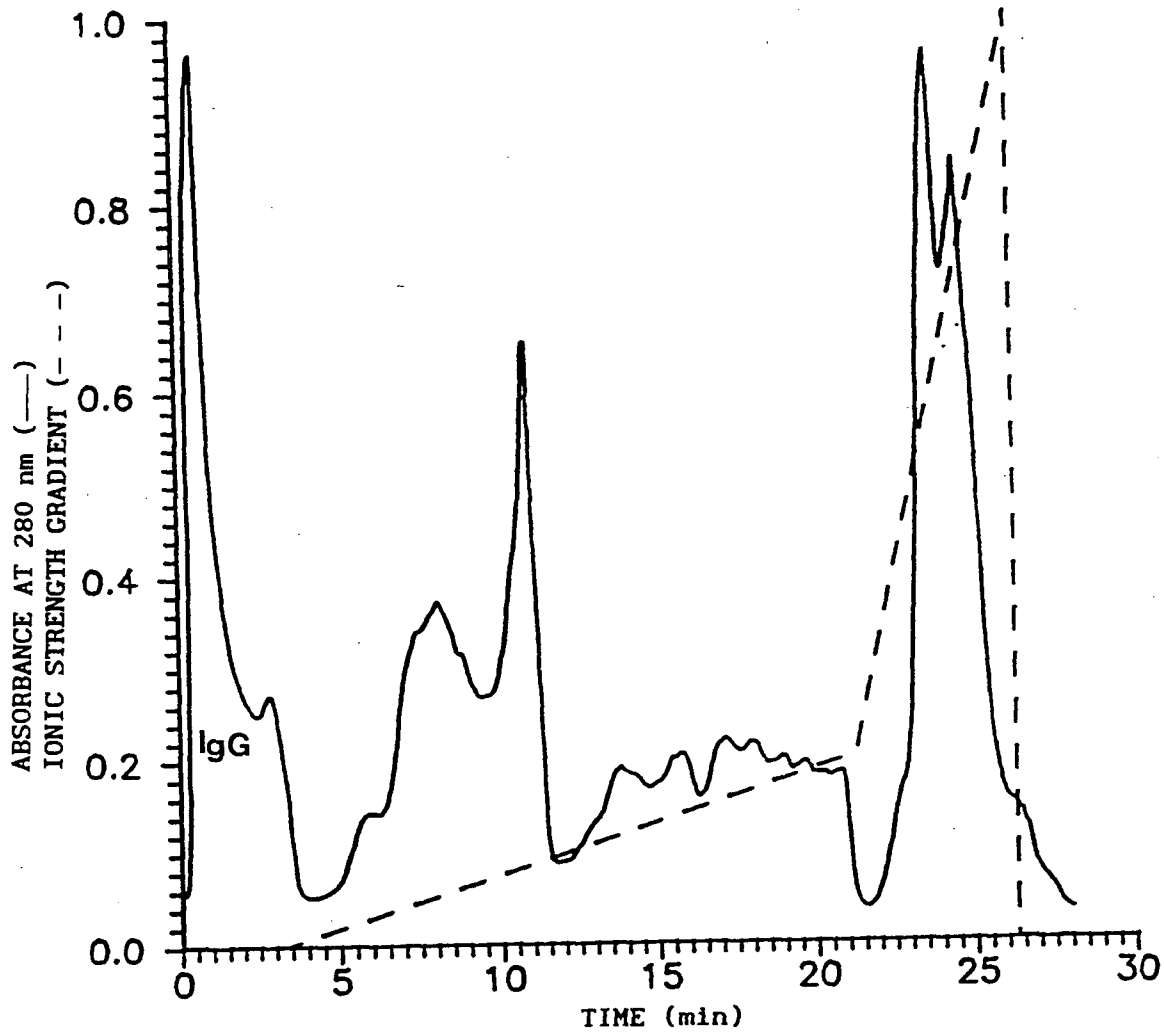


Fig. 14. FPLC profile of monoclonal mouse anti-NN glycophorin (mαNN glyc). The elution gradient (- - -) was 0-1M NaCl in 0.01M NaH_2PO_4/ Na_2HPO_4 buffer, pH 8. The IgG peak was identified by SDS-PAGE and ELISA. (Fig. 12, lanes 6 & 8). Only one class of IgG was detected by FPLC reflecting the monoclonal origin of the antibody.

were in 5,4,I (100 mM phosphate buffer) systems at 4°. Trypan blue modification of the sαmF$_c$ resulted in an increased partition coefficient which increased with the degree of modification. The modification of IgG with trypan blue required a lower degree of modification than BSA to obtain a similar partition coefficient suggesting that the trypan blue bound to IgG is more exposed to the phase system than that bound to BSA. Two different modified antibody preparations were examined, namely a 15:1 and 28:1 trypan blue to IgG mole ratio. The degree of modification was estimated from the ratio of dye absorbance to concentration of radiolabelled protein. However, the 15:1 conjugate had a partition coefficient of only 1.3 and did not have a significant effect on the partition of either antibody or erythrocytes. At higher concentrations of both native and antibody-conjugated dye, a decrease in the partition coefficient of erythrocytes was observed; most likely this was caused by aggregation of the dye and erythrocytes.

All experiments were carried out at 0.25 mg/mL of trypan blue-derivatized antibody, the maximum TB-sαmF$_c$ concentration at which no decrease in the partition coefficient was observed. Although the TB-sαmF$_c$ and mαNN glyc combination was able to increase the partition of human NN erythrocytes from 18% to 70% this was not as specific as the PEG-derivatized ligand (Stocks and Brooks, 1988) since the same ligand combination also increased the partition of rabbit erythrocytes from 8% to 20%. This is probably due to non-specific binding of the trypan blue-derivatized antibody to the rabbit erythrocytes. It is

Table 4. The effect of TB-sαmF$_c$ on the partition of other mouse IgGs.

3° Ligand[1]	2° Ligand[2]	1° Ligand[3]	PC	%UP	Control PC	%UP
TB-sαmF$_c$	mIgG		2.8	74	0.2	17
TB-sαmF$_c$	mαrIgG	rIgG	1.4	58	0.9[4]	47
TB-sαmF$_c$	mαpili		3.2	76	0.3	23

Concentrations of ligands are [1]0.15 mg/mL, [2]0.10 mg/mL and [3]0.05 mg/mL.

[4]The partition of rabbit IgG (rIgG) in the presence of mouse anti-rabbit IgG (mαrIgG) only.

unlikely that the mαNN glyc cross-reacts with the rabbit erythrocyte since it did not agglutinate rabbit erythrocytes in a microtiter assay.

Since one of the main advantages of using a second antibody as the affinity ligand is the potential to use the same second ligand with different primary ligands for different separation problems, the effect of the trypan blue-derivatized antibody on other potential primary ligands was examined. These were polyclonal non-specific mouse IgG (mIgG), monoclonal mouse anti-rabbit F_c fragment IgG (mαrF_c) with rabbit IgG (rIgG) and monoclonal mouse anti-pili IgG (mαpili). The results are summarized in Table 4. In all cases the trypan blue-derivatized antibody was able to alter the partition coefficient of the primary antibody, suggesting that this primary antibody could be used as a general ligand. The effect of TB-sαmF_c and mouse anti-pili IgG on the partition of ^{14}C labelled pili was examined. No partition change was observed but this may have been due to the availability of only small amounts of anti-pili antibody. The TB-sαmF_c and mαIgG were also used to increase the partition of a third antibody, non-specific rabbit IgG.

In summary trypan blue dye may be used as an affinity modifying agent and is comparable to a PEG-derivatized ligand in effectiveness. However a disadvantage is aggregation of the dye and non-specific binding of the dye to proteins and cells as illustrated by the effect of the free dye on human erythrocyte partition and a non-immunospecific increase in rabbit erythrocyte partition.

2. Separation of MBL-2(4.1) and MBL-2(2.6) cells by Immunoaffinity Partition. The initial approach taken to the separation of MBL-2(4.1) and MBL-2(2.6) cells is summarized in Fig. 15. The MBL-2 cell is immunospecifically coated with PEG through the binding of YE1.48.10., a rat MAb specific for MBL-2 cells and the binding of PEG-derivatized RG7/11.1, a mouse MAb specific for the F_c fragment of rat IgG2b, to the YE1.48.10. The first step in the separation was to generate and purify large quantities of MAb from both YE1.48.10. and RG7/11.1 hybridoma lines.

a. Production and purification of YE1.48.10. and RG7/11.1 monoclonal antibodies. The method used to purify the m α NN glyc for the previous

separation was ammonium sulfate precipitation of antibody-containing ascites fluid followed by FPLC (Stocks and Brooks, 1987; 1988). Ascites refers to a non-solid tumor produced intraperitoneally in rats or mice by injection of hybridoma cells after priming with pristane. It is rich in MAb (approximately 2mg/mL). However losses in MAb activity due to ammonium sulfate precipitation have been observed (Bruck *et al*, 1982; McGregor *et al*, 1983) and losses in activity previously observed by the author may well be due to this step of the purification (Stocks, 1986). For this reason ultrafiltration was used to concentrate the antibodies rather than ammonium sulfate precipitation.

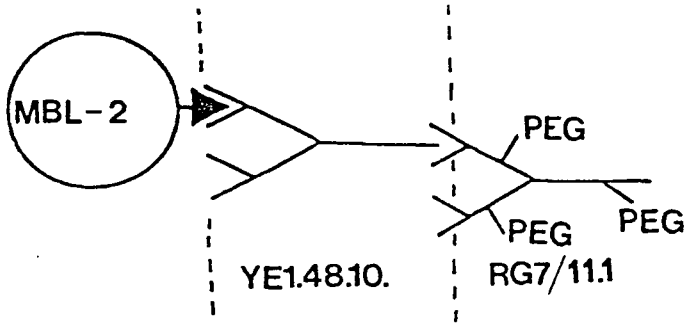


Fig. 15. Schematic diagram of the binding of a MBL-2 cell by YE1.48.10. MAb and PEG-derivatized RG7/11.1 MAb to immunospecifically coat the MBL-2 cell with PEG.

MAbs have traditionally been generated in rat or mouse ascites (Goding, 1983) but this is not practical for large quantities of MAb because of the large numbers of animals required. Also MAbs of ascites origin will be contaminated with non-specific mouse or rat antibodies. The alternative is to generate large quantities of cell culture medium but the addition of fetal bovine serum (FBS) to provide necessary growth factors also adds bovine antibodies to the medium and the large amount of bovine protein present in 10% FBS-containing medium makes the purification of a MAb of a typical concentration of 10 μg/mL difficult.

Therefore both hybridoma cell lines were adapted to growth in supplemented serum free medium (SSFM, Murakami *et al*, 1982; Stocks and Brooks, 1989). They continued to secrete antibody at the same rate as when plated in serum-containing medium and became confluent at a maximum

cell density of 1-2 x 10^6 cells/mL within 10 days of initially plating at 1-2 x 10^4 cells/mL. Typically they exhibited a lag of approximately three days prior to exponential growth when initially plated into SSFM (Fig 16). Cells maintained in SSFM exhibited a much shorter lag of approximately one day but they were not routinely maintained in SSFM as they began to deteriorate after several weeks in SSFM. Cell viability as estimated by erythrosin B dye uptake was close to 100% in SSFM until the cells became confluent.

Ultrafiltration was found to be an extremely effective method for both concentration and partial purification of the supernatant at 4°. The SDS-PAGE gels shown in Fig. 17 compare culture supernatants concentrated through different filters and assess the purity of insulin and transferrin added to the medium. It can be seen that both the transferrin and the insulin are not contaminated by any proteins detected by Coomassie blue-stained SDS-PAGE gels (Fig. 17, lanes 6 & 7). The addition of serum to the culture medium creates a more complicated separation problem as is shown by comparison of lanes 3 & 4, Fig. 17, where the unconcentrated serum-supplemented medium has similar protein concentrations as in the 20 fold concentrated SSFM. The large amount of IgG in lane 3, Fig. 12 is mostly bovine IgG originating from the serum. This is another advantage of using SSFM as it avoids the problem of quantitating the amount of bovine IgG relative to monoclonal IgG in a serum-containing preparation. Ultra-filtration through an Amicon XM100A filter which has a molecular weight cut-off of 100 000 removed a major protein band of approximate molecular weight 67 000 (Compare lanes 4 & 5, Fig. 17). This 67 kD band has a molecular weight corresponding to albumin and must originate from the hybridoma cells since the transferrin and insulin, the only additions to the medium, are seen to be relatively pure. The putative albumin may be that bound to the cell surface during culture in serum-containing medium which could dissociate from the cell surface in the SSFM. Transferrin was retained by the Amicon XM100A filter despite having a molecular weight of 77 000, significantly less than 100 000.

The concentrated SSFM was chromatographed on a mono Q anion exchange FPLC column and the elution profile is shown in Fig. 18. From the corresponding SDS-PAGE gels shown in Fig. 20 it can be seen that the

IgG is eluted in fractions 9 & 10 and the transferrin in earlier fractions. The two bands (MW ≈ 25-30 kD) visible in lanes 9 & 10 (Fig. 20) are a consequence of the secretion of κ light chain, as well as the λ light chain which forms the active antibody, by the YE1.48.10. hybridoma. This ability to synthesize κ chain originates from the myeloma half of the fusion partners (Galfrè and Milstein, 1981). The peak eluted in fractions 17 & 18, Fig. 18 did not contain any protein when examined by SDS-PAGE and is likely due to elution of the phenol red dye in the medium. The FPLC elution profile for the RG7/11.1 MAb is shown in Fig. 19. It is essentially the same as for the YE1.48.10. MAb; transferrin was eluted in fractions 2,3 & 6 whereas IgG was eluted mainly in fraction 10 (Fig. 19). The SDS-PAGE gels of the purified YE1.48.10. and RG7/11.1 are shown in Fig. 21. RG7/11.1 is seen to exist as a dimer which dissociates under reducing conditions (Fig. 21, lanes 4 & 5). Yields of antibody were approximately 30 ±5 mg per liter of SSFM for YE1.48.10. MAb and 15 ±5 mg per liter for RG7/11.1 MAb.

In summary this is an effective method to process one liter of culture supernatant per day, typically requiring 5-8 FPLC runs, each chromatographing approximately 5 to 10 mg of total protein. FPLC was the only step performed at room temperature as it only required 40 minutes and no loss in activity was detected by ELISA. For a more labile antibody this step could be performed at 4°. Both antibodies retained high activities, *e.g.* assuming the MAb concentration in SSFM to be in the typical range of 5-10 μg/mL (Goding, 1983), the minimum concentration at which MAb activity was still detectable in SSFM was 2-4 μg/mL, *i.e.* a 2-3 fold dilution of the antibody-containing SSFM. For the same antibody purified by this procedure the corresponding actual MAb concentration was similar:11 μg/mL, whereas the same antibody purified by ammonium sulfate precipitation showed a significant loss in activity: the minimum concentration at which MAb activity was detected was 330 μg/mL. These relatively high minimum concentrations at which activity was detected do not necessarily indicate a low affinity antigen as the surface antigen density on the ELISA plate was unknown. These values are only useful in terms of the comparison made here rather than an indication of absolute activity. For further details see Stocks and Brooks (1989).

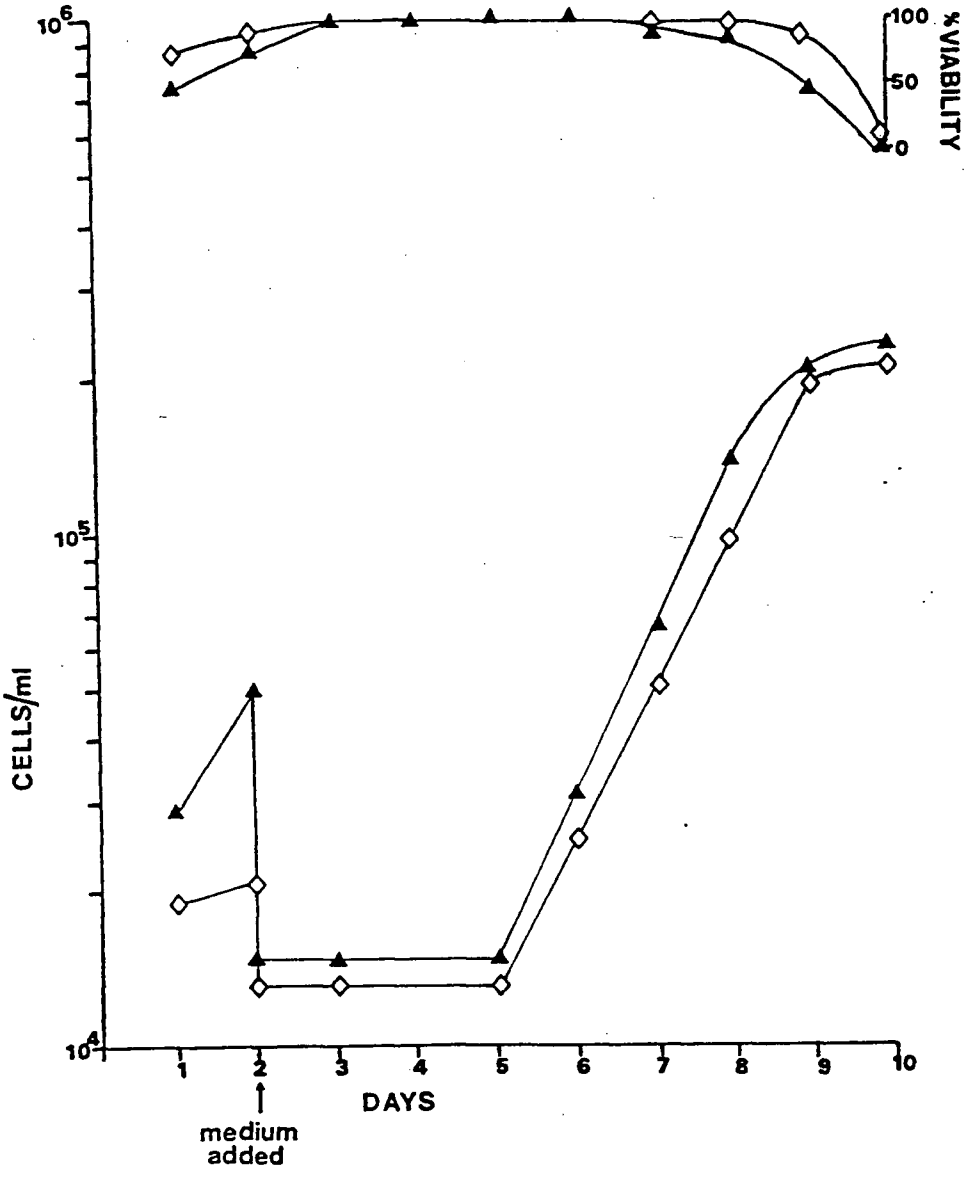


Fig. 16. Growth curves for YE1.48.10. (▲) and RG7/11.1 (◇) hybridoma cells. The cells were initially resuspended at approximately 10^4 cells/mL in 100mL of supplemented serum-free medium (SSFM) and a further 600 mL of SSFM added at day 2. The viability of both cell lines was estimated by erythrosin B dye uptake.

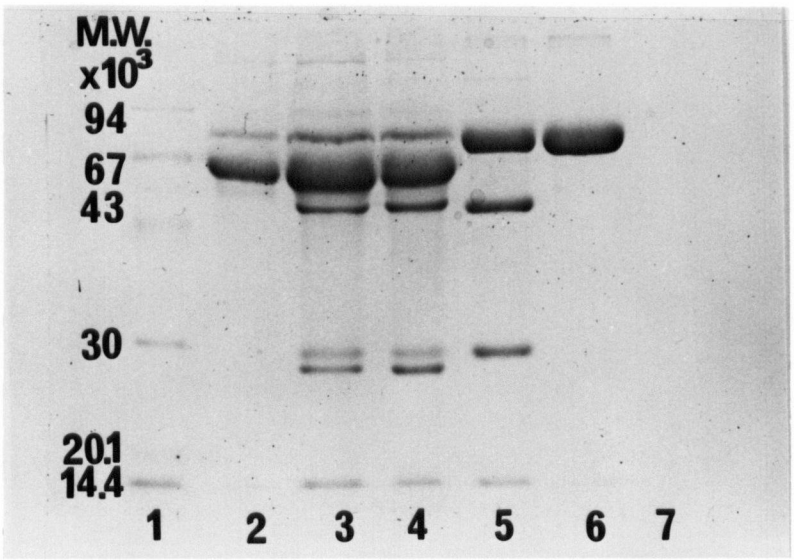


Fig. 17. 10% SDS-PAGE of serum-containing and serum-free hybridoma supernatants reduced with 5 mM 2-mercaptoethanol.

lane 1. Molecular weight standards.

lane 2. RPMI-1640 containing 5% fetal bovine serum (FBS). The major band at approximately 67 500 is probably albumin.

lane 3. Cell culture supernatant containing 5% FBS. Again the major band at 67 500 is albumin and the bands at 43 000 and 27 000 correspond to heavy and light chains of IgG. The two light chains are due to the synthesis of κ chain by the hybridoma cell (Galfré and Milstein, 1981).

lane 4. Supplemented serum-free medium (SSFM) cell culture supernatant concentrated 50 fold through an Amicon UM10 filter (MW cut-off = 10 000). The putative albumin (see text) and IgG bands are present as described in 2 and 3.

lane 5. SSFM cell culture supernatant concentrated 50 fold through an Amicon XM100A filter (MW cut-off = 100 000). The IgG and transferrin (MW = 77 000) are seen but the putative albumin (see text) is absent due to the ultrafiltration.

lane 6. Transferrin (5 μg, Sigma, MW = 77 000). This is free of major contaminants.

lane 7. Insulin (5 μg, Sigma, MW = 5 800). This illustrates the purity of the insulin.

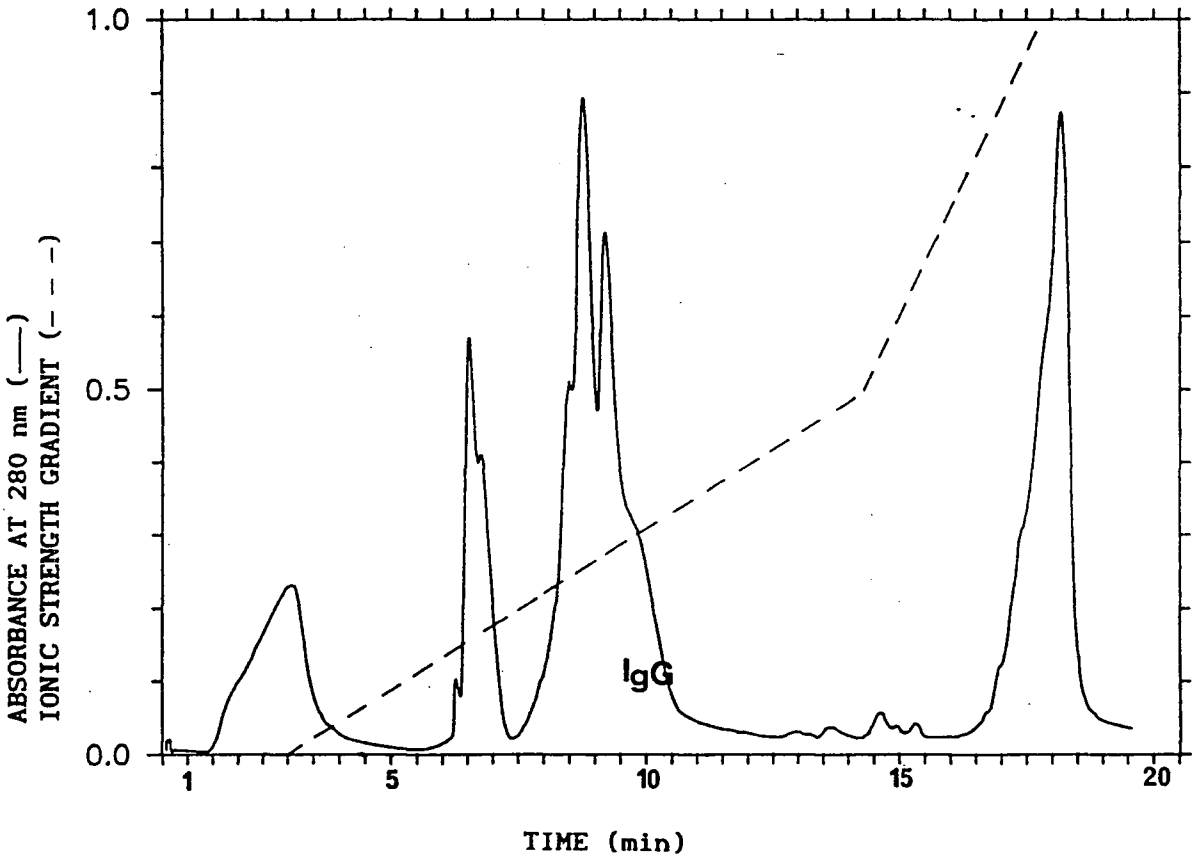


Fig. 18. FPLC profile of supplemented serum-free medium (SSFM) YE1.48.10. hybridoma culture supernatant concentrated by ultrafiltration through an Amicon XM100A filter. The flow rate was 1 mL/minute and 1 mL fractions were collected. The IgG peak is eluted in fractions 9 & 10.

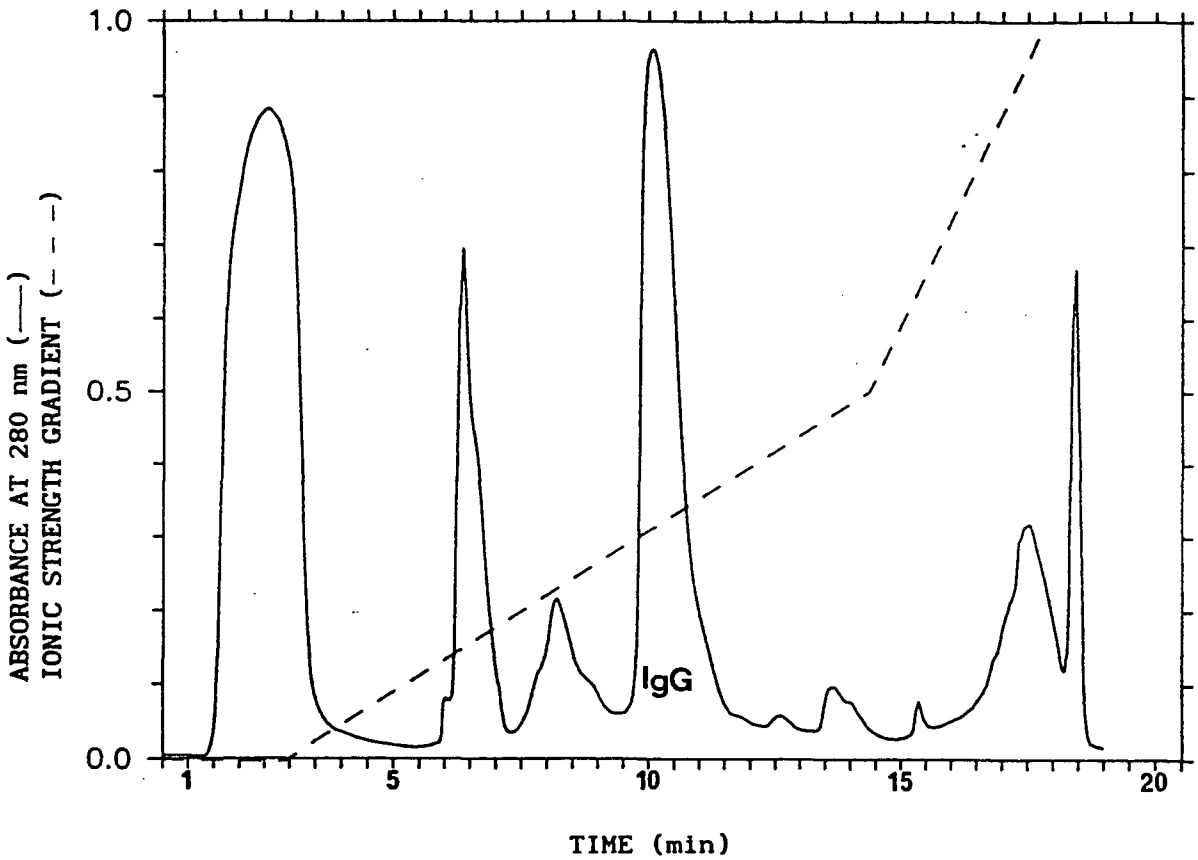


Fig. 19. FPLC profile of supplemented serum-free medium (SSFM) RG7/11.1 culture supernatant concentrated by ultrafiltration through an Amicon XM100A filter. The flow rate was 1 mL/minute and 1 mL fractions were collected. The IgG peak is eluted in fraction 10 while transferrin is eluted in fractions 2, 3 & 6.

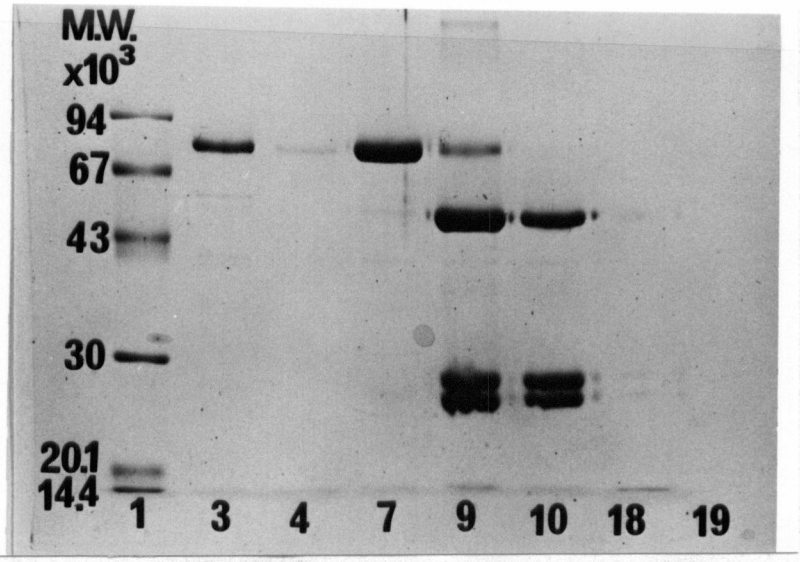


Fig. 20. 10% SDS-PAGE of FPLC fractions of YE1.48.10. hybridoma culture supernatant as shown in Fig. 18. Lane 1 is molecular weight standards and the other lane numbers correspond to the FPLC fraction as shown in Fig. 18. The bands at 77 000 are transferrin and those at 55 000 and less than 30 000 correspond to IgG heavy and light chain. The two light chains are due to secretion of κ chain by the hybridoma (Goding, 1983).

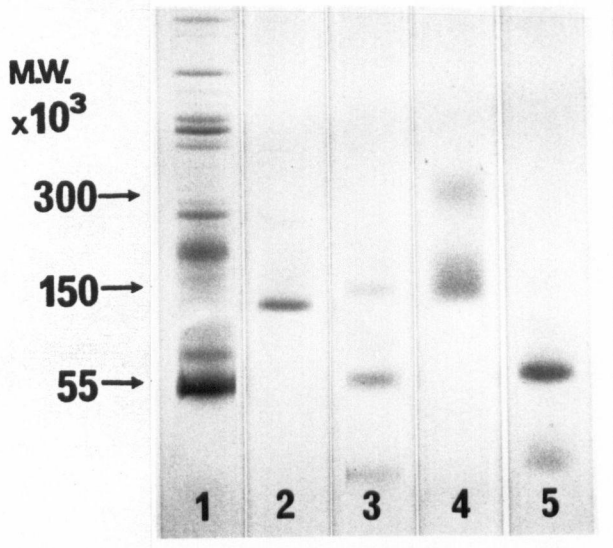


Fig. 21. 3% SDS-PAGE of FPLC fractions of RG7/11.1 and YE1.48.10. pure MAb. Lane 1 is molecular weight standards, 2 & 3 are non-reduced and reduced YE1.48.10. and 4 & 5 are non-reduced and reduced RG7/11.1. The band at 300 000 in lane 4 is a dimer of IgG and bands at approximately 150 000 are whole IgG. The heavy chains of IgG are at approximately 66 000 and the light chain is well below 66 000.

b. Characterization of the MBL-2 Cells. The known distinction between the two sub-lines of MBL-2, MBL-2(4.1) and MBL-2(2.6), is that MBL-2(4.1) has a higher surface density than MBL-2(2.6) of an antigen defined by the binding of rat MAb YE1.48.10. (Takei, 1986). Fluorescence microscopy of MBL-2 cells incubated with YE1.48.10. followed by staining with fluorescein-conjugated goat anti-rat IgG confirmed that more YE1.48.10. MAb molecules bound to MBL-2(4.1) than MBL-2(2.6) cells as shown in Fig. 22. The MBL-2(4.1) cells showed greater fluorescence than MBL-2(2.6) cells (compare 1 & 2, Fig. 22) whereas a lymphocyte control A6, a mouse hybridoma (Stocks and Brooks, 1988),showed no fluorescence (7, Fig. 22). Short incubation of either MBL-2 sub-line with trypsin almost completely removed the binding site for YE1.48.10. (3 & 4, Fig. 22) but Triton X, a non-ionic detergent, only slightly reduced the binding of YE1.48.10. (5 & 6, Fig. 22), although the antigen recognized by YE1.48.10. has been purified from EL4-BU cells by detergent solubilization (Takei, 1983).

In order to assess the feasibility and difficulty of the separation problem it was necessary to measure the equilibrium binding of YE1.48.10. MAb to MBL-2 cells as a function of MAb concentration and cell surface area. The cells were characterized by equilibrium binding studies using ^{125}I labelled YE1.48.10. antibody, and the surface area per cell was measured by digitization of a 69000-fold magnification photomicrograph of the two cell types. In measuring the cell surface area, three points on the cell circumference were digitized and used to describe a cross section of the sphere assumed to represent the lymphocyte. Using the expression $A = 4\pi r^2$, the surface area of a sphere corresponding to the two-dimensional photomicrograph was found to be 615 $\pm 30\ \mu m^2$. However accurate measurements of lymphocyte surface area have shown that the folding of the membrane generally gives rise to a total surface area 130% that of the corresponding sphere (Schmid-Schönbein *et al*, 1980). This would estimate the area of an MBL-2 cell at 800 $\pm 39\ \mu m^2$, but it is unlikely that all of this area would be available for ligand binding. The former measurement may be considered a lower limit and the latter an upper limit. The following calculations assume the lower value and thus the corresponding numbers of ligands bound per unit surface area represent maximum values. The density of MBL-2 cells was found to

be 1.0 g/mL by weighing accurate volumes of cell suspension at various hematocrits up to 60%. The number of packed cells per unit volume was 9 x 10^8 cells/mL after centrifugation at 300 g for four minutes. This calibration was used to estimate cell numbers from the hematocrit and was only used to estimate the number of cells in the pellet. It could not be considered a value for the cell volume since it does not take in to account the trapped volume of supernatant or cell deformation.

c. Analysis of Binding Experiments. Equilibrium measurements of ligand binding gave information on the moles of ligand bound per gram of cells at a known hematocrit and free ligand concentration. In order to convert this information to the number of moles of ligand bound at saturation (*i.e.* the number of binding sites) and the microscopic association constant (k_a) of the ligand-binding site interaction the following Scatchard analysis was employed (Cantor and Schimmel, 1980).

Assume a reaction of the type:

$$P + L = PL$$

where P = a binding site on the cell surface.
L = ligand.
PL = the ligand-binding site complex.

$$k_d = \frac{[P][L]}{[PL]} \quad \text{and} \quad \phi_1 = \frac{[PL]}{[P]+[PL]}$$

where k_d = the microscopic dissociation constant.
ϕ_1 = the fractional saturation of site 1.

$$\phi_1 = \frac{[P]([PL]/[P])}{[P](1+[PL]/[P])}$$

since $[PL]/[P] = [L]/k_d$

$$\phi_1 = \frac{[L]/k_d}{1+[L]/k_d} \qquad (25)$$

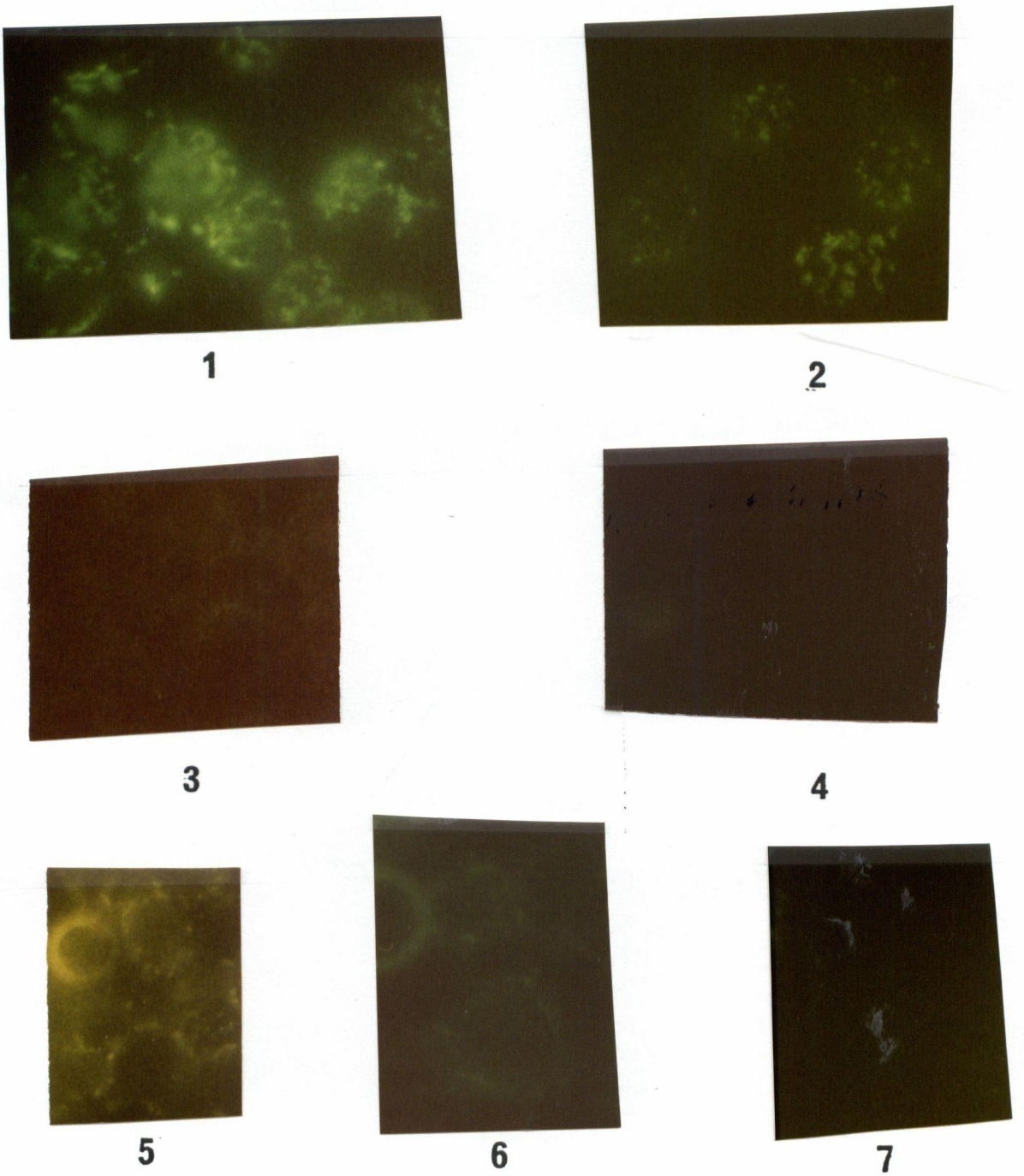


Fig. 22. MBL-2 cells stained with YE1.48.10. MAb and fluorescein-conjugated goat anti-rat IgG.

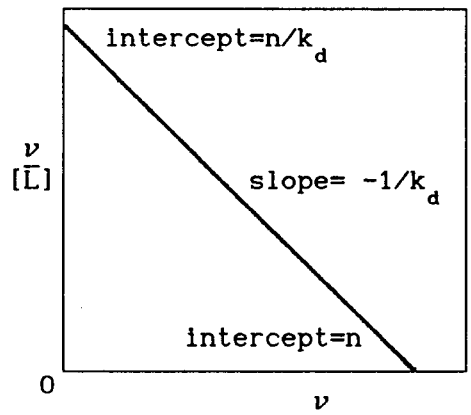
1. MBL-2(4.1) cells
2. MBL-2(2.6) cells
3. Trypsinized MBL-2(4.1) cells
4. Trypsinized MBL-2(2.6) cells
5. Triton X treated MBL-2(4.1) cells
6. Triton X treated MBL-2(2.6) cells
7. Lymphocyte control cells (A6, Stocks and Brooks, 1988)

A similar expression may be written for each site and assuming that all the sites are identical, the sum of all the species will give the total moles of ligand bound per unit surface area, ν, at equilibrium, *i.e.*

$\Sigma\phi_i = \nu$.

$$\text{Therefore } \nu = \frac{n[L]/k_d}{1+[L]/k_d} \quad \text{or in terms of } k_a \quad \nu = \frac{nk_a[L]}{1+k_a[L]} \qquad (26)$$

This is a convenient expression to relate ν and k (k_a is normally referred to as k when discussing antibody-antigen binding) when there are a maximum of n independent identical sites per unit area since the values for k and n may be calculated using various plots. A direct plot of ν vs [L] is one method to evaluate these parameters but requires data at high values of [L] which is difficult to measure and not practical when large quantities of costly MAb are required. Another possible plot is an inverse plot of $1/\nu$ vs $1/[L]$ but this is difficult to analyze statistically since the smaller, less certain measurements dominate the linear regression fits. The plot used to analyze this data was a plot of $\nu/[L]$ vs ν (Fig. 23) This allows a linear extrapolation to give n and does not weight the data at low values of ν or [L].



$$\frac{\nu}{[L]} = -\nu k_a + nk_a$$

Fig.23. Scatchard plot for independent identical binding sites.

Scatchard plots were constructed for the binding of ^{125}I-labelled biotin-derivatized and native YE1.48.10. MAb to MBL-2 cells as well as to erythrocytes and a mouse hybridoma control. A direct plot of the binding of YE1.48.10. to MBL-2(4.1) and MBL-2(2.6) is shown in Fig. 24.

Both MBL-2 sub-lines became saturated at 0.3 mg/mL which happens to be a convenient MAb concentration to use in an APTS.

The Scatchard plot corresponding to Fig. 24 is shown in Fig. 25. The errors in several of the points were estimated by taking the partial derivatives of ν and ν/L with respect to each measurement and multiplying by the sum of the errors of each measurement. The partial derivatives used in the calculation are summarized in Appendix 1. As can be seen in Fig. 25 the errors in each point are similar which made it possible to use linear regression analysis to fit the best line to the data. From the X-intercept and the slope of the graph it was possible to obtain values for ν and k as illustrated in Fig. 23. Similar plots were constructed for the two MBL-2 sub-lines using biotin-derivatized YE1.48.10 as shown in Fig. 26. Error analysis was carried out as before and the errors were sufficiently similar to allow linear regression analysis. The control Scatchard plots constructed using human erythrocytes and a mouse hybridoma line are shown in Fig. 27. No binding was observed within the limits of experimental error to trypsinized MBL-2 cells of either type. A summary of the information obtained from the plots is given in Table 5.

Table. 5. Summary of the information obtained by linear regression of the Scatchard plots in Figs. 25-27.

Cell type	Ligand	Molec. of ligand bound per cell	Molec. of ligand bound per cm^2	k_a (M^{-1})
MBL-2(4.1)	YE1.	$2.36 \pm 0.28 \times 10^6$	$3.83 \pm 0.23 \times 10^{11}$	$9.0 \pm 0.5 \times 10^8$
MBL-2(2.6)	YE1.	$7.96 \pm 1.61 \times 10^5$	$1.29 \pm 1.30 \times 10^{11}$	$8.6 \pm 0.3 \times 10^8$
MBL-2(4.1)	B-YE1.	$1.30 \pm 0.16 \times 10^6$	$2.10 \pm 0.13 \times 10^{11}$	$2.5 \pm 0.2 \times 10^8$
MBL-2(2.6)	B-YE1.	$6.67 \pm 1.37 \times 10^5$	$1.08 \pm 1.11 \times 10^{11}$	$3.2 \pm 0.08 \times 10^8$
Human-erythrocyte	YE1.	$2.47 \pm 0.61 \times 10^3$	*$1.76 \pm 0.43 \times 10^9$	$2.73 \pm 0.06 \times 10^9$
Mouse-hybridoma	YE1.	$8.20 \pm 1.87 \times 10^4$	$1.37 \pm 0.94 \times 10^{10}$	$4.4 \pm 0.5 \times 10^{10}$

YE1. = YE1.48.10. MAb.

B = biotin

*assuming the surface area of an erythrocyte to be $1.4 \times 10^{-6} cm^2$ (Evans and Fung, 1972).

The YE1.48.10. MAb is quite specific for the MBL-2 cells with respect to the hybridoma control, the binding to the mouse hybridoma being only 4% of that to MBL-2(4.1) cells and 11% of that to MBL-2(2.6) cells on a per cm^2 basis. The erythrocytes bind very small amounts of the YE1.48.10. MAb, *i.e.*, less than 1% of that bound to MBL-2(4.1) cells and 1.4% of that bound to MBL-2(2.6) cells on a per cm^2 basis. The surface antigen difference between the MBL-2 sub-lines is only a factor of 3. This small antigenic difference and low surface antigen density makes this a stringent separation problem. However tumor associated antigens (TAAs) are often of this order of surface density (Pimm *et al*, 1982; Price *et al*, 1982).

One of the objects of this work was to define and extend the limits of immunoaffinity partitioning sensitivity with respect to antigen density difference necessary for separation. Previous immunoaffinity partition separations of species-specific erythrocytes have used antibodies raised against the whole cell (Sharp *et al*, 1986; Karr *et al*, 1986) and in these cases large antigenic differences would be expected. A smaller surface antigen density was used as the basis for separation when rabbit anti-NN glycophorin was used as the primary ligand (Stocks and Brooks, 1988) but even in this case there was still a large surface antigen density difference of 0 and 5 $x10^{11}$ molecules per cm^2 as the basis for the separation.

A measure of the sensitivity of the partition coefficient to the number of PEG chains bound per unit area was obtained by Sharp (1985). He measured the binding of PEG 8000-palmitate to the erythrocyte surface and the corresponding partition coefficient. The binding of approximately 6 $x10^{11}$ molecules/cm^2 of PEG-palmitate was required to increase erythrocyte partition from 0 to 20% in the upper phase. The binding of 1 $x10^{11}$ molecules/cm^2 would have increased the upper phase partition from 0 to 6%. This implies that an immunospecific increase in the partition of even MBL-2(4.1) cells is approaching the limits of the technique as applied to date as this only binds 3.83 $x10^{11}$ molecules/cm^2 of YE1.48.10. MAb. Also MBL-2 cells are much larger than erythrocytes (615 μm^2 compared to 140 μm^2, Evans and Fung, 1972) and will have a larger free energy of adsorption at the interface. Another factor in the comparison is that the PEG 8000 ester of palmitate was used by Sharp

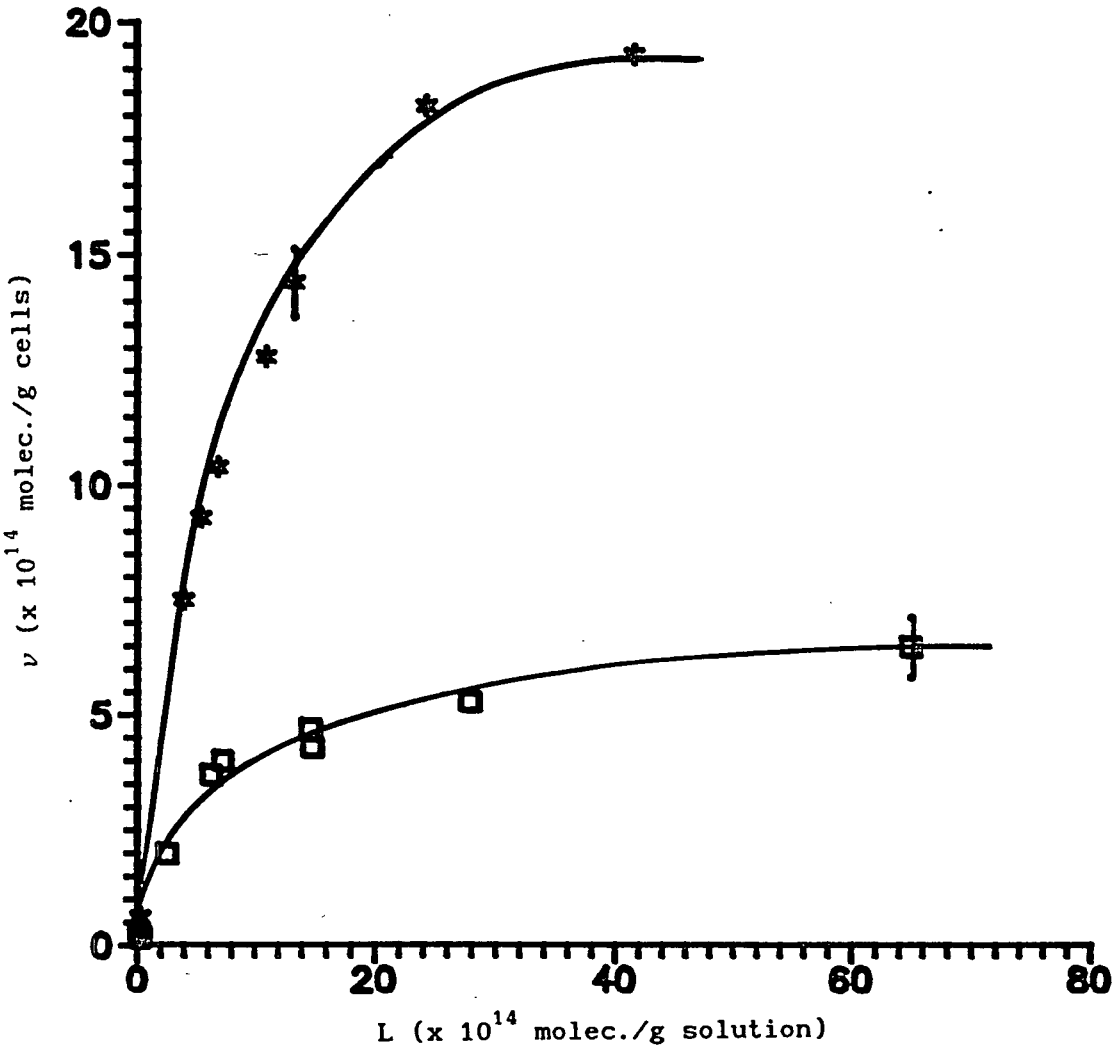


Fig. 24. A direct plot of the binding of YE1.48.10. MAb to MBL-2(4.1) (*) and MBL-2(2.6) (□) cells.

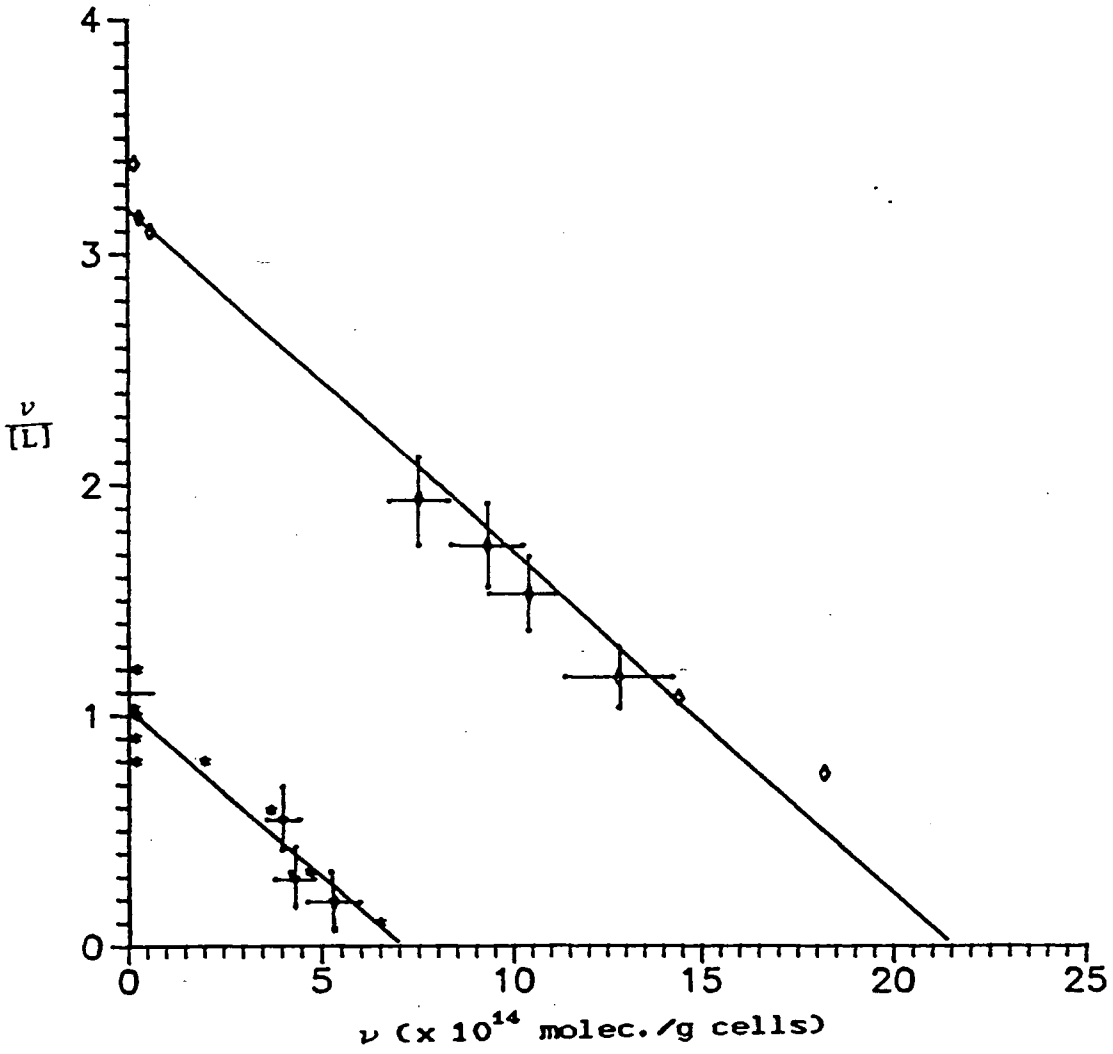


Fig. 25. A Scatchard plot of the binding of YE1.48.10. MAb to MBL-2(4.1) (◇) and MBL-2(2.6) (*) cells. The error bars were calculated as described in Appendix 1.

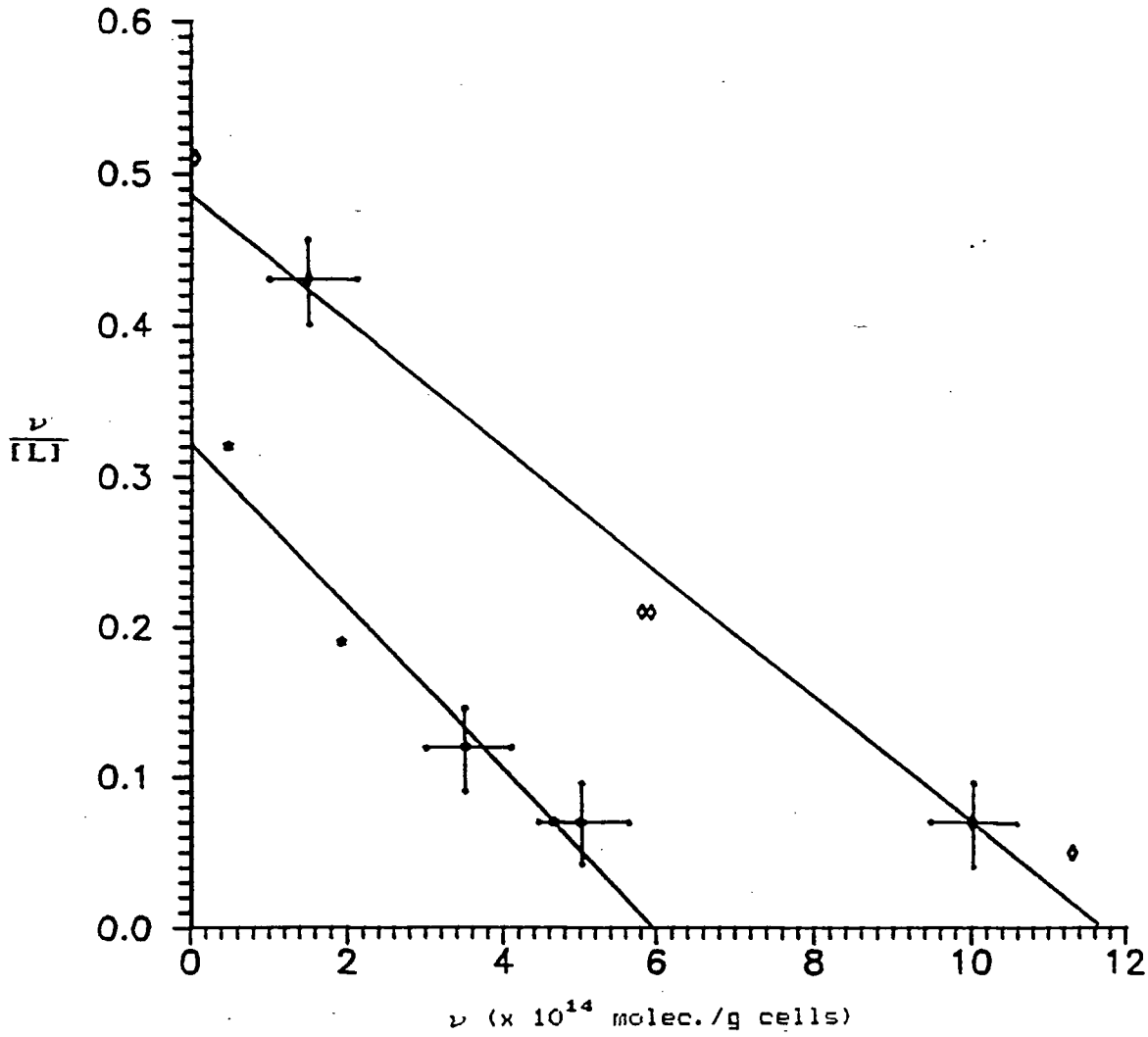


Fig. 26. A Scatchard plot of the binding of biotin-derivatized YE1.48.10. MAb to MBL-2(4.1)(◇) and MBL-2(2.6) (*) cells. The error bars were calculated as described in Appendix 1.

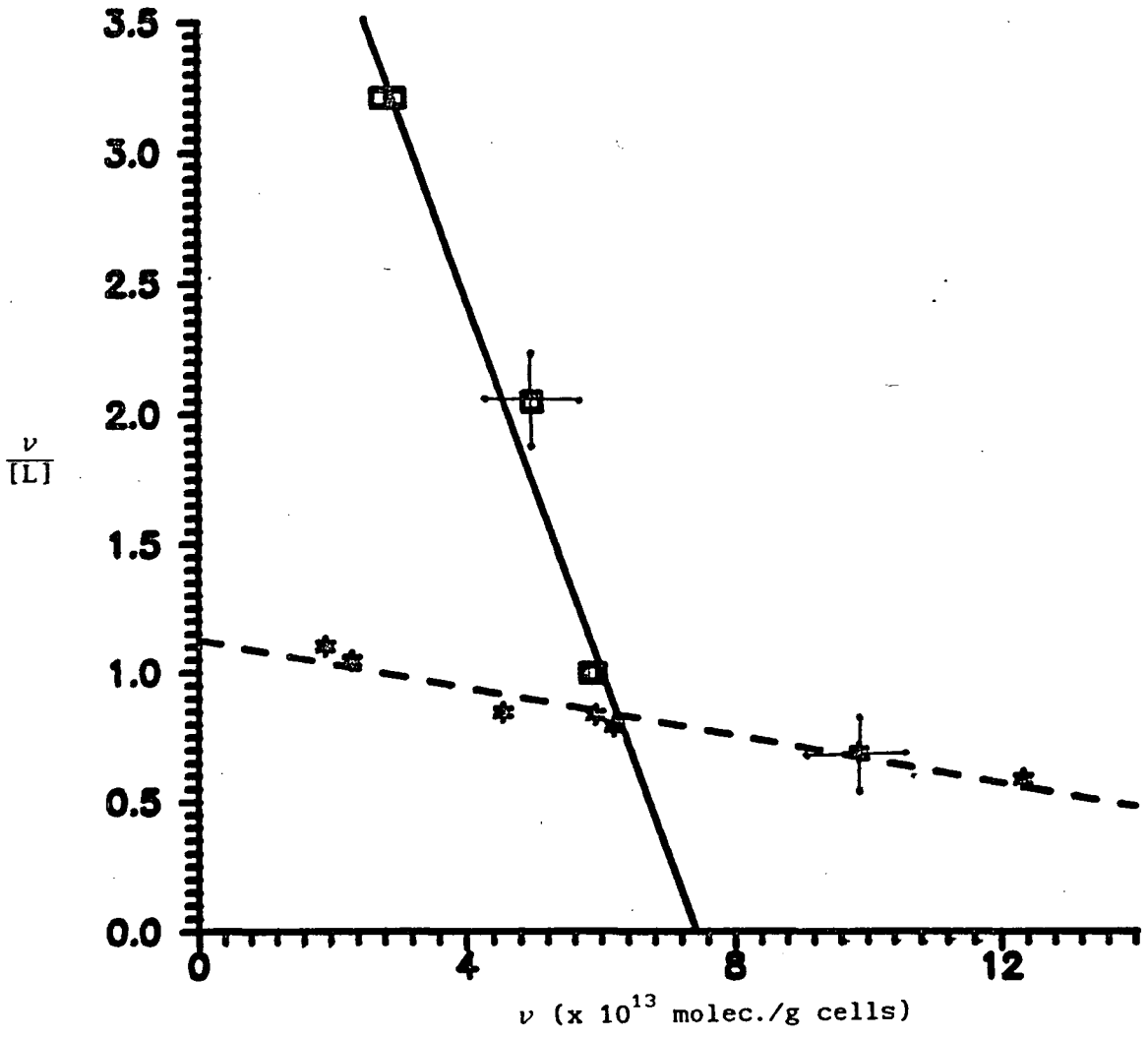


Fig. 27. A Scatchard plot of the binding of YE1.48.10. MAb to human erythrocytes (*) and mouse lymphocytes (□). The error bars were calculated as described in Appendix 1.

whereas the MAbs in this study are modified using the smaller PEG 1900 to preserve MAb activity meaning that the ligand partition coefficient will be lower. Offsetting this, however, is the fact that each IgG molecule is bound by approximately twenty PEG 1900 molecules in a typical affinity ligand compared to only one PEG 8000 in the case of the palmitate ester, although their effect is much lower than is theoretically expected for this level of derivatization (Sharp *et al*, 1986). Hence on the basis of past experience the MBL-2 separation problem seems to be on the limit of the resolution of immunoaffinity partition.

Binding studies were also done on the biotin-derivatized YE1.48.10. MAb. The biotin-YE1.48.10. bound to the cells at a slightly lower surface density than the native MAb and had a lower affinity for the MBL-2 cells. This is reflected in the k_a which is three fold less for the biotin-YE1.48.10. than the native YE1.48.10.(Fig. 26 and Table 5). If this is a real difference in binding it is possible that the increase in hydrophobic character of the MAb due to biotin-derivatization weakens the binding due to some kind of interaction with hydrophilic areas on the cell surface. The glycocalyx is known to be hydrophilic. Another possibility is that the sites are not identical; although the Scatchard plot is linear, the differences in binding energy may not be sufficient to affect the linearity.

d. Partitioning Studies on MBL-2 Cells.

i. The effect of phosphate and chloride. The partition of MBL-2 cells in the absence of ligands was examined in several phase systems with varying phosphate:chloride ratios at isotonic ionic strength and pH 7.2. The results are summarized in Table 6.

Table 6. The effect of different phase systems and phosphate on the partition of MBL-2 (4.1) and MBL-2 (2.6) cells at room temperature.

	% upper phase partition with 10 mM NaP added (μL)									
System	0		10		20		50		250	
Cell type	4.1[1]	2.6[2]	4.1	2.6	4.1	2.6	4.1	2.6	4.1	2.6
[3]5,4,I	2	2	7	9	3	2	4	3	6	7
5,4,II	0	0	2	2	-	-	1	1	4	2
5,4,III	2	3	4	4	1	2	2	2	2	3
5,3.4,I	31	32	63	61	51	58	60	62	74	78
5,3.4,II	27	27	23	25	16	19	20	21	59	52
5,3.4,III	7	5	3	5	7	8	3	4	8	9
[4]5,3.4,I	71	80								
5,3.5,I	54	52								
5,3.6,I	40	35								
5,3.7,I	7	10								
5,3.8,I	9	13								
5,3.9,I	5	9								
5,5,I	0.5	1								

[1]4.1 = MBL-2(4.1) cells and [2]2.6 = MBL-2(2.6) cells.
All experiments were in 2 mL of phase system.
[3&4]These two sets of data were obtained with different stock polymer solutions and MBL-2 cells at different stages of growth.

The results of partitioning experiments shown in Table 6 illustrate a problem encountered with MBL-2 cell partition throughout the study. The value of the % of cells returning to the upper phase varied widely in experiments carried out using cells harvested at different stages of growth with slightly different viabilities and different batches of phase system. MBL-2 cells are seen to have different partition coefficients in identical phase systems in experiments carried out at different times (3 & 4). Although the absolute value of K varied widely, the two cell types consistently had similar K values at similar stages of growth and the ligands all had a reproducible effect on cell partition. In this respect cell partition is very different from molecular partition in which the molecules do not change with time and repeated measurements of K will give consistent results. Control partitions were carried out with every experiment to be certain of the

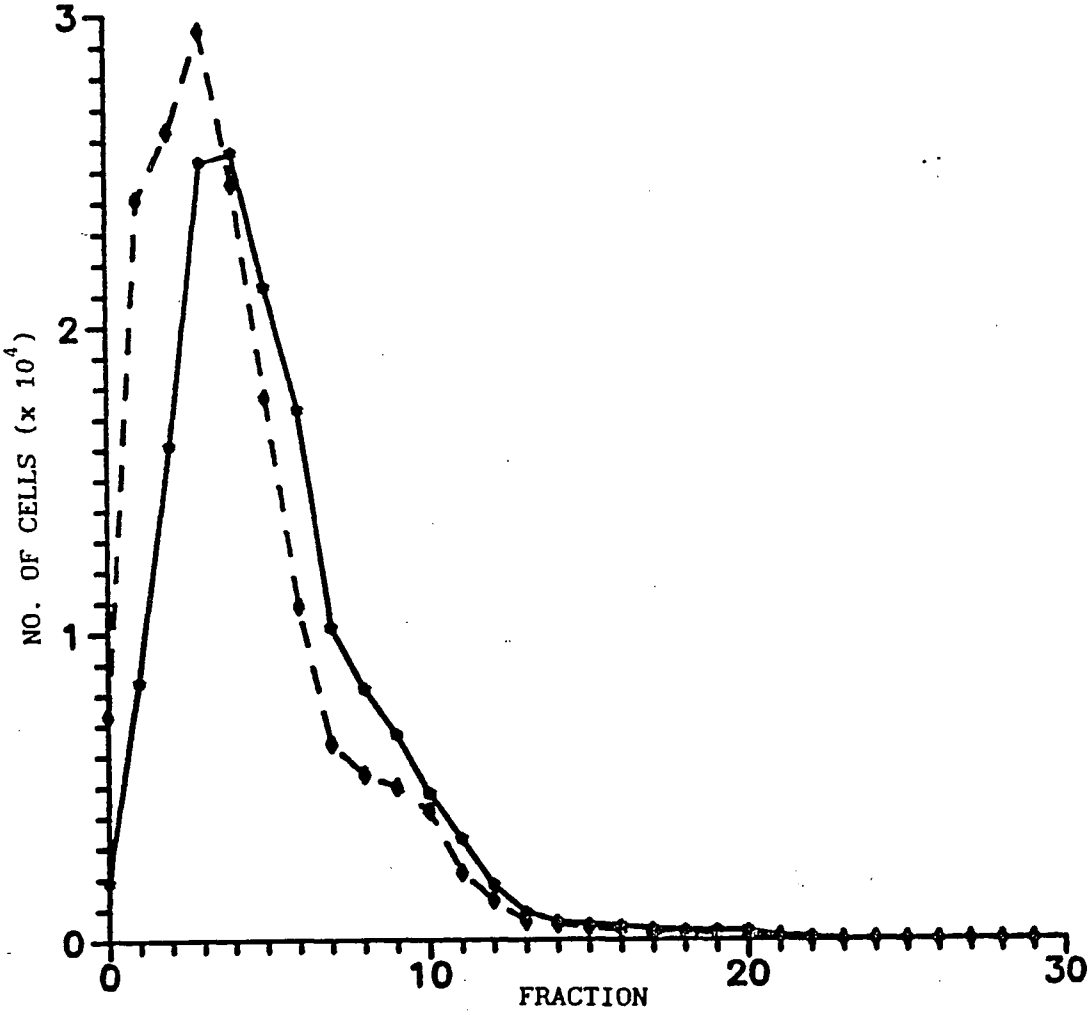


Fig. 28. A typical CCD profile of MBL-2(4.1) cells (——) and MBL-2(2.6) cells (- - -) in a 5,4,I phase system at room temperature.

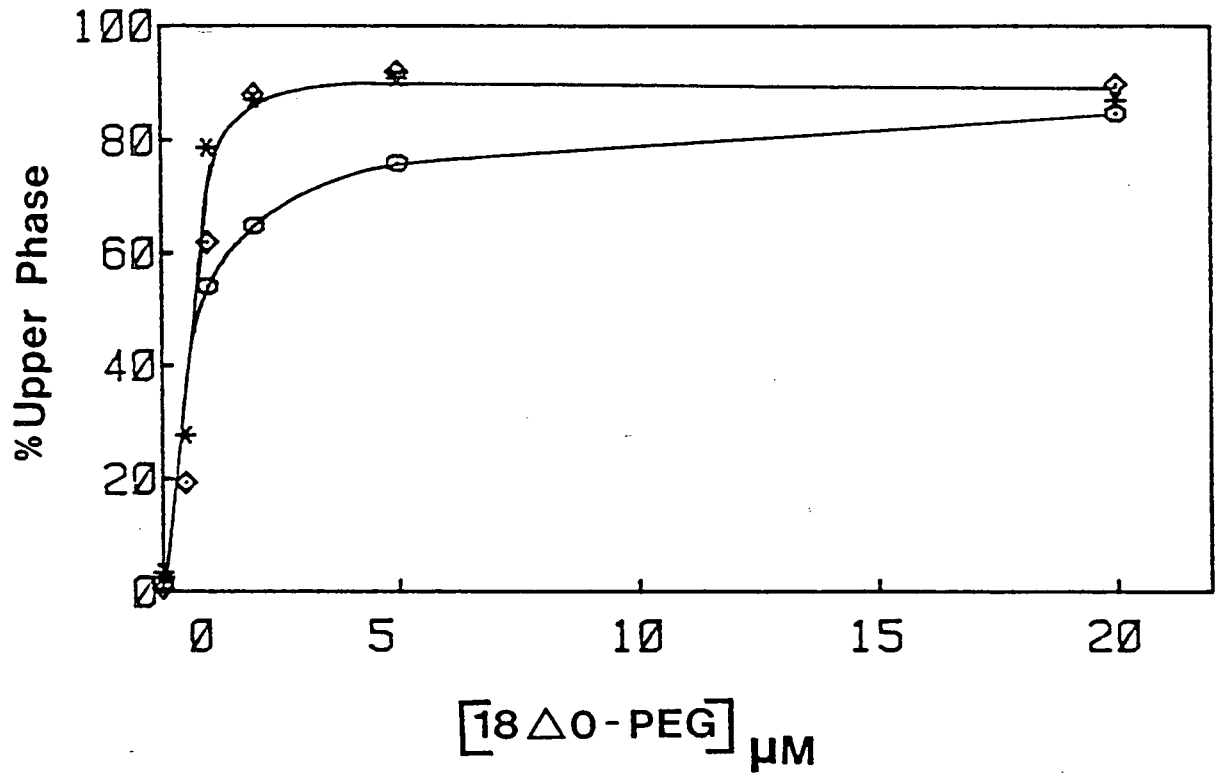


Fig. 29. The effect of PEG-linoleate on the partition of MBL-2(4.1) (*), MBL-2(2.6) cells (◇) and dead MBL-2(4.1) cells (0) in a 5,4,I phase system at 4°.

effect of the ligand and the two MBL-2 cell lines were thawed simultaneously and used in approximately the same passage. They did not always grow at the same rate and could not be described as identical with respect to their age distribution. It was found that in general the partition coefficient tended to increase with increasing age of the culture as well as did heterogeneity, reflected by the spread of the CCD (Fig. 28). However the rate of increase in K or the increase in spread of the CCD with number of cell passages was not consistent with the number of passages but varied from batch to batch of thawed cells.

As expected both MBL-2 sub-lines are sensitive to phosphate and phase composition, the upper phase partition increasing with phosphate concentration and decreasing with increasing tie line length (TLL). No significant differences between the partition of MBL-2(4.1) and MBL-2(2.6) were observed. The cell partition in the 5,4 systems was less sensitive to increasing phosphate concentration while cell partition in 5,3.4,I and 5,3.4,II systems was the most sensitive to phosphate concentration.

A CCD of the two MBL-2 sub-line showed slight differences between the two cell lines as shown in Fig. 28. However this small difference is likely a reflection of the heterogeneity of the populations than differences between the cells. The difference is insufficient to separate the cells.

ii. The effect of PEG-linoleate. The addition of PEG-linoleate to the system had the expected effect of increasing the upper phase partition of both MBL-2 cell sub-lines (Fig. 29). No differential partition behavior between the two MBL-2 sub-lines was observed although the ester was less efficient at increasing the partition of dead cells. For this reason all subsequent experiments were done using cells close to 100% viability.

As it seemed unlikely that the cells could be separated by their native partition characteristics or the use of fatty acid esters, the only reasonable alternative appeared to be immunoaffinity partition. The effect of various types of immunoaffinity ligands on the MBL-2 cells was investigated.

iii. The effect of PEG-YE1.48.10. This is the directly modified ligand approach as depicted in Fig. 2.a. In order to assay YE1.48.10. antibody activity by ELISA it was necessary to remove the antigen from MBL-2 cells by tryptic digestion and use the digest to coat the ELISA plate. Several liters of MBL-2(4.1) cells ($\approx 10^{10}$cells) were digested, the digest aliquoted and stored at -70°. The same digest was used throughout the study to coat ELISA plates at 10 μg/mL of total protein as this concentration gave the highest readings with a low background. The results of an ELISA using pure YE1.48.10. are shown in Fig. 30. The minimum concentration of YE1.48.10. at which binding was detected was 11 μg/mL.

The first immunoaffinity experiments were carried out with antibodies derivatized with cyanuric chloride-activated PEG 1900 (PCC).

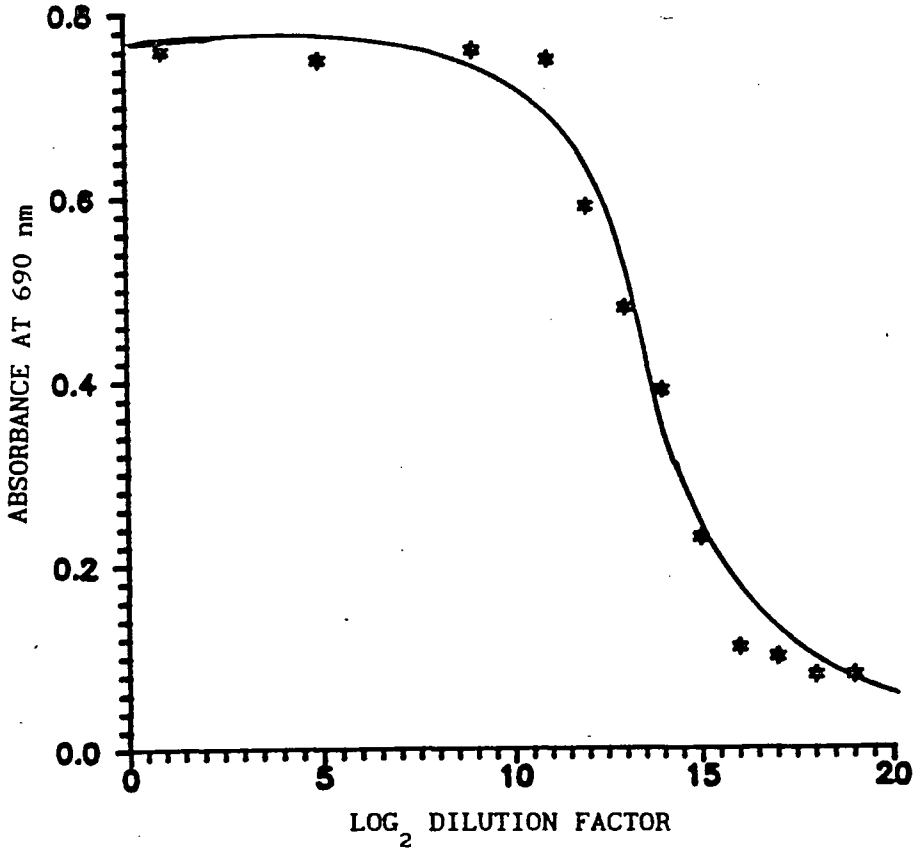


Fig. 30. Binding curve of a typical ELISA of pure YE1.48.10. The initial YE1.48.10. concentration was 22 mg/mL.

Previous studies (Sharp *et al*, 1986) have shown the 1:3 lysine:PCC ratio to be the best compromise between increasing the partition coefficient and minimizing the antibody activity loss.

The same batch of PCC was used throughout this study. It was stored at -70° under nitrogen and was found to be 26% modified by titration of the hydrolyzable chlorides. YE1.48.10. was modified using PCC at a modification ratio of lysine:PCC of 1:1, 1:3 and 1:5 which resulted in the modification of 4, 10 and 17 lysines per IgG molecule as estimated with fluorescamine and assuming 124 lysines per molecule (Edelman *et al*, 1966). At the two higher modification ratios the ligands partitioned strongly in to the upper phase. However none of these modified YE1.48.10. antibodies had any effect on the partition of either MBL-2 cell as shown in Table 7.

Table 7. The effect of PEG-YE1.48.10. on the partition of MBL-2 (2.6) and MBL-2 (4.1) cells at 4°.

No. of PEGs per IgG molecule	Partition coefficient of PEG-YE1.	% cell UP MBL-2(4.1)	MBL-2(2.6)	System
no IgG	-	7	5	5,3.4,III
4	5.7	6	6	" "
0	1	6	5	" "
no IgG	-	3	3	5,4,I
4	5.5	3	2	" "
0	1	2	1	" "
no IgG	-	5	6	5,3.4,III
10	10	6	6	" "
0	1	5	5	" "
no IgG	-	2	2	5,4,I
10	10	2	2	" "
0	1	2	2	" "
no IgG	-	4	4	5,3.4,III
17	14	5	4	" "
0	1	4	5	" "
no IgG	-	2	2	5,4,I
17	15	2	2	" "
0	1	1	2	" "

All partitioning was done at 2mg/ml ligand concentration.

iv. The Effect of PEG-RG7/11.1 and YE1.48.10. The use of a second MAb RG7/11.1, which was directed against rat IgG_{2b} and bound the primary MAb, YE1.48.10., was examined as the ligand. Binding studies on MBL-2 cells saturated with YE1.48.10. were carried out and the Scatchard plots are shown in Fig. 31. The underivatized antibody bound only weakly and at saturation only 6-12% of the cell-bound YE1.48.10. was bound by RG7/11.1. In the case of MBL-2(4.1) 1.3×10^5 molec./cell of RG7/11.1 were bound compared to 2.36×10^6 molec./cell of YE1.48.10. The corresponding numbers for MBL-2(2.6) cells are 9.6×10^4 and 7.96×10^5 molec./cell respectively. Unfortunately this was the only anti-rat $F_c(IgG_{2b})$ MAb available. As with the primary antibody described previously, a number of modification ratios were used and the degree of modification assayed with fluorescamine.

It is difficult to assess the activity of a PEG-derivatized antibody by an ELISA since a decrease in binding of the second, enzyme-conjugated, anti-MAb antibody could be due to decreased binding of the primary antibody or due to interference by the bound PEG in the binding of the second antibody. In order to avoid this problem an experiment was performed in which the inhibition of the binding of a second, enzyme-conjugated polyclonal mouse anti-rat F_c to YE1.48.10. by the PEG-derivatized or native primary antibody, RG7/11.1, was assayed. Thus there was an inverse relationship between the amount of RG7/11.1 antibody bound and the colorimetric enzyme-catalyzed reaction. The ELISA plate was coated using YE1.48.10., then native or PEG-derivatized RG7/11.1 was added. The second enzyme-conjugated antibody, polyclonal anti-rat F_c IgG, was added after the RG7/11.1 and BSA blocking agent. The concept is described in Fig. 32.

The PEG 1900 derivatized YE1.48.10. retained approximately half its original activity estimated by this assay (Fig.32), at the 10 PEGs per IgG level of derivatization, judged by the observation that at saturation it is about half as effective at inhibiting the binding of the HRP-conjugated antibody. This is a high retention of activity compared to previous results (Stocks and Brooks, 1988) but PEG-enzymes have retained higher activities (Abuchowski *et al*, 1977; Beauchamp *et al*, 1983).

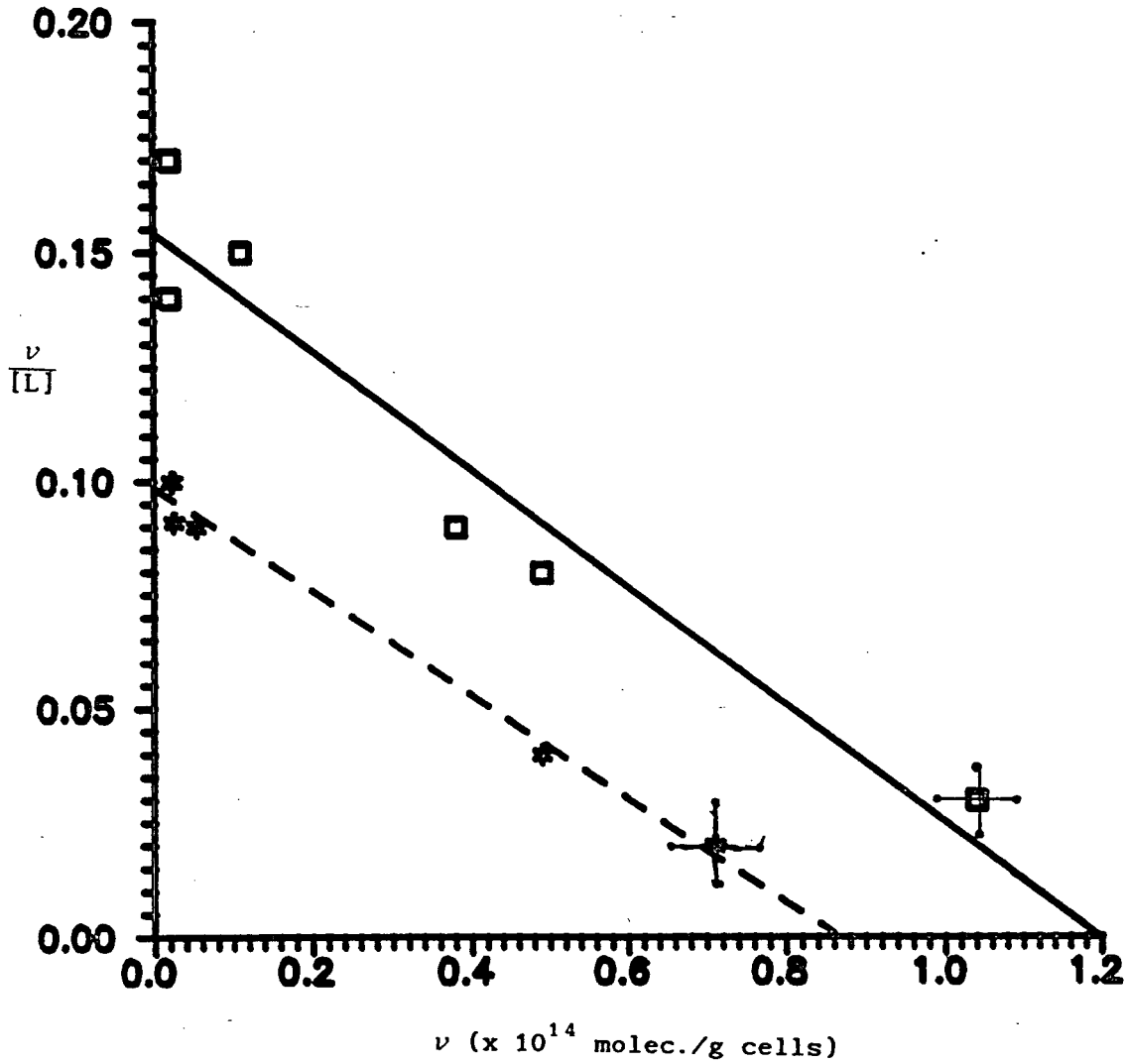


Fig. 31. Scatchard plots for the binding of RG7/11.1 MAb to MBL-2(2.6) (*) and MBL-2(4.1) (□) cells coated with YE1.48.10. MAb. The error bars were calculated as described in Appendix 1.

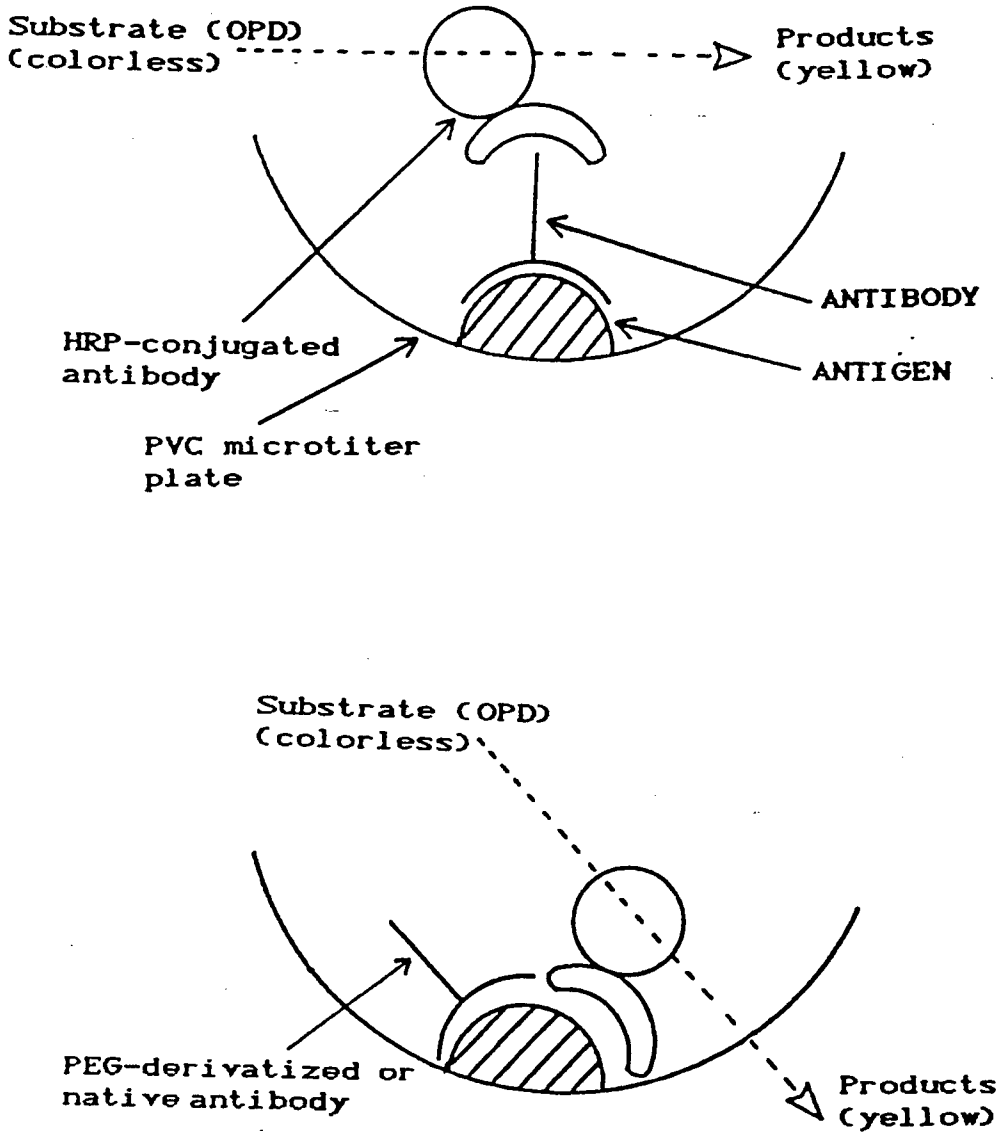


Fig. 32. Schematic diagram of an ELISA (top) and modified-ELISA (bottom) used to assay the binding of PEG-derivatized antibodies.

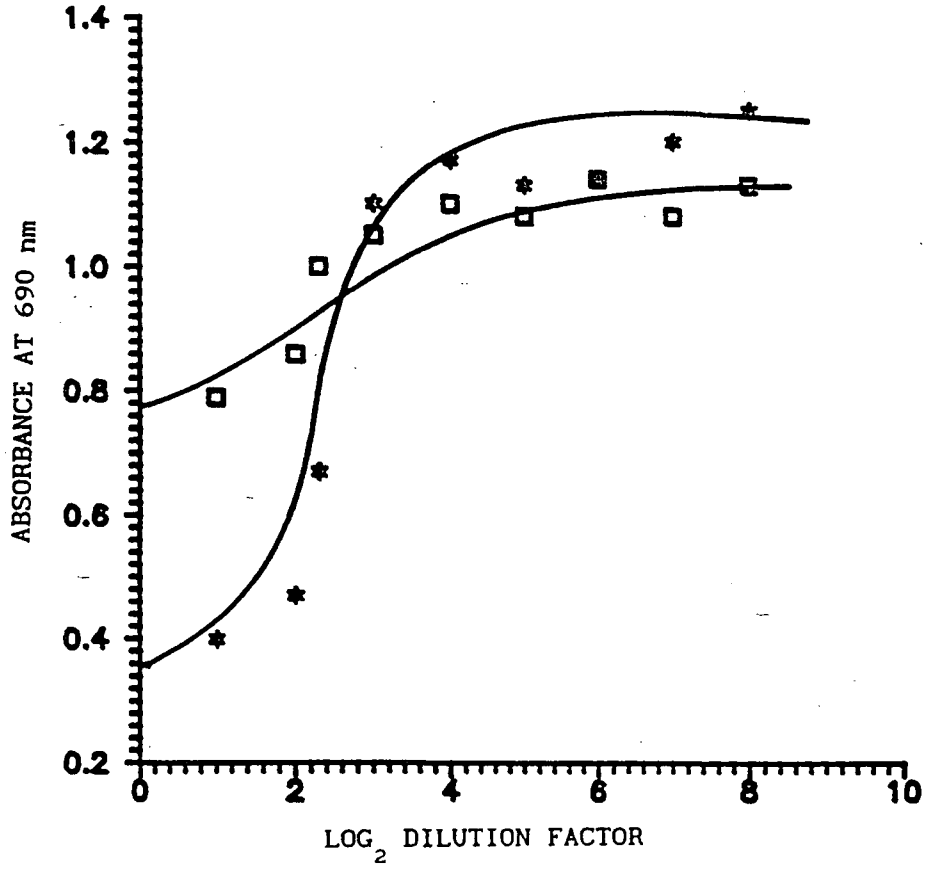


Fig. 33. Binding curves for native (+) and PEG 1900-derivatized RG7/11.1 (□) to YE1.48.10. assayed by an ELISA as described in Fig. 32. The PEG-RG7/11.1 was modified by the attachment of 10 PEGs per IgG molecule. Increasing values of absorbance indicate a decrease in ligand binding.

Partitioning experiments were carried out on cells which had been preincubated with YE1.48.10., then washed twice or with both antibodies present in the phase system and no preincubation period. The former practice was an attempt to economize on MAb as experiments with washing radiolabelled cells had shown that the YE1.48.10. antibody did not dissociate significantly under these conditions. However, as can be seen in Table 8, none of the antibody combinations had any effect on the partition of MBL-2 cells.

Table 8. The partition of MBL-2 (4.1) and MBL-2 (2.6) cells in the presence of PEG-RG7 11.1 and YE1 48.10. antibodies at 4°.

no. of PEGs per RG7/11.1	system	ligand concentration [1][PEG-RG7]	[2][YE1.48.10]	% UP [3](4.1)	[4](2.6)
-	5,3.4,I	-	-	70	72
0	"	0.45 mg/ml	preincub.	71	73
3	"	"	" "	72	73
10	"	"	" "	72	73
21	"	"	" "	68	71
23	"	"	" "	68	68
0	5,3.4,II	0.22 mg/ml	0.15 mg/ml	51	42
3	"	"	"	48	42
10	"	"	"	43	34
21	"	"	"	37	30
23	"	"	"	53	43
-	5,4,III	-	-	1	1
0		0.3 mg/ml	preincub.	1	1
3	"	"	" "	2	1
10	"	"	" "	2	1
21	"	"	" "	2	1
23	"	"	" "	1	1
-	5,4,II	-	-	11	8
0	"	0.3 mg/mL	preincub.	10	7
7	"	"	" "	5	4
19	"	"	" "	3	4
42	"	"	" "	2	4
46	"	"	" "	3	4

[1][PEG-RG7] = PEG-derivatized RG7/11.1 MAb concentration.

[2][YE1.48.10.] = YE1.48.10. MAb concentration.

[3](4.1) = MBL-2(4.1) cells.

[4](2.6) = MBL-2(2.6) cells.

v. The effect of Biotin-YE1.48.10. and PEG-avidin. The partition coefficient of biotin was measured using ^{14}C labelled biotin and found to be 1.4. The derivatization of avidin with PEG-1900 increased the partition of avidin from 0.6 to 11 in the case where 20 PEG molecules were bound per avidin. This PEG-avidin derivative was able to increase the partition of biotin from 1.4 to 15, an 11 fold increase, whereas native avidin decreased the partition of biotin from 1.4 to 0.31. These results are shown in Fig. 34.

The binding of the dye 2(4'-hydroxyazobenzene)benzoic acid (HABA) by an excess of avidin leads to an increase in ε_{500} from 480 to 34 500. The avidin-HABA complex may be dissociated by the addition of 4 moles of biotin per mole of avidin (Green, 1965). This reaction was used to measure the partition coefficient of biotin-derivatized proteins, avidin and PEG-avidin. The PEG-derivatization of avidin affected its binding capacity for biotin. This loss in binding ability could be estimated from the amount of biotin required to displace HABA from the avidin-HABA complex. There is evidence that HABA and biotin bind at the same site (Green, 1970) so this probably gives a good indication of the loss in binding ability caused by PEG-derivatization. In Fig. 35 an example of the decrease in affinity for avidin at two different PEG-modification ratios (*i.e.* 11 & 16 PEG 1900 per avidin) is given. From the slopes of the graphs it was estimated that 36% and 60%, respectively, of the biotin binding sites had been destroyed by PEG-modification. The partition coefficient of HABA was increased by including PEG-avidin in the phase system and this could be inhibited with biotin as is shown in Fig. 36.

The effect of biotin-derivatization and PEG-avidin or avidin on the partition of BSA was also investigated and the results are summarized in Table 9.

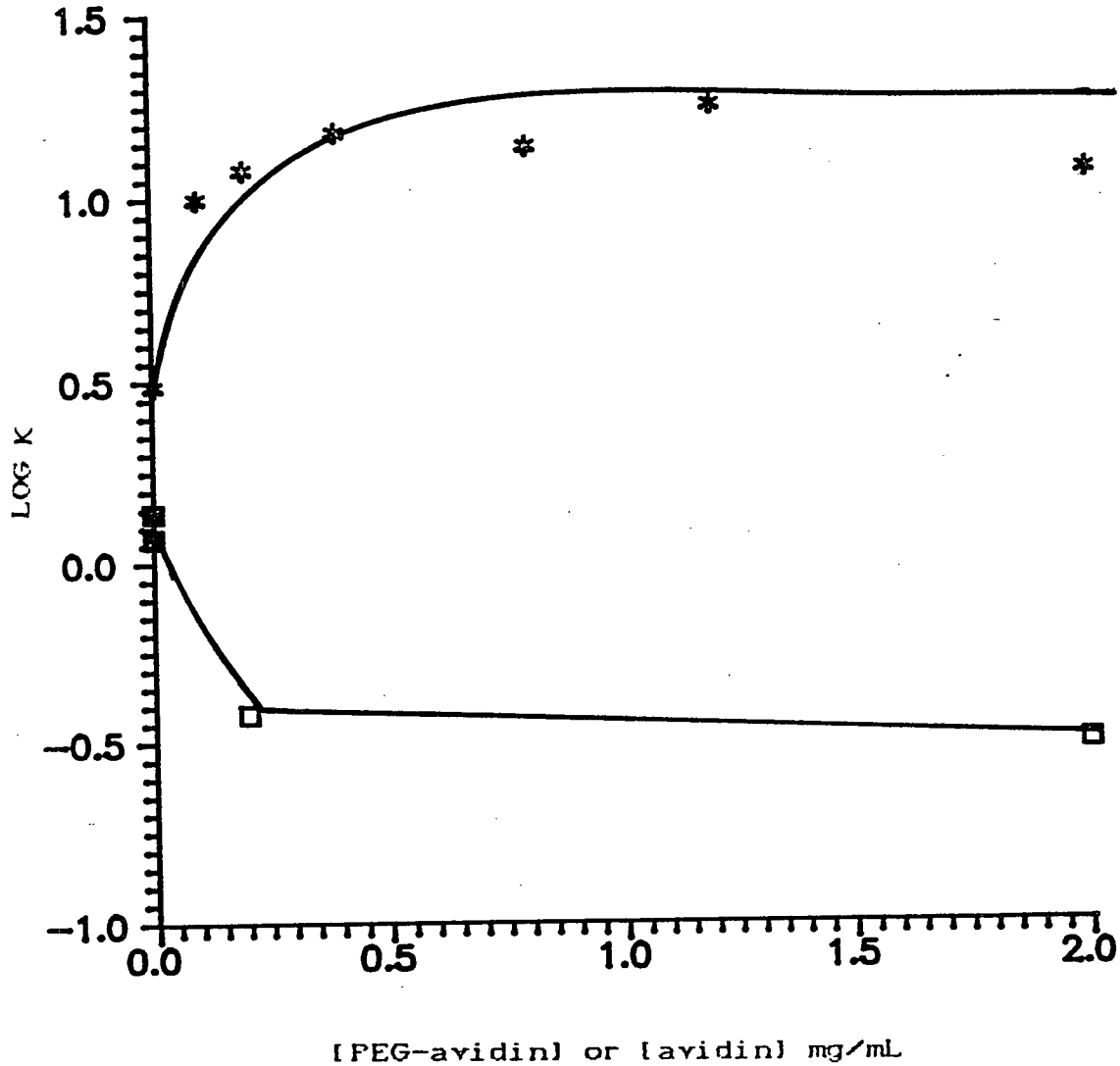


Fig. 34. The effect of PEG-avidin (*) and avidin (□) on the partition of biotin in a 5,4,I system at room temperature. The biotin concentration was 0.05 nM.

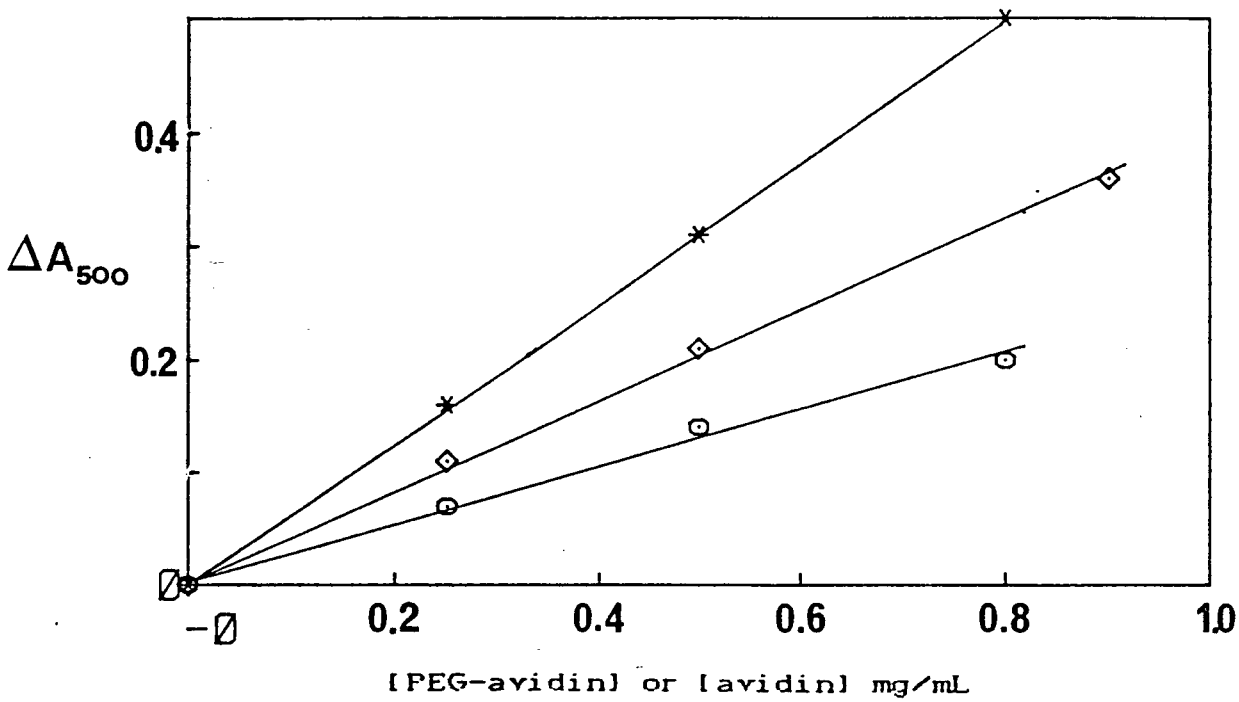


Fig. 35. The effect of PEG 1900 modification on the ability of avidin to bind 2(4'-hydroxyazobenzene) benzoic acid(HABA)

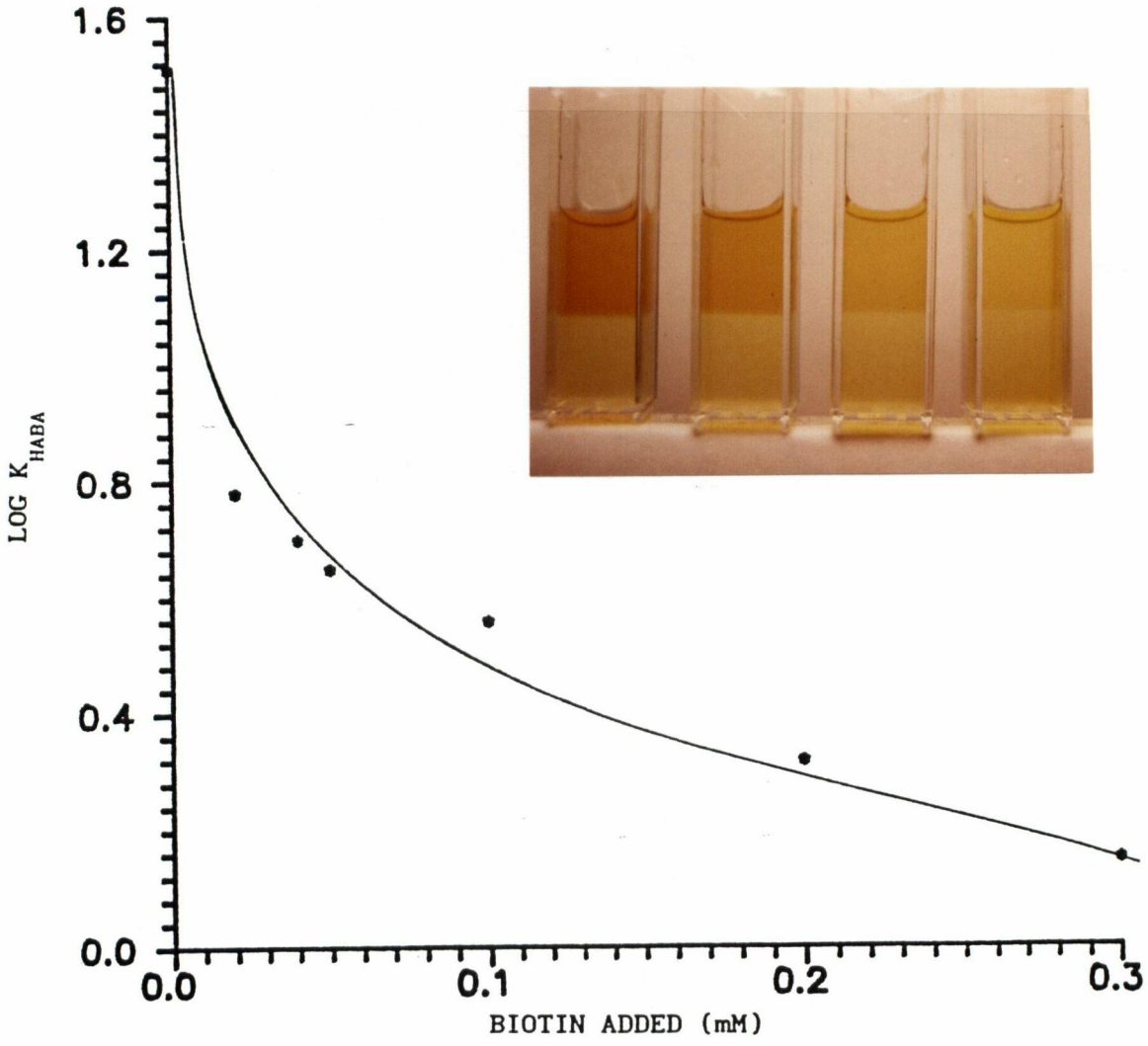


Fig. 36. The effect of biotin on the partition of 2(4'-hydroxyazobenzene) benzoic acid (HABA) in the presence of PEG-avidin (0.4 mg/mL). The concentration of HABA was 0.5 mM. Insert shows HABA in a 5,4,I system in the presence of PEG-avidin (0.4 mg/mL) and biotin concentrations from right to left of 0.3, 0.2, 0.1 and 0.05 mM.

Table 9. The effect of biotin-derivatization, PEG-avidin and avidin on the partition of BSA.

mol biotin/ mol BSA	% of biotin-BSA in upper phase (0.5 mg/ml)	% with PEG-avidin included (0.4 mg/ml)	% with avidin included (0.4 mg/ml)
0	50	53	52
42	84	100	22
37	77	85	20
26	72	80	20
21	70	71	17
15	65	67	14
7	55	57	14

All experiments were in a 5,4,I system at 4° and the mole ratio of biotin:BSA was assayed with fluorescamine.

Biotin-derivatization caused the BSA to partition in favor of the upper phase and the effect was further increased by the addition of PEG-avidin while native avidin decreased the effect. The larger the number of moles of biotin bound per BSA, the greater the partition into the upper phase. This illustrates the effect of increasing the molecular weight of the partitioned material since the biotin-BSA has a greater partition coefficient than native biotin.

Biotin-derivatized polyclonal sheep anti-mouse F_c IgG (sαmF_c) also partitioned into the upper phase as shown in Table 10. It was found that a large excess of N-hydroxysuccinimidobiotin was required to achieve a high modification ratio. This is also true of the PCC-protein reaction and is attributable to the competing hydrolysis reaction.

Even at these high modification ratios described in Table 10, no loss in activity of the sαmF_c was detected with an ELISA assay. However this is a polyclonal antibody and this will not necessarily be true for a MAb. In fact a Scatchard plot of biotin-YE1.48.10. showed some loss in YE1.48.10. activity at a lower modification ratio (Fig. 26). This may be because an ELISA is less sensitive than a binding assay or because of the polyclonal origins of the sαmF_c antibody.

Table 10. The effect of reaction ratio of N-hydroxysuccinimidobiotin on the degree of modification of IgG with biotin and the partition coefficient.

reaction ratio BNHS:IgG (mol)	product ratio biotin:IgG (mol)	% BNHS reacted	Activity by ELISA (% of original)	K
54	36	46	100	5.3
119	36	21	100	5.4
272	98	25	100	6.0
388	111	19	100	7.1
581	209	24	100	7.3

BNHS = N-hydroxysuccinimidobiotin

The next approach to the MBL-2 separation was the combination of biotin-derivatized YE1.48.10. and PEG-avidin as shown in Fig. 2.d. The biotin-derivatized YE1.48.10. had a lower affinity for MBL-2 cells than native YE1.48.10. as was discussed earlier. The biotin-YE1.48.10. partitioned into the upper phase at modifications ranging from 17, 27 and 63 biotins per IgG molecule (K = 6.7, 11.5 and 19 respectively) and the ELISA at all these levels of modification did not show any loss in activity despite the binding assay indicating some decrease in the amount bound. The addition of PEG-avidin in all cases was able to move the remaining biotin-YE1.48.10. into the upper phase while avidin shifted it all into the lower phase. An example of the dependence of the partition of biotin-YE1.48.10. (27 biotins per IgG molecule) on PEG-avidin and avidin concentration is shown in Fig. 37.

Although the MAb responded appropriately to biotin/PEG-avidin modification, the combination of these two ligands was unable to alter the partition of MBL-2 cells, although small but significant changes in partition were obtained with the biotin-YE1.48.10. alone. The results of partitioning experiments with both MBL-2 cells and YE1.48.10. are shown in Table 11. All the modified YE1.48.10. MAbs were able to increase the partition of both cell lines and the partition coefficient of MBL-2(4.1) cells was approximately twice that of MBL-2(2.6) cells in every case. However these differences are small compared to the partition of native cells and a separation on the basis of these partition coefficients would require a CCD of 200 transfers. The addition of PEG-avidin to the

system actually lowered the MBL-2 cell partition slightly with increasing PEG-avidin concentration. Even native avidin did not decrease the MBL-2 cell partition, although the differences may not be significant from PEG-avidin. This is shown in Fig. 38.

Table 11. Results of partitioning experiments using biotin-YE1.48.10. and MBL-2 cells.

Cell type	System	YE1.48.10. conc.(mg/mL)	Biotins per IgG	K (B-YE1)	% UP (cells)	K (cells)
MBL-2(2.6)	5,4,III	0.20	0	1.0	45	0.8
MBL-2(4.1)	5,4,III	0.20	0	1.0	45	0.8
MBL-2(2.6)	5,4,III	0.20	17	6.7	48	0.9
MBL-2(4.1)	5,4,III	0.20	17	6.7	63	1.7
MBL-2(2.6)	5,4,III	0.20	10	4.2	50	1.0
MBL-2(4.1)	5,4,III	0.20	10	4.2	62	1.6
MBL-2(2.6)	5,4,II	0.15	0	1.0	30	0.4
MBL-2(4.1)	5,4,II	0.15	0	1.0	31	0.4
MBL-2(2.6)	5,4,II	0.15	27	11.5	27	0.4
MBL-2(4.1)	5,4,II	0.15	27	11.5	46	0.9

The slight effect of the PEG-avidin and avidin in lowering the partition of MBL-2 cells was not examined in detail. If the effect is real it is difficult to explain. The amount of PEG added to the system would not change the interfacial tension of the system sufficiently to alter the cell partition. In fact the phase diagram of the system with all the ligands was measured and was almost identical to that without ligands. If the effect were due to agglutination of the cells by the avidin binding to more than one biotin, then the native avidin would be expected to have a much greater effect as it has more biotin binding sites available. No agglutination of the cells was observed at 40-times magnification.

The fact that some increase in cell partition is observed with the biotin-YE1.48.10. alone suggests that the PEG-avidin and avidin interfere with the binding of biotin-YE1.48.10. to the MBL-2 cell as the biotin-YE1.48.10. has no effect in the presence of these ligands. Table 12 shows the partition coefficients of B-YE1.48.10. with and without cells in the presence and absence of avidin or PEG-avidin. The partition coefficient of the biotin-YE1.48.10. is essentially the same in the

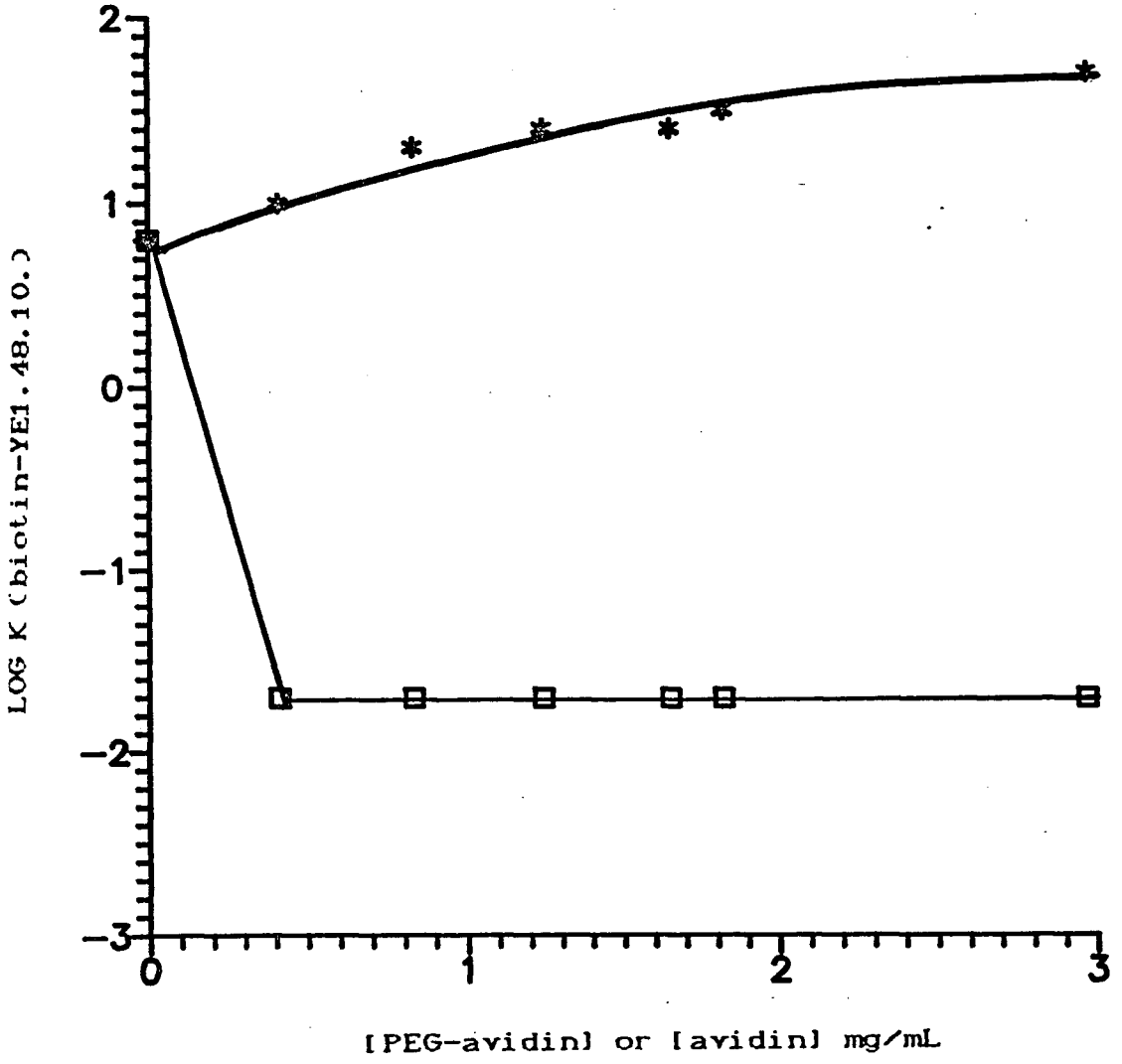
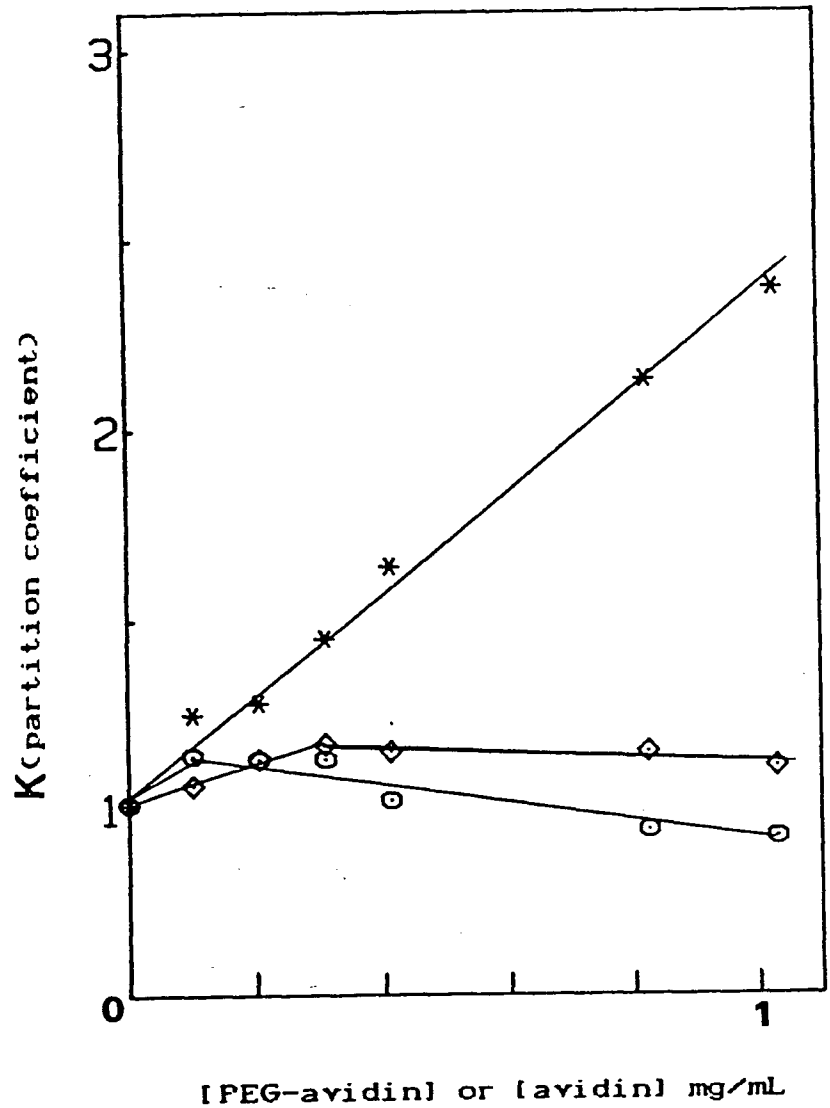


Fig. 37. The effect of PEG-avidin (✱) and avidin (□) on the partition of YE1.48.10 MAb. (0.3 mg/mL).



* K (biotin-YE1.48.10.) with PEG-avidin

⊙ K (MBL-2(4.1) cells with biotin-YE1.48.10.) and PEG-avidin

◇ K (MBL-2(4.1) cells with biotin-YE1.48.10.) and avidin

Fig. 38. The effect of avidin and PEG-avidin on the partition of MBL-2 cells in the presence of biotin-YE1.48.10. (0.3 mg/mL).

Table 12. The effect of PEG-avidin and avidin on the partition coefficient of biotin-YE1.48.10. in the presence of MBL-2(4.1) cells.

	without MBL-2(4.1) cells			with MBL-2(4.1) cells		
PEG-avidin conc. (mg/mL)	avidin conc. (mg/mL)	K (B-YE1)	log K (B-YE1)	K (B-YE1)	log K (B-YE1)	% UP MBL-2 (4.1)
-	-	[1]6.7	0.8	5.3	0.7	63
-	-	[2]11.5	1.6	9.8	1.0	62
-	-	[3]5.5	0.7	4.5	0.65	46
0.41	-	10	1.0	18	1.3	50
0.83	-	20	1.3	24	1.4	44
1.24	-	-	1.4	-	1.5	31
1.65	-	-	1.4	-	1.7	21
1.82	-	-	1.5	-	1.7	17
2.97	-	-	1.7	-	1.7	-
-	0.41	0.02	-1.7	0.02	-1.7	48
-	0.83	0.02	"	0.01	-0.2	40
-	1.24	0.02	"	0.01	"	45
-	1.65	0.02	"	0.01	"	45
-	1.82	0.02	"	0.01	"	56
-	2.97	0.02	"	0.01	"	-

% UP = % of cells partitioning into the upper phase.

All experiments used 0.2 mg/mL B-YE1.48.10.

[1]Biotins per YE1.48.10. = 17

[2]Biotins per YE1.48.10. = 27

[3]Biotins per YE1.48.10. = 15

presence or absence of cells plus PEG-avidin or avidin. If the biotin-YE1.48.10. and PEG-avidin or avidin were binding to the cells and the ligands were not strong enough to alter the cell partition, a decrease in K (biotin-YE1.48.10.) in the presence of cells compared to that in the absence of cells would be seen as the ligand became bound to cells at the interface. This was not observed. However as seen in the first three rows of Table 12, in the case where the partition is done in the absence of PEG-avidin or avidin, the K (biotin-YE1.48.10.) is decreased in the presence of MBL-2 cells relative to in the absence of cells. In the the presence of PEG-avidin and avidin however, the K (biotin-YE1.48.10.) is unaffected by the presence of MBL-2 cells

suggesting that under these circumstances the YE1.48.10. is not binding to the cell.

In summary the avidin-biotin system works well on a molecular level but did not affect MBL-2 cell partition. It seems possible that this is due to the binding of PEG-avidin or avidin to biotin located in or close to the antigen binding site of the antibody thus interfering with the cell-antibody binding.

As none of the upper phase partitioning ligands were able to alter the partition of MBL-2 cells by a sufficient amount it was decided to try a lower phase partitioning ligand. Since most of the MBL-2 cells are located at the interface in all the systems used and it is easy to optimize the systems for a lower phase partitioning ligand, cells partitioning into the lower phase may be separated from those at the interface.

3. The effect of polyacrylamide-derivatized antibodies. As discussed in the introduction, polyacrylamide has a strong affinity for the lower phase of a PEG/dx APTS. It was decided to graft polyacrylamide (PAA) onto antibody molecules, the advantages being that the antibody would partition more strongly into the lower phase than a PEG-ligand would into the upper phase. This supposition is based on the fact that dextran has a much larger lower phase partition than the corresponding PEG upper phase partition. For example in a 5,4 system the upper phase partition of PEG is 4.0 whereas the lower phase partition of dx is 28.3 (Albertsson, 1986). This means less polymer would need to be bound to the MAb thus decreasing the activity loss. Also because the reaction is specific for alcohols the sites of attachment are limited to serine hydroxyls and possibly threonine. The oligosaccharide hydroxyls are capable of initiating polymerization but are insignificant (approx. 8 per IgG) compared to the numbers of serine and threonine hydroxyls.

The optimization of the reaction conditions for grafting PAA onto protein was done using BSA and non-specific bovine IgG. A drawback of the polymerization is that since almost all ceric salts are insoluble, a buffer which did not precipitate the initiator could not be found and all polymerizations were carried out in water only. A minimum volume of $(NH_4)_2[Ce(NO_3)_6]$ in 1M HNO_3 was added to avoid protein precipitation and

denaturation. While it was possible in the cases of the two antibodies used in this study to maintain antibody activity this may not be true for all proteins. The increase in solution viscosity after polymerization was measured as this was a simple method of indicating that a reaction had occurred. PAA was grafted onto BSA, bovine IgG, rabbit anti-human erythrocyte IgG (rαhrbc) and YE1.48.10. The viscosity increases at various reaction ratios and partition coefficients for these derivatives are quoted in Table 13. One of the acrylamide:protein mole ratios was sufficiently high to produce a gel.

The PAA-protein derivatives were not characterized. Nitrogen analysis, the usual method of characterizing PAA bound to a solid support, was not feasible due to the nitrogen present in the protein, a radiolabelled acrylamide monomer was unavailable and an amino acid analysis encountered problems in the hydrolysis of the sample.

Hemagglutination assays (Table 14) using rabbit and human erythrocytes with all the PAA-rαhrbc derivatives did not show any decrease in agglutinating ability due to PAA derivatization nor did they agglutinate rabbit erythrocytes. The most highly modified rαhrbc antibody (1:100) showed a slight increase in agglutinating ability, probably due to the larger amounts of free PAA present. High molecular weight PAA is known to agglutinate cells non-specifically although in the present case this was not entirely non-specific since the rabbit cells were not agglutinated by the PAA-rαhrbc. In the case of the MBL-2 cells and PAA-YE1.48.10., again no loss in activity was observed at the 1:17 reaction ratio level as measured by microtiter but the gelled antibody had no agglutinating ability. The gel was resuspended by pipetting in order to carry out a hemagglutination assay. The PAA-YE1.48.10. did not agglutinate human erythrocytes or a control mouse lymphocyte. The PAA-YE1.48.10. agglutinated MBL-2 cells at much lower concentrations than PAA-rαhrbc, however. This is reflected in the partitioning experiments as no aggregation problems were encountered when using the PAA-rαhrbc but aggregation was a definite problem when using the PAA-YE1.48.10. This aggregation is antibody-mediated since none of the control cells aggregated as they would if this were an effect only of the PAA.

Table 13. A summary of polyacrylamide-grafted proteins synthesized with details of reaction ratios, viscosities and partition coefficients in a 5,4,I system.

Protein	Hydroxyl:Acrylamide reaction ratio (mol:mol)	Viscosity (cp) unreacted	reacted	K (native)	K (PAA-protein)
BSA	1:32	0.94	1.34	2.1	0.38
b IgG	1:5	0.98	2.31	1.5	0.16
b IgG	1:3	0.92	1.23	1.5	0.25
b IgG	1:1.6	0.91	0.99	1.5	0.28
rαhrbc	1:100	0.91	1.36	1.1	0.18
rαhrbc	1:25	0.90	1.04	1.1	0.21
rαhrbc	1:20	0.90	0.99	1.1	0.19
YE1.48.10.	1:17	0.90	0.97	1.2	0.05[1]
YE1.48.10.	1:260	0.91	gel	1.2	-

BSA = bovine serum albumin

b IgG = bovine IgG

rαhrbc = rabbit anti-human erythrocyte IgG

PAA = polyacrylamide

[1]measured in a 7,4,S phase system

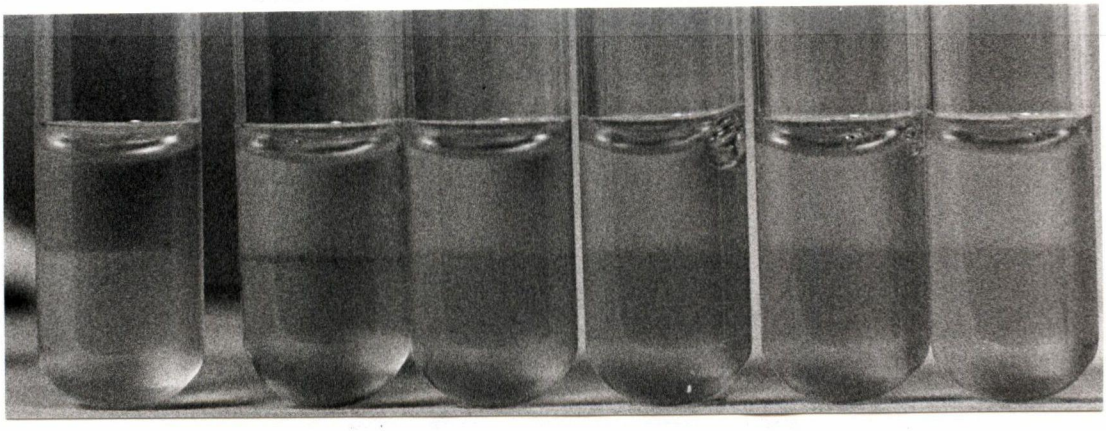
The PAA-rαhrbc was an extremely effective ligand (Figs. 39 & 40, Table 15) and obtained the most efficient immunoaffinity separation of erythrocytes reported to date. Even at concentrations as low as 0.08 mg/mL PAA-rαhrbc almost all the human erythrocytes were in the lower phase whereas the rabbit erythrocyte partition was unaltered. As shown in Fig. 40 a ligand concentration of 0.08 mg/mL increased the human erythrocyte partition from 8 to 86% whereas the rabbit erythrocyte concentration remained unaltered at 18%. Although a good separation could be obtained in a single partition step, a CCD was used to separate the cells with 100% purity. A 5,4,I system was found to give an efficient separation and was used for the CCD runs. Theoretical CCD profiles for the values of G of 0.9 and 0.22 for rabbit and human erythrocytes are shown in Appendix II. Theoretically, a CCD of 15 transfers would be sufficient to achieve a separation of 100% pure cell

Table 14. Hemagglutination assays of polyacrylamide-derivatized ligands.

Antibody	Hydroxyl:acrylamide reaction ratio (mol:mol)	Cell	Minimum agglutinating concentration (μg/mL)
rαhrbc	-	hrbc	2.0
PAA-rαhrbc	1:25	hrbc	2.0
PAA-rαhrbc	1:100	hrbc	0.4
rαhrbc	-	rrbc	none[1]
PAA-rαhrbc	1:25	rrbc	none
PAA-rαhrbc	1:100	rrbc	none
YE1.48.10.	-	MBL-2(4.1)	0.001
PAA-YE1.48.10.	1:17	MBL-2(4.1)	0.001
PAA-YE1.48.10.	1:260	MBL-2(4.1)	none
YE1.48.10.	-	hrbc	none
PAA-YE1.48.10.	1:17	hrbc	none
YE1.48.10.	-	m lymphocyte	none
PAA-YE1.48.10.	1:17	m lymphocyte	none

PAA = polyacrylamide rαhrbc = rabbit anti-human erythrocyte IgG
hrbc = human erythrocyte rrbc = rabbit erythrocyte
m lymphocyte = mouse lymphocyte control
[1]No agglutination at the highest concentration of antibody (0.5 mg/mL for rαhrbc and 1 mg/mL for YE1.48.10.).

populations but in order to allow for cell heterogeneity it was decided to use 20 transfers. Human erythrocytes were labelled using ^{51}Cr and the CCD profile of a mixture of rabbit and human erythrocytes in the presence of PAA-rαhrbc obtained in a CCD run and the corresponding theoretical profile is shown in Fig. 41. The rabbit and human erythrocytes were almost completely resolved. The broadening of the peaks compared to the theoretical peaks presumably reflects cell heterogeneity.



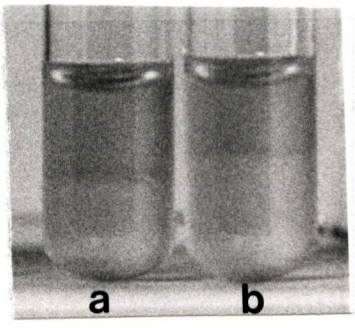


Fig. 39. The partition of human erythrocytes in a 5% dx, 4% PEG, 110 mM sodium phosphate buffer system showing (top) from left to right 0, 0.04, 0.08, 0.12, 0.16, 0.2 mg/ml polyacrylamide-derivatized rαhrbc. The bottom row shows the partition of rabbit erythrocytes in the same conditions with (a) and without (b) 0.2 mg/mL polyacrylamide-derivatized rαhrbc.

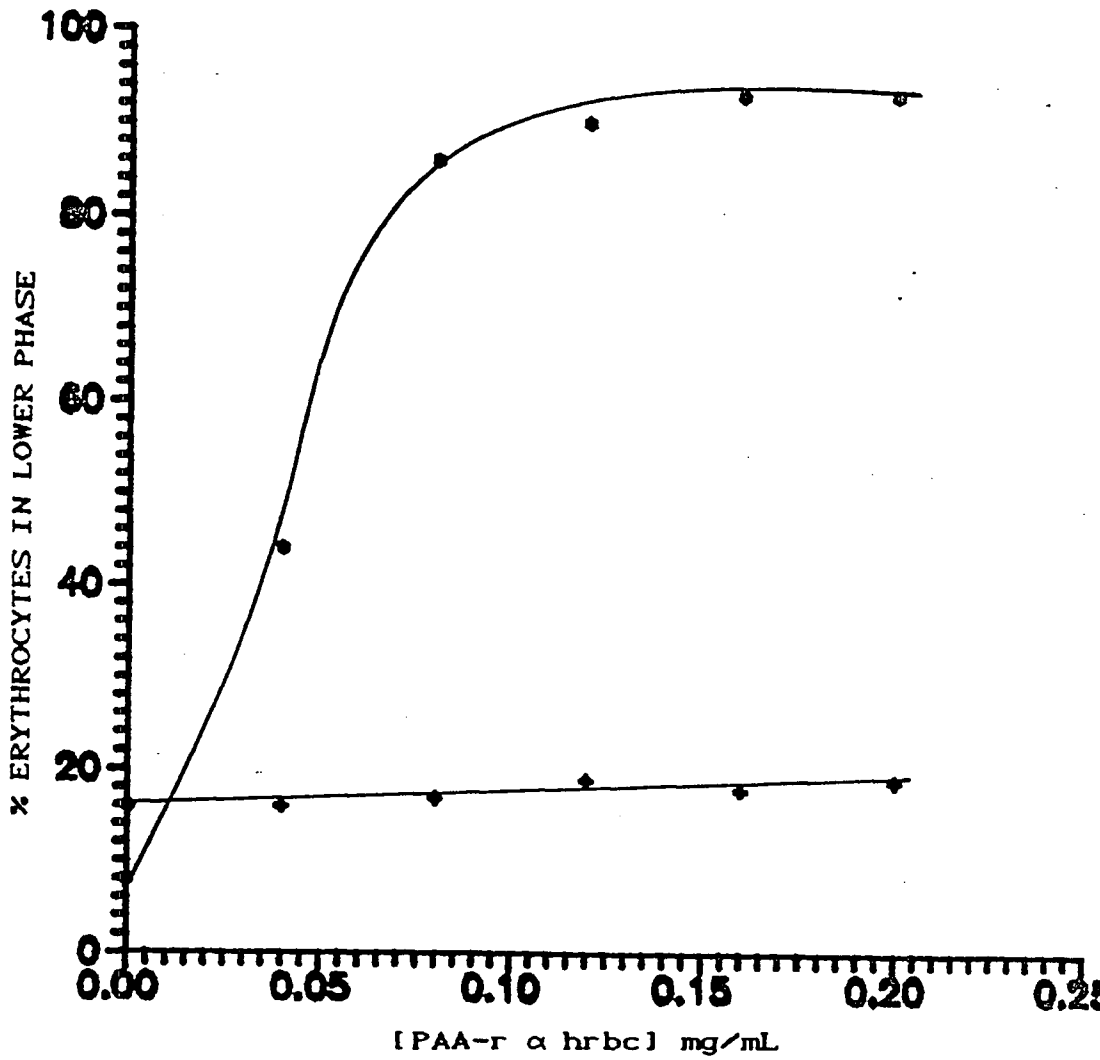


Fig. 40. The partition of human (*) and rabbit erythrocytes (+) in the presence of increasing concentration of polyacrylamide-derivatized rαhrbc antibody in a 5,4,I phase system at room temperature.

Table 15. The partition of human and rabbit erythrocytes in the presence of polyacrylamide-derivatized rαhrbc.

Ligand	Cell type	% LP in the system specified			
		5,3.4,I	5,3.4,III	5,3.4,I	5,4,III
-	hrbc	12	33	11	48
PAA-rαhrbc	hrbc	93	99	93	85
-	rrbc	16	22	31	37
PAA-rαhrbc	rrbc	19	39	29	46

PAA-rαhrbc = polyacrylamide-derivatized rαhrbc.

LP = lower phase.

All PAA-rαhrbc concentrations were 0.2 mg/mL.

The same approach was applied to the separation of the MBL-2 sub-lines. The partitioning of MBL-2 cells with PAA-YE1.48.10. was less straight forward than the erythrocyte partition. The MBL-2 cells tended to aggregate in a 5,4,I system even at extremely low ligand concentration. The resulting stabilization of the emulsion formed when the two phases were mixed meant that the separations were taking in excess of 45 minutes. The aggregation made it impossible to count the cells by impedance counting.

In order to avoid aggregation a system with the tonicity maintained using sorbitol (5,4,S) was used. Sorbitol is non-ionic and less likely to promote aggregation than high ionic strength phosphate buffer due to electrostatic repulsion between cells. The PAA-YE1.48.10. ligand had no effect on the partition of either MBL-2 cell in a 5,4,S system but changing to a 7,4,S system had a large effect. The two MBL-2 cell lines partitioned quite differently in this system, as shown in Fig. 42. The cells partitioned increasingly to the lower phase with increasing PAA-YE1.48.10. concentration. At a PAA-YE1.48.10. concentration of 0.2 mg/mL, 27% of the MBL-2(2.6) and 55% of the MBL-2(4.1) cells were in the lower phase.

At this point the partition of MBL-2 cells preincubated with PAA-YE1.48.10. then washed three times in PBS was measured. Under these conditions the number of cells in the lower phase was 7 and 14% for MBL-2(2.6) and MBL-2(4.1) cells respectively. The native lower phase

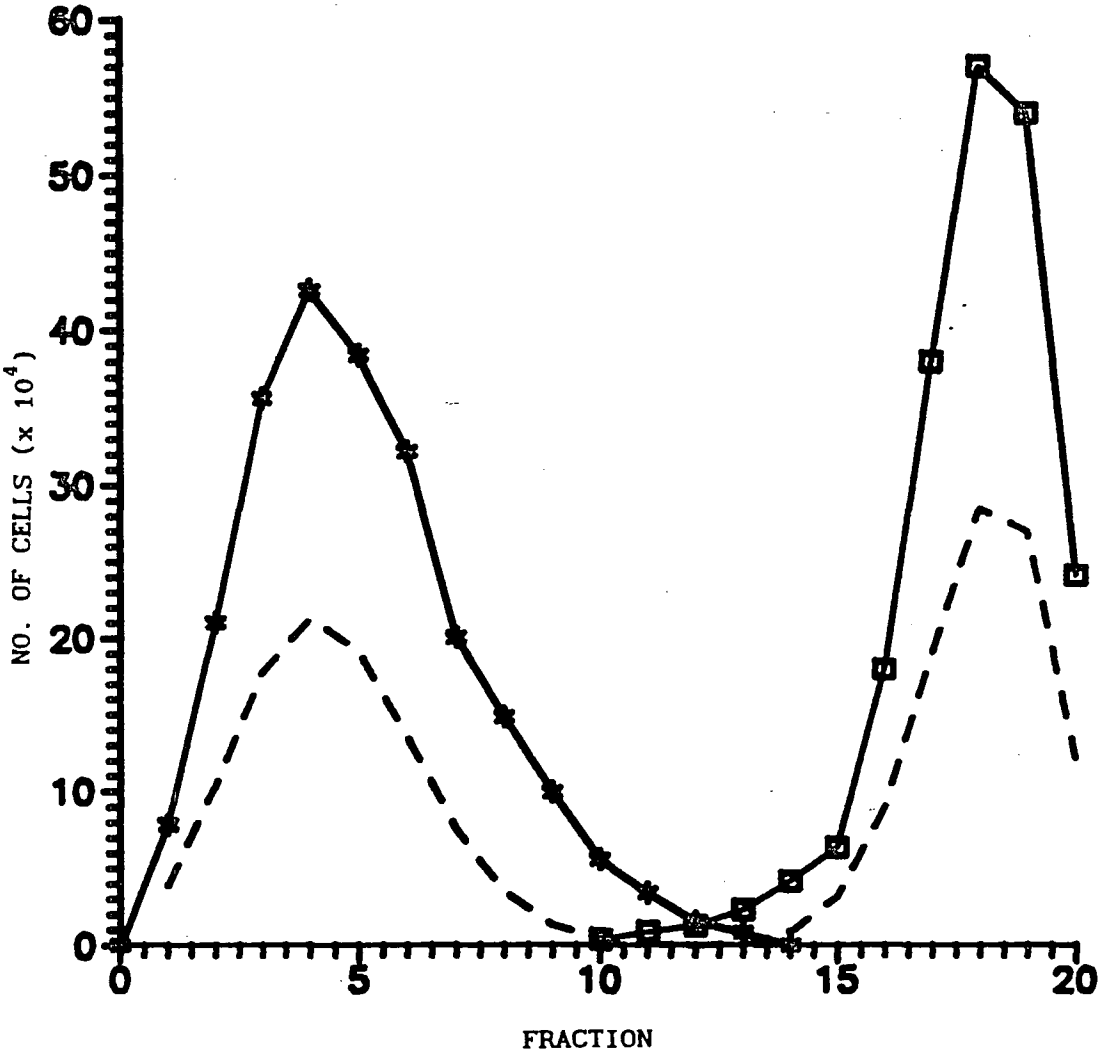


Fig. 41. CCD profile of rabbit (□) and human erythrocytes (*) in the presence of PAA-r α hrbc (0.08 mg/mL) in a 5,4,1 phase system at room temperature. G values are 0.9 and 0.22 for rabbit and human erythrocytes respectively. The dashed line (– –) represents the theoretical CCD plot for these G values.

cell partition was less than 2%. If the antibody had remained sufficiently bound during washing to cause the cells to partition strongly in to the lower phase, it would be possible to economize on costly MAb. However, this implies that the removal of the ligand from the cells after the separation would be relatively easy.

The separation was not as efficient as that of the human and rabbit erythrocytes, likely reflecting the higher antigen density on the human erythrocytes. It was necessary, therefore, to run a longer CCD to separate the two MBL-2 cell types. Theoretical CCD profiles for the appropriate G values are shown in Appendix II. A 60 transfer CCD was run and is shown, along with the corresponding theoretical plot, in Fig. 43. As expected the peaks are somewhat broader than the theoretical CCD due to cell heterogeneity but the two populations were resolved with 95% purity and by discarding overlapping cavities 14 to 24, the cells could be completely separated if this were more important than yield. The smaller area under the MBL-2(2.6) peak (Fig. 43) implies that fewer of these cells were present but in fact equal amounts of both cells were in the load mix. The MBL-2(4.1) cells were labelled with ^{14}C amino acids and the numbers of MBL-2(2.6) cells were obtained by impedance counting. Aggregation would decrease the numbers of cells counted by impedance counting but not the numbers of radiolabelled cells. The CCD was repeated with both cell lines labelled, MBL-2(4.1) with ^{14}C and MBL-2(2.6) with ^{125}I and is shown in Fig. 44. The profile is essentially the same as that obtained earlier (Fig. 43) but the areas under the peaks are similar, supporting the idea that the apparent loss of MBL-2(2.6) cells in the previous CCD was due to cell aggregation.

The effect of increasing dextran concentration is not easily explained in the context of current particle affinity partition theory. Increasing the dx concentration from 5 to 7% would increase the interfacial tension (γ_{TB}) from 3.8×10^3 to 13.5×10^3 erg/cm^2 (Albertsson, 1986). This would result in a greater free energy of interfacial adsorption, *i.e.* the opposite effect to that which is observed in this case. The other effect of increasing the dx concentration is to increase the values of K for both polymers. In this example the K_{PEG} is increased from 4.0 to 7.0 while K_{dx} only changes slightly. This favors an increase in the lower phase partition of both the cells and the ligand as is

observed. However as can be seen from eqn 16, the interfacial tension (γ_{TB}) in principle plays a stronger role in determining cell partition than the difference in cell surface free energy ($\Delta\gamma$).

In order to test the reproducibility of this separation, the entire procedure was repeated. The YE1.48.10. MAb was purified from a different batch of culture supernatant and the PAA-derivatization was carried out at the same 17:1 hydroxyl:acrylamide reaction ratio. The cells used for this separation had only been passed twice. The results of two CCDs performed on the radiolabelled MBL-2 cell lines are shown in Figs. 45 and 46. The G values for MBL-2(4.1) and MBL-2(2.6) cells were 0.19 and 0.41 respectively. The peaks are located in approximately the same fractions as in the previous separations shown in Figs 43 and 44 but they are much narrower, likely a reflection of less heterogeneity in the MBL-2 cells as a result of their being in only their second passage.

As these cell lines are changing constantly as illustrated by the inconsistent partition, it was important to ensure that they were, in fact, the same with respect to binding of the YE1.48.10. MAb. Large amounts of the original cell line were frozen in early stages of growth. These vials of original MBL-2 cells are immunologically similar to the MBL-2 cells used in the binding analysis. Binding assays which estimated the antigen density from cell pellet counts did not show evidence of any change in surface antigen density. In this repeat experiment (Figs. 45 and 46) efforts were made to reduce the variation in cell surface properties and to ensure that they were antigenically similar to the original cell line used in the binding analysis by using recently thawed cells.

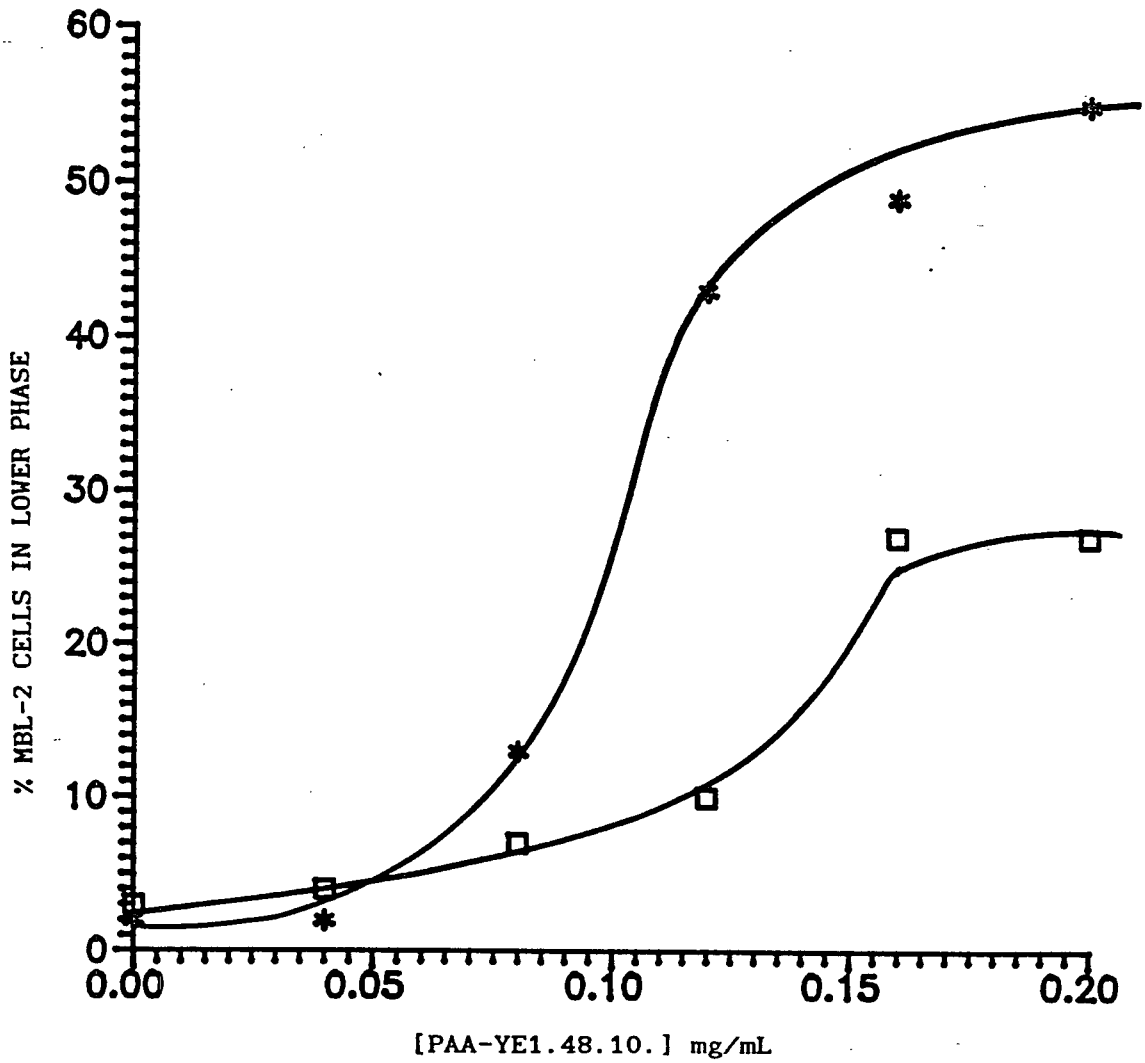


Fig. 42. The effect of increasing concentration of polyacrylamide-derivatized YE1.48.10. on the partition of MBL-2(4.1) (*) and MBL-2(2.6) (□) cells in a 7,4,S system at 4°.

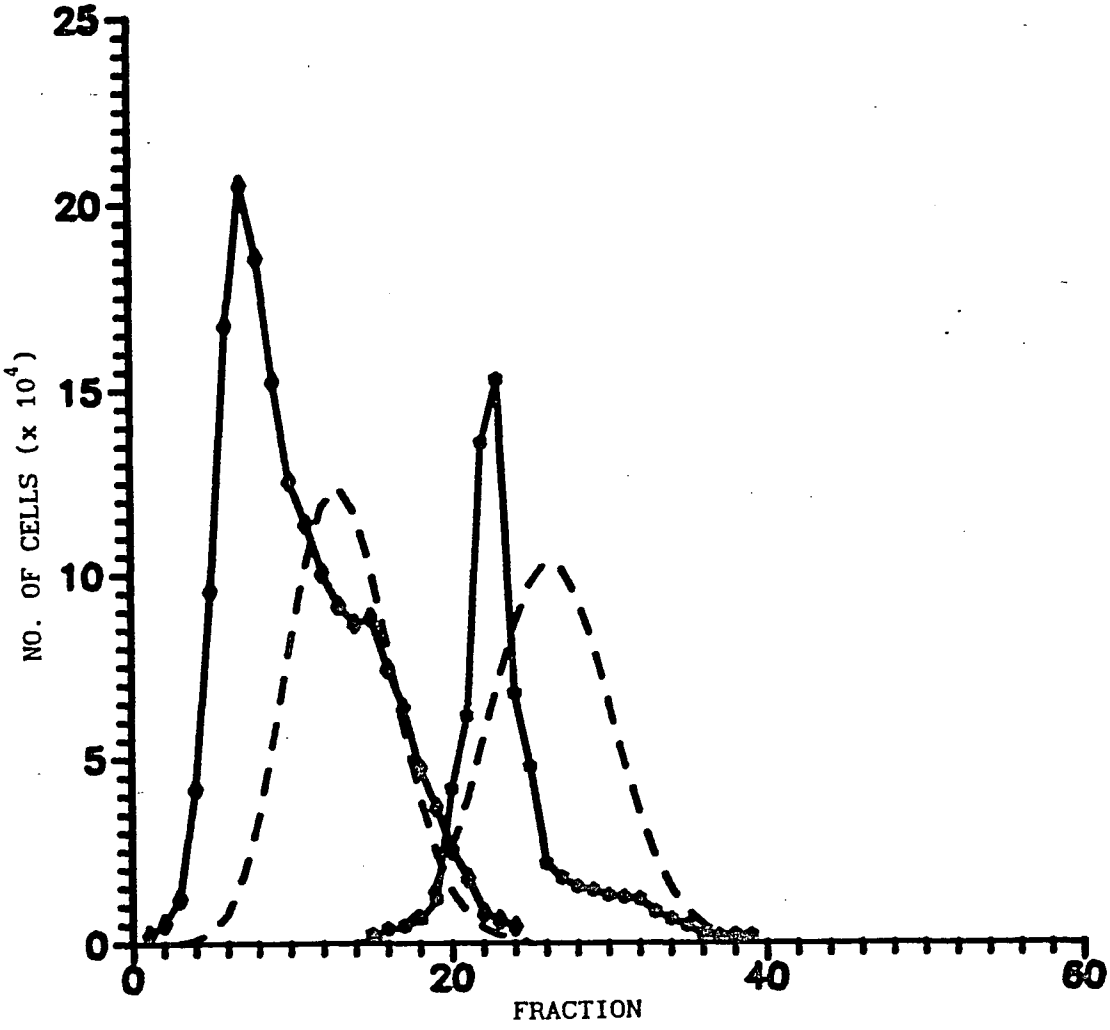


Fig. 43. CCD profile for MBL-2(2.6) (✽) and MBL-2(4.1) cells (◆) in a 7,4,S system at 4°. The G values are 0.22 and 0.44 for MBL-2(4.1) and MBL-2(2.6) cells respectively.The MBL-2(4.1) cells were labelled with ^{14}C and the MBL-2(2.6) cells were counted by impedance counting. The dashed line (– –) illustrates the theoretical CCD plot for these G values.

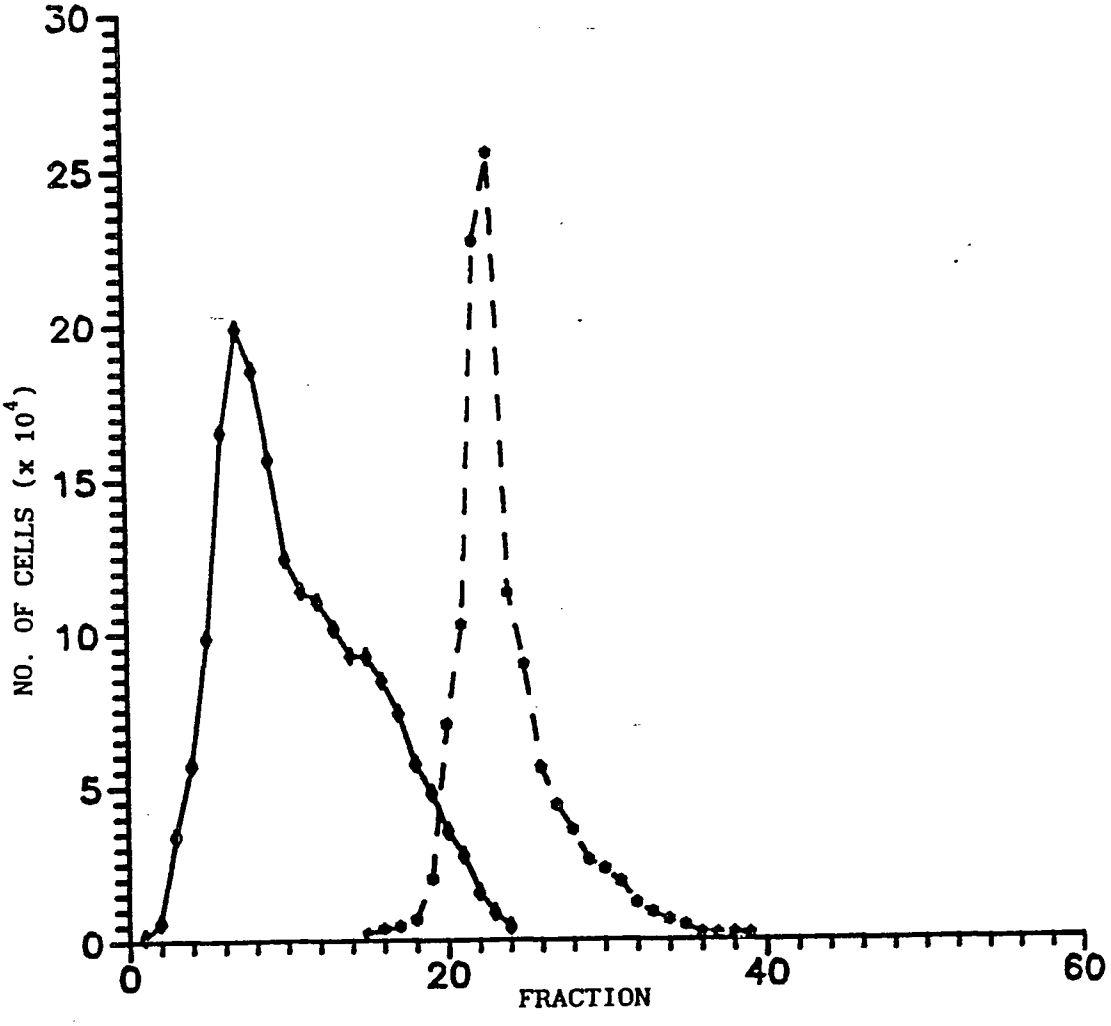


Fig. 44. CCD profile for MBL-2(2.6) (- - -) and MBL-2(4.1) cells (——) in a 7,4,S system at 4°. The G values are 0.22 and 0.44 for MBL-2(4.1) and MBL-2(2.6) cells respectively. (MBL-2(4.1) cells were labelled with ^{14}C and MBL-2(2.6) cells with ^{125}I.

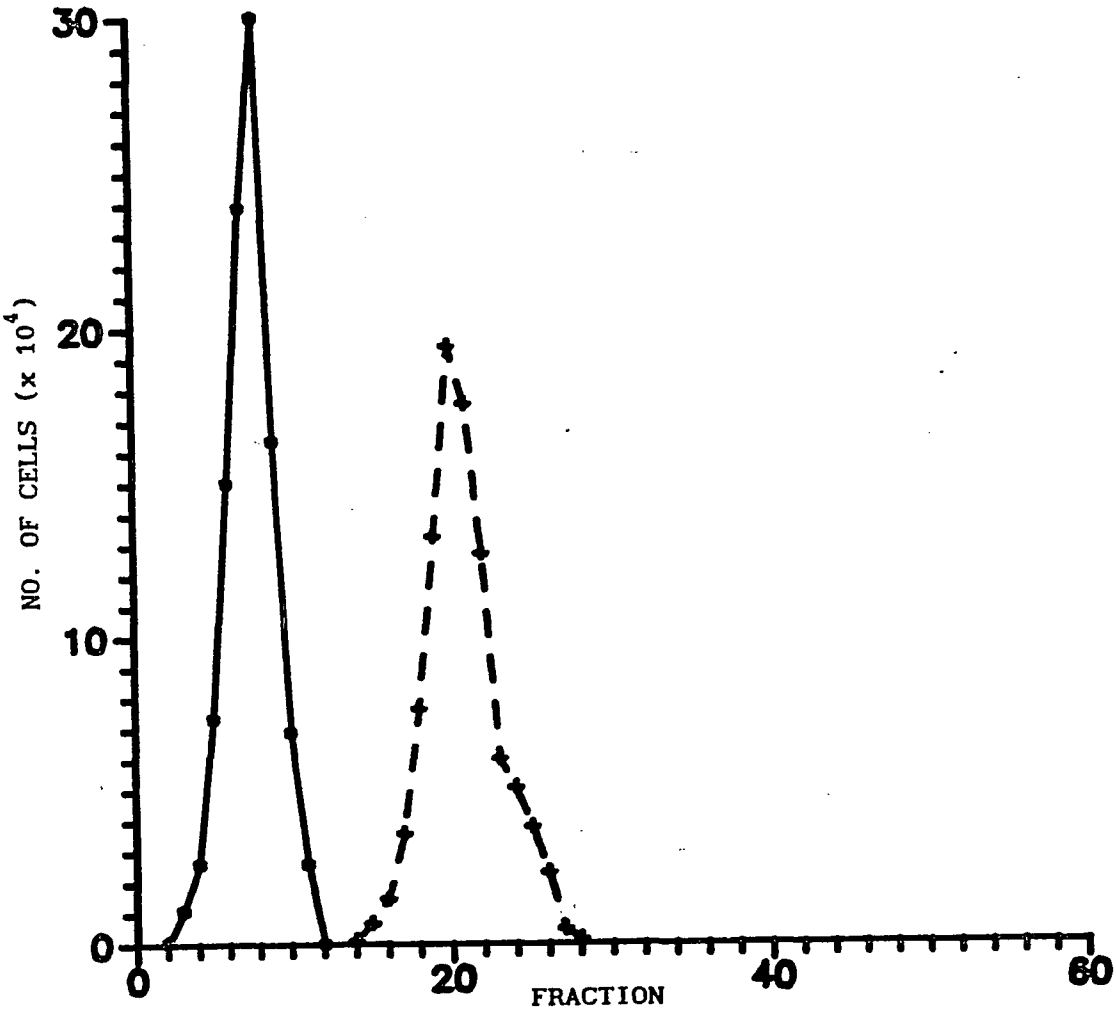


Fig. 45. CCD profile for MBL-2(2.6) (- - -) and MBL-2(4.1) cells (——) in a 7,4,S system at 4°. The G values are 0.18 and 0.41 for MBL-2(4.1) and MBL-2(2.6) cells respectively. (MBL-2(4.1) cells were labelled with ^{14}C and MBL-2(2.6) cells with ^{125}I. This CCD profile is different to those shown in Figs. 43 and 44 as it uses a different PAA-YE1.48.10. preparation and MBL-2 cells in an earlier stage of growth.

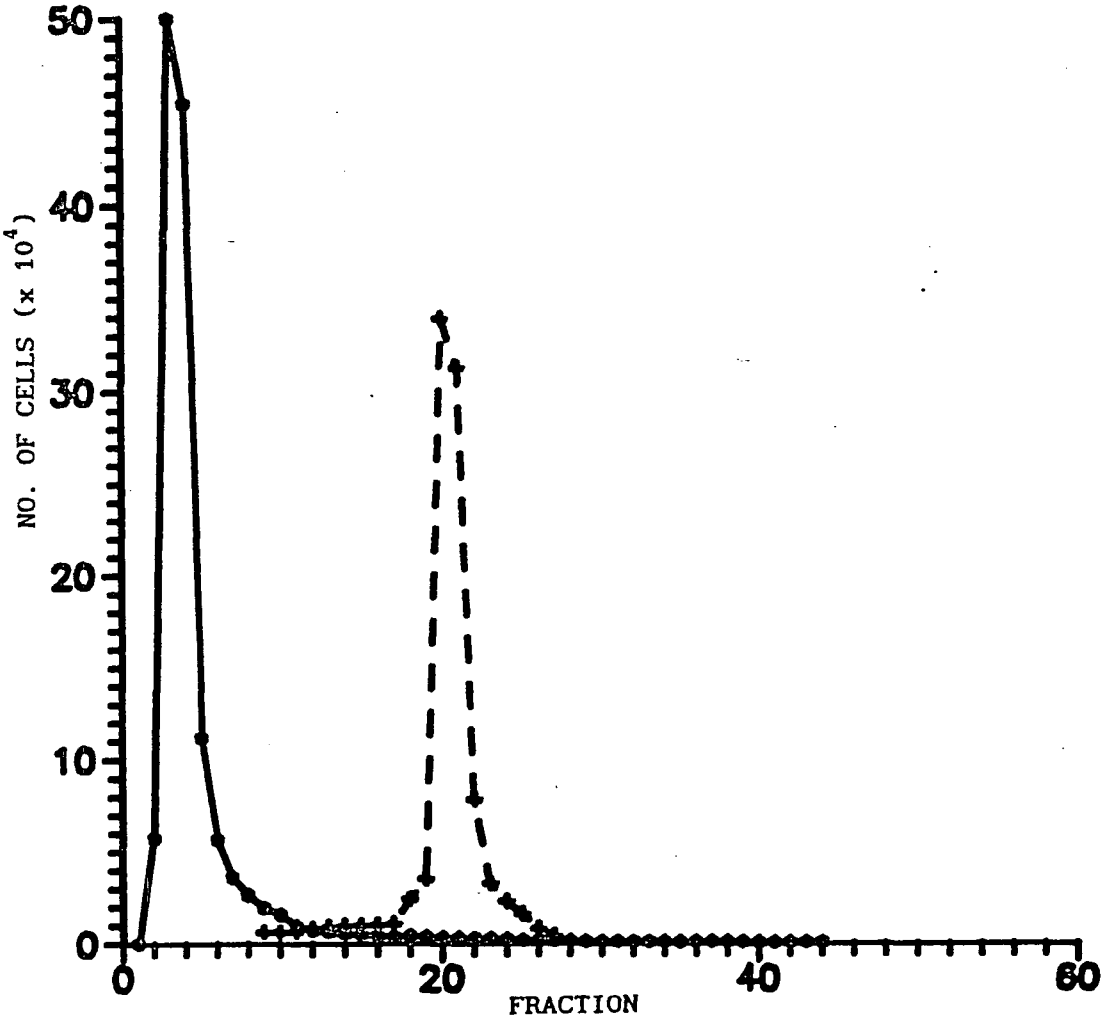


Fig. 46. CCD profile for MBL-2(2.6) (- - -) and MBL-2(4.1) cells (——) in a 7,4,S system at 4°. The G values are 0.18 and 0.41 for MBL-2(4.1) and MBL-2(2.6) cells respectively. (MBL-2(4.1) cells were labelled with ^{14}C and MBL-2(2.6) cells with ^{125}I. This is a repeat of Fig. 45.

CHAPTER 6

SUMMARY

Two different cell separation problems were accomplished in three ways by means of immunoaffinity partition. Rabbit and human erythrocytes were separated with a trypan blue-derivatized sheep anti-mouse F_c IgG and monoclonal mouse anti-NN glycophorin as well as with a polyacrylamide graft copolymer of rabbit anti-human erythrocyte IgG. The other separation was of two sub-lines of a transformed mouse lymphocyte line, MBL-2(2.6) and MBL-2(4.1).

Trypan blue was examined as an affinity ligand modifying agent. When compared with PEG 1900 for the modification of BSA, it was necessary to use a larger number of attached trypan blue molecules per IgG to achieve similar partition coefficients but the smaller molecular weight of trypan blue compared to PEG 1900 suggested that trypan blue modification may be less deactivating. However, a model separation of rabbit and human erythrocytes using trypan blue-derivatized sheep anti-mouse F_c was quite efficient, resulting in the increase from 18% to 70% of human erythrocytes in the upper phase. The upper phase partition of rabbit erythrocytes under the same conditions was also increased from 8% to 20%. Trypan blue modification seems to be less deactivating than PEG-modification as a similar experiment using a PEG 1900-modified antibody only increased erythrocyte partition from 10% to 30% in a system with a lower interfacial tension (Stocks and Brooks, 1988). However it is less specific because the rabbit erythrocyte partition was also increased from 8% to 20% while in the PEG-antibody experiment the rabbit erythrocyte partition was unaffected. The trypan blue-derivatized antibody was used in several other molecular affinity partition experiments proving it to be a general ligand, a major advantage of the second antibody immunoaffinity technique.

In an effort to achieve a clinically more useful separation by immunoaffinity partition and, more specifically, to model two clinical cell separation problems, namely bone marrow purging in combination with autologous bone marrow transplantation as leukemia therapy and the separation of fetal islet tissue for implantation as diabetes treatment, a lymphocyte separation was attempted. This was of two sub-lines of a

transformed mouse lymphocyte, MBL-2(4.1) and MBL-2(2.6), which differed in the surface density of an antigen recognized by the rat MAb, YE1.48.10. Binding studies showed that at saturation 2.4 x 10^6 molecules of YE1.48.10. were bound to a MBL-2(4.1) cell and 8 x 10^5 molecules of YE1.48.10. were bound to a MBL-2(2.6) cell. This small difference in surface antigen density represents an extremely stringent separation problem. Previous separations by immunoaffinity partition have been of species specific erythrocytes and the most stringent separation to date has been on the basis of a surface antigen difference of 0 and 5 x 10^{11} molecules of IgG per cm^2 (Stocks and Brooks, 1988). Experiments with erythrocytes and a PEG-palmitate ligand (Sharp, 1985) suggested that the MBL-2 separation would be close to the limits of immunoaffinity partition. The MBL-2 cells were indistinguishable by their native partition or with a PEG-linoleate ligand, a fatty acid ester commonly used to alter cell partition. PEG-derivatized YE1.48.10. did not have an effect on either MBL-2 cell despite ELISA assays suggesting that the antibody was still relatively active compared to previous PEG-antibody affinity ligands (Stocks and Brooks, 1988; Sharp *et al*, 1986). A second MAb, RG7/11.1, which bound to the F_c region of rat IgG was obtained. Unfortunately binding studies on RG7/11.1 showed that this MAb bound only approximately 10% of of the YE1.48.10. bound to the MBL-2 cell at saturation. As expected in light of the poor affinity of RG7/11.1 for YE1.48.10., a combination of PEG-derivatized RG7/11.1 and YE1.48.10. did not alter the partition of either MBL-2 cell.

The next system studied was a biotin-derivatized YE1.48.10. in combination with PEG-avidin. The biotin-derivatized YE1.48.10. remained quite active; at saturation 1.3 x 10^6 and 0.67 x 10^6 molecules per cell were bound to MBL-2(4.1) and MBL-2(2.6) cells respectively. Biotin derivatization alone actually caused the YE1.48.10. to partition into the upper phase and the addition of PEG-avidin moved almost all the ligand into the upper phase. Although the PEG-avidin, biotin-YE1.48.10. system worked well on the molecular level it did not achieve a separation of the MBL-2 cells. The biotin-derivatized YE1.48.10. alone did effect a small differential partition of the MBL-2 cells but a difference of this magnitude would require 200 CCD transfers to resolve the two MBL-2 sub-lines. The combination of PEG-avidin and biotin-

YE1.48.10 actually caused a slight decrease in MBL-2 cell partition.

Due to the inability of any of the upper-phase partitioning ligands to discriminate between the MBL-2 sub-lines it was decided to try a lower-phase partitioning ligand. APTS can easily be optimized such that all the cells are located at the interface thus a lower-phase partition is equally useful as an upper-phase partition. Polyacrylamide graft copolymers of rabbit anti-human erythrocyte IgG were used for the separation of rabbit and human erythrocytes. This effected a dramatic immunospecific separation at low antibody concentrations. The partition of human erythrocytes was altered from 12% to 93% lower phase partition while the partition of rabbit erythrocytes was unaffected. Larger differences were achieved in other systems but the results quoted also gave a low rabbit erythrocyte partition. This is the most efficient immunoaffinity partition separation of cells to date. Karr *et al* (1986) increased the partition of human erythrocytes from 14% to 58% with no effect on sheep erythrocyte partition with a PEG-derivatized antibody and Sharp *et al* (1986) increased human erythrocyte partition from 15% to 28% with no effect on rabbit erythrocytes. These separations required CCDs of 30 and 60 transfers respectively to resolve the cells completely whereas the polyacrylamide ligand achieved this in 20 transfers.

The same method was used to synthesize a polyacrylamide graft copolymer of YE1.48.10. Significant problems due to aggregation of MBL-2 cells were encountered in systems buffered by phosphate. This was avoided with the use of sorbitol. No effect of the ligand was observed in a 5,4,S system but increasing the dextran concentration to 7% optimized the system for this ligand. The polyacrylamide-derivatized YE1.48.10. increased the partition of MBL-2(2.6) from 8% to 26% and MBL-2(4.1) from 8% to 55% in the lower phase. This was sufficient to allow resolution of the MBL-2 cells in a CCD with 60 transfers. This result was surprising in light of the current particle affinity partition theory which predicts that effect of the increased surface tension in this system would dominate over the effect of the increased partition coefficient of PEG resulting in less not more cells partitioning in to the lower phase.

One useful outcome of the study was the development of a new method of producing and purifying large quantities of MAb by means of culture

in supplemented serum-free medium, ultrafiltration and FPLC (Stocks and Brooks, 1989). This method produces antibodies with a high retention of activity compared to those purified by ammonium sulfate precipitation.

Overall this study has shown that cells may be separated on the basis of small antigenic differences such as 3.83×10^{11} and 1.29×10^{11} molecules/cm^2. A polyacrylamide-derivatized ligand has a much stronger effect on cell partition than a ligand derivatized by trypan blue, PEG or biotin. As TAAs recognized by MAbs are within this order of antigen density this study supports the potential of immunoaffinity partition for use in bone marrow purging and fetal islet cell isolation.

GLOSSARY OF TERMS

Acinar cell - a secretory cell in an acinous gland.

Agarose - a linear polymer of alternating D-galactose and 3,6-anhydro-L-galactose.

Avidin - a basic glycoprotein (M ≈ 66,000) that binds biotin.
Biotin - 2'-oxo-3,4-imidazoline-2-tetrahydrothiophene-*n*-valeric acid (vit H), is bound tightly by avidin.

Complement system - 23 blood protein components activated by antibodies to bind and thereby eliminate foreign substances.

Concanavalin A (Con A) - a lectin and mitogen used to activate lymphocytes.

Confluent - a term which describes a cultured cell population which occupies all the available growth surface.

Dextran - poly α(1,6)-D-glucose.

F_c fragment - a region of the IgG molecule defined by papain digestion which is constant irrespective of antibody specificity (MW ≈ 50 000 - 75 000).

Ficoll - a synthetic copolymer of sucrose and epichlorohydrin.

Fluorescamine - 4-phenylspiro[furan-2H(3H),1'-phthalan]-3,3' dione.

HAT selection - a method of selecting hybridoma cell lines by growth in medium containing hypoxanthine, aminopterin and thymidine which only supports hybridoma growth.

Hypaque - sodium diatrizoate (3,5-Bis[acetylamino]-2,4,6-tri-iodobenzoic acid).

Hybridoma - a continuously growing cell line formed by the fusion of a malignant and normal cell.

Lectin - a group of antibody-like proteins found in plants which tend to bind to specific cell surface glycolipids or glycoproteins.

Leukemia - progressive proliferation of abnormal leukocytes.

Lymphoma - abnormally proliferative, generally neoplastic disease of the lymphoid system.

Mitosis - nuclear division in the somatic cells of eukaryotes.

Myeloma - a tumor consisting of a malignant form of plasma cell.

Neuraminic acid - 5-amino-3,5-dideoxy-D-glycero-D-galactonoulsonic acid.

Passage - the dilution of cell cultures which have become confluent in order to maintain a viable culture.

Polyethylene glycol (PEG) - a polymer of ethylene glycol.

Polyacrylamide (PAA) - a linear polymer of acrylamide.

Protein A - a protein made by most strains of *Staphylococcus aureus* which binds IgG.

Sialic acid - N and O acetyl derivatives of neuraminic acid.

Stem cells - usually immature, undifferentiated cells capable of rapid division and differentiation

T-, B-lymphocyte - thymus-dependent, thymus independent colorless nucleated blood cells.

Thalassemia - an inherited disorder of hemoglobin metabolism.

GLOSSARY OF SYMBOLS AND ABBREVIATIONS

A particle surface area

A_{500} absorbance at 500 nm

a_i the activity of i

ANLL acute non-lymphoblastic leukemia

APTS aqueous polymer two-phase system

BNHS N-hydroxysuccinimidobiotin

BSA bovine serum albumin

CCD counter current distribution

DMEM Dubellco modified Eagle medium

dx dextran

18ΔO-PEG PEG-linoleate

ELISA enzyme-linked immunosorbent assay

f molal activity coefficient

FACS fluorescent activated cell sorting

FBS fetal bovine serum

FITC fluorescein isothiocyanate

FPLC fast protein liquid chromatography

G distribution ratio in a CCD fraction = KV_t/V_b where K = partition coefficient and V_t/V_b is the volume ratio of upper to lower phase.

H hematocrit (%v/v packed cells)

HABA 4-hydroxyazobenzene-2'-carboxylic acid

HLA human lymphocyte antigens

HRP horseradish peroxidase

IgG immunoglobulin G

k Boltzmann's constant, 1.36×10^{-16} erg/molec.K°

K partition coefficient or critical point on a phase diagram

K_L partition coefficient of free ligand

K_o partition coefficient of unbound macromolecules

k_a or k microscopic association constant

k_d microscopic dissociation constant

L ligand

m_i density of i

M_i the concentration of molecules which have i of their sites occupied

M_n number average molecular weight

M_w weight average molecular weight

MAb monoclonal antibody

MBL-2 a transformed lymphocyte cell line which has two sub-lines, MBL-2(4.1) and MBL-2(2.6), defined by monoclonal antibody YE1.48.10.

MW molecular weight

m α NN glyc. monoclonal mouse anti-NN glycophorin IgG

N_A Avogadros number

P number of polymer segments (the ratio of polymer molecular volume to solvent molecular volume or a binding site on a cell surface or fraction of total amount of material in the upper phase of a CCD cavity

PAA polyacrylamide

PBS phosphate buffered saline

PCC 2(alkoxypolyethyleneglycoxy)-4,6 dichlorotriazine ("activated PEG")

PEG-S polyethylene glycol sulfonate

PEG X polyethylene glycol, mol. wt. approx. X g/mole

R molar gas constant, 8.314×10^7 erg/mol.K°

r α hrbc rabbit anti-human erythrocyte IgG

RG7/11.1 a mouse monoclonal antibody specific for rat F_c fragment

RPMI-1640 a defined cell culture medium

S specific activity of a radiolabelled antibody or cell

s α mF_c sheep anti-mouse F_c fragment IgG

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SSFM supplemented serum-free medium

TAA tumor associated antigen

TB trypan blue

TLL tie line length on a phase diagram

TMA-PEG trimethylamino polyethylene glycol

YE1.48.10. a rat monoclonal antibody specific for an antigen expressed on MBL-2 cells

w_i weight of sample i

χ Flory interaction parameter

ΔE_{TI} free energy of particle interface attachment

ΔG_m free energy of mixing

$\Delta\gamma$ surface free energy difference between phases

γ_{TB} interfacial tension between the phases

γ surface free energy

γ_i radioactive counts of sample i

Γ_i surface excess of the ith component

η_i viscosity of i

μ^o, μ standard state chemical potential, chemical potential

ϕ volume fraction

ϕ_i fractional saturation of site i

ν molecules of ligand bound per unit surface area

Appendix I

The error in a calculated quantity is estimated as the root mean square of the errors introduced by the basic measured quantities. The contribution to the final quantity y of error in measuring a basic quantity x is given by the partial derivative $\delta y/\delta x$. Thus the total error is given by:

$$\Delta y = \left[\left(\frac{\delta y}{\delta x_1} \cdot \Delta x_1\right)^2 + \left(\frac{\delta y}{\delta x_2} \cdot \Delta x_2\right)^2 + \ldots\ldots\ldots + \left(\frac{\delta y}{\delta x_n} \cdot \Delta x_n\right)^2 \right]^{\frac{1}{2}}$$

$$\text{or } \Delta y = \left[\sum_{i=1}^{n} \left(\frac{\delta y}{\delta x_i} \cdot \Delta x_i\right)^2 \right]^{\frac{1}{2}}$$

where $\delta y/\delta x$ are the partial derivative, Δx_i the measurement error and Δy the error in the calculated value (Mendenhall *et al*, 1981)

Partial Derivatives for ν:

$$\nu = \frac{[(\gamma_1(w_3-w_1)/(w_2-w_3)] - [(\gamma_2[(w_4-w_1) - (w_4-w_3)H]/(w_4-w_5)]}{(w_4-w_3).H.S}$$

Let $[(\gamma_1(w_3-w_1)/(w_2-w_3)] - [(\gamma_2[(w_4-w_1) - (w_4-w_3)H]/(w_4-w_5)] = [N]$

$$\frac{\delta\nu}{\delta H} = \frac{-\nu S + \gamma_2/(w_4-w_5)}{S.H}$$

$$\frac{\delta\nu}{\delta S} = -\nu/S$$

$$\frac{\delta\nu}{\delta\gamma_1} = \frac{1}{(w_4-w_3).H.S}\left[\frac{(w_3-w_1)}{(w_2-w_3)}\right]$$

$$\frac{\delta\nu}{\delta\gamma_2} = \frac{-1}{(w_4 - w_3).H.S}\left[\frac{(w_4 - w_1) - (w_4 - w_3)H}{(w_4 - w_5)}\right]$$

$$\frac{\delta\nu}{\delta w_1} = \frac{1}{(w_4 - w_3).H.S}\left[\frac{-\gamma_1}{(w_2 - w_3)} + \frac{\gamma_2}{(w_4 - w_5)}\right]$$

$$\frac{\delta\nu}{\delta w_2} = \frac{1}{(w_4 - w_3).H.S}\left[\frac{-\gamma_1(w_3 - w_1)}{(w_2 - w_3)^2}\right]$$

$$\frac{\delta\nu}{\delta w_3} = \frac{[N]}{(w_4 - w_3)^2.H.S} + \frac{1}{(w_4 - w_3).H.S}\left[\frac{\gamma_1}{(w_2 - w_3)}\left(1 + \frac{(w_3 - w_1)}{(w_2 - w_3)}\right) - \frac{\gamma_2 H}{(w_4 - w_5)}\right]$$

$$\frac{\delta\nu}{\delta w_4} = \frac{-[N]}{(w_4 - w_3)^2.H.S} + \frac{\gamma_2}{(w_4 - w_3)(w_4 - w_5)H.S}\left[1 - H - \frac{(w_4 - w_1) - (w_4 - w_3)H}{(w_4 - w_5)}\right]$$

$$\frac{\delta\nu}{\delta w_5} = \frac{-\gamma_2[(w_4 - w_1) - (w_4 - w_3)H]}{(w_4 - w_3).H.S\ (w_4 - w_5)^2}$$

Partial Derivatives for ν/L:

$$\frac{\nu}{L} = \frac{\{(\gamma_1(w_3 - w_1)/(w_2 - w_3)] - [(\gamma_2[(w_4 - w_1) - (w_4 - w_3)H]/(w_4 - w_5)\}(w_4 - w_5)}{(w_4 - w_3).H.\gamma_2}$$

$$\frac{\nu}{L} = \frac{1}{H}\left[\frac{\gamma_1}{\gamma_2}\ \frac{(w_3 - w_1)(w_4 - w_5)}{(w_2 - w_3)(w_4 - w_3)} - \frac{(w_4 - w_1)}{(w_4 - w_3)}\right] + 1$$

$$\frac{\delta(\nu/L)}{\delta H} = \frac{1}{H}\left(1 - \frac{\nu}{L}\right)$$

$$\frac{\delta(\nu/L)}{\delta \gamma_1} = \frac{1}{H\gamma_2}\,\frac{(w_3 - w_1)(w_4 - w_5)}{(w_2 - w_3)(w_4 - w_3)}$$

$$\frac{\delta(\nu/L)}{\delta \gamma_2} = \frac{-\gamma_1}{H\gamma_2^2}\,\frac{(w_3 - w_1)(w_4 - w_5)}{(w_2 - w_3)(w_4 - w_3)}$$

$$\frac{\delta(\nu/L)}{\delta w_1} = \frac{1}{H(w_4 - w_3)}\left[1 - \frac{\gamma_1(w_4 - w_5)}{\gamma_2(w_2 - w_3)}\right]$$

$$\frac{\delta(\nu/L)}{\delta w_2} = \frac{-1}{H(w_2 - w_3)}\left[\frac{\gamma_1(w_4 - w_5)(w_3 - w_1)}{\gamma_2(w_2 - w_3)(w_4 - w_3)}\right]$$

$$\frac{\delta(\nu/L)}{\delta w_3} = \frac{1}{H(w_4 - w_3)}\left[\frac{\gamma_1(w_4 - w_5)}{\gamma_2(w_2 - w_3)}\left(1 + \frac{(w_3 - w_1)}{(w_2 - w_3)} + \frac{(w_3 - w_1)}{(w_4 - w_3)}\right) - \frac{(w_4 - w_1)}{(w_4 - w_3)}\right]$$

$$\frac{\delta(\nu/L)}{\delta w_4} = \frac{1}{H(w_4 - w_3)}\left[\frac{\gamma_1(w_3 - w_1)}{\gamma_2(w_2 - w_3)}\left(1 - \frac{(w_4 - w_5)}{(w_4 - w_3)}\right) - \left(1 - \frac{(w_4 - w_1)}{(w_4 - w_3)}\right)\right]$$

$$\frac{\delta(\nu/L)}{\delta w_5} = \frac{-1}{H(w_4 - w_3)}\left[\frac{\gamma_1(w_3 - w_1)}{\gamma_2(w_2 - w_3)}\right]$$

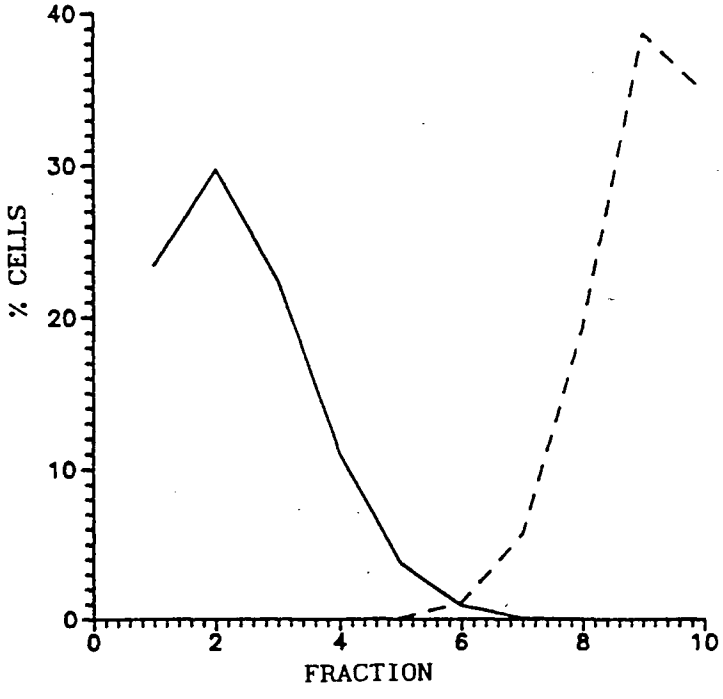
$$\frac{\delta(\nu/L)}{\delta S} = 0$$

Appendix II

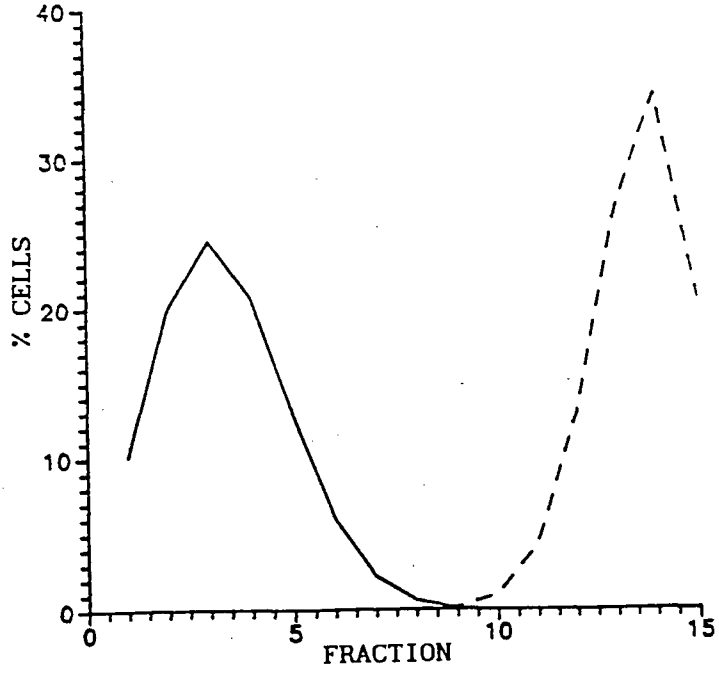
Theoretical counter current distribution profiles for the cell separations by CCD in this study.

1. Human (- - -, G = 0.22) and rabbit (——, G = 0.9) erythrocytes in the presence of polyacrylamide-derivatized rabbit anti-human erythrocyte.

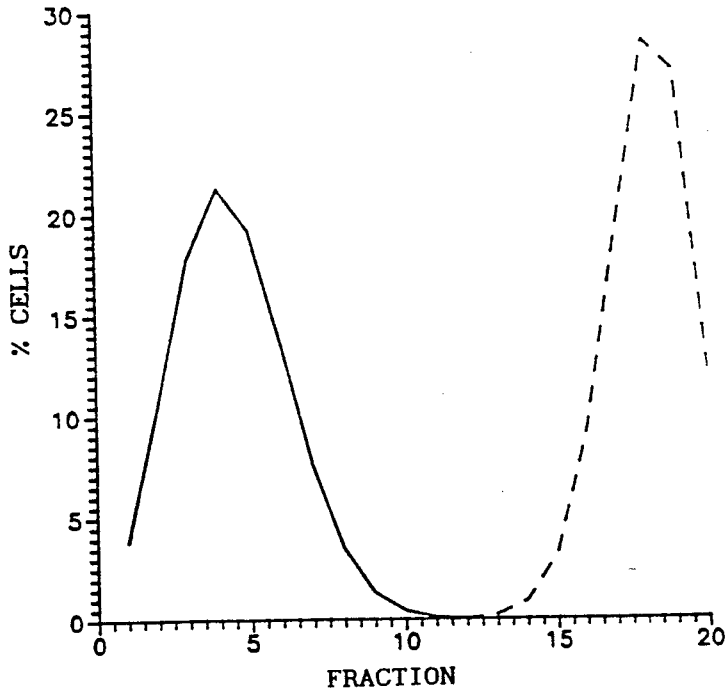
a. n = 10



b. n = 15

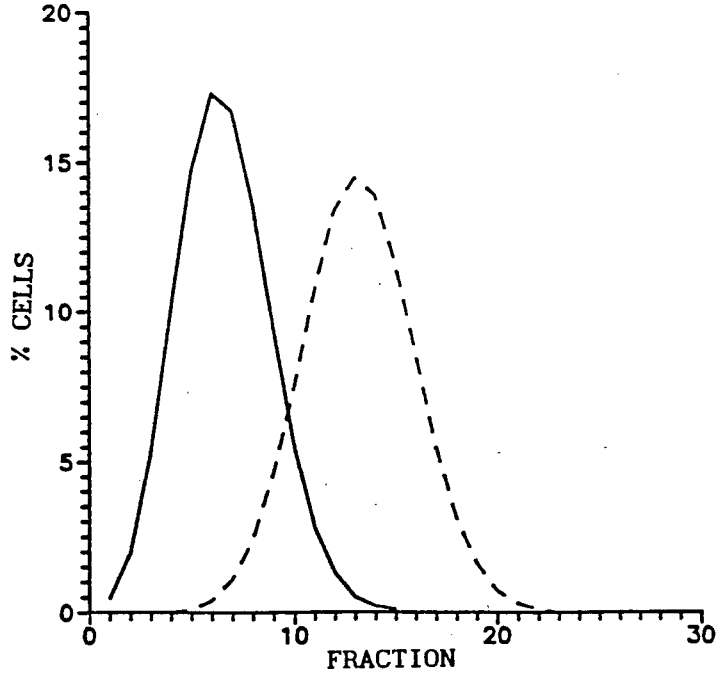


c. n = 20

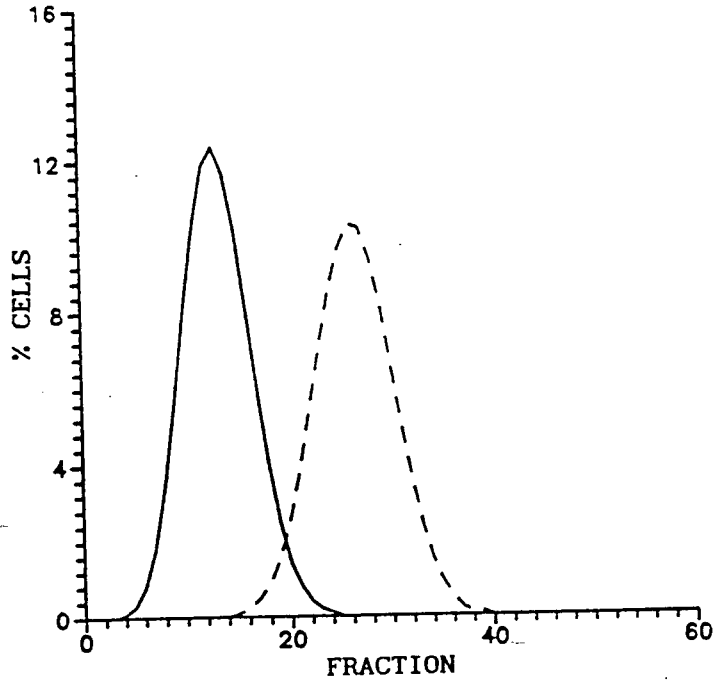


2. MBL-2(4.1) (——, G = 0.22) and MBL-2(2.6) (- - -, G = 0.44) cells in the presence of polyacrylamide -derivatized YE1.48.10. antibody.

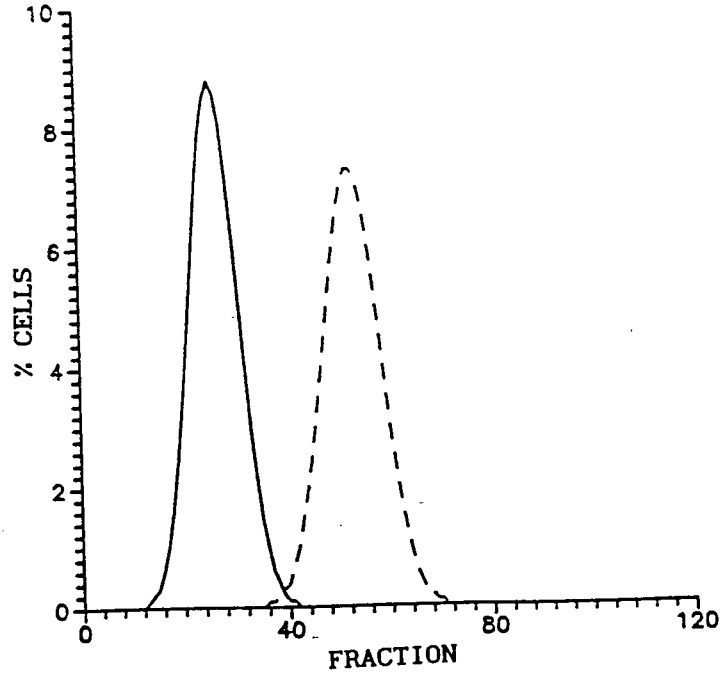
a. n = 30



b. n = 60


16
12
8
4
0
% CELLS
0
20
40
60
FRACTION

c. n = 120


10
8
6
4
2
0
% CELLS
0
40
80
120
FRACTION

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