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DEVELOPMENT OF A GENERAL LIGAND FOR IMMUNOAFFINITY
PARTITIONING IN TWO PHASE AQUEOUS POLYMER SYSTEMS

by

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Abstract

The partition of erythrocytes in a two phase aqueous polymer system composed of dextran T500, poly(ethylene glycol)8000 (PEG 8000) and buffer was studied and the effect of a combination of affinity ligands, namely rabbit IgG and PEG 1900 modified monoclonal IgG, was examined as a potential cell separation technique. Several hybridoma lines secreting mouse monoclonal IgG specific for the F_c receptor of rabbit IgG were produced by the fusion of immunised mice spleen cells and mouse myeloma cells. The monoclonal IgG was modified by cyanuric chloride attachment of PEG 1900. The modified monoclonal antibody partitioned predominantly into the PEG rich upper phase of a two phase aqueous polymer system containing PEG 8000 and dextran T500. The PEG-modified monoclonal IgG was used as an affinity ligand in the two phase polymer system to specifically increase the partition of rabbit anti-NN glycophorin A IgG. The rabbit IgG was used together with the PEG-modified monoclonal IgG to increase the partition of human erythrocytes. The same system had no effect on rabbit erythrocytes. In summary, it was demonstrated that a monoclonal antibody can be modified and used to alter cell partition in two phase aqueous polymer systems in an immunologically specific manner.

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A. INTRODUCTION

There are many existing procedures to identify and isolate pure molecular species from complex solutions but methods to separate sub-populations of cells from mixed suspensions are less well developed. Procedures using cell size, density or shape are less useful than those based on cell surface properties, as the latter are most likely to be associated with the function of the cell. For example, lymphocytes secreting different antibodies probably have similar size and geometry but different surface antigens. There are currently five main classes of cell separation methods based on membrane characteristics:

1. Cell electrophoresis or isoelectric focusing (1,2).
2. Individual cell sorting using fluorescent cell surface labels (3) or a light scattering signature (4).
3. Immunoabsorption on affinity columns (5,6).
4. Antibody-mediated complement lysis of contaminating cell sub-populations (7).
5. Partition and countercurrent distribution in two phase aqueous polymer systems (8,9).

Of these five classes, partition of cells in two phase aqueous polymer systems was examined in this study.

The fact that most mixtures of polymers show incompatibility under certain well defined conditions, in both organic and aqueous solvents, is a well established general phenomenon (10). Two types of phase separation can

occur as a result of this incompatibility: one polymer collects predominantly in one phase and the other polymer mostly in the other phase; or both polymers collect in the same phase, the other phase being mostly solvent (11). The term "coacervation" is used to describe the general phenomenon of phase separation of polymer mixtures and the case where oppositely charged polymers collect in the same phase is termed "complex coacervation". It is also possible to form a two phase system by combining a single neutral polymer or polyelectrolyte with a salt in a common solvent. The most commonly used phase systems for partitioning biological materials are those based on two polymers such as dextran/poly(ethylene glycol)(PEG) or dextran/ficoll.

Phase separation results from an unfavourable interaction energy between the two polymers. Solutes will partition selectively between the two phases or, in the case of particles, between the interface and one of the two phases, depending on the chemical potential of the solute or surface free energy of the particle in each phase or at the interface. Besides the incompatibility of the solute or particle with the phase polymers, other factors such as salt concentration or type, pH and the presence of affinity ligands (molecules which associate specifically with the material being distributed) will affect the partition coefficient of the particle or solute.

1. THE THEORY OF PHASE SEPARATION

The statistical thermodynamics describing phase separation of polymers was derived by Flory(12) and Huggins (13). The initial approach was to derive an expression describing the free energy change on mixing a single polymer and a solvent. Expressions for the enthalpic and entropic contributions to the free energy change were derived, then combined using the Gibbs-Helmholtz relationship, $\Delta G_m = \Delta H_m - T\Delta S_m$, to give the free energy of mixing.

In considering the enthalpy contribution, the polymer solution is represented by a number of lattice sites which can be occupied by a solvent molecule or a polymer segment. Each lattice site has coordination number, z . The enthalpy change on mixing the solvent and polymer is dependent on the energies of contact between the polymer segments and solvent molecules. The energy change associated with the formation of a one polymer segment-solvent molecule contact is given by,

$$\Delta W_{12} = W_{12} - (W_{11} + W_{22})/2 \quad \text{eqn [1]}$$

where W_{12} = energy associated with contact between solvent and polymer

W_{11} = solvent-solvent contact energy

W_{22} = polymer-polymer contact energy

Summing these individual contact energies for a particular occupancy of the lattice sites or composition gives the enthalpy of mixing of a polymer and solvent as,

$$\Delta H_m = z\Delta W_{12}n_1\phi_2 \quad \text{eqn [2]}$$

where z = lattice coordination number

n_1 = no. of solvent molecules on the lattice

ϕ_2 = fraction of sites occupied by the polymer segments

$$\phi_2 = n_2 P_2 / (n_1 + n_2 P_2)$$

where n_2 = no. of polymer molecules on the lattice

P_2 = no. of segments per polymer molecule

The term $z\Delta W_{1,2}$ is usually replaced by $kT\chi$ therefore

$$\Delta H_m = kT\chi_{1,2}n_1\phi_2 \quad \text{eqn [3]}$$

where k = Boltzmanns constant

T = absolute temperature.

The term $\chi_{1,2}$ is the Flory interaction parameter and represents the maximum interaction energy a solvent molecule can possess in a mixture, i.e. when completely surrounded by polymer segments.

The entropy of mixing is expressed in terms of the number of possible combinations of the solvent molecules and polymer segments on the lattice and is calculated from the Boltzmann relationship,

$$\Delta S_m = k \ln W \quad \text{eqn [4]}$$

Appropriate evaluation of W leads to the Flory-Huggins equation for the entropy of mixing a polymer and solvent on the lattice,

$$\Delta S_m = -k(n_1 \ln \phi_1 + n_2 \ln \phi_2) \quad \text{eqn [5]}$$

where $\phi_1 = n_1 / (n_1 + n_2 P_2)$.

Combining the enthalpy and entropy considerations gives the expression for the free energy of mixing a polymer and solvent,

$$\Delta G_m = kT(n_1 \ln \phi_1 + n_2 \ln \phi_2 + \chi_{12} n_1 \phi_2) \quad \text{eqn [6]}$$

The ϕ_1 and ϕ_2 terms for the fraction of lattice sites occupied by solvent molecules and polymer segments are practically represented by the volume fractions of the solvent and polymer respectively. This assumes that solvent molecules and polymer segments are interchangeable with no effect on the lattice, i.e. no volume change on mixing, the most restrictive aspect of the theory.

From the expression for ΔG_m , the theory can be extended to mixtures of more than two components. For example the free energy of mixing two polymers in a common solvent is given by,

$$\Delta G_m = kT[n_1 \ln \phi_1 + n_2 \ln \phi_2 + n_3 \ln \phi_3 + (n_1 + n_2 P_2 + n_3 P_3) (\phi_1 \phi_2 \chi_{12} + \phi_1 \phi_3 \chi_{13} + \phi_2 \phi_3 \chi_{23})] \quad \text{eqn [7]}$$

where 1 denotes solvent, 2 and 3 are the two polymer species and

$$\phi_i = (n_i P_i) / (n_1 + n_2 P_2 + n_3 P_3)$$

where $i = 1, 2$ or 3 = number of molecules of i on the lattice

$$\chi_{ij} = z \Delta W_{ij} / kT$$

$$\Delta W_{ij} = W_{ij} - (W_{ii} + W_{jj}) / 2$$

If two phases are present, then the equilibrium chemical potential of any component must be the same in each phase. The chemical potential of any of the three species is given by,

$$(\mu_i - \mu_i^\circ) = N_A (\partial \Delta G_m / \partial n_i)_{n_j} \quad \text{eqn [8]}$$

where μ_i = chemical potential of i when the volume fraction is ϕ_i

μ_i° = standard state chemical potential.

N_A = Avogadro's number.

As there must be the same value for the chemical potential, μ_i , of the species in each phase, then two values for, ϕ_i , the equilibrium volume fraction must exist. Generally, the value of μ_1 as a function of ϕ_2 will decrease as ϕ_2 is increased. Thus if the chemical potential of the solvent, μ_1 , is plotted as a function of one of the polymer concentrations, ϕ_2 , then two values of ϕ_2 on the curve will correspond to the same value of μ_1 if two phases are present. In this case, the plot of μ_1 against ϕ_2 must have a maximum, a minimum and an inflection point. Obviously the closer the maximum and minimum, the closer the composition of the two phases. The critical conditions for miscibility or appearance of two phases are represented when the maximum, minimum and point of inflection all coincide. Mathematically, this is described by,

$$\partial\mu_1/\partial\phi_2 = \partial^2\mu_1/\partial^2\phi_2 = 0$$

Combining the above expression with eqn [7] allows the critical conditions for phase separation to be calculated, i.e. the values of ϕ_i and x_{ij} which just produce phase separation in a two polymer one solvent phase system. If it is assumed that $x_{12}=x_{13}$, i.e. both polymers are equally soluble in the solvent, and that $P_2=P_3$ i.e. both have a similar number of segments per polymer molecule, then a solution of the above two equations gives the following values for critical conditions,

$$\phi_{2c} = \phi_{3c} = (1 - \phi_{1c})/2$$

$$\chi_{23c} = 1/P_2 \phi_{2c}$$

where c denotes critical conditions.

As P_2 is very large, this implies that phase separation will result even if the interaction energy between segments is only slightly positive.

2. THE PHASE DIAGRAM

In an aqueous mixture of two polymers, a two phase system will only arise when the constituents exceed a certain range of concentrations as defined above. This range may be represented on a phase diagram (Fig. 1.). The curved line dividing the two areas of the phase diagram is termed the binodial and all compositions of polymer A and B above the binodial will produce two phase systems. Points along the binodial or nodes (eg. 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 system with the same phase composition but the phase volumes will differ. The %w/w ratio of bottom to top phase is equal to the ratio between tie line lengths (eg. AC:BC). The binodial curve calculated by Flory-Huggins theory as outlined earlier agrees qualitatively with the experimental binodial curve suggesting that the Flory-Huggins theory provides a reasonable description of phase separation.

Fig.1. General Phase Diagram for a Two Polymer/Solvent 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.

3. THE THEORY OF MOLECULAR PARTITION

Due to the large size of the molecules, phase separation occurs even with very similar polymers. This means that there is a range of immiscible solutions in solvents with similar polarities. These immiscible solutions would be expected to be selective in partitioning substances which dissolve in the same solvents. Since aqueous solutions, which can be buffered and made isotonic for cells, will form two phase systems, partitioning in two phase aqueous polymer systems has potential for separating particles and macromolecules of biological origin. In addition many polymers do not have a deleterious effect on biological materials; in fact protective effects have been observed.

Using Flory-Huggins theory, it is easy to derive an expression for the partition coefficient of a third polymer added at low concentration to a two polymer phase system from eqn [7] (14). Assuming $\chi_{12} = \chi_{13} = \chi_{14}$, then the chemical potential of the third polymer can be calculated from,

$$(\mu_4 - \mu_4^0)/kT = 1 + \ln \phi_4 + P_4 \{ -\phi_1 + \phi_2(\chi_{24} - 1/P_2) + \phi_3(\chi_{34} - 1/P_3) + \chi_{12}(\phi_1 - \phi_1\phi_2 - \phi_1\phi_3) - \chi_{23}\phi_2\phi_3 \} \quad \text{eqn [9]}$$

where μ_4^0 refers to the standard state chemical potential of pure polymer before mixing with 1, 2 or 3.

If the above equation for each phase is equated, since at equilibrium the solutes in each phase must have the same chemical potential, and second order terms are dropped then,

$$K_4 = \exp P_4 \{ (\phi_1^T - \phi_1^B) (1 - \chi_{14}) + (\phi_2^T - \phi_2^B) (1/P_2 - \chi_{24}) + (\phi_3^T - \phi_3^B) (1/P_3 - \chi_{34}) \} \quad \text{eqn [10]}$$

where $K_4 = \phi_4^T / \phi_4^B$, the partition coefficient of 4 and T/B denote top/bottom phase respectively.

This expression is supported experimentally by several observations. The partition coefficient will become more one sided, the larger the molecular weight of the partitioned component or the greater the difference in polymer concentration between the two phases. An important point is that the partition of a solute is determined by the interaction energies of the partitioned solute and the phase polymers. Moreover, as the partition depends exponentially on these properties then partitioning would be expected to be a very sensitive separation procedure.

4. THE THEORY OF ELECTROLYTE PARTITION

Polymer phase systems need small amounts of salts or sucrose to act as a suitable environment for cells. Systems containing neutral polymers are little affected by the addition of most salts, eg. a dextran-PEG system has a constant volume ratio up to 0.8M NaCl and 0.3M phosphate but not a constant composition (Fig. 2.). The partitioning of cells, however, can be greatly affected by small amounts of salts if the salts partition unequally since in such cases a potential difference occurs between the phases.

The potential difference between phases will depend upon the difference in partition of the anion or cation,

Fig.2. Phase Diagrams for Dextran T500/PEG 8000/buffer systems. $T=22^{\circ}\text{C}$, $\text{pH}=7.16$. a) Buffer composition 10mM phosphate, 130mM NaCl. b) Buffer composition 110mM phosphate. All polymer compositions in %w/w (26).

according to the equation (15):

$$\Delta\psi = [RT/(z_+ + z_-)F][(\Delta\mu^\circ_- - \Delta\mu^\circ_+)/RT + \ln r_-/r_+] \text{ eqn [11]}$$

where $\Delta\psi = \psi^T - \psi^B$, the potential difference between the phases

z_+/z_- = nett charge of cation/anion

F = Faraday constant

$\Delta\mu^\circ_-/\Delta\mu^\circ_+$ = the difference in standard state chemical potential between phases for anion/cation

r_+ or r_- = f_+^T/f_+^B or f_-^T/f_-^B

f_+/f_- = activity coefficients for anion/cation.

The potential difference between the phases will be determined primarily by the degree to which the cation and anion differ in their intrinsic interactions with the phases as described by differences in their standard state chemical potentials. However, f_+ or f_- cannot be independantly measured. The predicted decrease in potential difference between phases with increased charge per ion is not observed experimentally, possibly due to higher charged ions interacting more strongly with the phase polymers, increasing the magnitude of the $\Delta\mu^\circ$ term. A highly charged molecule such as a protein can have a significant effect on the electrostatic potential between phases. When the salt is sufficiently in excess, the potential is determined by the electrolyte interactions with the phases but when the protein is in excess, the potential is determined by the protein interaction with the phase polymers and the cation-polymer interactions, expressed as follows (14),

$$\Delta\psi = [RT/(1+z_p)F][(\Delta\mu_p^\circ - \Delta\mu_\pm^\circ)/RT + \ln r_p/\ln r_\pm + (C_p^B/z_p C_p^B)(1-K_p/K_p)] \quad \text{eqn [12]}$$

where p denotes polymer and T/B denote top/bottom phase

$$K_i = C_i^T/C_i^B$$

It is easy to alter z_p by altering pH or $\Delta\psi$ by altering salt type or ionic strength.

5. THE APPLICATIONS OF AFFINITY PARTITIONING

There has been some investigation into the possibility of increasing the partition of a particular component of a mixture with an affinity ligand. An affinity ligand will partition predominantly into one of the phases while bound to one of the components of a mixture, thus increasing the partition of that particular component.

A simple example of an affinity ligand is the ester produced by conjugating palmitic acid to one of the terminal hydroxyls of PEG 8000. Small amounts of this ester in the phase system increase the partition of erythrocytes into the PEG rich phase of a dextran/PEG system (16,17). Other examples of affinity ligands include PEG-triethylamine which acts as a biospecific ligand used to purify cholinergic receptors specific for particular neurotransmitters (18).

Staphylococcus aureus has been modified with PEG for use as an affinity ligand to separate anti-*Staphylococcus aureus* antibodies (19). In this case an optimum degree of modification existed between increasing the partition of the *Staphylococcus aureus* and loss of antibody binding sites due

to steric interactions with the bound PEG.

The possibility of using biospecific affinity ligands to separate cells on the basis of cell surface antigens has been investigated (20). A model system was used to selectively partition human erythrocytes from a mixture with rabbit erythrocytes using a PEG-modified antibody in a dextran/PEG system. The increased partition of the human erythrocytes could be inhibited by native, unmodified antibody, demonstrating that the PEG-modified antibody was, in fact, an affinity ligand. It was also observed that despite a significant decrease in the ability of the antibody to agglutinate erythrocytes, usually an indication of a decrease in binding activity, it was still an effective affinity ligand. It was postulated that the haemagglutinating activity of the antibody was more sensitive to PEG-modification than the binding ability, i.e. the haemagglutinating activity may not give a good indication of the binding activity of the PEG-modified protein.

Synthesis of protein-PEG conjugates is an active area of research and although affinity partitioning is not the most common use of the conjugates, work done on other uses of the conjugates has resulted in a substantial increase in the methodology of PEG modification of proteins. For example, PEG-modified antigens administered to animals have resulted in immunosuppression (21). Attaching PEG to enzymes produces a decrease in enzyme antigenicity and

immunogenicity resulting in an increased serum lifetime which allows the conjugates to be used as drugs (22,23). Advantage of the increased serum lifetime effect is also taken in modifying haemoglobin for use as a blood substitute.

6. THE THEORY OF AFFINITY PARTITION

The previous discussions have centred around molecular partition as determined by the compatibility of the partitioned material with the phase polymers and the electrostatic potential difference between phases. Affinity partitioning is another method of manipulating the chemical potentials of the solute in each phase. If a ligand with a certain partition coefficient associates strongly with another molecule with a different partition coefficient, then the binding will change the partition coefficient of the complex.

The binding of a ligand to a macromolecule with n equivalent independent binding sites, i.e. the n microscopic equilibrium constants are all identical, can be considered in terms of the sequential addition of the ligand to the binding sites. If M_i equals the concentration of macromolecules with i sites occupied, then the total concentration of the macromolecule, M_{tot} is the sum of all the species of M_i ,

$$M_{\text{tot}} = \sum_{i=0}^n M_i$$

The partition coefficient of the macromolecule in the presence of ligand, K_m is

$$K_m = M_{\text{tot}}^T / M_{\text{tot}}^B$$

If the binomial theory is applied to the equation for M_{tot} then,

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

Therefore, the partition coefficient at any stage of binding is given by,

$$K_m = K_0 \frac{(1 + K_a^T L^T)^n}{(1 + K_a^B L^B)^n}$$

eqn [13]

where $K_0 = M_0^T / M_0^B$, the partition coefficient of unoccupied macromolecule

K_a^T, K_a^B = microscopic association constant for binding of ligand to a site in top, bottom phase

This model predicts that an affinity ligand will increase the partition of a bound molecule, providing it has a partition coefficient greater than 1. It cannot be assumed that the association constants in upper and lower phases are the same, as K_a , in general, will be increased in the phase in which the ligand is at lower concentration. When the ligand is bound to one of the phase polymers and thus

directed into the phase in which that polymer predominates, then it is implied that the standard state chemical potential differs in different phases, thus affecting K_a . This will decrease the effect of the affinity ligand by reducing the ratio, $K_a^T K_1 / K_a^B$.

The model assumes that the ligand interacts reversibly with the macromolecule, but affinity effects are also observed if one of the phase polymers is covalently linked to the affinity ligand. In this case K_a^T and K_a^B become very large and,

$$K_m = K_o K_1^n$$

In fact the dependence of K_m on the number of ligand binding sites measured experimentally is less than predicted theoretically. This is probably because the bound ligands do not behave ideally as assumed throughout the derivation. A more accurate model would take into account an activity coefficient for the complexed ligands which interact with each other as the amount of bound ligand increases.

7. THE THEORY OF CELL AND PARTICLE PARTITION

The theory developed for solute partition is not as useful to describe cell and particle partition. This is because a particle over a certain size does not diffuse freely between the phases, but tends to collect at the interface of the system where it has least free energy. In the case of a solute, the free energy of interfacial adsorption is small compared to that of a particle because a

particle will destroy a greater area of the interface on adsorption than a particle, thus having a greater free energy of adsorption than a solute. A theory can be written starting with the Boltzmann equation which relates the probabilities of a particle being in either of two compartments, designated 1 and 2, to the free energy required to move the particle between compartments, ΔE . However, the major drawback is the assumption of free diffusion.

$$K = n_1/n_2 = C_1/C_2 = \exp(-\Delta E/kT) \quad \text{eqn [14]}$$

Another problem with the use of the above equation is that the concept of volumetric concentration of particles is difficult to apply when the particles are partitioning between the interface and one of the phases. In fact numbers of particles should be considered (24).

If a particle is located at the liquid-liquid interface, then the interfacial area is reduced by the cross-sectional area of the particle. The decrease in area produces a proportionate decrease in free energy, thus stabilising 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 surface tension in a phase system of 5×10^{-3} erg/cm² would adsorb particles with a diameter over 320Å. The greater the surface tension, the smaller the particles adsorbed at the

interface.

The work done on moving a particle from the interface into the top phase is the sum of two components,

$$\Delta E_{TI} = A_{TB}\gamma_{TB} - A_B\Delta\gamma$$

where $A_{TB}\gamma_{TB}$ is a contribution from the nett energy change associated with the increase 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. Values for A_{TB} and A_B are obtained from contact angle measurements. The contact angle method results in the expression for particle partition as follows (25),

$$\ln K = -\Delta E_{TI}/kT \quad \text{eqn [15]}$$

or

$$\ln K = -\gamma_{TB}\Pi a^2(1-\Delta\gamma/\gamma_{TB})^2/kT \quad \text{eqn [16]}$$

where a = particle radius

$\Delta\gamma$ = difference in particle surface free energy in top and bottom phase.

γ_{TB} = interfacial tension between top and bottom phases

When typical values are substituted into this equation, it is found that ΔE_{TI} is very much larger than kT suggesting that few particles should partition into the upper phase. Experimentally this is not observed. However, qualitatively 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 dependance of K on ΔE_{TI} has been observed (26).

As indicated above the interfacial tension is extremely important in particle partition. To maximise the effect of the affinity ligand, a system with a low interfacial tension, in the order of 4×10^{-4} erg/cm², was used throughout this study. However, as the interfacial tension varies with tie line length ($\gamma_{TB} = TLL^a$, where $a = 3.4$ to 4.2 in different systems), this meant that the system was close to the critical point, making it a difficult system to manipulate. The system was affected greatly by small non-systematic errors such as the precise salt concentration, therefore all experiments were run simultaneously with controls. For example, NaCl has little effect on erythrocyte partition but phosphate has a large effect, especially in systems close to the critical point as used in this case. This is a consequence of both the increased tie line length (and therefore increased interfacial tension) and the electrostatic potential between phases that phosphate produces. Another problem resulting from the closeness of the system to the critical composition was that a small change resulted in the formation of a one phase system. If addition of the affinity ligand formed a one phase system, the addition of a small amount of PEG 8000 reformed the phases.

8. THE THEORY OF MONOCLONAL ANTIBODIES

In this study, it was aimed to produce an affinity ligand which combined the specificity of antibodies with the potential to be applied to the separation of many different cell types. The approach taken necessitated the use of a monoclonal antibody, the theory of which follows.

The majority of the exposed surface of a protein is antigenic (27,28) and a typical immune response will result in hundreds or thousands of clones of antibody-producing lymphocytes recognizing many different antigenic determinants. Of particular interest here are antibodies raised against human erythrocytes of M or N specificities.

The antigens of the MN blood group system are carried by the erythrocyte membrane sialoglycoprotein, glycophorin A. Erythrocytes from homozygotes carry molecules of MM or NN specificity while heterozygotes have both molecules in equal amounts (29). The M and N specificities seem to be due to different terminal amino acid sequences (30,31). Immunisation with the M or N antigen will result in many clones of antibody-producing lymphocytes, some recognizing antigenic determinants specific to M or N erythrocytes but others recognizing antigenic determinants common to M and N glycophorin A, i.e. a polyclonal response.

Despite the diversity of the overall immune response, each individual clone of lymphocytes is committed to secreting one antibody type, for instance IgG, specific for one antigenic determinant as described by the clonal

selection theory (Fig.3). Hybridoma technology uses cell culture techniques to propagate and select individual clones of lymphocytes to provide an "immortal" supply of antibody of the selected specificity.

The demonstration that it was possible to fuse two different plasma cell tumour lines with retention of both antibody products (32) was the start of hybridoma technology. However, prior to this, important relevant discoveries included, proof of the clonal selection theory (33), development of cell fusion techniques (34,35), the artificial induction of myelomas (36) and their adaptation to tissue culture (37). Myeloma or plasmacytoma cells arise from a tumor of malignant, antibody producing, plasma cells.

The Structure of Immunoglobulin G

Immunoglobulin G (IgG) is a symmetrical molecule made up of two identical glycosylated heavy chains (M_r 50,000 - 75,000) and two identical non-glycosylated light chains (M_r approx. 25,000) as shown in Fig. 4. The heavy chains are joined by two disulphide bonds to each other in the non-variable or F_c region of the molecule. Each light chain is joined by disulphide bonds to one of the heavy chains.

Enzymic digestion by papain splits the molecule into one F_c and two F_{ab} fragments. The F_{ab} fragment consists of one light chain and part of a heavy chain, it includes the F_v or variable region of the molecule which binds the antigen and is different in IgG molecules with different

Fig.3. The Clonal Selection Theory. Interaction of antigen($\blacktriangle$) with the receptor immunoglobulins on the surface of clone 2 leads to proliferated differentiation into cells which secrete antibody of the same specificity as the receptor.

Fig.4. Structure of the IgG Molecule. Reduction of the disulphide 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 as indicated during limited digestion.

specificities (38). The F_c fragment consists of only heavy chain and is constant irrespective of the specificity of the IgG molecule as it is not involved with antigen binding.

Although the F_c fragment lacks the ability to bind antigen, it possesses other biologically active sites, such as those concerned with complement fixation, membrane transmission, macrophage fixation and catabolic regulation.

The papain digestion of IgG produces fragments with retention of biological activity making it preferable over chemical degradation. However prolonged exposure leads to a range of sub-fragments.

Source of the Myeloma Cells

A cell line which secretes monoclonal antibodies is the result of the fusion of a myeloma or plasmacytoma cell and a spleen cell to produce a hybridoma. A schematic diagram of the procedure to produce hybridomas as described in the following pages is given in Fig. 5.

Mineral oil or 2,4,6,10-tetramethylpentadecane (pristane) are potent inducers of myeloma in BALB/c mice. Several of these myelomas have been adapted to continuous tissue culture lines (39) used to fuse with spleen cells and thus generate hybridomas. The most commonly used myeloma cell line is designated P3-NS1 Ag4-1 (NS1). This cell line synthesizes some IgG light chains but these are broken down internally and not secreted. The implication of this is that the gene for light chain synthesis is present in NS1 cells and may be expressed in the resulting hybridoma.

Another useful outcome of well characterised mouse myelomas has been an increased knowledge of immunoglobulin structure, biosynthesis and genetics (40).

Spleen cells used in the fusion are from immunised mice; these provide the genes coding for IgG synthesis of the selected specificity..

Fusion of Cells

Aqueous solutions of PEG will fuse almost all cell types at a sufficiently high concentration. 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 minimising cell damage. The concentration dependence seems to be related to the ability of the PEG to decrease the water activity of the solution since solutions inducing maximum fusion have virtually no water unassociated with the polymer (41). Many other polymers and viruses, in particular, Sendai virus which was used in early hybridoma work (42) will induce cell fusion.

When spleen and myeloma cells are treated with a fusogen, such as PEG 3500, their membranes fuse and multinucleate cells or heterokaryons are formed (42). At the next cell division the nuclei of the heterokaryons fuse and the cell then divides to produce daughter cells with an equal share of the genetic material and double the number of chromosomes of the parent cells.

Fig.5. Production of Hybridomas (see next page for legend).

Fig. 5. (previous page) Production of hybridomas. Spleen cells from immune mice are fused with myeloma (plasmacytoma) cells lacking in HGPRT using PEG 3500. The binucleate fusion products are called heterokaryons. At the next cell division, the nuclei fuse generating hybrid cells which grow in HAT medium as the genes for HGPRT synthesis have been supplied by the spleen cell half of the fusion. Unfused myeloma cells die in HAT medium, and unfused spleen cells will only survive a few days in culture. Hybrids are selected for the desired specificity and recloned by limiting dilution.

Fusion is a poorly controlled event and in addition to the required spleen-myeloma fusion, there will be spleen-spleen and myeloma-myeloma cell fusions or multiples of these. Although spleen cells will die within a few days in culture, the myeloma cells will continue to multiply and must be destroyed in order to culture a long term hybridoma line. Selection of hybridoma cells is usually by the HAT procedure.

HAT Selection

This is a method which selects hybridomas by culturing in a medium which only supports hybridoma growth and not myeloma growth. It depends on the fact that the main biosynthetic pathway to guanosine is blocked by aminopterin, a folic acid antagonist. There is an alternative "salvage

Fig.6. Metabolic pathways relevant to hybridoma selection in HAT medium. Cells lacking HGPRT are selected by growth in medium containing toxic guanine analogues, 6-thioguanine or 8-azoguanine

pathway" in which hypoxanthine or guanine are converted to guanine monophosphate via the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) as shown in Fig. 6.

Myeloma cells lacking in HGPRT are checked prior to fusion by growth in HAT medium, which contains hypoxanthine, aminopterin and thymidine. Cell lines which die in this medium are unable to convert hypoxanthine or guanine into guanosine since they lack HGPRT. After these myeloma cells lacking in HGPRT are fused with spleen cells which synthesize HGPRT, the hybridoma can synthesize HGPRT and thus survive in HAT medium but unfused myeloma cells will die.

Thymocyte Feeder Cells

Lymphoid cells often grow poorly or die at low cell population density. The reasons are not well understood but may be due to toxins from tissue culture vessels or requirements for ill defined growth factors. These problems can be partially overcome by culturing the lymphoid cells together with slow or non-growing cells termed feeder cells. Commonly used feeder cells include thymocytes (43), normal spleen cells (44) or peritoneal cells (45). The feeder cells do not have to be histocompatible with the hybridomas so thymocytes from cheaper Swiss mice were used in this study.

Recloning of Hybridomas

Often several cells grow in the same well of the cell culture plate. For an antibody to be monoclonal it must descend from a single spleen-myeloma cell fusion. Recloning ensures that the antibody is, in fact, monoclonal by diluting and replating the hybridomas such that each well contains a single cell. Even single clones should be recloned at least twice to ensure genetic stability. A hybridoma cell has a high probability of spontaneous chromosome loss associated with a loss of antibody producing ability. After recloning, the rate of chromosome loss is small but overgrowth by a non-producing mutant is always a potential hazard. The most common method of recloning is by limiting dilution (46).

If cells are grown in small numbers, the fraction of wells in the cell culture plate containing growth should follow the Poisson distribution,

$$F_0 = e^{-\lambda}$$

where F_0 is the fraction of cells with no growth and λ is the average number of clones per well. If $\lambda = 1$ then $F_0 = 0.37$, this means that to obtain 0.95 probability that wells with growth are monoclonal, at least 37% of the wells should have no clone growth.

The Types of Antibody Secreted by the Hybridoma

Although only 1% of spleen cells actively secrete antibody, about 10% of hybridomas secrete antibody. There seems to be a preference for myeloma cells to fuse with

activated B cells. An activated B cell is a lymphocyte which has been stimulated to produce antibody (47). Another possibility is that myeloma cells may activate non-secreting B cells to rapid secretion (48).

The original myeloma cell line, MOPC-21, used by Kohler and Milstein (49) secreted IgG, with κ light chains. This meant that the hybridoma secreted mixed molecules made up from various combinations of the two heavy and light chains (see Fig. 4 for details of chains). If association of the chains were random, only a small minority of IgG molecules would originate entirely from the spleen cells. A binding site made up of myeloma heavy or light chain (or both) would not be expected to bind antigen. This means that many secreted IgG molecules would be completely inactive, while some would possess only one active binding site and a minority would be completely active.

More recently the most commonly used fusion partner is NS1. NS1 cells synthesize light chains only so the antibodies secreted by a hybridoma arising from an NS1 cell fusion include more active molecules than a hybridoma from MOPC-21. If heavy and light chain pairing is random and rates of synthesis equal, then 25% of the IgG should have only spleen cell light chains (two binding sites), 50% should have one spleen cell and one NS1 cell light chain (one binding site) and 25% should have two NS1 cell light chains (0 binding sites), i.e. 75% of the secreted antibody would be active. Recently non-producing variants of myeloma

cells have been adapted to continuous cell culture (50). Hybridomas of these myeloma cells secrete 100% active antibody.

Aims of the Study

Although the PEG-modified IgG used by Sharp et al (20) showed a significant ability to increase the partition of erythrocytes, the system was limited in that the PEG-modified antibody could only be used to alter erythrocyte partition as the erythrocyte was the antigen used to raise the antibody. The modification of an antibody for use as an affinity ligand poses several problems with respect to the optimum degree of antibody modification. The optimum degree of modification is that which preserves the binding activity of the antibody, while still attaching sufficient PEG to make an effective affinity ligand with a high partition coefficient. Once one particular antibody is modified successfully there is no reason to expect a different antibody to respond to modification in the same way.

The diversity of the immune response gives rise to a heterogeneous mixture of antibodies directed against many different antigenic determinants of the antigen molecule. This causes batch variation in the purified antibody solution and each different antibody may respond differently to chemical modification leading to problems with both using and characterising the modified antibody. However a

monoclonal antibody simplifies these problems because it is a homogeneous sample of antibody molecules, all with the same specificity and the same response to chemical treatments. It is a pure chemical reagent which is rarely found in biological isolates.

In this study, a PEG-modified monoclonal antibody was used as a second affinity ligand in a system containing human erythrocytes as described in Fig. 7. The monoclonal antibody was directed against the F_c region of the rabbit IgG molecule, which is not involved in the binding of antigen and is constant irrespective of the antibody specificity. This meant that the first antibody specificity could be altered but the same PEG-modified monoclonal antibody could be used as the affinity ligand. By binding the second, monoclonal antibody to the F_c region of the rabbit IgG, it was hoped that the binding of the first antibody to the erythrocyte would be affected minimally. Another potential advantage of using the second antibody is that if the same antigenic determinant is repeated along the F_c fragment then several molecules of second antibody may bind each first antibody, thus increasing the amount of PEG bound and consequently the partition coefficient of the first antibody compared to direct modification of the first antibody.

Another interesting outcome of the study is the examination of the possibility of using monoclonal antibodies as affinity ligands. If the technique is

Fig.7a. A Schematic representation of PEG 1900 modified mouse monoclonal anti F_c fragment of rabbit IgG bound to an erythrocyte via anti-NN glycoporphin A rabbit IgG.

Fig.7b. The PEG 1900 coated erythrocyte will partition predominantly into the PEG 8000 rich upper phase of a 5,3,4 system.

successful, then there exists great potential to separate closely related cell types, such as subsets of lymphocytes, which are indistinguishable using conventional polyclonal antibodies. A summary of the potential advantages of using a second, monoclonal antibody are as follows:

1. The same monoclonal antibody can be used with different first antibodies.
2. A monoclonal antibody is a pure reagent.
3. The second antibody technique may attach more PEG to the erythrocyte than by directly modifying the antibody.
4. A monoclonal antibody can be selected to bind the F_c region of IgG which should have less effect on the binding site of the IgG than direct modification with PEG.

9. ANALYTICAL TECHNIQUES USED IN THE STUDY

Fast Protein Liquid Chromatography (FPLC)

Separation by ion exchange chromatography is obtained by reversible adsorption. Different substances have different affinities for the ion exchanger due to charge differences, which can be controlled by varying pH and ionic strength. Other types of binding such as Van der Waals or polar interactions may occur but have a small effect.

Initially the ion exchanger, which can be either an anion or a cation is at equilibrium with its counter ion. On application of the sample, counter ions are exchanged for the sample ions. The sample ions are then eluted by an increasing ionic strength gradient of counter ions.

The FPLC system is similar to an HPLC system except that it is adapted for separation of biological materials by using a medium pressure, in the order of 0-4 MPa, and a slower flow rate, in the order of 2ml/minute. Also no potentially denaturing metal ions are found within the system. The mono Q column used with the FPLC system in this study consists of a quaternary ammonium salt anion exchanger ($-\text{CH}_2\text{N}^+(\text{CH}_3)_3$) bound to a monodisperse beaded resin with a particle diameter of $10\mu\text{m}$.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A polyacrylamide gel is a latticed structure with pores of molecular dimensions. The pore size can be controlled by the monomer (acrylamide) concentration used when the gel is polymerized. If a protein is positioned in the gel and a voltage applied across the gel, it will migrate towards the anode with a mobility dependent on its net charge. If the pores are of a similar size to the protein, they can impose frictional resistance to the passage of the protein. If sodium dodecyl sulphate (SDS), an anionic detergent, is in the gel or the buffer, the binding of SDS by the protein renders all the molecules similarly charged. This results in separation by a sieving effect, dependent mostly on size.

In this study, slab gels containing SDS in the buffer only and rod gels containing SDS in both the running buffer and the gel were used. This meant that separation was mostly size dependent.

Diffusion of the protein will result in spreading of the protein bands and a decrease in resolution. This can be minimised by using a stacking gel. The stacking gel has a larger pore size and a lower pH than the running gel. The sample contains protein and an anion, in this case glycine, which migrates slower than the protein in the stacking gel and faster in the running gel. When a voltage is applied across the stacking gel, the ions become stacked in order of their relative mobilities, independent of their original concentrations, reducing the thickness of the sample starting zone.

The ion mobility or conductivity (κ) within a gel depends on the current (i) and the electric field (E), i.e. $E=i/\kappa$. The pH of the stacking gel is close to the pI of the glycine and consequently it has a low mobility. In the running gel, however, the glycine is above its pI and has a high mobility, resulting in a low electric field in regions where glycine dominates. Each protein band enters the gel in the order in which it was stacked. A low electric field is present both before and after the protein band due to the high mobility of the glycine. This means that the trailing edge of the protein will migrate quickly in the high electric field of the protein band directly in front of it, catching up to the leading edge of the protein band. The leading edge of the protein band will be slowed as it moves into the low electric field caused by the glycine. This has the effect of sharpening the protein band.

Fig.8. The Enzyme-linked Immunosorbent Assay (ELISA)

The Enzyme-linked Immunosorbent Assay (Fig.8.)

The ELISA is based on the fact that polyvinyl chloride (PVC) surfaces will tightly adsorb nanogram amounts of protein. The antigen-containing solution is pipetted into a PVC microELISA plate which contains 96 250ul wells and left overnight at 4°C. Unbound material is washed out and the antibody solution, specific for the bound antigen, is added. Again unbound antibody is washed out and any remaining non-specific protein binding sites are saturated with bovine serum albumen(BSA). Finally a revealing agent is added to give an indication of the amount of antibody bound. This is an enzyme-antibody conjugate specific for the primary antibody. When the enzyme substrate is added to the plate, formation of the products of the reaction catalysed by the enzyme indicate that the primary antibody has bound to the antigen.

B. MATERIALS AND METHODS

1. IMMUNISATION OF THE RABBITS

Two adult New Zealand White male rabbits were injected subcutaneously with NN glycophorin A (50 ug) in complete Freund's adjuvant (1:1 emulsion). The NN glycophorin A was isolated from human blood by John Cavanagh by phenol extraction of the lithiumiodo salicylic acid extract of human erythrocyte ghosts (51). The rabbits were reinjected one month later with NN glycophorin A (50ug) in phosphate buffered saline (PBS, 16.7mM Na_2HPO_4 , 3.3mM NaH_2PO_4 , 130.4mM NaCl, pH 7.2). After one week approximately 30ml of blood was drawn from an ear vein. A continuous source of antigen was maintained by booster injections once a month.

2. PURIFICATION OF THE RABBIT ANTI-NN GLYCOPHORIN A IMMUNOGLOBULIN G

The blood was allowed to clot at room temperature and the serum obtained by decantation and centrifugation. A solution of saturated $(\text{NH}_4)_2\text{SO}_4$ was added dropwise to the slowly stirred serum at room temperature. At about 40% saturation most of the immunoglobulins were precipitated and the suspension was stirred slowly for about 30 minutes. If too much $(\text{NH}_4)_2\text{SO}_4$ was added the sample would be contaminated with transferrin and albumin. The suspension was centrifuged at 10,000g for 10 minutes and the pellet washed three times with 40% saturated $(\text{NH}_4)_2\text{SO}_4$ solution.

The pellet was dialysed against water and the precipitated lipoprotein and IgM removed by centrifugation. The IgG was dialysed against 10mM phosphate buffer, pH 8 and separated from the remaining IgM and other contaminating proteins on a Pharmacia FPLC system using a mono Q anion exchange column with a 0-1M NaCl ionic strength gradient elution in 10mM phosphate, pH 8 (Fig.9.). SDS-PAGE was used to check the purity of the IgG (Fig.15.) and identify the fractions.

3. MICROTITRE OF THE ANTI-NN GLYCOPHORIN A IMMUNOGLOBULIN G

The ability of the anti-NN glycophorin A IgG to agglutinate human erythrocytes was assayed via a microtitre experiment. This also gave an indication of the relative affinities of the antibody for MN, NN and MM erythrocytes.

Two fold serial dilutions of 50 ul of IgG solution (1mg/ml) were made up in PBS in a microtitre plate. An equal volume of 1% haematocrit fresh erythrocyte suspension with known NN, MN or MM specificity was added to each well of the microtitre plate. The suspensions were mixed and examined after four hours. In the wells of the microtitre plate containing agglutinating concentrations of IgG the cells were clumped together in a button at the bottom of the well, whereas in those containing sub-agglutinating IgG concentrations the cells were uniformly distributed over the bottom of the wells. The results are shown in Table 1., p.73. The concentration dependence of the binding of erythrocytes by anti NN glycophorin A IgG was also examined

Fig.9. Fast Protein Liquid Chromatography profile of the serum proteins of an immunised rabbit partially purified by precipitation with $(\text{NH}_2)_4\text{SO}_4$ on a mono Q column with an ionic strength gradient of 0-1M NaCl in 10mM phosphate buffer, pH 8. The peaks were detected by absorption at 280nm and identified by SDS-PAGE.

using an ELISA (Fig.16).

4. FRAGMENTATION OF RABBIT IMMUNOGLOBULIN G BY PAPAIN

This is an adaptation of the methods of Porter (52) and Utsumi (53). Rabbit IgG(10mg) in phosphate buffer(50mM), pH 8 containing cysteine(10mM) and EDTA(2mM), was incubated with activated mercuripapain(15 mins, 37° C) in the same buffer at 37° C. The mercuripapain was obtained from BDH. After 16-20 hours the reaction was stopped by irreversible alkylation of the enzyme sulphydryl groups using iodoacetamide, final concentration 20mM, and the mixture was held on ice, protected from light, for one hour. The mixture was dialysed against sodium acetate (10mM), pH 5.5 at 4°C, then fractionated on the FPLC mono Q column with an ionic strength gradient from 0.01-1M sodium acetate, pH 5.5 (Fig. 10). The fractions were examined with SDS-PAGE (Fig.11).

The F_c fragment was also crystallised from the digest mixture by dialysis against sodium borate(10mM), pH 8.6 (54). This yielded a purer sample of F_c fragment but the yield was too low for preparative use.

5. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

Two types of SDS-PAGE were used, slab gels with no SDS in the gel and rod gels with SDS. All concentrations are weight ratios unless otherwise specified. The slab gels consisted of 10% acrylamide:N,N'-methylene-bis-acrylamide

Fig.10. FPLC profile of the papain digest of IgG on a mono Q column with an ionic strength gradient of 0.01-1M sodium acetate, pH 5.5. The peaks were detected by absorption at 280nm and identified by SDS-PAGE.

Mol. Wt. Std.	1	2	3	4	IgG ₁	IgG ₂
94 K —						
67 K —	—		—		—	—
45 K —						
30 K —	==	—	==	—	—	—
20 K —						
14.4 K —	—		—			

Fig. 11. Diagram of 10% slab gel of reduced rabbit IgG and the papain digest of IgG fractionated by FPLC.

lane 1. Whole papain digest.

lane 2. F_{ab} fragment.

lane 3. F_c fragment.

lane 4. F_c fragment crystallised by dialysis against Na₂B₄O₇ (0.01M, pH 8.6)

(30:0.8) resolving gel with a 5% acrylamide:

N,N'-methylene-bis -acrylamide (bis) stacking gel. The rod gels were 3% acrylamide:bis (30:1.2). Both types of gel were used for native and reduced protein.

Method for Slab Gels

This is an adaptation of the methods of Davis (55) and Ornstein (56). The stock solutions were made up as follows:

1. Acrylamide:bis (30:0.8) - Acrylamide solution (60g in 200ml of water) containing charcoal was heated to 60°C, then held at 56°C for 30 minutes protected from light. The solution was filtered and bis (1.6g per 200ml) was added. The solution was stored at 4°C in the dark.
2. 1.875M Tris(hydroxymethyl)aminoethane (tris-base), pH 8.8.
3. 1M [Tris(hydroxymethyl)aminomethane hydrochloride] (tris-HCl), pH 6.8.
4. Sample preparation solution (10% SDS) - The following were combined: SDS(0.41g), glycerol(12.3ml), tris-HCl (1.28ml), water(12.3ml) and bromophenol blue dye to colour.
5. Running buffer - Glycine(57.6g) and SDS(4g) were added to 4L of tris-base(0.05M), pH 8.3.
6. Coomassie stain - Coomassie blue dye(0.4g) was dissolved in isopropanol(30ml), water(130ml) and acetic acid(30ml).
7. Destain solution - An aqueous solution of 35%v/v ethanol and 10%v/v acetic acid was used.

Pouring and running the gel

Acrylamide:bis(10ml), tris-base(6ml), 0.2M Ethylenediaminetetraacetate(EDTA, 0.3ml) and water(13.4ml) were mixed and degassed. N,N,N',N'-Tetramethylethylenediamine (TEMED, 0.015ml) and freshly made 10% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ (0.3ml) were added and the resolving gel was poured into a Hoeffler gel electrophoresis apparatus. Water was layered over the gel surface and polymerization was complete within 30-40 minutes.

The previous procedure was repeated to prepare the stacking gel using, acrylamide:bis(2.5ml), tris-HCl(1.88ml), 0.2M EDTA(0.15ml), water(10.3ml), TEMED(7.5ul), and 10% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ (0.15ml). The water was removed from the surface of the resolving gel, a sample comb inserted and the stacking gel poured. Again polymerization was completed in 30-40 minutes.

Each protein sample (10-20ug) was mixed with an equal volume of sample preparation solution and reduced with mercaptoethanol(5 ul) at 100°C for one minute. The samples were applied to the gel, air bubbles removed and a constant current of 75mA was applied until the tracking dye reached the end of the gel. The gel was stained for two hours, destained over several hours, soaked in 10% glycerol several hours, then dried six hours on a Biorad 224 gel drier.

Rod Gels

The acrylamide system is a modification of that used by Fairbanks et al (57). Stock solutions were made up as follows:

1. 30% Acrylamide:bis (30:1.2) was made up as for slab gels
2. 10x Buffer - 1M tris-HCl, 2M sodium acetate, 0.2M EDTA, pH 7.4
3. Coomassie blue stain - as for slab gels
4. Destain solution - as for slab gels
5. Running buffer - 100ml 10x buffer, 50ml SDS(4%) were made up to 1L with water
6. Sample preparation solution - 20mM tris-HCl, 2mM EDTA, 2% SDS, 14% glycerol, 20mg/ml pyronin Y, pH 8. For reducing conditions, 50mM mercaptoethanol was used.

The reagents were mixed as follows, the mixture was degassed prior to addition of $(\text{NH}_4)_2\text{S}_2\text{O}_8$.

- a. 30% Acrylamide:bis (3ml)
- b. 10x Buffer (3ml)
- c. 4% SDS (1.3ml)
- d. 0.5%v/v TEMED (1.5ml)
- e. Water (18ml)
- f. 1.5% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ (3ml)

The mixture was cast as rod gels in 125x5mm inside diameter tubes. The rods were mounted in a Bio-rad model 150A electrophoresis chamber and run at an initial current of 0.5mA per tube until the sample entered the gel, then the current was increased to 8mA per tube. When the tracking dye

reached the end of the gel, the gels were removed and the tracking dye marked with Indian ink. The gels were stained and fixed for one hour in Coomassie stain, then destained one hour. Final clearing was in 7% acetic acid for one hour.

6. IMMUNISATION OF BALB/C MICE

BALB/c mice were injected subcutaneously with 80 ug of rabbit IgG in complete Freund's adjuvant. At two week intervals two further subcutaneous injections of rabbit IgG in incomplete adjuvant were administered. After the first three injections, a trial bleed from the retro-orbital sinus was checked for production of IgG against rabbit IgG by an ELISA assay. Finally an intraperitoneal boost of the F_c fragment of rabbit IgG was administered four days prior to fusion.

The initial injections were of whole IgG even though a monoclonal antibody against the F_c fragment was required. This was due to the possibility of the antigenic determinants of the F_c fragment being altered by the papain digestion. The monoclonal antibody was required to recognize the F_c region of the whole IgG molecule. The final boost of F_c fragment was to preferentially stimulate lymphocytes secreting anti- F_c fragment antibodies over those secreting antibodies directed against other areas of the IgG molecule.

7. CULTURE OF NS1 CELLS

Preparation of Stock Solutions

- a. Heat inactivated fetal calf serum (FCS). The FCS was incubated one hour at 56°C, centrifuged at 500g then filtered through 0.45 and 0.22um sterile Nalgene filters. The FCS was stored at -20°C.
- b. L-Glutamine. A solution of L-glutamine (30mg/ml) was autoclaved, then filtered through a sterile 0.2 um membrane filter. It was stored in 1ml aliquots at -20°C.
- c. Growth medium. RPMI-1640 was obtained from Fisher, made up as directed and filtered through 0.45 and 0.22um sterile Nalgene filters. RPMI-1640 (100ml) was mixed with heat inactivated FCS (10ml) and L-glutamine (1ml).
- d. Serum-free medium. As above without FCS.

Thawing and Culture of NS1 Cells

The NS1 cells were thawed over five minutes at room temperature, then added to cold serum-free medium (10ml). A 100 ul aliquot was stained with erythrosin B dye and the viability of the cells was checked by counting the dead stained cells on a haemocytometer. Erythrosin B only stains dead cells. If the viability of the cells was over 80%, the cells were centrifuged (300g, 5min) and the medium aspirated off. The cells were resuspended in growth medium to give a final concentration of 10^5 cells per ml. Incubation was in T25 flasks at 37°C, 5% CO₂ and 100% humidity. At a population density of approximately 10^6 cells per ml, the cells were divided and an equal volume of fresh growth

medium added.

Freezing of NS1 cells

Approximately 10^6 cells were resuspended in 1ml of freezing medium (10%v/v DMSO, 40%v/v FCS, 50%v/v RPMI-1640). The cells were frozen initially at -70°C overnight, followed by long term storage in liquid nitrogen.

Selection of HAT Sensitive NS1 Cells

This is necessary to ensure that only hybridomas survive after fusion. The HAT medium was made up by adding 1ml of 100xHAT (10mM hypoxanthine, 0.04mM aminopterin, 1.6mM thymidine) to 100ml of growth medium. NS1 cells were plated into HAT medium and all died within 24 hours.

8. PREPARATION OF CELLS FOR FUSION

The NS1 cells were grown from frozen storage. Approximately 10^7 cells were used in the fusion.

The Thymocyte Feeder Cells

The thymuses were removed from six week old Swiss mice. Two to three thymuses were placed in serum free medium and washed well. The thymocytes were teased out of the thymus with forceps, then drawn up and down in a syringe to break up clumps. The cell suspension was transferred to a test tube, allowed to settle, then transferred again to leave any large clumps of cells behind. The cells were counted and resuspended in 130ml of HAT medium. The final thymocyte concentration was 5×10^6 cells per ml.

The Spleen Cells

The spleen was removed from an immunized BALB/c mouse and washed with serum-free medium. The spleen was teased apart in 5ml of serum-free medium and passed through a sterile sieve. This was repeated and large pieces of spleen were ground with a syringe plunger. The suspension was centrifuged (300g, 5min) and the medium was aspirated off. Erythrocytes were lysed in 5ml of NH_4Cl (0.17M) for 5 min, then centrifuged (300g, 5min). The NH_4Cl was aspirated off and the cells were resuspended in 5ml of serum-free medium. Approximately 10^8 cells were used in the fusion.

Preparation of the PEG 3500 Solution

PEG 3500 (10g) was autoclaved, cooled, then serum-free medium (10ml) was added while the PEG 3500 was still liquid. The pH was slightly alkaline so the solution was pink in colour.

9. FUSION PROTOCOL

The spleen cells (10^8 in 5ml) and NS1 cells (10^7 in 1ml) were mixed, centrifuged (300g, 5min) and the medium was aspirated off. Sterile PEG 3500 (1g/ml) in serum-free medium was added (1ml, 0.1ml every 6 seconds) with stirring, then stirred for one minute. Next serum-free medium was added (5ml, 0.2ml every 12 seconds for the first 2ml, then 0.5ml every 30 seconds for the next 3ml) with stirring. The cells were resuspended by pipetting and the final volume made up to 10ml. The cells were centrifuged (300g, 5min) and

resuspended in thymocyte "feeder" medium (5ml). The remaining feeder medium was added to make a final volume of 130ml. The cells were plated into 96 well plates, 3 drops per well. Lipopolysaccharide (LPS, 0.025mg/ml, 1drop per well), a growth stimulant was added to half the plates to make a comparison. The cells were incubated at 37°C, 5% CO₂, 100% humidity for four days, then each well was fed with thymocytes in HAT medium. After one week HAT selection was terminated and the cells were cultured in HT medium (100xHT, 10mM hypoxanthine, 1.6mM thymidine) for several days to ensure the complete removal of aminopterin prior to culture in normal growth medium.

The inhibition constant of aminopterin for dihydrofolate reductase is less than 10^{-9} M (58) and the concentration of aminopterin in HAT medium is 4×10^{-7} M therefore the concentration must decrease 400 fold before the enzyme regains activity. Aminopterin is metabolised extremely slowly and the main route of removal from cell cultures is by dilution. For this reason slow growing cell lines were cultured in HT medium for several weeks.

10. THE ENZYME-LINKED IMMUNOSORBENT ASSAY(ELISA)

Goat anti-mouse IgG-horseradish peroxidase (IgG-HRP) was obtained from Sigma. A 96 well PVC microELISA plate was coated with antigen solution (1ug/ml in 15mM Na₂CO₃, 35mM NaHCO₃, pH 9.6) at 200ul per well and left at 4°C overnight. The antigens used in this study were rabbit IgG, F_c and F_{ab}

fragments of rabbit IgG, and NN,MM or MN glycophorin A. The plate was washed three times with PBS-Tween (0.5% Tween 80) and blotted dry. An aliquot of antibody solution was added (200 ul per well) and incubated for one hour at 37°C. The antibody solutions used were monoclonal antibody in ascites fluid, culture supernatant or FPLC purified monoclonal antibody and rabbit IgG. The antibody solution was removed and the plate was washed three times with PBS-Tween. A solution of BSA (0.5%) in PBS-Tween was added (200 ul per well) as a blocking agent and incubated 30 minutes at 37°C. The BSA solution was removed and the plate was washed three times with PBS-Tween. A solution of goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) in PBS was added to each well (200 ul per well). The anti-mouse IgG-HRP conjugate was a 1:8000 dilution of a 0.5mg/ml stock solution and the enzyme:anti-mouse IgG molar ratio was 4:1. The plate was incubated for two hours at 37°C. The excess conjugate was removed by washing the plate three times with PBS-Tween. O-Phenylenediamine solution (OPD, 0.04% in 25mM citric acid, 51mM Na₂HPO₄, pH 5), containing 0.12% H₂O₂ added just prior to use, was added to each well (200ul per well). The colour was allowed to develop in the dark until the reaction was stopped by addition of H₂SO₄ (4M, 50 ul per well). The absorbance of each well was measured at 492nm with a 690nm reference beam on a Titertek Multiskan MC.

11. RECLONING BY LIMITING DILUTION

Both cells producing anti-rabbit Fc and anti-whole rabbit IgG were recloned. An aliquot of cells was diluted 100 times into HT medium and counted. The cells were diluted in HT medium to a concentration of approximately 10 cells per ml and plated into a 96 well cell culture plate. The cells were grown as before with thymocyte feeder cells, then examined by ELISA for F_c specificity.

12. ANTIBODY PRODUCTION AND PURIFICATION

Monoclonal Antibody Production via Ascites Fluid

In most cases injection of 10^6 - 10^7 histocompatible hybrid cells into mice will result in tumor formation within two to four weeks. Intraperitoneal injection of hybridomas often results in the development of ascites containing 5-15mg/ml of antibody, a similar level to that found in the serum. Ascites yield per mouse is 2-5ml whereas serum yield is only 0.5-1ml, therefore generation of ascites is a rapid method to produce monoclonal antibodies.

BALB/c mice were injected with pristane (0.5ml per mouse) intraperitoneally (I/P) and three to four days later injected with 10^6 hybridoma cells I/P in 1ml of serum-free medium. After seven to ten days, the ascites fluid was drained by inserting a needle I/P and this was repeated every two days until death of the mouse. Each mouse typically yielded 5-6ml. The ascites was centrifuged to remove the cells, then stored at -20°C until purification.

Fig.12. FPLC profile of monoclonal IgG precipitated by saturated $(\text{NH}_4)_2\text{SO}_4$ solution. Elution was with a .0.01-0.3M phosphate ionic strength gradient, pH 8 on a mono Q column. A single peak was obtained due to the monoclonal origin of the antibody.

Purification of Antibodies from Ascites Fluid and Cell Culture Supernatant

Ascites fluid (typically 2mg/ml of IgG) or cell culture supernatant (typically 10ug/ml of IgG) were precipitated with $(\text{NH}_4)_2\text{SO}_4$ and washed three times with 40% saturated $(\text{NH}_4)_2\text{SO}_4$ as described earlier. The pellet was dialysed against 10mM phosphate, pH 8 and purified on a FPLC mono Q column, with a 0.01-0.3M ionic strength gradient and a flow rate of 2ml/min. Typically one major peak was eluted with traces of murine proteins in the case of the ascites preparation (Fig.12).

13. FREEZING AND THAWING OF HYBRIDOMA CELLS

Approximately 10^7 cells were resuspended in 1ml of hybridoma freezing medium (90%v/v FCS, 10%v/v DMSO) and frozen at -70°C overnight. The vials were stored in liquid nitrogen.

The cells were thawed over five minutes, then added to cold HT medium. Often the cells were slow to begin to divide and required thymocyte feeder cells.

14. CHARACTERISATION OF THE PURIFIED MONOCLONAL ANTIBODY BY A WESTERN BLOT

The specificity of the monoclonal antibody for heavy and light chains of IgG and the papain digest fractions was examined using the Western blot technique which identifies proteins after electrophoretic separation (59). The proteins

are eluted electrophoretically from the polyacrylamide gel onto a membrane made of nitrocellulose which binds protein tightly. The membrane is then exposed to antibody. Usually the developing antibody is radioactive but in the present case the bound antibody was revealed with HRP anti-mouse IgG conjugate in a similar way to the ELISA.

Polyacrylamide gels of IgG₁, IgG₂, F_{ab} and F_c fragments were run in a 10% slab gel as described earlier. Two pieces of S-S filter paper, a nitrocellulose sheet and a Scotchbrite pad were soaked in degassed transfer buffer (tris-base(12.1g), glycine(57.6g), methanol(800ml), made up to 4L, pH 8.3). A "sandwich" was built up in the following order: scotchbrite pad, filter paper, nitrocellulose paper, gel, filter paper and scotchbrite pad (Fig.21b). The sandwich was clipped together and placed in a trans-blot cell such that the nitrocellulose paper was between the gel and the anode. The cell was filled with transfer buffer and the proteins were transferred over sixteen to twenty four hours at 12v with cooling. Immediately after transfer the nitrocellulose sheet was immersed in a solution of tris-BSA (0.9%w/v NaCl, 5%w/v fraction V BSA, 10mM tris-HCl, pH 7.4) and incubated at 37°C for 90 minutes to saturate remaining binding sites on the nitrocellulose. The nitrocellulose was then immersed in tris-BSA containing monoclonal antibody (50 ug/ml) and incubated for 90 minutes at 37°C. During both incubations the blotted side of the nitrocellulose was facing upwards. After incubation the nitrocellulose was

washed with shaking for ten minutes in tris-saline (0.9%w/v NaCl, 10mM tris-HCl, pH 7.4), twenty minutes in two lots of tris-saline containing 0.05%w/v Nonidet P-40 and ten minutes in tris-saline. This was followed by immersion in a solution of anti-mouse IgG-HRP conjugate, a washing step, then OPD solution (as described for ELISA). Finally the nitrocellulose was washed with tris-saline and air-dried at room temperature. The dark bands were marked in case of fading.

15. AFFINITY CHROMATOGRAPHY OF ANTIBODIES

a. Preparation of the Sepharose-Protein Conjugate

CNBr-activated Sepharose 4B was obtained from Pharmacia. Anti-rabbit IgG (goat) was obtained from Cappel. CNBr-activated Sepharose 4B (1g) was swollen and washed in HCl (0.001M, 200ml). Rabbit IgG or anti-rabbit IgG (30mg) in NaHCO₃ buffer (0.1M, pH 8.3) containing NaCl (0.5M) was added to the swollen gel and mixed, end-over-end for 2 hours at room temperature. The suspension was washed with NaHCO₃ buffer (0.1M, pH 8.3), then mixed end-over-end in glycine buffer (0.2M, pH 8.0) for 2 hours at room temperature. The suspension was washed 5 times with a cycle of NaHCO₃ (0.1M, pH 8.3) followed by CH₃COONa (0.1M, pH 4.0). The conjugate was stored at 4°C in buffer containing sodium azide (3.6mM).

b. Use of the Sepharose-Protein Conjugate

A batch process was used for convenience. The sample, either rabbit IgG or monoclonal antibody, was added to the

Sepharose suspension and incubated at 37°C for 2 hours, then washed with PBS. The suspension was resuspended in HCl (0.001M) for 15 minutes and the supernatant collected in buffer. This step was repeated and the combined supernatant was dialysed against the required buffer.

16. MODIFICATION OF THE MONOCLONAL ANTIBODY WITH PEG 1900

The usual approach to preparing protein-PEG conjugates is to synthesize an "activated" PEG with a reactive functional group that will readily couple with a protein functional group, usually a primary amine. In this study, PEG 1900 was activated using cyanuric chloride (60).

Synthesis of 2(Alkoxypolyethyleneglycoxy)-4,6 dichlorotriazine (PCC)

A reaction scheme is given in Fig. 13. NaH (0.18g) was suspended in anhydrous benzene (50ml) and the system was purged with nitrogen. PEG-1900 (10g) in anhydrous benzene (50ml) was added over fifteen minutes. The reaction mixture was stirred for one hour under nitrogen flow and one hour under a stationary nitrogen atmosphere. A four fold molar excess of cyanuric chloride (3.9g) was added and refluxed for twelve hours. The reaction mixture was poured into petroleum ether (b.p.frac.60-80°C, 150ml) at 0°C 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°C. The reprecipitation was repeated three times.

Fig.13. Reaction scheme for protein modification with PEG via an activated cyanuric chloride derivative of PEG.

The PCC was vacuum dried and stored at -70°C under nitrogen. The reaction efficiency was determined by assaying the hydrolysable chlorides of the PCC.

Determination of Hydrolysable Chlorides of PCC

The Buchler Cotlove Chloride Titrator uses the coulometric principle of titration. Silver ions are generated at the anode which react with the chloride ions and precipitate out. When all the free chloride has reacted, the increase in current is detected as the end point.

Of four samples of approximately 40mg of PCC, two were hydrolysed in $\text{Na}_2\text{B}_4\text{O}_7$ (0.1M, pH 10) for at least two hours. The other two samples were dissolved in the same buffer, pH 9, immediately prior to titration. A solution of nitric acid (0.1M) and 10% acetic acid (4ml) and 0.62% gelatin (200ul) was added to each sample, a blank and a NaCl standard. The free chlorides were titrated using the chloridometer.

Reaction of PCC with the Monoclonal Antibody

Previous studies on modification of antibodies with PCC for use as affinity ligands found that a 3:1 molar ratio of PCC:lysine in the reaction mixture gave the best compromise between a high partition coefficient and retention of binding activity in the modified antibody (20). For this reason the 3:1 ratio was used throughout this study and it was found more effective than the 1:1 ratio which was also tried.

Monoclonal IgG (approximately 2-8mg/ml final concentration) was made up in $\text{Na}_2\text{B}_4\text{O}_7$ (0.1M, pH 9) and PCC

was added to give a 3:1 PCC:lysine molar ratio (assuming 90 lysines per IgG molecule (61)). After forty minutes at room temperature, the reaction was stopped by freezing the mixture or removal of the unreacted PEG by dialysis or ultrafiltration, although most of the PCC was hydrolysed anyway. The degree of primary amine modification was assayed by trinitrobenzenesulphonic acid (TNBS) or 4 phenylspiro[furan-2H(3H),1'-phthalan]-3,3' dione (fluorescamine).

17. DEGREE OF PEG 1900 MODIFICATION OF THE MONOCLONAL ANTIBODY

As PCC reacts principally with the primary amines of a protein, the amount of PEG attached can be estimated by assaying the primary amines before and after reaction with PCC. The most commonly used method is the TNBS assay (62), a colourimetric procedure. Another assay uses fluorescamine which forms stable fluorophors with primary amines.

The TNBS Assay

To 1ml of protein solution (0-1mg/ml) were added 4% NaHCO_3 , pH 9.5 (1ml) and 0.1% TNBS (1ml). The samples were vortexed then incubated at 37°C for two hours. After incubation 10% SDS (1ml) and HCl (0.5M, 1ml) were added to each sample, vortexed and the absorbance read on a Hewlett Packard 8450A uv/vis spectrophotometer by taking the average absorbance over a 330-340nm range.

The Fluorescamine Assay

Fluorescamine reacts rapidly with primary amines at pH 8-9 to yield highly fluorescent products (63, Fig.14a). The reaction is complete within seconds and the excess reagent hydrolysed within a minute (64). The reagent is water insoluble so is added to a buffered protein solution in acetone or dioxane. The addition of organic solvents to polymer solutions often precipitates the polymer. This is the case with dextran and the assay cannot be used for protein estimation in dextran-containing solutions such as the dextran-rich phase of two phase systems. Obviously amine-containing buffers cannot be used and the best buffers are phosphate or borate salts.

The widely used ninhydrin method for detection of amines uses the fluorescent ninhydrin derivatives of phenyl acetaldehyde, which is formed *in situ* from phenylalanine and ninhydrin, and primary amine (65). Fluorescamine, however, reacts directly with primary amines to form the same fluorophors (66).

To 1.5ml of protein solution (0-10ug) in 0.1M phosphate, pH 8, fluorescamine (0.5ml, 0.3mg/ml) in spectroscopic grade acetone was added while vortexing. After a minimum of seven minutes the fluorescence of the solution was measured on a Turner model 430 spectrofluorometer with an excitation wavelength of 390nm and emission detected at 475nm. A standard immunoglobulin solution was assayed simultaneously as the fluorescence reading depends on the

Fig.14a. The reaction of fluorescamine with primary amines to yield a highly fluorescent derivative.

Fig.14b. The reaction of TNBS with primary amines to yield a derivative with absorbance at 335nm.

machine sensitivity setting.

18. ASSAY OF THE BINDING ACTIVITY OF THE PEG-ANTIBODY CONJUGATE

The ELISA showed a negative result for the PEG-modified antibody. This could be due either to the PEG-modified antibody no longer binding the rabbit IgG, or the HRP-anti mouse IgG conjugate no longer binding the PEG-modified monoclonal antibody. To compare the binding of rabbit IgG by PEG-modified and native monoclonal antibody, the antibody was removed from the microELISA plate by dissociating the antigen-antibody bond and assaying the antibody concentration by fluorescamine.

The method was the same as the ELISA, except the BSA blocking step was omitted, until the addition of the HRP-anti mouse IgG conjugate. Instead of adding the conjugate, 10% dioxane in HCl (0.01M, 200 μ l per well) was added to dissociate the rabbit IgG-monoclonal antibody bond (67). After fifteen minutes at room temperature, the contents of each well was pipetted into 1.1ml phosphate (0.1M), pH 8 and 0.2ml NaOH (0.01M). The fluorescamine assay was used to estimate the bound protein and an ELISA assay was run simultaneously. The NaOH was added to maintain pH 8 since the rate of the fluorescamine reaction is pH dependent.

19. SINGLE TUBE PARTITION EXPERIMENTS

The Two Phase System

The following stock solutions were required to make up the 5% dextran, 3.4% PEG 8000 phase system. Dextran T500 ($M_w=494,000$, $M_n=181,200$) was obtained from Pharmacia and PEG 8000 ($M_w=6,000-7,000$) was obtained from Union Carbide

1. Dextran. Dextran T500 (22g) was made up to 100g with water and stirred for two hours. The %w/v was determined by polarimetry, using a Steeg and Reuter polarimeter, on a 12.5 times dilution of the stock solution using $[\alpha]_D^{25} = +199^\circ$

2. PEG. PEG 8000 (30g) was made up to 100ml with water. The %w/w was determined by measuring the refractive index of a 6.25 times dilution of the stock solution using a Bausch and Lomb "Serum Protein" Meter. ($\%w/w = RRI/SRRI \cdot \rho \cdot 6.25$ where RRI = refractive index of PEG solution relative to water and $SRRI$ = the specific refractive index of PEG, ρ = density of PEG solution)

3. NaCl (0.6M)

4. Sodium phosphate buffer (0.3M, pH 7.2)

The stock solutions and water were mixed to give a final overall composition of 5%w/v dextran, 3.4%w/w PEG, 130mM NaCl and 10mM phosphate (a 5,3.4 system). The phases were allowed to separate overnight or by centrifugation (ten minutes at 200g), then stored as upper and lower phase separately.

Partition of the Native and Modified Antibodies

Various amounts of antibody (up to 200 μ l), both native rabbit IgG and monoclonal IgG or PEG-modified monoclonal IgG, were added to 4ml of 5,3.4 total phase system. If the resulting dilution of the phase system formed one phase, stock PEG (approx.30%w/w) was added to reform the phases (10-20 μ l). The phases were mixed twice at eight minute intervals and after eight minutes, 20 μ l samples were taken from the upper phase. The system was made into one phase by the addition of phosphate buffer (200 μ l) and 20 μ l samples of the total phase system were taken. The protein in the upper phase and total phases was assayed by fluorescamine.

Preparation of Erythrocytes for Partitioning

Blood was collected into 0.38% $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ (final concentration) from human or rabbit donors. The erythrocytes were washed three times in 10 volumes of PBS, then fixed in 1% glutaraldehyde for later use or used immediately. Fixed cells were washed three times in ten volumes of PBS before use.

Partition of Cells

A small aliquot of fresh or fixed cells was washed and resuspended in upper phase. The cell concentration in the load mix was determined by impedance cell counting on a Particle Data Inc. Electrozone Celloscope and was typically 2×10^8 cells per ml. Approximately 10^7 cells were added to 1ml of phase system from the load mix. Other additives included various combinations of antibodies or buffers which

resulted in the formation of one phase. An aliquot was removed for counting the total cell concentration in the phase system and 10-20 ul of stock PEG solution was added to reform the phases. The systems were mixed twice at eight minute intervals, settled, then the top phase was sampled and the cells counted. The % of cells returning to the upper phase was calculated and expressed as a partition coefficient.

Fig.15. 3% SDS-PAGE of native and reduced anti-NN glycoprotein A rabbit IgG and mouse monoclonal IgG.
 lane 1. Standard molecular weight markers.
 lane 2. Native mouse monoclonal IgG.
 lane 3. Reduced mouse monoclonal IgG.
 lane 4. Native rabbit IgG.
 lane 5. Reduced rabbit IgG.

C. RESULTS AND DISCUSSION

1. RABBIT ANTI-NN GLYCOPHORIN A

The rabbits responded well to the NN glycophorin A immunisation and yielded approximately 40mg of IgG on each 40ml bleed. Precipitation of IgG by $(\text{NH}_4)_2\text{SO}_4$ resulted in the co-precipitation of contaminating proteins, mostly IgM and fibrinogen. This was minimised by adding the $(\text{NH}_4)_2\text{SO}_4$ as a solution rather than a solid to avoid high local concentrations causing precipitation of contaminating proteins. The washing step with 40% saturated $(\text{NH}_4)_2\text{SO}_4$ also reduced the level of contaminating proteins. Dialysis against water precipitated lipoproteins and the bulk of the IgM. Final purification on the FPLC mono Q column (Fig.9) shows that the IgG was eluted as two peaks at 8% and 12% NaCl. The first peak is IgG₂ and the second peak is IgG₁, which is the more basic of the two IgG classes thus binding to the column more strongly and requiring a greater ionic strength for elution. The class of an IgG molecule is determined by the heavy chain structure and defined by size and electrophoretic mobility. Throughout this study IgG₂ was used due to the possibility that the monoclonal antibody recognized a heavy chain determinant only present on IgG₂, as this was the class used in the BALB/c mouse immunisation. In fact no difference was observed in the affinity of the monoclonal antibody for IgG₂ over IgG₁. The IgM bound strongly to the column and was eluted at 55% NaCl.

The mono Q column is a strong anion exchanger compared to a DEAE cellulose column which explains the high concentration of NaCl required to elute the IgG and IgM from the mono Q column compared to the former.

The Activity of the Anti-NN Glycophorin A IgG

The activity of the IgG was assayed by direct haemagglutination in a microtitre plate (Table 1).

Blood type	Initial rabbit IgG conc.(mg/ml).	Maximum erythrocyte agglutination dilution	Minimum erythrocyte aggregation conc. (mg/ml)
NN	4.0	2^{-9}	0.008
MN	4.0	2^{-8}	0.016
MM	4.0	2^{-5}	0.125

Table 1. Results of microtitre of rabbit anti-NN glycophorin IgG and human erythrocytes of NN,MM or MN specificities. Erythrocytes were added at 1% haematocrit. Protein concentrations detected by OD₂₈₀.

The anti-NN glycophorin A IgG caused aggregation of NN erythrocytes at a concentration fifteen times as dilute than that which caused agglutination of MM erythrocytes and twice

as dilute as that which caused agglutination of MN erythrocytes. This degree of cross reactivity was expected with a polyclonal antibody because the M and N antigens share many common antigenic determinants. Previous studies by Sharp (20) used rabbit IgG with a minimum aggregation concentration of 0.03mg/ml compared to the value of 0.008mg/ml for anti-NN glycophorin A IgG and NN erythrocytes (Table 1). This may be because the IgG in this study was raised against isolated NN glycophorin A, a known surface antigen, whereas the antigen used by Sharp was raised against whole erythrocytes. The possibility of contaminating antigens to dilute the cell surface antigens is far greater in a sample of erythrocytes than in a purified homogeneous protein sample. Also the shear forces in passing an erythrocyte through a needle during immunisation could rupture the cells exposing new antigens.

The binding of anti-NN glycophorin A IgG to glycophorin A was also examined using an ELISA (Fig.16). The glycophorin A (1 ug/ml) adsorbed to the microELISA plate was bound by rabbit anti-NN glycophorin A, which was in turn bound by monoclonal anti-rabbit Fc fragment and revealed by the HRP-anti mouse IgG conjugate. Since the monoclonal antibody was used well above saturation level, the absorbance at 492nm gives an indication of the degree of binding of glycophorin A by anti-NN glycophorin A IgG. At the minimum aggregation concentration of 0.008mg/ml from the microtitre results (Table 1), the ELISA absorbance is 0.3, only just

Fig. 16. Concentration dependence of binding of MN and NN glycophorin A by anti-NN glycophorin A rabbit IgG. The microELISA plate was coated at 1 μ g/ml and the initial rabbit IgG concentration was 1mg/ml. The bound antibody was revealed using monoclonal mouse anti-rabbit F_c fragment IgG, followed by HRP-anti-mouse IgG conjugate and OPD. The ELISA absorbance is proportional to the bound antibody.

above background absorption. This demonstrates that only small amounts of IgG need to bind the erythrocyte to cause aggregation.

The ELISA was revealed by binding the monoclonal antibody to the rabbit IgG, followed by the HRP-anti mouse IgG conjugate. This demonstrated that the monoclonal antibody would bind the rabbit IgG which was bound to glycophorin A, suggesting binding would also occur when erythrocytes were used.

SDS-PAGE of the Rabbit IgG

The SDS-PAGE rod gels of IgG are shown in Fig.15 in two different arrangements to facilitate comparisons of both the native and reduced IgG and the monoclonal and polyclonal IgG. The native rabbit IgG ran as two bands in SDS-PAGE (Fig.15, lane 4). The more intense band has an approximate molecular weight of 150,000 so is likely to be whole IgG. The less intense band has an approximate molecular weight of 300,000 and is likely to be a dimer of IgG. The probability that the band at 300K is a dimer is supported by the fact that the reduced IgG runs as two bands, corresponding to heavy and light chains of IgG (Fig.15, lane 5). Presumably the dimer dissociates on reduction. However, no contaminating fibrinogen or IgM bands were observed so the samples were assumed to be homogeneous. No differences were observed in SDS-PAGE of IgG₁ and IgG₂ (Fig.11).

2. PAPAIN DIGESTION OF RABBIT IMMUNOGLOBULIN G

Papain cleaves polypeptides and proteins, non-specifically at the peptide bond one residue removed towards the C terminus from a phenylalanine residue (68), e.g.

Limited digestion allows cleavage below the hinge region of IgG but above the heavy chain disulphide linkage (Fig.4). However prolonged exposure of F_c fragments to papain yields a range of sub-fragments. Papain is a thiol protease which means that the cysteine sulphydryl in the active site must be in the reduced form for activity. Due to this the reaction was carried out under reducing conditions and the IgG was dissociated into heavy and light chains. The reaction was monitored by FPLC but it was not immediately obvious from the FPLC profile whether the two peaks were F_c and F_{ab} or IgG heavy and light chains. It was found that stopping the digestion prior to complete fragmentation of the IgG yielded the most F_c fragment. If the digestion was continued to completion, a range of low molecular weight sub-fragments was formed. Remaining undigested IgG was removed during FPLC separation of the fragments (Fig.10).

FPLC Fractionation of the Papain Digest Fractions

The papain digest fractions were fractionated on the mono Q anion exchanger with an ionic strength gradient of 0.01-1M sodium acetate, pH 5.5 (Fig.10). The F_{ab} fragment only bound to the column at low concentrations and in

preparative chromatography was eluted by the starting buffer. The F_c fragment was eluted last and was unavoidably contaminated with whole IgG when examined by SDS-PAGE (Fig.11). Subsequent to this part of the study, Cleardin et al.(69) used the FPLC mono Q column to separate mouse IgG papain digest fragments and found a similar contamination with whole IgG. The F_c fragment from the FPLC can only be considered enriched in F_c fragment. Papain did not bind to the column and was eluted in the void volume. Crystallisation of the F_c fragment yielded a homogeneous sample which ran as one band in SDS-PAGE (Fig.11) but yields were too low for preparative use.

As the hybridomas were selected as F_{ab} non-binding as well as F_c binding, the prescence of whole IgG in the F_c fraction was not a problem. This was demonstrated by the Western blot (Fig.21a) discussed later.

3. PRODUCTION AND SELECTION OF HYBRIDOMAS

Fusion and growth of clones proceeded as described earlier. Half the clones were cultured in medium containing LPS, a mitogen which stimulates lymphocyte proliferation (71). The cells cultured in LPS were slightly slower to begin dividing than those without LPS, but divided more rapidly after the initial lag phase. However no difference in the total number of clones was observed with or without LPS.

P3-NS1-Ag4-1 MYELOMA CELLS

Fig.17. Cell cultures showing dividing myeloma and hydridoma cells, fibroblast contamination and thymocyte feeder cells.

Initially 960 wells were plated with the fusion products and thymocyte feeder cells. Of these 240 showed hybridoma clones, many wells contained from two to ten clones in a single well. One hundred clones were examined by ELISA for anti-rabbit IgG activity, fifty of which were positive. These fifty clones were cultured for one week, then examined for F_c specificity. Ten clones bound the F_c fragment but not the F_{ab} fragment.

Over the next two weeks three clones were lost as a result of overgrowth by fibroblasts (see later in discussion) and two cell lines stopped producing antibody, presumably due to spontaneous chromosome loss. The five remaining clones were designated A6,B4,D1,E4 and F1 by the normal convention of naming cell lines after the coordinates of the wells in which they originated.

Ten of the anti-whole IgG producing clones were recloned by limiting dilution as it was not known if the antibody was cross reacting or several clones were producing different antibodies in the same well, i.e. the antibody was not monoclonal. Although F_0 was 0.54, well above the 0.37 required to ensure 95% probability that growth originated from one cell (see recloning in introduction), no further clones were found to be F_c specific so it was assumed that the antibodies were cross reactive. All the anti-rabbit F_c producing clones were recloned to ensure genetic stability.

During the early stages of culture, fibroblast contamination was observed in the cell cultures (Fig.17).

Fig.18. 3% SDS-PAGE of native purified monoclonal antibodies.

lane 1. Polyclonal rabbit IgG.

lane 2. Monoclonal A6 from cell culture supernatant.

lane 3. Monoclonal A6 from ascites.

lane 4. Monoclonal B4 from cell culture supernatant.

lane 5. Monoclonal B4 from ascites.

lane 6. Monoclonal E6 from ascites.

lane 7. Molecular weight markers.(70)

The thymuses of very young mice are rich in fibroblasts and the source of the contamination was the four week old mice used as thymocyte donors. Fibroblasts will grow continuously in culture and have the potential to overgrow clones. They settle to the bottom of the cell culture well so repeated careful transfer will remove hybridomas from fibroblasts.

Several aliquots of each anti- F_c producing cell line were stored in liquid nitrogen and two samples of each anti-whole IgG producing cell line.

4. PURIFICATION OF THE MONOCLONAL ANTIBODY

The monoclonal antibody was purified from both culture supernatant and ascites fluid by precipitation with $(NH_4)_2SO_4$, followed by FPLC purification on the mono Q column with a 0.01-0.03M phosphate, pH 8, ionic strength gradient (Fig.12). The protein was eluted as one peak with little or no contamination. Any murine contaminants from the ascites were removed by this procedure.

The monoclonal antibodies ran as single bands when non-reduced in SDS-PAGE (Fig.18), with approximate molecular weights of 146,000. The cell line B4 either died or mutated while in the mouse peritoneum (Fig.18, lane 5), as the protein purified from B4 ascites had a M_r in excess of 700,000 which had no affinity for rabbit F_c fragment when assayed by ELISA. It could be non-specific IgM produced by the ascites donor.

Fig.19. Concentration dependence of the binding of rabbit F_c by unpurified monoclonal anti-rabbit F_c . The microELISA plate was coated with 1ug/ml of rabbit F_c . The monoclonal antibody was unpurified in cell culture supernatant (approx. 10ug/ml) initially diluted 200 fold. The highest dilution with no apparent decrease in binding is 6400 fold (1.5ng/ml).

Fig.20. Concentration dependence of the the binding of rabbit F_c by purified monoclonal antibody. The plate was coated at 1ug/ml and the initial monoclonal antibody concentration was 0.5mg/ml. The maximum dilution with no apparent decrease in binding is 15ug/ml.

The monoclonal antibody purified from ascites had a greater molecular weight than that purified from cell culture supernatant (compare lanes 2 & 4 with lanes 3 & 6, Fig.18). This could either be due to the ascites originating antibody being bound to a low molecular weight contaminant or the culture supernatant originating antibody being cleaved by non-specific proteases. The latter seems most likely as there is a blurring of the protein band which is characteristic of proteolysis.

5. ACTIVITY OF THE PURIFIED MONOCLONAL ANTIBODY

The activity of the monoclonal antibodies decreased after purification. This is demonstrated by comparing an ELISA of purified and non-purified antibody (Figs.19 & 20). The non-purified antibody could be diluted significantly compared to the purified antibody before binding below saturation level of the rabbit F_c . This loss in activity may have been due to non-specific proteases in the antibody solution or to the effects of precipitation with $(NH_4)_2SO_4$. Samples of antibody were left at room temperature with and without protease inhibitor, PMSF (72,73), for one to two days. No further decrease in activity was observed in samples with or without protease inhibitors suggesting that perhaps the precipitation with $(NH_4)_2SO_4$ caused the loss in activity. No further loss in activity occurred during storage at $-70^\circ C$.

Precipitation with $(\text{NH}_4)_2\text{SO}_4$ is considered a gentle, effective and simple method of reducing the protein load on the ion exchange column. The $(\text{NH}_4)_2\text{SO}_4$ ions remove water of hydration from the protein, exposing hydrophilic ionic groups thus decreasing the protein solubility. Such a procedure seems unlikely to cause a large loss of activity but several studies of monoclonal antibodies suggest that they may be particularly susceptible to denaturation by $(\text{NH}_4)_2\text{SO}_4$ solution (74,75).

Another possibility is that degradation and corresponding loss of activity occurred during purification, prior to removal of proteases by FPLC.

The Specificity of the Monoclonal Antibody by the Western Blot Technique

The results are shown in Fig.21a. The monoclonal antibody recognizes the F_c fragment and heavy chain, but not the F_{ab} fragment or light chain of rabbit IgG. This is as expected since the heavy chains include the F_c region of the IgG molecule. The light chain is not recognized by the monoclonal antibody since this is not part of the F_c fragment. The monoclonal antibody does not recognize the small fragments ($M_r < 15K$) arising from the prolonged exposure of rabbit IgG to papain.

Mol. Wt. Std.	1	2	3	4	IgG ₁	IgG ₂
94K —						
67K —						
45K —						
30K —		—			—	—
20K —						
14.4K —	—		—			

Fig.21a. A Western blot of monoclonal anti-F_C fragment against papain digest fragments of rabbit IgG and whole IgG. Bands recognized by the antibody are indicated ( )

lane 1. Papain digest mixture.

lane 2. F_{ab} fragment.

lane 3. F_C fragment.

lane 4. F_C fragment by crystallisation.

Fig.21b. Schematic representation of electrophoretic transfer of protein bands from a polyacrylamide gel to nitrocellulose paper.

6. SYNTHESIS AND ANALYSIS OF THE PEG-MONOCLONAL ANTIBODY

Analysis of PCC

The PCC synthesis was in two steps, first the formation of the sodium salt of PEG-OH followed by a nucleophilic displacement of one of the chlorides of cyanuric chloride (Fig.13). The substitution of the first chloride deactivates the other two chlorides to nucleophilic substitution. The degree of substitution of cyanuric chloride was assayed by hydrolysis of the remaining chlorides and titration against AgNO_3 . The results are shown in Table 2.

Sample	Sample Wt. (mg)	Titre (mM Cl^-)	Titre for 100% yield	% Free Cl^- in sample	Mod ⁿ of cyanuric chloride
PCC	37.3	82	364	22.5	
PCC	42.1	90	411	21.9	
PCC (H)	37.2	188	363	51.8	30.7%
PCC (H)	39.9	210	389	54.0	
NaCl Std. (107mM)	0.1	107	107		
Blank	-	-			

(H)=hydrolysed sample

Table 2. Results of Free Chloride Titration of PCC 1900.

The titration results in Table 2 indicate that 30.7% of the PEG 1900 was modified to the extent of one cyanuric chloride molecule added. The PCC was stored under nitrogen at -70°C . Slow hydrolysis occurred and maximum storage time was one year.

Reaction of PCC with the Monoclonal Antibody

The primary amines of the antibody effected a nucleophilic substitution of one of the remaining chlorides on the PCC (Fig.13). The remaining chloride is much less reactive but there does appear to be some reaction at this site cross-linking the PEG to the protein (76). The maximum reaction time is 40 minutes because the competing hydrolysis reaction destroys the free PCC in that time. There is also some evidence that the remaining chloride of the PCC-protein conjugate is hydrolysed (77).

The degree of modification of the monoclonal antibody was estimated by assaying the primary amines before and after the linkage reaction using the fluorescamine and TNBS assays.

7. RESULTS OF THE TNBS AND FLUORESCAMINE ASSAYS

Both fluorescamine and TNBS assays gave linear standard curves for native and modified antibody (Figs.22a,22b). The correlation coefficients for both assays are significant at the 0.95 confidence level and the regression equations are shown. The degree of modification was calculated from the gradients of the standard curves.

Fig.22a. Linear regression analysis of fluorescamine standard curves for native and PEG-modified mouse IgG. The regression equations are $Y=0.02+0.368X$ for native mouse IgG and $Y=0.047+0.24X$ for PEG-modified mouse IgG, where Y = fluorescence intensity and X = protein concentration (ug/ml). The correlation coefficients are 0.95 and 0.96 respectively.

-- Native mouse IgG

◇_◇ PEG-modified mouse IgG

$$\text{Deg.Mod}^n(\text{fluorescamine}) = 1 - (0.240/0.368) = 0.35$$

$$\text{Deg.Mod}^n(\text{TNBS}) = 1 - (1.248/1.754) = 0.29$$

The TNBS assay was found to produce systematically lower results than the fluorescamine assay. This is most likely due to TNBS reacting with other nucleophiles on the protein, such as sulphydryl groups (78). PCC will also react with other nucleophiles, however it is unlikely to react to the same extent as TNBS. The TNBS assay has several practical disadvantages in that the assay uses large amounts of protein (in the order of milligrams) and is not easily reproducible. The fluorescamine assay, however, uses small amounts of protein (in the order of micrograms) and is reproducible. It has been observed that protein amino groups may fail to react with TNBS and some of the trinitrophenylated groups on proteins are in environments which perturb their absorption spectrum (79). This may account for some of the problems with reproducibility. The fluorescamine assay has a 80-95% yield of primary amine fluorescent derivatives (80).

Another advantage of the fluorescamine assay is that free PEG up to a concentration of 0.08% final concentration in the assay mixture does not interfere. This means the free PEG does not have to be removed from the sample prior to the assay. The assay therefore can be used to estimate concentrations of protein in phase systems. Free PEG does interfere with the TNBS assay and must be removed from samples prior to assay (81).

Fig.22b. Linear regression analysis of TNBS standard curves for native and PEG-modified mouse IgG. The regression equations are $Y=0.092+2.165X$ for native mouse IgG and $Y=0.032+1.547X$ for PEG-modified mouse IgG, where Y = absorbance and X = protein concentration (mg/ml). The correlation coefficients are 1.00 and 0.99 respectively.

-- Native mouse IgG

◇--◇ PEG-modified mouse IgG

The mole ratio of PEG 1900:IgG can be calculated using a nominal ratio of 90 lysines per mole (61). The degree of modification of 35% (by fluorescamine) represents the attachment of 31-32 molecules of PEG 1900 to each molecule of IgG. However, this does not take into account any PEG 1900 bound to other groups.

8. THE ACTIVITY OF THE PEG-MODIFIED ANTIBODY

The binding activity of the modified antibody was assayed as described earlier. The amount of modified antibody bound was assayed by fluorescamine and compared with the results of an ELISA run simultaneously. The results are shown in Fig.23. Although the amounts of antibody bound were very small, it appeared that the native monoclonal antibody had a 50 times greater affinity for the F_c fragment than did the PEG-modified antibody. The controls of native antibody examined by ELISA were consistent with the amount of antibody bound according to the fluorescamine assay.

The amounts of antibody bound were too small to estimate accurately with the fluorescamine assay, in the order of 0.2ng or less, but an indication of a significant loss of binding ability was apparent.

The inactivation of the antibody was most likely due to steric interactions caused by the bulk of the PEG rather than the blockage of the amine groups. This is suggested by the fact that modification with PEG 200 had little effect on rabbit anti-human erythrocyte agglutinating ability, whereas

Fig.23. Results of a binding assay for PEG-modified monoclonal IgG to rabbit IgG. A microELISA plate was coated at 1 ug/ml and the amount of native and modified protein bound to the rabbit IgG was measured by the fluorescamine assay. All concentrations are the amount of monoclonal antibody reacted with the bound rabbit IgG.

1. PEG 1900 modified monoclonal antibody (0.8mg/ml)
2. PEG 1900 modified monoclonal antibody (0.4mg/ml)
3. Native monoclonal antibody (0.9mg/ml)
4. Native monoclonal antibody (0.6mg/ml)

fluorescent units
ELISA absorbance

PEG 5000 destroyed agglutinating ability at the same degree of modification (20).

However because the partition coefficient depends exponentially on the amount of ligand bound, even small amounts of bound ligand can have a significant effect.

9. PARTITIONING OF NATIVE AND MODIFIED PROTEINS

Partitioning was performed as described using a 5,3.4 system. The partition coefficients of the monoclonal antibody and rabbit IgG, native or PEG modified shown are in Table 3. The partition of the rabbit IgG was increased from 1.0 to 1.94 by addition of 29% PEG 1900 modified monoclonal IgG but no significant increase in the partition of rabbit IgG was observed on addition of 25% modified monoclonal IgG. However, the 25% modified monoclonal antibody itself had a lower partition coefficient than the 29% modified monoclonal antibody and would be expected to have a lesser effect on the partition coefficient of the rabbit IgG. This reflects the loss in activity of the modified monoclonal antibody. The results in experiments 1 and 2 show that the modified monoclonal antibody itself has a partition coefficient 5 to 10 times that of the native antibody, yet the partition of the rabbit IgG is increased 2 times demonstrating that the binding is not as great as initially hoped for.

Table 3. Partition of Modified and Native Antibodies detected by fluorescamine

Expt.	Conc. of 25% PCC-m/c in the Phase ¹ System ¹ (ug/ml)	Conc. of 29% PCC-m/c in the Phase ¹ System ¹ (ug/ml)	Conc. of Native m/c in the Phase ¹ System ¹ (ug/ml)	Conc. of rabbit IgG in the Phase ¹ System ¹ (ug/ml)	Borate buffer ² added ² (ul)	Phosphate buffer ² added ² (ul)	Partition Coefficient of PCC-m/c or m/c	Partition Coefficient of rabbit IgG	% of total protein in the upper Phase
1	13	-	-	-	-	-	5.3 ±0.15	-	84
2	-	13	-	-	-	-	10.0 ±0.1	-	91
3	13	-	-	20	-	-	5.3 ³	1.1 ±0.1	66
4	-	13	-	20	-	-	10 ³	1.9 ±0.2	75
5	-	-	-	20	75	-	-	1.0 ±0.05	50
6	-	-	13	-	-	35	1.0 ±0.1	-	51
7	-	-	13	20	-	-	1.0 ±0.1 ⁴	1.0 ⁴ ±0.1	50
8	-	-	-	-	75	35	-	-	-

1. Detection was by O.D.²⁸⁰

2. Borate buffer (0.1M sodium borate, pH 9.0)
Phosphate buffer (0.01M sodium phosphate, pH 8.0)

3. Values assumed from expts. 1 & 2

4. Values assumed from expts. 5 & 6

The degree of modification of the monoclonal antibody with PEG 1900 was assayed by fluorescamine.
PCC-m/c = monoclonal antibody modified by attachment of PEG 1900
m/c = monoclonal antibody

10. EFFECTIVE ATTACHED PEG 1900

As defined earlier (p.16), the partition coefficient of a macromolecule bound to an affinity ligand, assuming reversible binding, can be expressed by eqn [13]. In the case of the PEG-modified monoclonal antibody, the ligand is covalently bound PEG 1900. Therefore, the association constants, K_a^T and K_a^B , are very large and the same in either phase. Eqn [13], under these conditions, simplifies to,

$$K_m = K_o K_1^n$$

Or, if n ligand molecules with partition coefficient K_1 are covalently linked to a protein with partition coefficient K_p , if each ligand molecule is fully exposed to the system and if it is assumed that all components exhibit ideal behaviour, then the partition coefficient of the modified protein, K_{p1} , can be expressed by (14),

$$K_{p1} = K_p K_1^n$$

The K_1 for radiolabelled PEG 1900 was measured as 1.88 ± 0.05 . If K_p is taken as 1.0 (Table 3) and the appropriate experimental partition coefficient is used for K_{p1} , then the number of effective attached ligands can be calculated as

shown in Table 4.

PCC:lysine molar ratio	Partition coefficient	Attached PEG mol/mol	Effective attached PEG mol/mol
0	1.0	-	
1	5.0	22-23	2-3
3	10.0	26-27	3-4

Table 4. Calculation of the Effective Amount of PEG 1900 Attached to the Monoclonal Antibody from the Partition Coefficient.

The effective amount of attached PEG 1900 was found to be seven to eight times smaller than the actual values measured by fluorescamine. A possible reason for this discrepancy is that increasing amounts of bound PEG 1900 may have less effect as they are increasingly hidden from the phase system by already attached PEG. The theory outlined earlier assumes ideal thermodynamic behaviour, which apparently does not apply when more than one ligand is bound per macromolecule. The expression should also include an activity coefficient to allow for the apparent shielding of the inner PEG molecules from the phase system.

This hypothesis is supported by the charge shielding effects demonstrated by DEAE cellulose ion exchange chromatography of PEG-modified IgG at pH 9 (data not shown). The pI of IgG is 8, so at pH 9 it is negatively charged and

binds to the column in low salt. The modified IgG's with less lysines would be expected to bind more strongly. However, only a small amount of PEG 1900 bound to the column under these conditions and most was eluted in the void volume. The native IgG actually bound to the column at a greater ionic strength than the modified IgG. From this evidence it seems likely that the bound PEG reduces the magnitude of the interaction between the phase system and the individual bound PEG molecule. It also seems possible that the same steric shielding prevents the antibody from closely associating with the rabbit IgG, accounting for the loss in activity.

The calculation of the amount of modified monoclonal antibody bound to the rabbit IgG from the partition coefficient in a similar way, requires several more assumptions. Starting with the expression,

$$K_m = K_o K_l^n (K_a^T / K_a^B)^n \quad \text{eqn [17]}$$

and assuming that $K_a^T = K_a^B$, then a value for n can be calculated from $K_{pl} = K_p K_l^n$

However, the exclusion of the ligand from one of the phases implies that the standard state chemical potential of the ligand is different in the two phases since the standard state chemical potential depends on the type and magnitude of the interaction between the solute and solvent. The standard state chemical potential is likely to be lower in the phase in which it is most concentrated since the chemical potential in each phase must be equal and the

chemical potential is dependent on the sum of the standard state chemical potential and the natural logarithm of the solute activity. This means that K_a is likely to be greater in the bottom phase than the top phase, thus reducing the ratio, K_a^T/K_a^B , suggesting that the calculated value for n is likely to be smaller than the actual value.

Using the equation, $K_{pl} = K_p K_1^n$, the effective amount of attached modified monoclonal antibody is approximately 0.3 mole per mole of rabbit IgG. Although this is likely to be a low estimate, it again reflects the loss in binding activity of the modified monoclonal antibody. No increase in the partition coefficient of the rabbit IgG was observed on adding native monoclonal IgG.

11. PARTITION OF HUMAN ERYTHROCYTES IN THE ABSENCE OF ANTIBODY

Both fresh and fixed erythrocytes were used initially in partitioning experiments. Glutaraldehyde fixation of erythrocytes is due to the reaction of membrane and intracellular proteins, mostly haemoglobin, with glutaraldehyde. This maintains the cells in their original condition as regards size, morphology and spatial relationship of organelles and macromolecules for extended periods of time (82). Fixed cells were more convenient to use and provided a reproducible supply of cells, for these reasons they were used in all the experiments reported in this thesis. Another advantage of working with fixed cells

was the decreased tendency of fixed cells to aggregate in the phase system compared to fresh cells. This is thought to be due to the cells decreased ability to deform.

The addition of 0.01M phosphate, pH 8 used to buffer the proteins had a significant effect on the partition of erythrocytes (Table 5). For this reason, it was especially important to run controls with every experiment. As can be seen from Table 5 phosphate had a far greater effect than NaCl. All the systems had similar total ionic strengths of 84mM $\pm$ 4mM.

0.1M Na ₂ B ₄ O ₇ , pH 9 added	0.01M phosphate added	Fraction of cells in upper phase	Partition coefficient
75ul	-	3%	0.03
-	35ul	9%	0.10
75ul	35ul	11%	0.12

Table 5. The Effect of Buffers on Erythrocyte Partition

The increase in erythrocyte partition is due to the phosphate ions partitioning to the lower dextran rich phase causing an electrostatic potential between the two phases (83) and increasing the surface tension. The NaCl and most likely the Na₂B₄O₇ ions, since they have a small effect on

erythrocyte partition, partition equally between the two phases and the system without phosphate is electrically neutral. The phosphate containing system (10mM) is about 2mV more positive in the upper phase (83). Due to this potential the phosphate containing system is charge sensitive and the negatively charged erythrocyte will partition into the upper phase (83).

Because the systems used were close to the critical point, small unavoidable fluctuations in conditions caused the partition of erythrocytes to vary slightly from experiment to experiment. To make the partition reproducible, sufficient stock solution of PEG (approximately 30%w/w) was added to the controls and experiments in each run to make the control partition coefficient approximately 10% so that any increase in partition could be observed clearly.

The effects of antibodies on erythrocyte partition are summarised in Table 6. It seems that only the combination of rabbit IgG and PEG-modified monoclonal antibody increased the partition of erythrocytes. All other combinations of monoclonal antibody and/or rabbit IgG had no effect or decreased erythrocyte partition. The small decrease in erythrocyte partition caused by the native rabbit IgG with or without monoclonal antibody was probably due to the aggregation of erythrocytes by the rabbit IgG increasing the rate of settling to the interface or bottom phase. The decrease in partition caused by the native monoclonal

antibody is statistically insignificant. The modified monoclonal antibody had no effect in the absence of rabbit IgG. Although this is a small increase in partition, it is sufficient to produce separation in a counter current distribution apparatus with twenty transfers.

12. THE EFFECT OF THE ANTIBODIES ON ERYTHROCYTE PARTITION

Expt.	Amount of antibody added	Amount of buffer added	% cells in upper phase	P.C.	Ratio of partition increase
1	PCC-m/c rabbit IgG	-	31 $\pm$ 4	0.45	3.75
2	-	phosphate borate	11 $\pm$ 2	0.12	1
3	PCC-m/c	borate	11 $\pm$ 2	0.12	1
4	m/c	phosphate	7 $\pm$ 1	0.08	-0.7
5	rabbit IgG	borate	7 $\pm$ 1	0.08	-0.7
6	m/c rabbit IgG	-	10 $\pm$ 2	0.11	0.9

P.C. = Partition Coefficient

PCC-m/c = PEG 1900-modified monoclonal antibody

m/c = native monoclonal antibody

In all cases the following amounts were used: 91 μ l phosphate buffer (0.01M, pH 7.2), 73 μ l borate buffer (0.1M, pH 9.0), 0.37 mg native or cyanuric chloride modified monoclonal antibody and 0.48 mg rabbit IgG.

The above are the average results taken over 10 experiments.

Table 6. The Effect of Antibodies on Erythrocyte Partition

The theoretical treatment applied to the effect of an affinity ligand on a solute cannot be applied to particle affinity partition because the number of binding sites is much greater and the concept of chemical potential of the particle does not apply. The change in surface free energy of a particle on binding a ligand, i , is obtained instead by integrating the Gibbs equation (84),

$$d\gamma = -\sum \Gamma_i d\mu_i \quad \text{eqn[18]}$$

where Γ_i = surface excess of the i th component.

$d\mu_i$ = the change in chemical potential of the i th component on binding.

To find the difference in surface free energy difference of an erythrocyte between top and bottom phases on adding a ligand, eqn [18] must be integrated from 0 to the ligand concentration and the surface free energy difference for an erythrocyte in the top phase subtracted from that in the bottom phase. If it is assumed that the binding of the ligand does not affect the particle surface area and only the ligand has a significant effect on the particle surface free energy, then this gives,

$$d\Delta\gamma = \Gamma_1^B d\mu_1^B - \Gamma_1^T d\mu_1^T \quad \text{eqn [19]}$$

where 1 refers to the ligand.

The chemical potential of the ligand in either phase is given by $\mu = \mu^\circ + kT \ln C$.

Assuming that the surface excess of ligand, Γ_1 , obeys the Langmuir binding isotherm (85) for n independent binding

sites per unit area (cm^2), i.e. $n^T = (nC^TK^T)/(1+C^TK^T)$ for the top phase, then the integration and evaluation of the limits gives the following (84),

$$\Delta\gamma_1 - \Delta\gamma_0 = nkT \ln(1+C^BK^B)/(1+C^TK^T) \quad \text{eqn [20]}$$

where n = the saturation value of bound ligand

$\Delta\gamma_1, \Delta\gamma_0$ = surface free energy difference between the phases in the presence, absence of a ligand

Knowing that $C^T/C^B = K_1$ then,

$$\Delta\gamma_1 - \Delta\gamma_0 = nkT \ln(n^TK^B)/(n^BK^TK_1) \quad \text{eqn [21]}$$

If it is assumed that $K^T = K^B$ and that the ligand is an irreversibly bound complex of modified monoclonal antibody and rabbit IgG (these assumptions were discussed earlier), then the total number of bound ligands can be estimated from the erythrocyte surface free energy change on binding the ligand. This is estimated from the erythrocyte partition coefficients with and without ligand. These were measured experimentally and are listed in Table 6. The equation for particle partition is,

$$K = n_1/n_2 = \exp(-\Delta E/kT) \quad \text{eqn [14]}$$

This expression assumes that cell partition is a thermal equilibrium process. In fact this is not the case as discussed on p.17-20. Characteristic erythrocyte partition energies were found to be larger than predicted by this equation, in the order of $2-20 \times 10^4 kT$ per cell by K. Sharp (26). The value of 10^5 therefore is used to scale the Boltzmann constant throughout the cell surface free energy

calculations.

It is straightforward to show from thermodynamic considerations that (26):

$$\Delta E = (A\gamma_{TB}/4kT)(1-\Delta\gamma/\gamma_{TB})^2 B \quad \text{eqn [22]}$$

where A = particle surface area

γ_{TB} = interfacial tension between top and bottom phase

$\Delta\gamma$ = difference in cell surface free energy between the phases

therefore

$$\ln K = (A\gamma_{TB}/4bkT)(1-\Delta\gamma/\gamma_{TB})^2 \quad \text{eqn [23]}$$

where b denotes the empirical factor of 10^5 to scale the Boltzmann constant to allow for the non-thermal distribution of erythrocytes. The surface area of an erythrocyte is $1.4 \times 10^{-6} \text{ cm}^2$ and the interfacial tension of the phase system used was $4 \times 10^{-4} \text{ erg/cm}^2$.

a. Calculation of $\Delta\gamma_0$, the erythrocyte surface free energy difference without ligand. The value for K_0 is 0.12 from expt. 2, Table 6. Substituting these values into eqn [23] gives,

$$\begin{aligned} \ln 0.12 &= (1.4 \times 10^{-6} \times 4 \times 10^{-4}) / (4 \times 10^5 \times 1.38 \times 10^{-16} \times \\ &\quad 298) \{1 - \Delta\gamma_0^2 / (1.4 \times 10^{-6})^2\} \\ \Delta\gamma_0 &= 1.11 \times 10^{-5} \text{ erg/cm}^2 \end{aligned}$$

b. Calculation of $\Delta\gamma_1$, the erythrocyte surface free energy with the maximum bound ligand. The value for K_1 is taken from expt. 1, Table 6.

$$\begin{aligned} \ln 0.45 &= (1.4 \times 10^{-6} \times 4 \times 10^{-4}) / (4 \times 10^5 \times 1.38 \times 10^{-16} \times \\ &\quad 298) \{1 - \Delta\gamma_1^2 / (1.4 \times 10^{-6})^2\} \end{aligned}$$

$$\Delta\gamma_1 = 0.68 \times 10^{-5} \text{ erg/cm}^2$$

By substituting these values for $\Delta\gamma^0$ and $\Delta\gamma_1$ into eqn [21] and assuming that $K_a^T = K_a^B$ and the number of bound ligands is above saturation, i.e. $n^T = n^B$, then n , the effective number of ligands bound, can be estimated. The value of K_1 is taken from expt. 4, Table 3.

The correction factor for k is no longer required because the ligand is in solution and the principles of thermal equilibrium can be applied.

$$(0.68 - 1.11) \times 10^{-5} = n \times 1.38 \times 10^{-16} \times 298 \times \ln 1/1.9$$

$$n = 1.63 \times 10^8 \text{ cm}^{-2}$$

As the surface area of an erythrocyte is $1.4 \times 10^{-6} \text{ cm}^{-2}$, then the effective number of ligands bound per cell = $1.63 \times 10^8 \times 1.4 \times 10^{-6}$

$$= 228 \text{ ligands bound per cell}$$

The saturation level of the erythrocyte for rabbit IgG was found to be 8×10^5 molecules per cell in PBS (personal communication - J. Janzen). Although a direct comparison between K_a for the rabbit IgG in PBS and phase system cannot be made, this suggests that many rabbit IgG molecules are bound to the erythrocyte and a greater increase in partition could result by increasing K_a for the modified monoclonal antibody.

13. THE EFFECT OF ALTERING THE RATIOS OF ANTIBODIES

PCC-m/c added (ul)	Rabbit IgG added (ul)	P.C.	Control P.C.	Ratio of partition increase	Effective attached PEG (mol/mol)
35	65	0.25	0.1	2.5	1.5
65	35	0.59	0.12	4.9	2.5
70	30	0.42	0.12	3.5	2.0
80	20	0.18	0.08	2.25	1.3

P.C. = Partition Coefficient

The antibody concentrations used were as follows:

0.37mg/ml PCC-m/c in 0.1M borate (pH 9.0)

0.48mg/ml rabbit IgG in 0.01M phosphate (pH 7.2)

Controls contained only buffer in the same amounts as the experiment.

The above results are combined from two experiments done in duplicate.

Table 7. The Effect of Altering the Relative Amounts of Antibodies on the Partition of Human Erythrocytes

The results in Table 7 demonstrate that the partition of erythrocytes increases with increasing PEG-modified monoclonal IgG concentration up to a maximum ratio of 65:35 modified monoclonal IgG:rabbit IgG. This was interpreted as indicating that the decreasing rabbit IgG concentration

limited the number of binding sites for the modified monoclonal antibody. The optimum ratio (w/w) found within this range of modified monoclonal IgG to rabbit IgG was 2. The polyclonal antibody was found to be 8% active by affinity chromatography. This is a consequence of it's polyclonal origins. A similar degree of activity was found by binding studies with erythrocytes (results not shown). This suggests that, for the preparations used, the optimum ratio within this range of modified monoclonal antibody : active rabbit IgG is actually closer to 65:3 or 17:1(w/w). If the modified antibody were more active, then this ratio would decrease as more modified monoclonal antibody bound to rabbit IgG.

Preincubation of the two antibodies at 37°C, with or without erythrocytes, did not alter the erythrocyte partition, the increase was seen when all components were present. This is a true equilibrium as the result was independent of the order in which the reagents were added.

14. THE EFFECT OF THE ANTIBODIES ON RABBIT ERYTHROCYTE
PARTITION

PCC-m/c added (ul)	Rabbit IgG added (ul)	Erythrocyte species	% of cells in the upper phase	Partition coefficient
65	35	human	35	0.53
-	-	human	10	0.11
65	35	rabbit	12	0.14
-	-	rabbit	12	0.14

All antibody concentrations and controls are as defined for Table 7.

Table 8. The Effect of Antibodies on Human and Rabbit Erythrocyte Partition

The combination of modified monoclonal antibody and rabbit IgG had no effect on the partition of rabbit erythrocytes, whereas the partition of human erythrocytes was tripled under the same conditions as shown in Table 7. The same result was obtained using a mixture of radiolabelled human erythrocytes and rabbit erythrocytes. In this case the upper phase became enriched with human cells. This verifies the specificity of the technique and confirms that the partition increase of human erythrocytes by modified monoclonal

antibody and rabbit IgG is not due to non-specific binding.

15. SUMMARY

The partition of erythrocytes was increased significantly by a combination of PEG 1900-modified monoclonal IgG and anti-erythrocyte rabbit IgG. However, this partition increase was similar in magnitude to that obtained by direct modification of the rabbit IgG (20). Although the use of the monoclonal antibody has several intrinsic advantages, most importantly its versatility and homogeneity, it was also hoped that the second antibody technique might result in a greater partition increase.

The monoclonal antibody was demonstrated to undergo a significant loss in binding activity on modification with activated PEG 1900. As the partition coefficient is dependent on the exponent of the number of bound ligands, any increase in the binding activity of the modified monoclonal antibody should result in a large increase of the partition coefficient. The power law dependence of partition also means that the greatest increase in partition would be observed if the control partition was close to 1. In this case, $(\exp)-\Delta E/kT$ tends to 1 therefore $-\Delta E/kT$ tends to 0 and the system will be sensitive to a small change in cell surface free energy on binding the ligand. If the control partitions had been manipulated to approximately 1 instead of 0.1, possibly a greater partition increase would have been observed.

There are at least two possible approaches by which the partition coefficient may be increased. One is to protect

the antibody binding site during the modification reaction or to enrich the proportion of active molecules remaining after modification. The other approach is to look at alternative chemistry to cyanuric chloride attachment of the PEG. Other methods of modifying proteins have been extensively documented and there is evidence in some cases, for instance enzyme modification, that some methods allow a higher retention of enzyme activity than cyanuric chloride attachment. Such methods include:

1. Formation of a 1,1'-carbonyldiimidazole activated PEG subject to nucleophilic attack by protein primary amines (23).

2. Formation of a PEG ester with succinamide followed by a nucleophilic substitution of the PEG succinate with protein primary amines (78).

3. Reaction of a PEG salt with dithiocarbonate, followed by nucleophilic displacement of a C-S bond by protein primary amines (22).

4. A direct route via reductive amination of PEG aldehydes by protein primary amines (86).

All of these methods involve modification of the primary amines. Glass has developed a method using cysteine sulphydryl groups (87) and Pollak has reacted a PEG amine and protein in the presence of a carbodiimide hydrochloride to yield amide linkages (88).

If the coupling chemistry is crucial to retention of activity, it implies that the chemical nature of the group

modified determines the effect on protein activity. If the loss of binding activity is due instead to steric interference between bound PEG and the antigen, as suggested by the evidence quoted in the thesis, it is unlikely that the coupling chemistry is the most important factor in maintaining activity. Hence, although investigation into alternative ways of modifying the antibody may prove fruitful, it seems that the steric interference with binding is the root of the problem. For this reason I would suggest that protection of the active site during modification or enrichment of active molecules is the direction that future research should take.

One method by which this might be done would be to modify the whole complex of monoclonal and rabbit IgG in solution, then dissociate the bond and separate the antibodies by chromatography or gel filtration. The problem with this method is that an ion exchange column cannot be run under dissociating conditions and the two molecules are too similar in size to be separated by gel filtration. The F_c fragment alone is not pure, only enriched so if this were used the sample would still be contaminated with rabbit IgG and pose the same problem, although gel filtration chromatography may resolve the fragments.

A more productive approach might be to immobilize the antigen on a bead, bind the antibody, couple the PEG, then dissociate the antigen-antibody bond and collect the modified antibody.

CONCLUSION

It was demonstrated that a monoclonal antibody raised against the F_c fragment of rabbit IgG and modified with cyanuric chloride attachment of PEG 1900 in combination with rabbit IgG raised against NN glycophorin A could increase the partition of human erythrocytes in a two phase aqueous polymer system containing PEG and dextran. The partition was not altered by any of the components alone nor was the partition of rabbit erythrocytes affected by the combination of all three components.

It seems that monoclonal antibodies and two phase aqueous polymer systems are potentially useful for biologically specific cell separations.

GLOSSARY OF TERMS

Antigenicity - an indication of the reactivity of an antigen with an antibody.

B Cells - lymphocytes localised in lymphoid organs and the spleen. They bind antigens by means of surface antibodies which stimulates the B cell to divide and differentiate. Initial stimulation causes IgM secretion, followed by other immunoglobulins.

Blast transformation - stimulation of the lymphocyte provoking transformation to a rapidly dividing state.

Catabolic regulation - control of degradative metabolic processes.

Complement fixation - a chain reaction initiated by C₁ component of complement binding to the F_C fragment of bound antibodies. The complement system results in cytolysis of foreign particles.

Dextran - poly $\alpha(1,6)$ D glucose.

Ficoll - a synthetic copolymer of sucrose and epichlorohydrin.

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

κ light chain - one of two types of IgG light chain, the other is λ . The chains differ considerably in amino acid sequence and antigenicity.

Immunogenicity - a measure of the effectiveness of a foreign substance to provoke an immune response.

Immunosuppression - inhibition of the immune response

Macrophage fixation - stimulation of macrophage production. Macrophages destroy foreign particles, process the antigen for the immune response and act as secretory cells.

Membrane transmission - formation of holes in the cell membrane of the antigen by a membrane-attack complex formed as a result of complement fixation. Lysozyme completes the antigen destruction by rupturing the inner cell membrane.

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

Neuraminic acid -

5-amino-3,5-dideoxy-D-glycero-D-galactonoulosonic acid.

PEG - poly(ethylene glycol)

Plasmacytoma - see myeloma.

Sialoglycoprotein - protein bound to carbohydrate and sialic acid. Sialic acids are the N and O acetyl derivatives of neuraminic acid.

GLOSSARY OF SYMBOLS AND ABBREVIATIONS

A	particle surface area
F	Faraday constant
f	molal activity coefficient
K	partition coefficient
K_a	association constant
k	Boltzmann's constant, 1.36×10^{-16} erg/molecule.K°
M_n	number average molecular weight
M_w	weight average molecular weight
N_A	Avogadro's number
P	number of polymer segments (the ratio of polymer molecular volume to solvent molecular volume)
PEG X	poly(ethylene glycol), mol. wt. approx. X g/mole
R	Molar gas constant, 8.314×10^7 erg/mole.K°
z	lattice coordination number
χ	Flory interaction parameter
Γ_i	surface excess of the ith component
γ	surface free energy
γ_{TB}	interfacial tension between the phases
ΔE_{TI}	free energy of particle/interface attachment
ΔE_{TB}	free energy of particle transfer between phases
ΔG_m	free energy of mixing
ΔH_m	enthalpy of mixing
ΔS_m	entropy of mixing
$\Delta \gamma$	surface free energy difference between phases
$\Delta \gamma_1, \Delta \gamma_0$	surface free energy difference between phases in the presence, absence of a ligand

$\Delta\mu_{i,j}^{\circ}$ difference in standard state chemical potentials
between the phases

μ°, μ standard state chemical potential, chemical potential

ϕ volume fraction

$\psi, \Delta\psi$ Galvani, or inner potential, potential difference
between the phases

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