AN IMMUNOCHEMICAL INVESTIGATION OF THE Wr^a AND Wr^b BLOOD GROUP ANTIGENS.

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ABSTRACT

This thesis describes the production of a monoclonal antibody to the low incidence blood group antigen Wr^a which is thought to be allelic to Wr^b (a high frequency antigen known to be associated with glycophorin A and band 3).

The main approach was to immunise mice with Wr(a+) red cells and select appropriate antibodies by screening them against a panel of red cells by haemagglutination. One anti-Wr^a antibody (BGU1-WR) was found from the 1074 hybridomas screened. BGU1-WR belongs to immunoglobulin subclass IgG1 and has an affinity constant of 1.82×10^7. This antibody and a previously described anti-Wr^b monoclonal antibody was used to investigate the nature of the antigens.

Haemagglutination studies showed that both antigens are resistant to proteinase treatment, probably do not involve sialic acid and do not require intact disulphide bonds on native red cells.

The site number of the Wr^a and Wr^b antigens was determined by direct binding of ^{125}I-labelled antibody to red cells. Wr(a+b+) cells were shown to have approximately 70,000 Wr^a and 150,000 to 350,000 Wr^b sites per cell.

The possible expression of these antigens on other cells was investigated using flow cytometry. The Wr^a antigen was not found on leucocytes from a Wr(a+) donor or on 19 cell lines of different cell and tissue origin of unknown Wr^a phenotype, including 2 erythroid-like lines. Wr^b was only found on 2 myeloid-like cell lines.

SDS-PAGE, immunoblotting and immunoprecipitation were used to investigate the molecular structure of the antigens. Neither antibody recognises the denatured, SDS-treated antigens under the wide variety of conditions used. Numerous immunoprecipitation experiments showed that anti-Wr^b immunoprecipitates glycophorin A and band 3. Under identical conditions anti-Wr^a did not immunoprecipitate any detectable component of the red cell membrane.

Thus the Wr^a antigen appears to differ from the Wr^b antigen casting doubt on the antithetical relationship of these antigens.

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ABBREVIATIONS.

AABB	American Society of Blood Banks.
AET	2-aminoethylisthiouronium bromide.
AGG	Direct agglutination test.
AIHA	autoimmune haemolytic anaemia
BSA	bovine serum albumin.
DAB	diamino benzidine.
DAF	decay accelerating factor.
DMEM	Dulbecco's minimal Eagles medium.
DMS	di-methyl-suberimerimidate.
DMSO	dimethylsulphoxide.
EBV	Epstein-Barr virus.
EDTA	ethylenediaminotetraacetic acid
ELISA	enzyme linked immunoabsorbant assay.
En^aFR	En^a ficin resistant.
En^aFS	En^a ficin sensitive.
En^aTS	En^a trypsin senstive.
FCS	fetal calf serum.
FITC	fluorescein isothiocyanate.
Fuc	L-fucose.
Gal	D-galactose.
GalNAc	N-acetyl-D-galactosamine.
GlcNAc	N-acetyl-D-glucosamine.
GPA	glycophorin A.
GPC	glycophorin C.
G3PD	glucose 3 phosphate dehydrogenase.
GPS	glutamine, penicillin and streptomycin.
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid.
HDN	haemolytic disease of the newborn.
HGPRT	hypoxanthine guanine phosphoribosyl transferase.
HM	hybridoma medium.
HMT	hypoxanthine, methotrexate and thymidine.
IAT	indirect antiglobulin test.
Ig	immunoglobulin.
ISBT	International Society of Blood Transfusion.
K	functional affinity constant.
MEM	Eagle's minimal essential medium.

M_r	relative molecular mass.
NHS-biotin	N-hydroxysuccinimidobiotin.
nt	not tested.
PAGE	polyacrylamide gel electrophoresis.
PAS	periodic Schiffs reagent.
PBAST	PBS containing albumin and Tween-20.
PBS	phosphate buffered saline.
PEG	polyethylene glycol.
PMSF	phenylmethane sulphonyl fluoride.
RAM	rabbit anti-mouse immunoglobulin.
RDE	receptor destroying enzyme - (neuraminidase).
RIA	radioimmune binding assay.
RPMI/OS	RPMI medium without FCS.
SDS	sodium dodecyl sulphate.
TRIS	tris(hydroxymethyl)aminomethane.
$X63_{sec}$	P3/X63-Ag8 cell line.

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CHAPTER 1.

INTRODUCTION.

Blood group antigens are components on the red cell membrane surface which are detected by or stimulate the production of specific antibodies. Our understanding of the structure of these antigens has been greatly assisted by the use of monoclonal antibodies. Monoclonal antibodies have also been used to show that certain of these antigens are expressed on cells other than erythrocytes, which has in some cases helped to expand our knowledge of their structure and genetic control.
This thesis describes the production of a monoclonal antibody which recognises the low incidence red cell antigen Wr^a and the use of this antibody to investigate the nature of Wr^a. The properties of Wr^a are compared with those of the high frequency antigen Wr^b in the hope that this comparison will shed some light on the proposed antithetical relationship of these two antigens and their relationship to glycophorin A.

1.1. Blood Group Antigens.

The first red cell antigens to be described were those of the ABO system in 1901, when Landsteiner discovered that the red cells of some individuals were agglutinated by serum from others (Landsteiner, 1901). These person to person differences were subsequently shown to result from genetically determined polymorphisms. Polymorphism in this context can be defined as "the occurrence of two or more alleles for a given locus in a population where at least two alleles appear with frequencies of more than one percent" (Bodmer and Cavalli-Sforza, 1976).
Since 1901 over 300 inherited red cell antigens have been discovered. Some are polymorphic, others are either of high incidence (greater than 99%) or low incidence (less than 1%) and yet others are monomorphic in a given population.

1.1.1. Discovery of Blood Group Antigens.

The large number of red cell antigens that are known can be attributed to the availability of red cells from blood samples and the involvement of the antigens in haemolytic disease of the newborn (HDN) and transfusion reactions. After the discovery of the ABO antigens a deliberate attempt was made to find other polymorphic genetic markers on red cells. Landsteiner and Levine (1927 a and b) injected animals with human red cells in the hope of producing agglutinating antibodies which defined polymorphic red cell antigens. This work resulted in the

discovery of the M and N antigens. The introduction of the antiglobulin test (Coombes *et. al.*, 1945) which can detect non-agglutinating antibodies, led to the discovery of many more antigens recognised by human immune antibodies. Cells coated with an antibody are washed and agglutination is facilitated by the addition of an anti-human immunoglobulin. This method detects alloantibodies which are made by individuals immunised by an antigen not present on their own red cells, during pregnancy or transfusion. The antibodies usually came to light during compatibility testing prior to transfusion, antenatal testing or during investigations of HDN. Other red cell antigens were detected using seed or animal extracts which agglutinate human and animal cells due to the carbohydrate binding lectins present in them. For example, the agglutinin found in *Dolichos biflorus* has anti-A_1 specificity and can be used to distinguish between the A antigen variants, A_1 and A_2 (Bird, 1952). In recent years monoclonal antibodies have led to the discovery of some previously unknown red cell antigens. The MER2 red cell polymorphism was detected in such a way when monoclonal antibodies, produced following immunisation of mice with cells of a human small-cell lung carcinoma line, were shown to react with some red cells and not others (Daniels *et. al.*, 1987).

1.1.2. Significance of Blood Group Antigens.

1.1.2.1.. Clinical Significance.

Certain blood group antigens are of considerable clinical significance particularly in relation to transfusion and haemolytic disease of the newborn (HDN), the most important being those of the ABO and Rh systems. Transfusion reactions occur when a recipient is given red cells which react with an antibody present in their serum, although compatibility testing of donor and patient blood prior to transfusion normally prevents this. Some antibodies such as anti-A and anti-B occur naturally and are always found in the plasma of individuals whose cells do not express the relevant antigen. Most other antibodies are produced following stimulation by exposure to the antigens. Red cell antigens are also important in diseases such as autoimmune haemolytic anaemia (AIHA) where a patient produces autoantibodies which recognise antigens on their own red cells thus causing accelerated red cell destruction and anaemia. Haemolytic disease of the newborn (HDN) occurs when a mother's red cells lack antigens carried by those of her fetus. The most common cause of severe HDN were antibodies to the Rh antigen D. A D negative mother can become exposed to the red cells of a D positive baby, usually during birth, and form anti-D. During subsequent pregnancies IgG anti-D can cross the placenta and react with the red cells of a subsequent D positive baby, resulting in anaemia. This was a relatively common

occurrence due to the high immunogenicity of the D antigen, 80% of D negative individuals transfused with D positive blood form anti-D, although a much lower percentage (less than 10%) of D negative women produce anti-D following exposure to D positive cells during pregnancy (Mollison, 1972). Now the incidence of HDN has been greatly reduced due to the administration of anti-D to D negative mothers soon after delivery. This removes D positive fetal red cells and prevents formation of maternal anti-D. Antigens other than those from the ABO and Rh systems can cause transfusion reactions and HDN but to a lesser extent than those from the ABO and Rh systems.

1.1.2.2. Genetical Significance.

Blood group polymorphisms have made a significant contribution to the field of human genetics. They provided the first examples of single gene genetic markers that were not associated with inherited diseases or affected by environment. The *Xg* locus was the first non-disease locus to be located on the X chromosome (Mann *et. al.*, 1962). The Lutheran blood group system and secretor loci provided the first example of human autosomal linkage (Mohr, 1951) and the first example of a human autosomal gene assignment was the Duffy blood group locus to chromosome 1 (Donahue *et. al.*, 1968).

Red cell antigens are useful genetic markers in parentage disputes and forensic work. The gene frequencies of alleles encoding red cell antigens are different in different populations, for example the Ge antigen has a high frequency in most populations but is polymorphic in Melanesian populations (Booth *et. al.*, 1970). Such variation has been exploited in population and physical anthropology studies.

1.1.3. Classification of Blood Group Antigens.

Inherited blood group antigens have been assigned to blood group systems, collections or series by the ISBT working party (Lewis *et. al.*, 1990 and 1991). There are 21 blood group systems, examples of which include the ABO, MNS and Rh systems. A system is composed of antigens which are controlled by a single gene locus or by tandemly arrayed highly similar genes, as is the case with the MNSs system (section 1.3.3.1). Collections are composed of antigens which have been shown to be related by serological, biochemical or genetic means but for which not enough evidence is available for them to warrant system status. Antigens which are not polymorphic and have not yet been assigned to systems or collections are classified according to their frequencies into the high or low incidence series.

1.1.3.1. Low Incidence Antigens.

Low incidence antigens were originally identified by serological techniques using polyspecific human antisera. Some low incidence antigens have been shown to be part of known blood group systems. When haplotypes can be distinguished and are reasonably polymorphic, such as in the Rh and MNS systems, the antigens can be classified as part of these systems by family studies. Such assignments have been confirmed by biochemical studies for many low incidence MNS antigens.

It is often difficult to show that other low incidence antigens are part of an established blood group system. For example, the antigens (K, Kp^a, Kp^c, Js^a, Wk^a, Ul^a) in the Kell system are low incidence antigens in some populations but polymorphic in others. Studies of populations in which two of these markers were polymorphic (for example, of K which is polymorphic in whites but low incidence in blacks and of Js^a which is polymorphic in blacks but a low incidence antigen in whites) suggested linkage disequilibrium between their genes. Family studies of K and Js(a+) propositi, deliberately sought, showed that Js^a was part of the Kell blood group system (Stroup *et. al.*, 1965). Later biochemical studies confirmed this observation.

Such an approach is unrewarding for antigens which are of low incidence in all populations and unless a low incidence antigen is antithetical to a high incidence antigen family studies are rarely informative. However, serological data supported by biochemical information can suggest an assignment of a low incidence antigen to a blood group system. For example, the low incidence antigen An^a has been located on glycophorin D by immunoprecipitation and immunoblotting (Daniels *et. al.*, 1990) and the nucleotide sequence of RNA from an An^a individual has revealed a point mutation in the glycophorin D gene sequence (King *et. al.*, 1991). This has resulted in An^a becoming part of the Gerbich blood group system, associated with glycophorins C and D. Other low incidence antigens, Wb, Ls^a, and Dh^a, have also been shown to be part of this system (Reid *et. al.*, 1985; MacDonald *et. al.*, 1990; Spring *et. al.*, 1990).

Low incidence antigens not known to be part a blood group system are often called "private" antigens. Race, Sanger and Cleghorn (Race and Sanger, 1975) classified private antigens as being Mendelian dominant characters, defined by specific antibodies; they must have an incidence of less than 1 in 400 in a given population and have been shown to be independent from the known blood group systems.

Thirty-six Private antigens exist which have been numbered as the 700 series by the ISBT working party (Lewis *et. al.*, 1990 and 1991).

Private antigens can be of clinical significance as some have been shown to cause HDN or transfusion reactions. Transfusion reaction, however, is a rare occurrence due to compatibility testing, and the antibodies to low incidence antigens do not cause any problems in supplying compatible blood because so few donors carry these antigens.

1.1.3.2. Antibodies to Low Incidence Antigens.

Antibodies to low incidence antigens occur as immune antibodies in the sera of mothers of babies suffering from HDN or patients who have had transfusion reactions. However, "naturally occurring" antibodies also exist, that is non-red cell immune antibodies, which occur in serum of individuals who have never been exposed to red cells carrying private antigens. AIHA patients often produce antibodies to a large number of low incidence antigens as well as autoantibodies in their serum. More occasionally normal healthy donors provide multispecific antisera, for example the serum of a donor named Tillet has been shown to contain at least anti-Mi^a, -Vw, -Mt^a, -Bp^a, -Gf, -Go^a,- Mo^a, -Or^a, -RH32, -Tr^a, -Wr^a, -Evans, and -Pt^a (Contreras *et. al.*, 1978). Reasons for the occurrence of these antibodies in apparently unexposed individuals is as yet unknown.

1.1.4. Nature of Red Cell Antigens.

The molecular nature of many red cell antigens is now known. Some antigens are carbohydrate structures carried on membrane proteins or lipids. Other antigens are dependent on the amino acid sequence of the proteins themselves.

Further discussion of the nature of red cell antigens is difficult without a description of the red cell membrane components which follows in the next sections.

1.2. Erythrocyte Membrane Structure.

The human erythrocyte membrane has been well studied due to the availability of red cells and the ease with which membranes can be prepared by hypotonic lysis and washing of red cells (Dodge *et. al.*, 1963). The structure and abundance of various membrane components is well understood and many red cell antigens have been assigned to particular red cell membrane components.

The red cell membrane is composed of approximately 45% lipid, 45% protein and 10% carbohydrate (Anstee, 1986). Phospholipid makes up about 65% of the membrane lipid and is distributed asymmetrically in the lipid bilayer, sphingomyelin and phosphotidylcholine are in the outer leaflet and phosphotidyl ethanolamine and phosphatidyl serine in the inner leaflet (Bretscher, 1973). A further 30% of the lipid is cholesterol which is located in the hydrophobic core of the membrane. The remaining lipid is made up of extracellularly located glycolipid. Membrane carbohydrate is all located extracellularly covalently linked to proteins as O- or N- glycans or to lipids as glycolipids.

1.2.1. Red Cell Membrane Proteins.

Red cell membrane proteins have been well characterised using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and staining procedures (Fairbanks *et. al.*, 1971). The protein bands stained with Coomassie Brilliant Blue have been named according to their migration on SDS-PAGE gels (Steck, 1974); some also have more informative names based on their function. Table 1.1. lists the major red cell membrane proteins, their apparent molecular weights (M_r) and synonyms used to describe them. Proteins with a high sialic acid content are poorly detected using Coomassie Brilliant Blue staining. The major sialic acid containing red cell membrane proteins are therefore detected by periodic acid-Schiff (PAS) staining (Fairbanks *et. al.*, 1971). The various nomenclatures used to describe these proteins are given in Table 1.2. Throughout this thesis the "glycophorin" nomenclature of Furthmayer *et. al.* (1978) is used.

Red cell membrane proteins can be described as peripheral or integral membrane proteins (Singer, 1974). The peripheral proteins are easily extracted from the lipid bilayer by treatments such as pH manipulation. The major peripheral proteins form the cytoskeleton (section 1.2.1.2). The integral membrane proteins are strongly associated with the lipid bilayer due to hydrophobic interactions between the protein and the hydrophobic core of the bilayer. These proteins can only be removed with the use of strong detergents or solvents. Blood group antigens are carried on integral membrane

proteins such as the glycophorins and band 3. These proteins are described in more detail below.

TABLE 1.1.
RED CELL MEMBRANE PROTEINS DETECTED BY COOMASSIE BRILLIANT BLUE STAINING.

POLYPEPTIDE	APPARENT M_r	SYNONYM
BAND 1	260,000	SPECTRIN α-CHAIN
BAND 2	225,000	SPECTRIN ß-CHAIN
BAND 2.1	215,000	ANKYRIN
BAND 3	93,000	ANION TRANSPORT PROTEIN
BAND 4.1a	80,000	
BAND 4.1b	78,000	
BAND 4.2	72,000	
BAND 4.5	55,000	GLUCOSE TRANSPORTER
BAND 5	43,000	ACTIN
BAND 6	35,000	G3PD*
BAND 7	29,000	

This table lists the major proteins detected by Coomassie Brilliant Blue staining following SDS-PAGE of red cell membranes. The numerical nomenclature based on electrophoretic mobility of Steck (1974) is shown as well as more informative synonyms for some proteins. Data also taken from Bennett (1985).

* - G3PD - Glyceraldehyde 3 phosphate dehydrogenase

TABLE 1.2
PAS STAINING OF RED CELL MEMBRANE PROTEINS.

FURTHMAYER	ANSTEE *et. al.*	APPARENT M_r
GLYCOPHORIN A	SIALOGLYCOPROTEIN α	37,000
GLYCOPHORIN B	SIALOGLYCOPROTEIN δ	25,000
GLYCOPHORIN C	SIALOGLYCOPROTEIN ß	35,000
GLYCOPHORIN D	SIALOGLYCOPROTEIN γ	27,000

This table lists the major sialic acid containing proteins of the red cell membrane as detected by Periodic acid Schiff staining after SDS-PAGE. The synonyms sialoglycoprotein (Anstee *et. al.*, 1979) and glycophorin (Furthmayer *et. al*, 1978) have both been used to describe these proteins. The "glycophorin" nomenclature is used throughout this thesis. The molecular weight data was taken from Dahr (1986).

1.2.2. The Red Cell Membrane Cytoskeleton.

The major red cell peripheral proteins interact together to form the cytoskeleton as illustrated in Figure 1.1. The cytoskeleton interacts with the integral proteins in the lipid bilayer to provide a tough but flexible membrane.

The major component of the cytoskeleton is spectrin (bands 1 and 2). Spectrin is composed of two polypeptides, the α-chain with an apparent molecular weight of 260,000 and the ß-chain with an apparent molecular weight of 225,000. The α and ß-chains are encoded by different genes. That for the α-chain is located on chromosome 1q21 (Burns and Sherman, 1989) and that for the ß-chain on chromosome 14q24.1-24.2 (Cox *et. al.*, 1991). The amino acid sequence of both chains has been deduced by cDNA sequencing (Sahr *et. al.*, 1990; Winklemann *et. al.*, 1990). The α-chain can be divided into 22 segments. Seventeen of these are highly conserved repeat units, eleven of 106 and five of 105 amino acid residues. Three of the remaining segments, are less highly conserved but appear to be related to the other repeat units. The N-terminal 22 amino acids, segment 10 and segment 22 (the C-terminal 150 amino acids) are unrelated to the conserved repeat sequences. The ß-chain was shown to be composed of three domains. The N-terminal domain binds actin and shows homology to the actin binding region of dystrophin. The second domain is made up of 17 repeat units related to those of the α-chain. The third domain represents the C-terminus which is unrelated to the repeat units.

The α and ß-chains associate side by side to form heterodimers. The NH_2-terminus of the α-chain and the COOH-terminus of the ß-chain of the two heterodimers then associate at a contact site to form tetramers (Morrow *et. al.*, 1980). The tail region of the tetramers are associated with actin and band 4.1 which in turn link the cytoskeletal mesh to the lipid bilayer via glycophorin C. A second linkage point is provided by ankyrin which binds both spectrin and band 3.

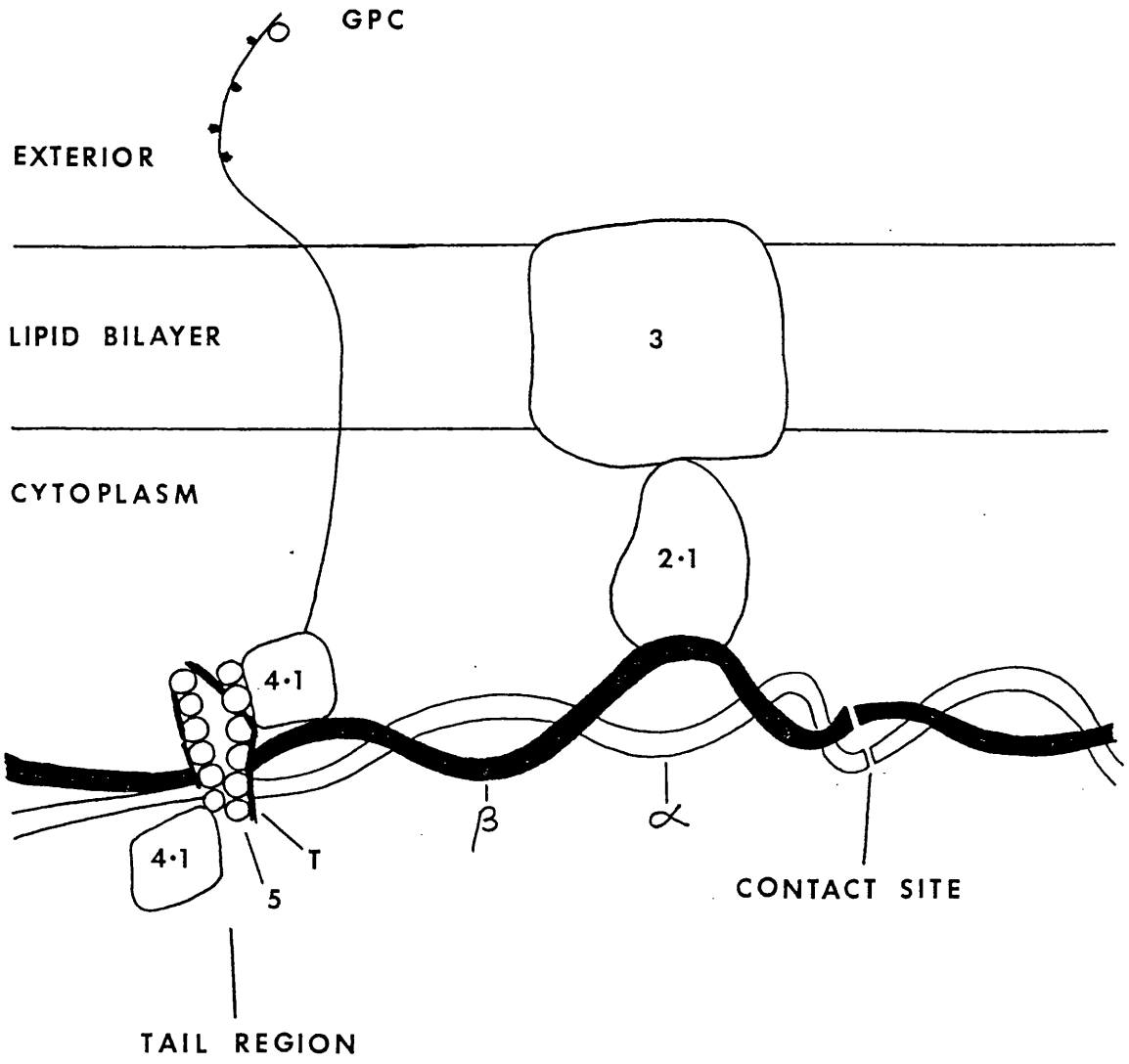
Ankyrin (band 2.1) has an apparent molecular weight of 215,000. The binding site for ankyrin on the spectrin molecule is located near the midregion of the tetramers on the ßchain (Bennett and Stenbuck, 1980). The amino acid sequence of ankyrin has been deduced by isolation and sequencing of cDNA clones of the molecule (Lux *et. al.*, 1990a; Lambert *et. al.*, 1990). These reports showed that the molecule consists of three domains; the N-terminal domain, which is involved in the interaction of the molecule with band 3 and is composed almost entirely of 23 highly conserved 33 amino acid repeat units; the spectrin binding domain and the C-terminal regulatory domain. The ankyrin gene has been assigned to chromosome 8p11.2 (Lux *et. al.*, 1990b)

Band 4.1 provides a link between the integral membrane proteins and the cytoskeleton by binding spectrin, actin and the C-terminal end of glycophorin C. The amino acid

sequence of band 4.1 has been determined and the gene has been mapped to chromosome 1p32-ter (Conboy *et. al.*, 1986a; Conboy *et. al.*, 1986b). Two isoforms of band 4.1 are visualised on SDS-PAGE gels with an apparent M_r of 80,000 is called band 4.1a and the other with an apparent M_r of 78,000, band 4.1b. These have been shown to be the result of alternative mRNA splicing of the band 4.1 gene (Conboy *et. al.*, 1988).

Actin (band 5) has an apparent molecular weight of 43,000 and associates to form short filamentous oligomers of 12-17 units (Pinder and Gratzer, 1983). Tropomyosin has been discovered in erythrocyte membranes and may help to stabilise these short actin filaments or regulate their association with spectrin (Bennett, 1985). This erythroid specific tropomyosin is composed of two polypeptides with an apparent M_r of 29,000 and 27,000. This is thought to be the result of tissue specific mRNA splicing of the tropomyosin gene (Palek and Lambert, 1990).

FIGURE 1.1.
RED CELL MEMBRANE CYTOSKELETON.



Adapted from Bennett (1985).

This figure illustrates the association of peripheral red cell membrane proteins in the cytoskeleton and their association with the integral membrane proteins.

GPC - glycophorin C; 3 - band 3; 2.1 - ankyrin (band 2.1); 4.1 - band 4.1; 5 - actin (band 5); α- spectrin α-chain; ß - spectrin ß-chain; T - tropomyosin.

1.3 Red Cell Antigen-Carrying Membrane Components.

The major integral membrane proteins carry blood group antigens on their external portions. Table 1.3 lists these proteins, the antigens which are associated with them and their abundance in the red cell membrane.

1.3.1. The Anion Transport Protein (band 3).

The structure of band 3, or the anion transport protein is shown in Figure 1.2. The molecule is highly glycosylated containing approximately 8% carbohydrate. Heterogeneity of glycosylation means the protein appears as a diffuse band on SDS-PAGE gels (Markowitz and Marchesi, 1981) with an apparent M_r of 88,000 to 98,000. The gene encoding band 3 is located on chromosome 17q21-qter (Showe *et. al.*, 1987). The amino acid sequence of the protein has been deduced from cDNA clones (Tanner *et. al.*, 1988b) and has shown the protein to consist of a cytoplasmic C-terminal domain, 14 membrane spanning regions and a cytoplasmic N-terminal domain. The glycosylation of the molecule takes the form of a single highly branched poly-N-acetyl lactosaminyl N-glycosidically linked oligosaccharide (N-glycan) (Fukuda and Fukuda, 1981) which is located on an asparagine residue at amino acid residue 642 (Tanner *et. al.*, 1988b). AB(H) antigens are expressed at the non-reducing terminii of this oligosaccharide and are described in more detail later (1.3.6.).

Band 3 associates in the membrane as a dimer or possible tetramers. The N-terminal region is involved in the anchorage of integral membrane proteins to the cytoskeleton as it has binding sites for ankyrin and band 4.2. This region also binds glycolytic enzymes and haemoglobin (Low, 1986). The C-terminal membrane spanning domain is involved in anion exchange. Chloride ions are exchanged for bicarbonate ions in a 1:1 ratio allowing the red cell to carry bicarbonate ions derived from CO_2 from respiring tissues to the lungs.

Abnormalities in the band 3 protein can lead to greater rigidity of erythrocytes. A deletion of amino acid residues 400 to 408 gives rise to ovalocytes; such cells are more rigid than normal cells (Schofield *et. al.*, 1992). Band 3 has been shown to be associated with glycophorin A in the red cell membrane (Nigg *et. al.*, 1980).

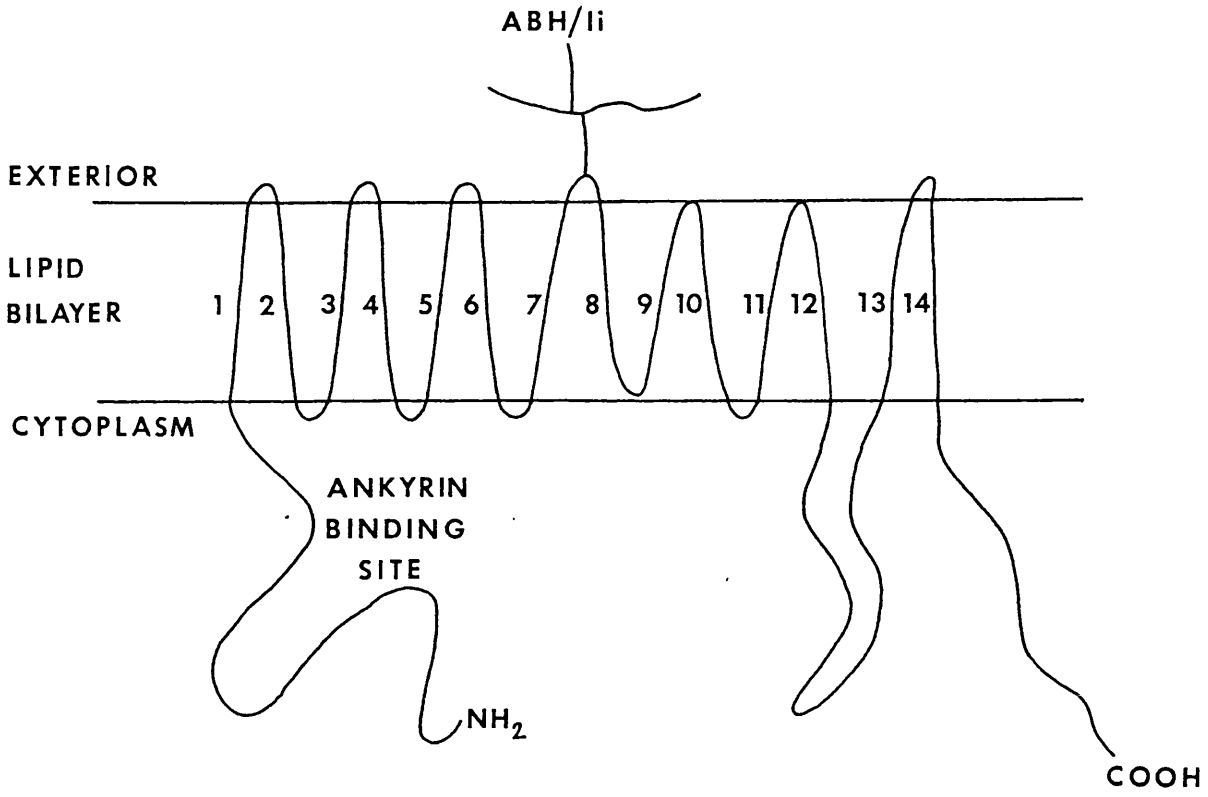
TABLE 1.3.
MAJOR INTEGRAL RED CELL MEMBRANE PROTEINS.

PROTEIN	COPIES PER CELL X 10^5	BLOOD GROUP ANTIGENS.
ANION TRANSPORT PROTEIN (BAND 3)	10	ABH, Ii
GLYCOPHORIN A	10	MN
GLUCOSE TRANSPORTER (BAND 4.5)	5	ABH, Ii
GLYCOPHORIN B	2.5	"N", Ss
Rh POLYPEPTIDES M_r 30,000	1-2	Rh ANTIGENS
M_r 45-100,000	1-2	Rh ANTIGENS
GLYCOPHORIN C	0.6-1.2	Ge ANTIGENS
GLYCOPHORIN D	0.15-0.2	Ge ANTIGENS

TAKEN FROM ANSTEE (1990a).

This table lists the abundance of the major red cell proteins and the blood group antigens they carry.

FIGURE 1.2
ANION TRANSPORT PROTEIN (BAND 3).



ADAPTED FROM ANSTEE (1990a).

This figure illustrates the structure of the anion transport protein. The numbers denote the putative membrane spanning domains.

1.3.2. Glucose Transporter (band 4.5).

The glucose transporter or band 4.5 appears as a diffuse band on SDS-PAGE gels with an apparent molecular weight of 55,000. The amino acid sequence has been deduced from cDNA clones (Mueckler *et. al.*, 1985). The N- and C-terminals of the protein are located in the cytoplasm and the molecule has 12 membrane spanning regions which facilitate the transport of glucose across the membrane. The molecule has a single complex N-linked oligosaccharide which carries the AB(H) antigens.

1.3.3. Red Cell Membrane Glycophorins.

The glycophorins have a high sialic acid content and are detected on SDS-PAGE gels after PAS staining. Molecular weights, abundance and discussion of nomenclature are given elsewhere (Section 1.2.1.1, Tables 1.2 and 1.3). Glycophorins A and B occur in the membrane as monomers and dimers; both can be seen on SDS-PAGE gels since the dimers are only partially dissociated by SDS treatment. Heterodimers between glycophorins A and B can also be visualised and are assumed to occur in the membrane.

1.3.3.1. Glycophorins A and B.

The structure of glycophorins A and B are shown in Figure 1.3. They are transmembrane proteins with a C-terminal cytoplasmic domain, a hydrophobic region which spans the lipid bilayer and a glycosylated N-terminal domain.

Glycophorins A and B are coded for by separate, closely linked genes (Siebert and Fukuda, 1986), which are located on chromosome 4 q28-q31 (Cook *et. al.*, 1981; Rahuel *et. al.*, 1988). Tate and Tanner (1988) isolated almost full length cDNA clones of glycophorins A and B. These showed that the two genes are very similar with almost identical N-terminal leader sequences. The genomic organisation of glycophorins A and B has been deduced (Kudo and Fukuda, 1989) and is shown in Figure 1.4. Glycophorin A has 7 exons and Glycophorin B has 5. Exons 1, 2, 4 and 5 of glycophorin A correspond to exons 1, 2, 3 and 4 of glycophorin B. A sequence is present in the glycophorin B gene which corresponds to exon 3 of glycophorin A. This sequence is not expressed due to a mutation at the splice site and is called a pseudoexon (ψB) (Huang *et. al.*, 1991). Southern blot analysis has revealed the presence of a third glycophorin gene related to glycophorins A and B (Tate *et. al.*, 1989). The genomic organisation of this gene is also shown in figure 1.4. Kudo and Fukuda (1990) and Vignal *et. al.* (1990) cloned and sequenced this gene and named it *GYPE*. It was shown to have 4 exons capable of encoding a polypeptide of 59 amino acids which was predicted to have a molecular weight of approximately 20,000. Northern blot analysis indicated that the gene was expressed in an erythroid specific

manner. Whether or not the product is found in red cell membranes is not yet certain. Anstee (1990b) has proposed a candidate protein (see below).

Glycophorins A and B are highly glycosylated with numerous O-glycans, about 15 and 11 respectively. Glycophorin A also carries a single N-glycan. Heterogeneity of O-glycosylation in glycophorin A has been reported. Dahr *et. al.* (1985) reported that only 40% of normal glycophorin A molecules are sensitive to digestion with chymotrypsin and suggested that this is due to variability of O-glycosylation of the threonine at position 37. Gardner *et. al.* (1989) also supported heterogeneity of glycosylation in this region when monoclonal antibodies recognising different epitopes on glycophorin A gave variable estimates of the number of antibody binding sites.

The M and N blood group antigens are carried on glycophorin A and are caused by differences in the amino acid residues present at positions 1 and 5. Glycophorin A with the M antigen has serine at position 1 and glycine at position 5, whereas these are replaced with leucine and glutamic acid respectively, to form the N antigen.

Individuals are known to exist who lack glycophorin A {En(a-)}, glycophorin B (U-) or both glycophorins A and B (M^kM^k). Studies on En^a have shown that anti-En^a, the immune antibody produced by En(a-) individuals, is heterogeneous and the terms En^aTS, En^aFS and En^aFR are used to describe the En^a antigens on glycophorin A (Issitt *et. al.*, 1981): En^aTS refers to the trypsin-sensitive region of glycophorin A from amino acid residues 1-39; En^aFS, the ficin-sensitive region between the trypsin and ficin cleavage sites, amino acid residues 48-56 and En^aFR, the ficin resistant region of glycophorin A , amino acids 62-70.(see figure 1.3)

Glycophorin B has leucine and glutamic acid at positions 1 and 5 of its N-terminus thus forming the N antigen. This is referred to as "N" to distinguish it from N antigen carried on glycophorin A. Glycophorin B also carries the S and s antigens which are caused by amino acid substitutions at position 29, methionine for S and threonine for s. The U antigen has been shown to be associated with amino acids 33-39 of glycophorin B (Dahr and Moulds, 1987), though the formation of the antigen is thought to depend on an association of glycophorin B with an Rh protein.

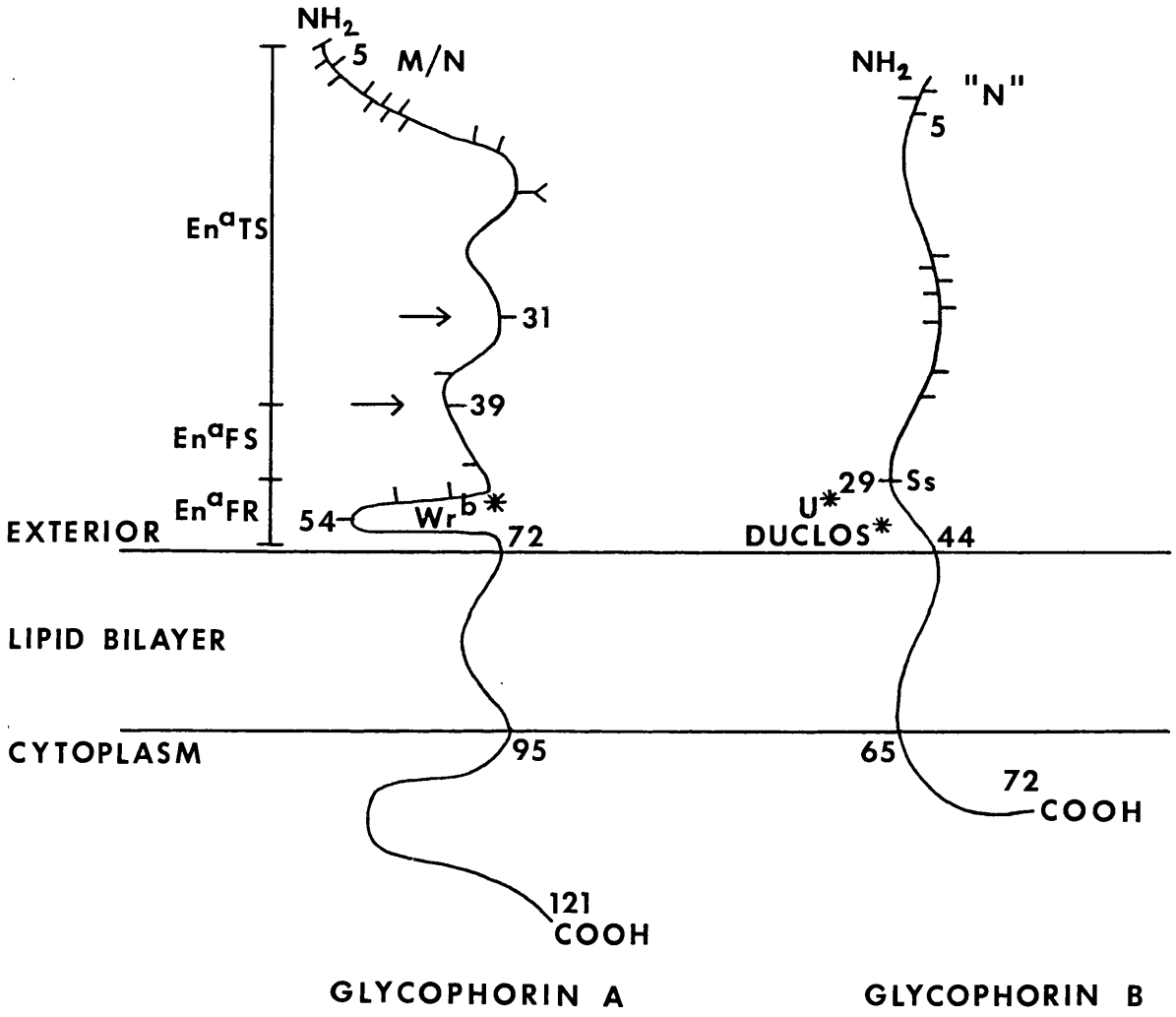
Glycophorin E, should it exist, from its cDNA sequence would have serine at position 1 and glycine at position 5 and thus be expected to express M antigen. Anstee (1990b) reports observing a band with an apparent M_r of 20,000 on SDS-PAGE immunoblots stained with a monoclonal antibody which appeared to have anti-M specificity. This band appeared in addition to bands representing glycophorin A, in membranes from M positive individuals and also appeared alone in membranes from N homozygous individuals and a M^k homozygote, who lacks glycophorins A and B. Anstee (1990b) proposes that this band may represent glycophorin E.

The function of glycophorins A and B is unknown. The red cells of En(a-), U- and M^kM^k individuals are known to function normally. Glycophorin A has been shown to be a receptor for microorganisms such as encephalomyocarditis virus (Alloway and Burness., 1986). Also, the attachment site for the malaria parasite *Plasmodium falciparum* is thought to involve the O-glycans located on glycophorins A and B, as cells lacking glycophorin A or B have been shown to be refractory to *in. vitro.* invasion by the parasite (Pasvol *et. al.*, 1982).

The high frequency antigen Wr^b is known to be associated with glycophorin A. This is discussed in section 1.4 and in chapter 6.

Hybrid proteins, formed from glycophorin A and B sequences have been shown to exist. These often give rise to the formation of novel amino acid sequences and hence new epitopes which are recognised as low frequency antigens of the MNS system (Huang *et. al.*, 1991). Study of the DNA from individuals with variant glycophorins has confirmed that recombination occurs between the two glycophorin genes. Recombination of the Lepore and Anti-Lepore type have been identified. In some cases gene conversion has been postulated. The pseudoexon sequence of *GYPB* has also been shown to be expressed when the *GYPA* gene has provided the required sequences to replace the mutated splice site, thus giving rise to novel antigens.

FIGURE 1.3.
GLYCOPHORINS A AND B.



ADAPTED FROM ANSTEE (1990a).

This figure shows a diagrammatic representation of the glycophorin A and B molecules and their location in the red cell membrane.

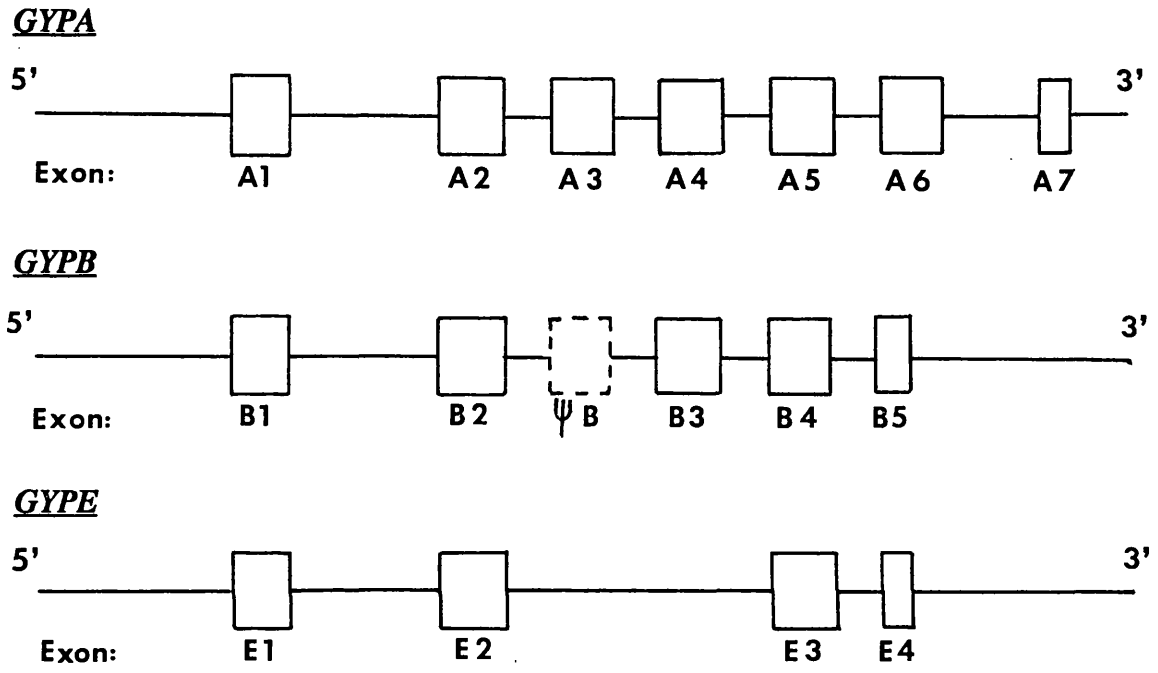
* - antigens probably formed by an interaction with other red cell membrane components.

→ - trypsin cleavage site.

Y - N-glycan.

ı - O-glycan.

FIGURE 1.4.
GENOMIC ORGANISATION OF *GYPA*, *GYPB* AND *GYPE*.



TAKEN FROM TIPPETT *et. al.* (1992).

This figure shows the genomic organisation of the *GYPA*, *GYPB* and *GYPE* genes.

1.3.3.2. Glycophorins C and D.

Two other red cell membrane proteins are called glycophorins, glycophorin C and glycophorin D, but are not related to glycophorins A and B. The high sialic acid content and some similarities observed in early biochemical analyses led to this misleading nomenclature.

Glycophorin D has been shown to be very similar to glycophorin C. Reid *et. al.* (1987) showed that various monoclonal antibodies and a polyspecific rabbit antiserum against glycophorin C also immunoprecipitated glycophorin D.

The molecules are single polypeptides which cross the lipid bilayer once and are glycosylated in the extracellular domain. Glycophorin C is four times as abundant as glycophorin D as shown in Table 1.3. The amino acid sequence of glycophorin C has been deduced from protein sequencing (Dahr *et. al.*, 1982; Dahr and Beyreuther, 1985) but that of glycophorin D has not. The two polypeptides are known to be products of the same gene (Le van Kim *et. al.*, 1987), located on chromosome 2 at position 2q14-q21 (Mattei *et. al.* 1986). Tanner *et. al.* (1988b) have shown that the mRNA sequence at the initiation site for glycophorin C is a poor initiation sequence and that a better sequence occurs at the next methionine codon which corresponds to amino acid 22. It is thought that glycophorin D is produced when leaky transcription at the downstream methionine codon results in transcription beginning at the codon corresponding to amino acid 22 of the glycophorin C gene.

Glycophorin C and possibly glycophorin D are important in maintaining red cell shape as they provide a link between the cytoskeleton and the membrane. The cytoplasmic domain of glycophorin C is known to interact with the cytoskeletal protein, band 4.1 as described in section 1.2.1.2. Individuals with a total deficiency of glycophorins C and D (Leach phenotype - see below) have elliptocytic red cells (Anstee *et. al.*, 1984a).

Glycophorins C and D carry the Gerbich blood group antigens. The high incidence antigens Ge2 and Ge3 originally comprised the Gerbich system. Ge2 is carried on glycophorin D (Reid *et. al.*, 1987) located at the N-terminus, and Ge3 is carried on glycophorins C and D (Dahr *et. al.*, 1987). Human anti-Ge3 recognise determinants on glycophorins C and D whereas some murine monoclonal antibodies recognise epitopes only on glycophorin C.

Different types of Gerbich-negative red cells exist which lack the Ge2 and Ge3 antigens. Red cells of the Leach phenotype have a total deficiency of glycophorins C and D (Anstee *et. al.*, 1984b), which has been shown to be an inherited condition (Daniels *et. al.*, 1986). Tanner *et. al.* (1988a) showed that an individual of the Leach phenotype has a deletion in the 3' portion of the *GYPC* gene which encodes the membrane domain of the protein. The resulting product of this gene would thus not be

expected to appear in the red cell membrane. Two other Gerbich-negative cell types, the Yus and Gerbich types, have also been shown to have a total deficiency of normal glycophorins C and D. However, unlike those of the Leach phenotype, red cells of such individuals carry abnormal glycophorin C-related components in their membranes. The Yus type glycophorin has a slightly higher M_r (32,000-36,5000 as compared to 30,500-34,500) and is trypsin sensitive (Anstee *et. al.*, 1984b). Both the Yus and Gerbich types of abnormal glycophorin have increased N-glycosylation compared to normal glycophorin C and D (Reid *et. al.*, 1987). Both types have been shown to be the result of internal deletions of the *GYPC* gene. Exon 2 is missing in the Yus type and exon 3 in the Gerbich type (High *et. al.*, 1989).

Several low incidence antigens have been shown to belong to the Gerbich blood group system as they are associated with abnormal glycophorin C or D molecules. For example, individuals whose red cells carry the Wb antigen have an abnormal form of glycophorin C with an apparent M_r about 2,700 less than that of normal glycophorin C. This appeared to be the result of the absence of the N-glycosidically linked oligosaccharide normally found on glycophorin C (Reid *et. al.*, 1985; MacDonald and Gerns, 1986).

1.3.4. The Rh Related Polypeptides.

The Rh blood group system is complex and made up of a large number of related antigens. The main antigens are D, C, c, E, and e. C is antithetical to c and E is antithetical to e. No antithetical antigen to D has been recognised. Antigen combinations of D or no D, C or c and E or e are always inherited together in families. This led Race and Fisher to postulate that the antigens were coded for by three closely linked genes. Wiener, in contrast, postulated that antigens were produced from multiple alleles of the same gene. A number of high and low incidence antigens are associated with the Rh system; over 40 have been listed (Lewis *et al.*, 1990). The *RH* locus is therefore complex and its exact genetic nature is far from understood. The *RH* gene locus has been located on chromosome 1p36-34 (Povey *et. al.*, 1985).

Some Rh antigens are known to be phospholipid dependent (Hughes-Jones *et. al.*, 1975; Plapp *et. al.*, 1980) suggesting that phospholipid may be involved in orientating the antigens in the membrane. Rh antigens are known to be associated with several polypeptides in the red cell membrane. Some are known not to be controlled by the *RH* locus. The LW polypeptide, glycophorin B, the Fy glycoprotein and a glycoprotein recognised by murine monoclonal antibodies of the BRIC-125 type, are all thought to be involved, as antigens carried by them show various degrees of reduced expression in Rh_{null} cells, which lack all Rh antigens (Anstee, 1990a). These molecules are thought to form a complex in the membrane with polypeptides whose

expression is controlled by the *RH* locus. Two groups of Rh polypeptides have been recognised which are co-precipitated by Rh antibodies.

1.3.4.1. Rh polypeptides of M_r 30,000.

Moore *et. al.* (1982) and Gahmberg (1983), using anti-D sera immunoprecipitated a component with an apparent M_r of 30,000 from radioiodinated red cells or membranes of appropriate Rh phenotype. Components of around 30,000 have also been immunoprecipitated using anti-c and anti-E. A monoclonal antibody, R6A which does not react with Rh_{null} red cells has immunoprecipitated a similar component with an apparent M_r of 34,000 (Ridgwell *et. al.*, 1983a). All these polypeptides have been shown to be absent in Rh_{null} cells. It is not known how many different molecules with a molecular weight of around 30,000 exist nor which antigens they carry. The 30,000 polypeptides, transverse the lipid bilayer and interact with the cytoskeleton and the D polypeptide, unlike similar membrane components, is unglycosylated. Avent *et. al.* (1990) and Cherif-Zaher *et. al.* (1990) have isolated cDNA clones which correspond to the 30,000 M_r protein, The amino acid sequence deduced from these clones suggested that they correspond to the polypeptide thought to carry the C and c and /or the E and e antigens.

1.3.4.2. Rh Polypeptides of M_r 40,000-100,000.

A second group of polypeptides with an apparent M_r of 45,000-100,000 have been shown to be co-precipitated with the 30,000 polypeptides in immunoprecipitation studies using anti-D, -c, and -E sera (Moore and Green, 1987). A polypeptide with a similar M_r has also been immunoprecipitated with the murine monoclonal antibody R6A (Avent *et. al.*, 1988b). These are highly glycosylated and have ABH activity. This group of proteins presumably associates with the 30,000 polypeptides in the membrane but, as Avent *et. al.* (1988a) point out, it is impossible to tell from these immunoprecipitation experiments, whether both groups of proteins carry the Rh antigens or whether the antigens are formed as a result of the association between the molecules.

1.3.5. Minor Red Cell Membrane Proteins and Their Associated Blood Group Antigens.

A number of blood group antigens have been located on minor red cell membrane proteins. These are listed in Table 1.4 with their relative molecular weights, abundance in the red cell membrane and the blood group antigens they carry.

TABLE 1.4

MINOR BLOOD GROUP-ACTIVE RED CELL MEMBRANE PROTEINS.

PROTEIN	GENE	APPARENT M_r	COPIES PER CELL X 10^3	BLOOD GROUP ANTIGENS
Duffy	*FY*	38,000-90,000	12	Fy antigens
Decay Accelerating factor	*DAF*	70,000	6-14	Cromer related antigens
Kell	*KEL*	93,000	3-6	Kell antigens
LW	*LW*	40,000-47,000	3-5	LW antigens
CD44		80,000	3-6	In^a/In^b
Lutheran	*LU*	85,000 78,000	1.5-4	Lutheran antigens
Ok^a	*OK*	35,000-69,000		Ok^a
12E7 protein	*MIC2*	32,500 29,000		12E7 and is associated with Xg^a
C4*	*CH/RG*	200,000		Chido/Rogers.*

This table lists the minor red cell membrane proteins, their abundance, apparent M_r and the red cell antigens they carry. Data taken from Anstee (1990a); Lewis *et. al.* (1990); Williams *et. al.* (1988) and Banting *et. al.* (1985).

* - These proteins are adsorbed onto red cells.

1.3.6. Carbohydrate Antigens.

Some red cell antigens have been identified as complex oligosaccharides. The antigens are generally found as part of many different glycoproteins and glycolipids closely associated with the red cell membrane. Oligosaccharide antigens are formed by the sequential action of specific glycosyltransferase enzymes. The addition of the terminal immunodominant monosaccharide units is mediated by the primary gene products of the blood group genes. Table 1.5 lists the structures of some common carbohydrate antigens and the genes which control them. The ABO and Hh systems are the most well known and are discussed further below.

1.3.6.1. ABO and Hh Systems.

The ABO antigens were first recognised as red cell determinants (Landsteiner, 1901) but have since been shown to be widely distributed in human tissue (Szulman, 1960 and 1961). Red cell, ABO antigens are predominantly found on glycoprotein molecules such as band 3 (section 1.3.1), the glucose transport protein (section 1.3.2.) (Fukuda and Fukuda, 1987) and the glycophorins (Takasaki and Kobato, 1976), as well as on less abundant red cell glycoproteins such as the Rh-related glycopeptides of apparent M_r 45,000-100,000 (Moore and Green, 1987). A significant proportion of the antigens have also been detected on a wide range of glycolipid structures (Koscielak *et. al.*, 1973). The ABO blood group determinants are derived from three major backbone structures termed type 1 (Galß1-3 GlcNAc), type 2 (Galß1-4 GlcNac) and type 3 (Galß1-3 GalNAc -Ser/Thr). Red cell precursors synthesise only type 2 and 3 based oligosaccharides but the cells can adsorb glycoplipids, including those that contain type 1 determinants, from the plasma.

The A and B antigens are synthesised by the enzymic transfer of α-GalNAc and α-Gal respectively to the *O*-3 position of galactose of the precursor H structure. Individuals of blood group O produce a truncated, enzymically inactive protein and so retain an unmodified H antigen. Production of the H antigen, which is determined by a gene on chromosome 19 encoding an α-2-fucosyl transferase, is genetically independent from the *ABO* gene located on chromosome 9.

The ABO genes are allelic and their coding sequences differ in only a few single base pair substitutions. Thus four amino acid changes between the A and B gene encoded proteins, cause differences in sugar nucleotide specificity. The *O* gene was shown to differ from the *A* gene by only a single base deletion early in the coding sequence, resulting in a frameshift mutation and early termination of transcription (Yamamoto *et. al*, 1990).

Thus molecular biology has provided the definitive proof of Bernstein's hypothesis (1924) that the ABO antigens were controlled by genes at the same locus.

TABLE 1.5
SOME OLIGOSACCHARIDE DERIVED RED CELL ANTIGENS.

ANTIGEN	TERMINAL OLIGOSACCHARIDES	GENES
TYPE 1 H	ßGal(1,3)ßGlcNAc-R ↑1,2 αFuc	*Se,H*
TYPE 1 A	αGalNAc(1,3)ßGal(1,3)ßGlcNAc-R ↑1,2 αFuc	*Se,H,A*
TYPE 1 B	αGal(1,3)ßGal(1,3)ßGlcNAc-R ↑1,2 αFuc	*Se,H,B*
TYPE 2 H	ßGal(1,4)ßGlcNAc-R ↑1,2 αFuc	*H*
TYPE 2 A	αGalNAc(1,3)ßGal(1,4)ßGlcNAc-R ↑1,2 αFuc	*H,A*
TYPE 2 B	αGal(1,3)ßGal(1,4)ßGlcNAc-R ↑1,2 αFuc	*H,B*
Le^a	ßGal(1,3)ßGlcNAc-R ↑1,4 αFuc	*Le*
Le^b	ßGal(1,3)ßGlcNAc-R ↑1,2 ↑1,4 αFuc αFuc	*Se,H,Le*
P1	αGal(1,4)ßGal(1,4)ßGlcNAc(1,3)ßGal(1,4)Glc-Cer	*P1*
Pk	αGal(1-4)ßGal(1,4)Glc-Cer	
P	ßGalNAc(1-3)αGal(1-4)ßGal(1,4)Glc-Cer	

This table lists the terminal oligosaccharides of carbohydrate composed red cell antigens and the genes involved in their synthesis.
Adapted from Tippett (1984).

1.4. THE Wr^a AND Wr^b RED CELL ANTIGENS.

The low incidence antigen Wr^a was originally discovered as the result of an investigation of a case of HDN (Holman, 1953). Wr^a is an autosomal dominant character which has a frequency of about 0.08% in English donors (Cleghorn, 1961). This frequency has been reported to be slightly lower, 0.01% in Caucasian, New York State blood donors (Greendyke and Banzhof, 1977).
Several family studies have shown that the Wr^a antigen is not part of 11 blood group systems; the ABO, MNS, P, Rh, Lutheran, Kell, Lewis, Duffy, Kidd, Dombrock and Xg systems (Cleghorn, 1961; Kornstad, 1961; Metaxas and Metaxas-Buhler, 1963; Pavone *et. al.*, 1977; Race and Sanger, 1975). The only hint of linkage of Wr^a to another blood group antigen was provided by a family in which the rare antigen "super" Sd^a was segregating in addition to Wr^a (Lewis *et. al.*, 1973). No further informative families have become available for study. More recent genetic studies have also excluded Wr^a from the Scianna, Landsteiner-Weiner, Chido/Rogers and XK blood group systems (Lewis *et. al.*, 1990b). These authors also reported linkage data that excludes the *WR* gene from regions comprising about 10% of the genetic map of the human genome. It is important to note, for reasons explained below, that the *WR* gene was excluded from chromosome 4q28-q32, the chromosomal location for the *GYPA* gene which controls the expression of the M and N blood group antigens.

Serological studies identified a possible antithetical antibody to anti-Wr^a (Adams *et. al.*, 1971). An antibody to a high frequency antigen, present in the serum a Wr(a+) patient, Mrs MF, was assumed to be anti-Wr^b. This assumption was made because Mrs MF was a possible Wr^aWr^a homozygote since the expression of Wr^a on her cells was stronger than that on the cells of other Wr(a+) individuals, including other members of her family. The family of Mrs MF was small and was compatable with this hypothesis. However, the red cells of all the other family members tested, reacted with the antibody in her serum. Those with the Wr^a antigen were therefore, assumed to be Wr(a+b+) heterozygotes. The low incidence of the Wr^a antigen make it unlikely that further Wr(a+b-) individuals are common (only one would be expected in the total population of Great Britain). Another such individual has been identified but unfortunately had no other family members available for study (Schutt, Daniels, Tippett and Dahr, unpublished observation).
In 1975 red cells of the rare En(a-) phenotype (which lack the M and N antigen carrying glycophorin A molecule) were observed to be Wr(a-b-) (Issitt *et. al.*, 1975). This was an unexpected phenotype since family studies had shown that the Wr^a

antigen was independent of the MNS blood group system. This finding therefore cast doubt on the proposed relationship between the Wr^a and Wr^b antigens.
However, further data supporting the allelic nature of the Wr^a and Wr^b antigens was provided by Wren and Issitt (1988) using an enzyme-linked antiglobulin test (ELAT). They showed that Wr(a+b+) red cells bind a little over half the amount of anti-Wr^b bound by Wr(a-b+) cells and that Wr(a+b+) red cells bind about half the amount of anti-Wr^a bound by Wr(a+b-) red cells. These results thus provided evidence of dosage of the Wr^a and Wr^b antigens.

1.4.1. The Occurrence of anti-Wr^a.

Antibodies which detect the Wr^a antigen can occur as immune antibodies produced following exposure to red cells carrying the antigen, and result in cases of HDN or in transfusion reactions. However, most examples of human anti-Wr^a are naturally occurring in the multispecific sera of AIHA patients or healthy donors. The frequency of donors with anti-Wr^a in their serum is surprisingly high; 1 in 80 North London donors have anti-Wr^a in their serum, (Lubenko and Contreras, 1992). Some correlation between the occurrence of anti-Wr^a produced by individuals whose immune systems are active has been reported. For example, Greendyke and Banzhaf (1977) found that the incidence of individuals with anti-Wr^a in their serum increased progressively in the following groups of individuals; random blood donors; postpartum women within 24 h delivery, patients who received a large single transfusion during open-heart surgery; patients undergoing haemodialysis and random persons with other alloantibodies in their serum.
Anti-Wr^a is very rarely the sole atypical antibody present. Antibodies which detect other low incidence antigens are often found in the same sera, anti-Sw^a, -Pt^a, and -Bp^a for example. This means that investigation of the Wr^a antigen using such sera is often complicated by the additional antibodies.

1.4.2. The Nature of the Wr^a and Wr^b antigens.

Very little information is available concerning the nature of the Wr^a antigen. This is in contrast to the Wr^b antigen, which has been shown, without doubt, to be associated with glycophorin A. All red cell types known to lack normal glycophorin A (ie. En(a-) or M^kM^k cells) and some red cells with hybrid glycophorin molecules are Wr(a-b-) (Vengelen-Tyler *et. al.*, 1981; Langley *et. al.*, 1981). Ridgwell *et. al.* (1983b) provided more evidence for this association when they immunoprecipitated glycophorin A from Wr(a-b+) red cells using a murine monoclonal anti-Wr^b. The Wr^b antigen was shown to be associated with the En^aFR region of glycophorin A.

Dahr *et. al.* (1986) showed that Wr^b and En^aFR are labile lipid dependent antigens associated with glycophorin A when they inhibited human anti-Wr^b and anti-En^aFR with amino acid residues 62-72 of glycophorin A incorporated into liposomes. The lipid dependence of Wr^b was also reported by Reardon (1985) when she showed that treating red cell membranes with phospholipase A_2 gave a marked reduction in reactivity with two monoclonal anti-Wr^b antibodies.

The association of Wr^b with glycophorin A conflicted with information that the Wr^a antigen was independent of the MN blood group system (see above) and casts doubt on an allelic relationship between the Wr^a and Wr^b.

It is possible, however, that an allelic relationship could be explained by post translational glycosylation of the glycophorin A molecule. The region associated with Wr^b is, however, not glycosylated and this seems to rule out the possibility that Wr^a and Wr^b could be formed by the action of transferases controlled by allelic genes.

The limited amount of data already published about the nature of the Wr^a antigen have suggested that its properties differ from those of the Wr^b antigen. For example, Dahr *et. al.* (1986) showed that different conditions were necessary to isolate the Wr^a and Wr^b antigens from red cell membranes.

Some authors have also suggested that involvement of the red cell membrane protein, band 3, which is known to be associated with glycophorin A in the red cell membrane (Nigg *et. al.*, 1980), may be necessary for Wr^a and Wr^b antigen expression (Dahr *et. al.*, 1986; Wren and Issitt, 1988; Telen 1987).

The properties and relationship of the Wr^a and Wr^b antigens are discussed further in chapter 6.

The amino acid sequence of the glycophorin A molecule from the Wr(a+b-) red cells of Mrs MF. has been shown to be identical to that from a Wr(a-b+) individual (Dahr *et. al.*, 1986)

1.5. Monoclonal Antibodies.

Köhler and Milstein's method for producing murine monoclonal antibodies (Köhler and Milstein, 1975) has been widely used to produce large quantities of structurally identical antibody molecules recognising a single epitope. Antibody secreting B lymphocytes are taken from the spleen of an immunised mouse. These will not survive in culture so are fused to a mouse myeloma cell to produce a hybridoma. Hybridomas have the antibody secreting properties of the lymphocytes and the ability of the myeloma cells to grow in culture.

Human monoclonal antibodies can also be produced. B lymphocytes from peripheral blood or if available from the spleens of individuals known to be producing a particular antibody are transformed with Epstein-Barr Virus (EBV). The lymphocytes can then grow in culture and secrete the desired antibody. The Rh antibody anti-D has successfully been produced in this way (Koskimies, 1980, Boylston *et. al.*, 1980). The cultured lymphocytes are difficult to clone but fusion to mouse myeloma cells has been shown to produce stable mouse-human hybridomas secreting monoclonal antibodies (Thompson *et. al.*, 1986a and b).

1.5.1. Blood Group Antigen Specific Monoclonal Antibodies.

Many monoclonal antibodies which define red cell antigens have been produced. Monoclonal antibodies are being used with increased frequency as reagents in routine blood grouping laboratories. There are many advantages and some disadvantages to using monoclonal antibodies in routine serology as summarised by Issitt (1989). The main advantage is that large quantities of a reagent with identical specificity can be produced; there is no batch to batch variation in specificity which can occur with conventional polyclonal sera. The other major advantage is that monoclonal antibody preparations contain no unwanted specificities. This is a particular advantage over some sera used to identify low incidence antigens which can contain many different antibodies of specificities (see section 1.1.3.2). The disadvantages of using monoclonal antibodies result from the antibodies being specific for a single epitope. Polyclonal sera can contain many antibodies which recognise many different epitopes of the same antigen, a monoclonal antibody which recognises only one of these epitopes may therefore have a slightly different specificity to the polyclonal reagent. A further disadvantage is that epitopes are small and may occur as part of two separate quite unrelated proteins. Both of these problems may be overcome by using a combination of monoclonal antibodies which recognise different epitopes of the same antigen.

1.5.2. Contributions of Monoclonal Antibodies to our Understanding of Red Cell Antigens.

Many monoclonal antibodies with red cell antigen specificity are proving invaluable in furthering our biochemical and genetic knowledge of red cell antigens. For example, monoclonal antibodies have been used to deduce the nature of the membrane component associated with the Cromer-related antigens; a group of antigens including, Cr^a, Wes^a, Wes^b, Tc^a, Tc^b, Tc^c, Dr^a and Es^a, known to be absent from rare Inab red cells (Daniels *et. al.*, 1982). Spring *et. al.* (1987) identified two monoclonal antibodies which did not react with Inab cells but did not recognise any of the known Cromer-related antigens. Immunoblotting and immunoprecipitation experiments revealed that the monoclonal antibodies reacted with a membrane glycoprotein with an apparent M_r of 70,000. These authors also showed that the glycoprotein was present on peripheral blood leucocytes, platelets and several haemopoietic cell lines. The glycoprotein was subsequently shown to be the complement regulatory molecule, decay-accelerating factor (DAF) (Telen *et. al.*, 1988). These studies thus revealed the function of the red cell membrane component associated with the Cromer related antigens.

Other monoclonal antibodies have revealed new antigens. One such antibody defined the MER 2 antigen and hence revealed a new blood group polymorphism, the first to be assigned to chromosome 11 (Daniels *et. al.*, 1987).

Another monoclonal antibody which facilitated the assignment of an antigen to a red cell membrane component also allowed the chromosomal location of the gene encoding that antigen to be deduced was an antibody which recognised the high frequency antigen Ok^a. Williams *et. al.* (1988) showed that the antibody reacted with a series of heterogeneous components on the red cell membrane which had apparent M_rs of 35,000-68,000 using immunoblotting studies. Tissue distribution studies showed that the antigen was expressed on a wide variety of human tissues but not on any mouse tissue. Somatic cell hybrid and DNA transfection studies then showed that the antigen carrying component was a product of a single gene located on chromosome 19 at position 19p13.2-pter. It is unlikely that this information would have been deduced without the monoclonal antibody since it is difficult to isolate sufficiently pure reagents for such studies from polyclonal sera.

Monoclonal antibodies which react with particular portions of red cell membrane components can be useful in deducing more about red cell antigens. The LICR-LON R1.3, R10 and R18 anti-glycophorin A monoclonal antibodies (Anstee and Edwards, 1982) and antibodies with similar specificities have proved invaluable in deducing the nature of rare hybrid glycophorin molecules and the antigens they carry.

1.6. Aims of This Project.

The primary aim of this project was to produce a monoclonal antibody to one of the many low incidence red cell antigens. The Wr^a antigen was chosen as the first antigen of study as, although rare, fresh Wr(a+) red cells were reasonably easy to obtain through the kindness of Dr Marcela Contreras of the North London Blood Transfusion Centre. The Wr^a antigen was also of interest due to the controversy of its proposed antithetical relationship to the high incidence antigen Wr^b. Any anti-Wr^a monoclonal antibody produced was to be used to investigate the unknown nature of the Wr^a antigen using an variety of immunochemical techniques. The characteristics of the antigen could then be compared with those of the Wr^b antigen in the hope that this would shed some light on the antithetical relationship between the antigens. The anti-Wr^a monoclonal antibody would also be used to examine the tissue distribution of the antigen and may provide some clues as to why so many individuals never apparently exposed to the antigen produce antibodies to it.

CHAPTER 2.

MATERIALS AND METHODS.

2.1.MATERIALS.

2.1.1 Chemicals.

Chemicals were obtained from BDH unless otherwise stated.

Phosphate buffered saline (PBS) used throughout the work was 10mM NaH_2PO_4, 10mM Na_2HPO_4, 0.15M NaCl, pH 7.3.

2.1.2. Blood Samples.

Blood samples were obtained from many blood transfusion centres but especially from the North London Blood Transfusion Centre. Table 2.1. lists the phenotypes of donors specifically mentioned in this thesis. Blood samples were stored at 4^oC. Alternatively, samples were prepared for long term storage at -30^oC. Samples were centrifuged at 350xg for 5 min at 4^oC. The plasma was removed and an equal volume of freezing fluid (3 parts of 5% tri-sodium citrate mixed with 2 parts of glycerol) was added. After mixing, samples were stood at room temperature for 30 min and then stored between -20^oC and -40^oC.

Frozen cells were recovered, after thawing, by dialysis in PBS at room temperature for at least 1 h. Cells were then washed three times in PBS and used as described.

Washed red cells were stored in red cell preservative (South West Regional Transfusion Centre) at 4^oC for up to 7 days.

2.1.3. Cell Lines.

The cell lines used throughout this project are shown in Table 2.2.

2.1.4. Monoclonal Antibodies.

The monoclonal antibodies used in this project are shown in Table 2.3.

TABLE 2.1
RED CELL PHENOTYPES OF DONORS.

DONOR	Wr a b	ABO	Rh	MN	S s	P_1	Lu a b	K k	Kp a b	Le a b	Fy a b	Jk a b	Xg^a
.C.	+ +												
L.C.	+ +	O	R_1r	MN	+ +	+	- +	- +	- +	- +	+ -	+ -	
R.D.	+ +	O	R_1R_2	M	- +	+	- +	- +	- +	+ -	- +	+ +	
B.L.	+ +	O	rr	MN	+ +	++	- +	- +	- +	- +	+ +	+ +	
I.P.	+ +	O	R_2r	N	+ +	+	- +	- +	- +	- +	+ +	+ -	
G.R.	+ +	O	R_1R_1	M	+ -	+	- +	- +	+ +	- +	+ +	- +	
A.V.	+ +	O	R_1r	MN	- +	-	- +	- +	- +	- +	+ +	+ +	+
G.W.	+ +	O	rr	MN	+ +	++	-	- +		- +	+ +	+ +	
B.W.	+ +	O	rr	MN	+ -	++	-	+ +		- +	+ +	+ -	
I.W.	+ +	O	R_1r	MN	+ +	+++	- +	- +	- +	- +	+ +	+ +	
S.W.	+ +	O	rr	N	+ +	+	- +	- +	- +	+ -	- +	+ +	+
M.Fr	+ -	O	rr	MN									
C.G.	- +	O	rr	M	+ -	+	-	-		- +	- +	- +	+
S.R.	- +	A_2	R_1R_2	N	- +	-	-	-	-	- +	+ +	+ +	+
P.T.	- +	O	rr	MN	- +	+	- +	-	-	- +	+ +	- +	+

TABLE 2.2
CELL LINES USED IN THIS THESIS.

LINE	TYPE	MEDIUM	REFERENCE
P3/NS1/ 1-Ag4-1	Non secreting myeloma	RPMI -HMT	Köhler and Milstein (1976)
P3/X63 -Ag8	Secreting myeloma	RPMI -HMT	Köhler and Milstein (1975)
HEL	Erythroleukemic	DMEM	Martin and Papayannopoulou (1982)
K562	Erythroleukemic	DMEM	Lozzio & Lozzio (1975)
HL60	Myeloid-like	DMEM	Collins *et. al.* (1977)
U937	Myeloid-like	DMEM	Anderson & Spiegelberg (1981)
MOLT-4	T-ALL	DMEM	Minowadu *et. al.* (1973)
RAJI	Burkitt's lymphoma	RPMI -HMT -HM	Pulvertaft (1965)
DAUDI	Burkitt's lymophoma	RPMI -HMT -HM	Klein *et. al.* (1968)
6897	Lymphoblastoid cell line	RPMI -HMT -HM	unpublished
HENSON	Lymphoblastoid cell line	RPMI -HMT -HM	unpublished
WISEMAN	Lymphoblastoid cell line	RPMI -HMT -HM	unpublished
BARKER	Fibroblast	MEM	unpublished
WISEMAN	Fibroblast	MEM	unpublished
T47D	Breast	DMEM	Keydar *et. al.* (1979)
MCF7	Breast	DMEM	Soule *et. al.* (1973)
HT29	Enterocyte	DMEM	Fogh *et. al.* (1977)
HEPG2	Hepatoma	DMEM	Knowles *et. al.* (1980)
RAG	Mouse	MEM	Klebe *et. al.* (1970)
A23	Hamster	MEM	Westerveld *et. al.* (1971)
FAZA	Rat	MEM	Weiss *et. al.* (1981)
B95.8	Marmoset	RPMI -HMT -HM	Miller and Lipman (1973)

Abreviation - = without

TABLE 2.3

MONOCLONAL ANTIBODIES USED IN THIS THESIS.

NAME	SPECIFICITY	REFERENCE
BGU1-WR	ANTI-Wr^a	this thesis
BRIC-14	ANTI-Wr^b	RIDGEWELL *et. al.* 1983b
LICRLON-R7	"	ANSTEE AND EDWARDS, 1982
10-22 4-21	" "	ANSTEE, 1988 "
LICRLON-R1.3	ANTI-GPA and B	ANSTEE AND EDWARDS, 1982
LICRLON-R10	ANTI-GPA En^aTS	ANSTEE AND EDWARDS, 1982
BRIC-127	"	not published
LICRLON-R18	ANTI-GPA En^aFS	ANSTEE AND EDWARDS, 1982
BRIC-256	"	not published
BRIC-130 BRIC-132	ANTI-BAND 3 "	WAINWRIGHT *et. al.* 1989 "
BRIC-126	ANTI-CD47	not published
GERO	ANTI-Ge	DANIELS *et. al.* 1983
mlac2 mlac3 mlac6 mlac9	ANTI-lactase " " "	MAIURI *et. al.* (1991) " " "

2.2. METHODS.

2.2.1. Tissue Culture Methods.

2.2.1.1. Stock Solutions.

The following stock solutions were prepared, frozen in small aliquots and added to medium as required.

2.2.1.1.1.Glutamine, Penicillin and Streptomycin (GPS). A 100 x stock solution was prepared by adding Crystapen Benzyl penicillin (sodium) BP at 6 $mgml^{-1}$ and streptomycin sulphate BP at 10 $mgml^{-1}$ to 200mM glutamine.

2.2.1.1.2. Hypoxanthine, Thymidine and Methotrexate (HMT). A 100 x stock solution containing 10^{-2}M hypoxanthine (Sigma) and $1.6x10^{-3}$M thymidine (Sigma) in deionised water was filter sterilised and added to an equal volume of a sterile 100 x stock solution of 10^{-3}M methotrexate (Lederle).

2.2.1.1.3. Hybridoma Medium (HM). A 100 x stock solution was prepared containing $4.5mgml^{-1}$ sodium pyruvate (Sigma), $1mgml^{-1}$ bovine insulin (Sigma) and $1.32mgml^{-1}$ oxaloacetic acid. This was filter sterilised.

2.2.1.2. Growth of Hybridomas.

All hybridomas were grown in RPMI 1640 (Gibco). A ten fold stock solution was diluted in sterile distilled deionised water. 3 ml of a 5.3% $NaHCO_3$ solution and 1 ml of the GPS, HM, and HMT stock solutions, prepared as described above were added to every 100 ml of medium. The pH was brought to approximately 7.3. with 1 M NaOH. Fetal calf serum (FCS) (Imperial Laboratories) which had been batch tested to ensure it supported the growth of hybridomas was added to give a final concentration of 20%. Hybridomas were fed with medium warmed to 37°C and were grown in a moist atmosphere containing 5% CO_2 at 37°C.

2.2.1.3. Growth of Other Cell Lines.

Table 2.2 lists the cell lines used in this project and the medium in which each was grown. Lymphoblastoid cell lines were grown in RPMI with the addition of 10% FCS and GPS. Fibroblasts and rodent cell lines were grown in Eagle's Minimal Essential Medium (MEM) (Gibco) with the addition of 10% FCS, 1% Hepes (0.297% Hepes; 0.25% NaOH; pH 8.0), 1% Pyruvate, 1% non essential amino acids (Gibco) and GPS. Tumour cell lines were grown in Dulbecco's minimal essential medium

(DMEM) (Gibco) with the addition of 10 or 20% FCS and GPS. Some lines (MCF7 and T47D) also required the addition of insulin at a concentration of $10\mu gml^{-1}$ in the medium.

Adherent cell lines were removed from the surface of flasks by trypsin treatment when cells were harvested or transferred to two or more flasks for further growth. Medium was removed from flasks and approximately 2ml of versene containing 0.25% trypsin added. This was incubated for approximately 5 min at 37°C until the cells were coming away from the surface. Medium containing FCS was then added to neutralise the trypsin and the cells transferred to new flasks or harvested.

2.2.1.4. Production of Antibody Containing Supernatant.

The bulk culture of hybridomas was carried out in 260ml ($80cm^2$) tissue culture flasks (Nunc). When cells were growing well medium was doubled every 1.5-3 days and the contents of flasks divided when full. Rapidly dividing cells were preserved by suspending in 1 ml of a 5% solution of dimethyl sulphoxide (DMSO) in FCS, freezing slowly and storing in liquid nitrogen. Frozen ampoules were thawed quickly in warm water, the cells washed in medium and grown as described above.

Antibody containing supernatant was prepared by leaving medium in contact with a dense culture of cells for at least 48 h. The medium was then removed, centrifuged at 225xg to remove any cells and stored at -20°C. The remaining cells were either regrown or discarded. Occasionally supernatant was required with a lower FCS concentration. In this case FCS concentration in the medium was gradually decreased over a period of days from 20% to 15%, 10% and finally 5%. The cells were then grown for at least 48 h in the 5% FCS medium before harvesting.

Lymphoblastoid cell lines grown for antibody production were grown in the same way except that HM and HMT were not added to the RPMI medium.

2.2.1.5. Mycoplasma Testing.

All cells grown were tested on arrival in the laboratory and occasionally during growth for the presence of mycoplasma using a Hoeschst staining technique. Cells which adhere to a petri dish can be stained directly but nonadherent cells such as hybridomas and lymphoblastoid cells lines needed to be grown for 48 h in a dish containing fibroblasts. Any mycoplasma present in the nonadherent cells could then infect the fibroblasts and be detected. Adherent cells were fixed with 3 parts methanol and 1 part glacial acetic acid in the petri dishes for 2 min. The fixative solution was removed and a second volume added and the dishes incubated for five min. The dishes were then dried and stained in a freshly prepared solution of $0.1\mu gml^{-1}$ Hoechst 33258 (Sigma) in PBS for 10 min. Dishes were washed 4 times in distilled water, a drop of phosphate

citrate buffer (0.1M citric acid, 0.2M Na_2HPO_4, pH 5.5) was added and the dishes were covered with a coverslip. Dishes were examined under a fluorescent microscope. A mycoplasma infection could be detected as fluorescent particles present in the cytoplasm of the fibroblasts.
Where possible infected cells were discarded.

2.2.2. Monoclonal Antibody Production.

2.2.2.1. Production of Hybridomas.

Hybridomas were produced using a method based on that devised by Köhler and Milstein (1975). Intact red cells were used to immunise approximately six week old female BALB/c or BALB/tk mice. The red cells were either freshly collected, washed and suspended in sterile saline or were suspended in freezing fluid (3:2 solution of 5% trisodium citrate:glycerol) after storage at -30°C. Some immunisations used papain treated cells, this enzyme treatment was carried out as described in 2.2.3.4.1. Further details of the immunisation protocols are given in Tables 2.4. and 2.5. Mice were injected intraperitonally, the initial immunisation being 0.5ml or 0.1ml of the red cell suspensions. Mice were subsequently boosted once, twice or three times using 0.1ml of the cell samples. The length of time between each boost varied from fusion to fusion and is shown in Tables 2.4. and 2.5.
Three days after the final boost, fusion of spleen cells to myeloma cells was carried out as described by Swallow *et. al.* (1985). The spleen was removed from the immunised mouse and the cells teased out aseptically into 10ml of RPMI medium which did not contain FCS or HMT (RPMI/OS). These were washed twice centrifuging at 225xg and then suspended in RPMI/OS. Spleen cells (approximately 10^8) were then mixed with approximately 10^7 confluent P3NS1/1-Ag4-1 myeloma cells which had been washed and resuspended in RPMI/OS. The cell mixture was spun at 400xg for 7 min, the supernatant removed and the pellet resuspended. 0.8ml of a 1:1 solution of polyethylene glycol (PEG) 1500 in RPMI/OS was then added carefully over 1 min. This was incubated at 37°C for 45 s. 1ml of RPMI/OS was added over 1 min followed by a further 19ml over 5 min. The cells were centrifuged at 400xg for 7 min and the supernatant removed. 40ml of RPMI containing 20% FCS but without HMT was then used to gently resuspend the cells. The suspension was plated into four 96 well plates using 100μl per well. Feeder cells were prepared from the spleen of a mouse which had not been immunised. The cells were washed three times in RPMI/OS and suspended in 80ml RPMI containing 20% FCS but without HMT. 200μl of this was added to each of the wells containing fused cells. In some fusions feeder cells were replaced by a 10% solution of BM-combined H1 medium (Boehringer

Mannheim) in order to compare the two. The results of this comparison are described elsewhere (3.1.8.). The fused cells were then incubated at 37°C, 5% CO_2 in a moist atmosphere. The medium was gradually changed to selective medium containing HMT over the next 5 days. Culture was then continued in this medium which selects hybridomas as the methotrexate poisons the endogenous purine and pyrimidine biosynthesis pathways. Cells which have the enzyme hypoxanthine guanine phosphoribosyl transferase are HGPRT+ and are able to utilise the salvage pathway and grow in HMT medium. The myeloma parent is HGPRT- and the immune mouse spleen cells (HGPRT+) do not normally divide in culture. Therefore only the hybridomas which have the HGPRT gene from the spleen cells will grow in HMT medium.

After the first five days the medium was changed every two to three days. When hybridomas completely covered the bottom of a well they were transferred to a 24 well plate. Spent culture medium was harvested from confluent wells after at least 36 h in contact with the cells and was screened for antibodies as described below.

TABLE 2.4
IMMUNISATION PROTOCOLS.

FUSION STRAIN	MOUSE	DONOR	SUSPENSION	LENGTH OF BOOSTS IN DAYS
1	Balb/c	A.V.	20% in sterile saline	21; 18
2	Balb/c	A.V.	20% in sterile saline	18; 14
3	Balb/c	G.W.	20% in sterile saline or 50% in freezing fluid	34; 26; 14
4	Balb/c	G.W.	50% in freezing fluid	14; 11
5	Balb/c	P.T.	50% in freezing fluid	7; 24
6	Balb/c	P.T.	50% in sterile saline	7; 24
7	Balb/c	S.W.	50% in sterile freezing fluid	25
8	Balb/tk	S.W.	50% in sterile saline	25

This table gives details of the immunisation protocols of mice used for fusions 1 to 8 carried out in this thesis. The phenotypes of the immunising red cells are given in Table 2.1. Fusions were always carried out three days following the final boost. Red cells suspended in sterile saline for fusions 1 and 2 were freshly collected or had been stored at -30^{o}C in freezing fluid. In the latter case they were dialysed to PBS before immunisation. In other fusions cells which had been stored at -30^{o}C were not dialysed but remained in the freezing fluid.

TABLE 2.5

IMMUNISATION PROTOCOLS USING PAPAIN TREATED RED CELLS.

FUSION STRAIN	MOUSE	DONOR	SUSPENSION IN DAYS.	LENGTH OF BOOSTS
9	Balb/tk	I.W	20% sterile saline	8; 24
10	Balb/tk	I.W	20% sterile saline	15
11	Balb/tk	I.W	20% sterile saline	14
12	Balb/tk	I.W	20% sterile saline	14
13	Balb/tk	I.W	20% sterile saline	15
14	Balb/tk	I.W	20% sterile saline	12

This table gives details of the immunisation protocols using papain treated red cells of mice used for the fusions 9 to 14 carried out in this thesis. The phenotypes of the immunising red cells are given in Table 2.1. Fusions were always carried out three days following the final boost.

2.2.2.1.1. Screening Hybridoma Supernatants. All supernatants were screened by haemagglutination using techniques descibed in section 2.2.3. Some were also screened by ELISA (2.2.4.3.), some by RIA (2.2.4.2.) and some using immunoblotting (2.2.4.8.). All of the supernatants were tested with the red cells used for the immunisation protocol and cells not carrying the Wr^a antigen either by haemaaglutination or RIA. Those supernatants which reacted only with the cells used for immunisation were screened further against a panel of red cells in order to deduce the specificity of the antibody.

2.2.2.1.2. Subcloning Hybridomas. Subcloning of interesting hybridomas was carried out by plating cells at limiting dilution in soft agar as described by Swallow *et. al.* (1985). A feeder layer of washed mouse spleen cells in 0.7% Bacto-agar (Difco) in RPMI 1640 containing 11.5% FCS, $8x10^{-5}$M ß-mercaptoethanol, GPS and HMT was placed in 60 x 15mm petri dishes. A second layer containing approximately 1,000 hybridomas per dish in 0.35% Bacto-agar in the same medium was added. The cells were then grown for about two weeks until discrete colonies could be picked. These colonies were transferred to 96 well plates, grown up and tested for antibody production. Positive clones were subcloned once more to ensure they were monoclonal. The monoclonal lines were then grown up for antibody production and frozen down as described in section 2.2.1.4.

2.2.2.2. Production of Antibody Secreting Lymphoblastoid Cell Lines.

Blood samples were collected in heparin and left to stand at room temperature overnight. White cells were separated by spinning the sample through a lymphocyte separation medium (Flow Laboratories) gradient. Epstein-Barr virus (EBV) containing supernatant was harvested from the EBV-secreting B95.8 marmoset cell line. EBV attaches to B lymphocytes specifically (Steel *et. al.*, 1977). White cells were then suspended at a concentration of approximately 2.0×10^6 ml^{-1} in a 1:1 mixture of EBV containing supernatant and fresh RPMI containing 20% FCS and $5\mu gml^{-1}$ phytohaemagglutinin (Sigma). T-cells are stimulated by phytohaemagglutinin and hence grow and act as feeder cells in the initial stages of culture. Phytohaemagglutinin was omitted from the medium after two weeks, at which point only transformed B-lymphocytes grew. Cyclosporin-A, which depletes T-lymphocytes, was sometimes used instead of phytohaemagglutinin to treat some cultures. In these cases the Balb/c peritoneal macrophage feeder cells were required to initiate growth of the B-lymphocytes. In both cases cells were plated out into 96 well plates and fed every 3 or 4 days. Spent culture medium was removed from confluent cultures after at least 36 hours in contact with the cells and was screened as described in section 2.2.2.1.1.

2.2.3. Haemagglutination Methods.

The serological techniques used were adapted from those described by Race and Sanger (1958). For all tests red cells were washed three times in PBS, centrifuging at approximately 500xg for 5 min. Incubation was either at 4°C, room temperature or 37°C depending on the antibodies used. However, when screening supernatants from fusions, samples were incubated at room temperature for 30 min, followed by 30 min at 4°C. Agglutination was read macroscopically and recorded as follows:-

-	No agglutination.
w	Very small agglutinates, just visible.
(+)	Small agglutinates.
+	Clearly visible agglutinates.
++	Large agglutinates.
$++^{v}$	All cells agglutinated in large clumps.
$++^{vv}$	All cells agglutinated.

2.2.3.1. Tube Direct Agglutination Test (AGG).

A 10μl volume of a 3% suspension of red cells in PBS was incubated with 10μl of monoclonal antibody or serum in a tube for 1 h at room temperature for murine antibodies and at 37°C for human ones. Tubes were then spun at 500 xg for 15 s. Tests were read by gently shaking and rolling the tubes and scoring agglutination as described above.

2.2.3.2. Tube Indirect Antiglobulin Test (IAT).

A 10μl volume of an approximately 12% suspension of red cells in PBS was incubated with 10μl of murine monoclonal antibody for 1 h. Sensitised red cells were then washed twice with PBS. A drop (approximately 30μl) of a 1 in 60 dilution of Rabbit anti-mouse Ig (RAM) (Dako) was then added. Tubes were immediately spun at 500xg for 30 s then read and recorded as for the tube agglutination test (2.2.3.1.).

Human sera and monoclonal antibodies were also tested in the same way except that one drop of anti-human Ig (Blood Products Laboratory [diagnostics]) was used instead of RAM. Also, when polyclonal sera were used the red cells were washed four times before the addition of the antiglobulin reagent.

2.2.3.3. Microtitre Plate Test.

U or V-well microtitre plates (Sterilin) were pretreated by washing in 0.2% Tween 20, followed by five washes in distilled water. Plates were then dried before use.

A 10μl volume of a 1% suspension of red cells in Tween/albumin/PBS (0.001% Tween 20, 3% bovine serum albumin [BSA] in PBS) was incubated with a 10μl volume of monoclonal antibody for 1 h. The plate was centrifuged at 100xg for 1 min and shaken gently on a plate shaker (Denley) until samples were resuspended. Agglutination was scored as described above.
Plates were then washed twice in Tween/albumin/PBS, centrifuging at 100xg for 5 min. One drop of RAM, diluted 60 fold in Tween/albumin/PBS was added to each well and plates centrifuged at 100xg for 1 min. Indirect agglutinates were then read and recorded as described above.

2.2.3.4. Enzyme Treatment of Red Cells.
Red cells treated with enzymes as described below were used in serological or immunochemical tests in the same way as untreated cells.

2.2.3.4.1. Papain Treatment. A pestle and mortar was used to grind 2g of papain (BDH) with 100ml of 0.067M phosphate buffer, pH 5.4. This was filtered and 10ml of 0.5M cysteine added to activate the enzyme. The solution was diluted to 200ml and incubated at 37^{o}C for 1h. This was then stored in small quantities at -20^{o}C.
To treat red cells, 1 volume of the papain solution was added to 2 volumes of packed, washed red cells with 7 volumes 0.067M phosphate buffer, pH 7.7 and incubated at 37^{o}C for 10 min. The cells were washed once in PBS before use.

2.2.3.4.2. Ficin Treatment. A 1% solution of ficin (Sigma) was prepared in Hendry's buffer, pH 7.3 to 7.5 (1 part 0.514% NaH_2PO_4 to 4 parts 0.445% Na_2HPO_4) and stored at -20^{o}C. One volume of this solution was mixed with one volume of packed, washed red cells and 8 volumes of PBS. This was incubated at 37^{o}C for 15 min. The red cells were washed once in PBS before use.

2.2.3.4.3. Trypsin Treatment. One volume of packed, washed red cells was mixed with four volumes of a 2.5mgml^{-1} solution of trypsin (Sigma) in 0.067M phosphate buffer, pH 7.7. This was incubated at 37^{o}C for 30 min. Red cells were washed three times in PBS before use.

2.2.3.4.4.Alpha-Chymotrypsin Treatment. One volume of packed, washed red cells was mixed with four volumes of a 5mgml^{-1} solution of alpha-chymotrypsin (Sigma) in 0.067M phosphate buffer, pH 7.7. This was incubated for 1 h at 37^{o}C. Red cells were washed three times before use.

2.2.3.4.5. Pronase Treatment. One volume of packed, washed red cells was mixed with four volumes of a 2.5mgml^{-1} solution of pronase (Boehringer Mannheim) in 0.067M phosphate buffer, pH 7.7. This was incubated for 30 min at 37^{o}C. Red cells were washed three times before use.

2.2.3.4.6. Bromelin Treatment. One volume of washed, packed red cells was mixed with one volume of a 0.5% or 0.1% solution of bromelin (Sigma) and 7 volumes of 0.067M phosphate buffer, pH 7.7. This was incubated for 15 min at 37^{o}C. Red cells were washed 3 times in PBS before use.

2.2.3.4.7. Neuraminidase (RDE) Treatment. Receptor destroying enzyme from *Vibrio cholerae* (RDE) (Koch-Light) was diluted ten fold in electrolyte (0.145M NaCl, 0.005M $CaCl_2$). One volume of this was mixed with one volume of packed, washed red cells and 3 volumes of electrolyte. This was incubated for 1 h at 37^{o}C. Red cells were then washed twice in PBS before use.

2.2.3.4.8. Phospholipase A_2 Treatment. Red cell membranes, prepared as described in section 2.2.4.4. were treated with various concentrations of Phospholipase A_2 (Sigma). 200μl of membranes were suspended in 1.2ml of 0.15 M glycine; 80mM NaCl; 2mM $CaCl_2.H_2O$, pH 6.7. This was incubated for 1 h at 30^{o}C and the reaction stopped by washing twice with 0.15M glycine, 80mM NaCl; 3mM EDTA, pH 6.7.

2.2.3.5. Chemical Modification of Red Cells using 2-Aminoethyl-isothiouroniumbromide (AET).

One volume of red cells was mixed with 4 volumes of a 6% AET (Calbiochem) solution, pH 8.0. This was incubated for 20 min at 37^{o}C with mixing every 5 min. Red cells were then washed 5 to 7 times in PBS until the supernatant was clear.

2.2.3.6. Heat Treatment of Red Cells.

A 50% suspension of red cells in PBS was incubated at 45^{o}C or 56^{o}C for 10 or 30 min. Cells were then washed in PBS until the supernatant was clear.

2.2.3.7.Investigation of the Effect of pH on Agglutination.

The effect of pH on agglutination was investigated by haemagglutination. Red cells suspensions and antibody dilutions were either made with phosphate buffered saline, pH 6.3 (nine volumes of 0.9% NaCl were mixed with one volume of phosphate buffer, pH 6.3 (0.1M Na_2HPO_4 mixed with 0.1M KH_2PO_4 to pH 6.3) or borate buffered saline, pH 8.3 (nine volumes of 0.9% NaCl were mixed with one volume borate buffer [0.1M KCl, 0.1M H_3BO_3 adjusted to pH 8.3 with 0.1M NaOH, then diluted two fold with distilled water]).

2.2.3.8. Estimation of Titres of Monoclonal Antibodies.

Monoclonal antibodies were serially diluted in 2% BSA in PBS and each dilution tested in tubes. The lowest dilution at which agglutination or indirect agglutination could be scored as "+" was taken as the titre. Where titrations were tested in microtitre plates the antibody was diluted in Tween/albumin/PBS instead of 2% BSA in PBS.

2.2.3.9. Absorption Experiments.

Absorption experiments were carried out by incubating a dilution of monoclonal antibody with an optimum amount of red cells or red cell membranes for 1 h. This was then centrifuged and the supernatant titrated. The amount of antibody remaining in the supernatant was assessed by haemagglutination or using an ELISA to detect antibody (2.2.4.3.3.). The optimum dilution of antibody and the amount of red cells or red cell membranes used for inhibitions was determined in initial experiments.

2.2.4.Immunochemical Methods.

2.2.4.1. Ouchterlony Immunodiffusion Assay.

Immunodiffusion was carried out to determine the immunoglobulin subclass of monoclonal antibodies. An approximately 5mm gel was made using 3ml of a 1% solution of HSA agarose in PBS in petri dishes. A circle of six wells surrounding a central well were cut and 10μl of the test antibody were placed in the central well. 10μl of anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, anti-IgA and anti-IgM (Miles Laboratories) were placed in the surrounding wells. Diffusion was allowed to occur at 4^{o}C over 48 h. The gels were transferred to Gelbond film (Marine Colloids) and squashed under a glass plate until firmly attached to the Gelbond. This was washed three times for 1 h in PBS then twice for 20 min in distilled water at 4^{o}C. The diffusion bands were visualised by staining gels for 1 min in a 0.2% solution of Coomassie Brilliant Blue in 10% acetic acid then destaining in 10% acetic acid for 5 min.

2.2.4.2. Radioimmunoassay (RIA).

RIA was used to screen supernatants harvested from fusions 7 and 8. Flexible V-well plates (Linbro) were blocked with 150μl of Tween/albumen/PBS (0.001% Tween 20, 1% BSA in PBS) per well for at least 90 min at 4^{0}C. 50μl of culture supernatant and 50μl of a 0.2% red cell suspension in Tween/albumen/PBS were added and incubated with gentle shaking for 2 h at 4^{o}C. Plates were washed four times in Tween/albumen/PBS by completely filling wells, centrifuging plates at 400xg for 3 min and flicking off the supernatant. A dilution of ^{125}I-labelled anti-mouse Ig (5-20μCi per μg antibody protein) (Amersham) in Tween/albumen/PBS, which gave 200,000 counts per 50μl was made and 50μl added to each well. Plates were incubated with gentle shaking at 4^{o}C for 90 min. Plates were washed four times in Tween/albumen/PBS as described above and dried. Individual wells were cut out and counted in a gamma-counter (LKB). Wells which gave counts at least three times that of the negative controls were classed as positive.

2.2.4.3.Enzyme Linked Immunoabsorbent Assay (ELISA).

2.2.4.3.1. ELISA to Detect Red Cell Components Using Preprepared Red Cell Membranes. Flat-well microtitre plates (Nunc) were used and all incubations were at 4°C. 30μl of red cell membranes made as described in section 2.2.4.4, diluted four fold in 10mM Tris/HCl, 6% sucrose, pH 7.5 were incubated in each well of a plate overnight. Excess membranes were removed and wells blocked with 3% BSA in PBS for 2 h. Plates were washed five times in PBS. 50μl of monoclonal antibody were added to each well and the plates incubated for 2 h. After a further five washes in PBS, 50μl of a 1 in 500 dilution of peroxidase-conjugated Rabbit anti-mouse Ig (RAM) (DAKO) in 3% BSA in PBS were added per well, and the plates incubated for 2 h. Plates were washed five times in PBS and stained with 50μl ortho-phenylenediamine dihydrochloride [0.04% orthophenyl diamine (Sigma) in 0.07M citric acid/phospate buffer containing 0.012% H_2O_2] per well. The reaction was stopped after 5 min by addition of 30μl of 1M HCl per well and the absorbance at 492nm read on an ELISA reader (Anthos Labtec Instruments). Wells which gave an absorbance three times greater than background were classed as positive.

2.2.4.3.2. ELISA to Detect Red Cell Components Using Red Cells Membranes Prepared *In Situ.* All incubations were carried out at room temperature in a damp box. Flat-well microtitre plates (Sterilin) were treated with poly-L-lysine. 75μl of $3ugml^{-1}$ poly-L-Lysine (Sigma) were added to each well of a plate and incubated for 1 h. Plates were washed three times in PBS/Tween (0.055% Tween 20 in PBS). 0.25% suspensions of washed packed red cells in PBS were prepared and 75μl added to wells. Plates were spun at 60xg for 1 min and the supernatants removed. Plates were then washed three times in blocking buffer (0.5% casein in 10mM tris buffered 0.15M NaCl, pH 7.4). Red cells were lysed by flooding wells with distilled water, incubating for 3 min and removing the water. This was repeated once. Membranes were sometimes fixed at this stage by addition of 75μl of 0.25% glutaraldehyde (BDH) in PBS and incubating for 5 min. All plates were then washed three times with blocking buffer, then blocked by filling the wells with blocking buffer and incubating for 30 min. The blocking buffer was removed, 75μl of monoclonal antibody added per well and the plates incubated for 1 h. The plates were washed three times with PBS-Tween and 75μl of a 1 in 500 dilution of RAM peroxidase in blocking buffer were added per well and plates incubated for 1 h. The plates were then washed three times in PBS-Tween then stained and read as in section 2.2.4.3.1.

2.2.4.3.3. Detection of Immunoglobulin. Flat-well microtitre plates (Nunc) were used and all incubations were at 4°C. 50μl of a 1 in 500 dilution of rabbit anti-mouse Ig (RAM) (Dako) in PBS were incubated in wells overnight. The plates were blocked with 3% BSA in PBS for 2 h and washed five times with PBS. 50μl of monoclonal antibody were added to the wells and incubated for 2 h. The plates were then washed five times with PBS. 50μl of a 1 in 500 dilution of RAM peroxidase in 3% BSA in PBS were added to each well and incubated for 2 h. Plates were washed five times with PBS and stained with orthophenyl diamine and read as in section 2.2.4.3.1.
This ELISA technique was also used to measure the residual antibody in absorption experiments. Monoclonal antibody was incubated with red cells or red cell membranes for 1 h. The cells or membranes were removed by centrifugation. The supernatant was titrated and the antibody detected using this ELISA technique. The amount of monoclonal antibody, red cells and membranes used varied depending on the antibody.

2.2.4.4. Preparation of Red Cell Membranes.
Red cell membranes were prepared using the method of Dodge *et. al.* (1963). Red cells were washed three times in phosphate buffered saline (PBS), centrifuging at 500xg for 5 mins. This was followed by one wash in 0.1M phosphate buffer, pH 7.3 (0.1M Na_2HPO_4 mixed with 0.1M NaH_2PO_4 until pH was 7.3). Cells were lysed in cold 5mM phosphate buffer, pH 7.3 and centrifuged at 11,200xg for 30 min at 8°C. This was followed by three or four washes in 5mM phosphate buffer, pH 7.3, centrifuging at 11,200xg for 20 min until the final wash, when the membranes were centrifuged for 40 min. Phenylmethane sulphonyl fluoride (PMSF) in iso-propanol was added to membranes to give a final PMSF concentration of 2mM and membranes were stored at -70°C.

2.2.4.5. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

2.2.4.5.1.Preparation of SDS-PAGE Gels. SDS-PAGE was carried out using a Biorad gel electrophoresis tank. 5-15% gradient gels were prepared by mixing 5% and 15% acrylamide solutions made up as in Table 2.5 in a BRL gradient former and running this into a slab gel mould (195mm x 160mm x 1.5mm) using a peristaltic pump. A layer of distilled water was added to exclude air and allow the acrylamide to polymerise. The water was removed and a 3% gel, prepared as in Table 2.6 added to form the stacking gel.

2.2.4.5.2. Running SDS-PAGE Gels. Membrane, cell extract or marker samples were added to an equal volume of either reducing or non-reducing dissolving buffer and boiled for 3 min.

Reducing dissolving buffer :-

0.5ml 1.87M Tris-Cl pH 8.8, 2.5ml 10% SDS,
1.25ml glycerol, 0.625ml 2-ß-mercaptoethanol,
8.125ml distilled water and a few grains of bromophenol blue
and pyronin Y.

Non-reducing dissolving buffer:-

5ml 10% SDS, 2ml 100mM Tris-Cl pH 8.0,
2ml distilled water,
1ml glycerol,
0.019g EDTA and a few grains of bromophenol blue and pyronin Y.

Samples were loaded onto gels set up in the gel apparatus containing tank buffer (0.192M glycine, 0.025M Tris and 0.1% SDS, pH 8.3). Gels were run for approximately 4 h with the cross-over power pack set to limit at 180 V, 45 mA per gel, until the pyronin Y tracking dye had reached the bottom of the gel. Alternatively the power pack was set to limit at 36 V, 12 mA per gel when gels were run overnight. The voltage was increased to 180 V the next morning, until the gel had finished running.

TABLE 2.6.
COMPOSITION OF SDS-PAGE GELS.

	ACRYLAMIDE CONCENTRATION		
	5%	**15%**	**3%**
glycerol	0	1.8	0
distilled water	10.55	3.02	7.5
acrylamide/bis*	2.79	8.36	1.25
1.87M Tris/HCl pH 8.8	3.34	3.34	/
1.0M Tris/HCl pH 6.8	/	/	1.25
Temed	15μl	15μl	10μl
10% SDS	200μl	200μl	100μl
10% ammonium persulphate	50μl	25μl	50μl

This tables lists the components of acrylamide solutions used to make gradient 5 -15% SDS-PAGE gels and the 3% stacking gel. Volumes shown in ml except where stated.

* - Acrylamide/bis solution made from preweighed acrylamide and bis acrylamide in ratio of 37.5:1 (Biorad) made up to give a total acrylamide concentration of 30%.

2.2.4.6. Staining SDS-PAGE Gels.

2.2.4.6.1.Coomassie Brilliant Blue Stain. Gels were fixed in 50% methanol in distilled water for 30 min. They were then stained in 0.1% Coomassie Brilliant Blue in 7.5% acetic acid, 5% methanol in distilled water for 30 min. The gels were destained by washing in several changes of 7.5% acetic acid, 5% methanol until a satisfactory background had been obtained.

2.2.4.6.2. Silver Stain. Gels were silver stained using a kit from Koch Light. All solutions used were prepared as directed in the kit instructions using distilled deionised water. Incubations were carried out at room temperature , with continuous shaking for the times recommended in the instructions for gels < 2 mm thick. However, half the volumes of prepared solutions recommended for gels of this size were found to stain the gels sufficiently. The gels were first incubated in two fixing solutions and then pretreatment solution. The gels were washed once in distilled deionised water. They were then incubated with the silver stain solution and washed a further four times with distilled deionised water. The stain was then developed using the developer solution and the reaction stopped using the stopper solution when colour had developed. Gels were then washed two or three times in distilled deionised water.

2.2.4.7. Electroblotting of SDS-PAGE Gels.

Gels were electroblotted onto nitrocellulose filters (Schleicher & Schull) using a Biorad electroblotting apparatus filled with TGM buffer (0.025M Tris, 0.192M glycine, 20% methanol, pH 8.3). Gels were blotted at 120 mA, 33 V for 18 h or at 400 mA for 2-4 h. The buffer was cooled with a water coil.

2.2.4.8. Immunostaining of Nitrocellulose Filters.

The following procedure was carried out at 4^{o}C. Filters were blocked for at least 2 h in blocking buffer (PBS containing either 3% BSA, 5% Marvel powdered milk or 0.15% or 0.005% Tween 20).

The filters were incubated overnight with monoclonal antibody in plastic bags. The volume of antibody used varied depending on the stength of the antibodies and quantities available. Antibodies were diluted in the appropriate blocking buffer where necessary. Filters were given five, 5 min washes in PBS and then incubated with peroxidase-conjugated antibody for 1.5-3 h in plastic bags. Where a murine monoclonal antibody was used this was a 1 in 500 dilution of RAM peroxidase in the appropriate blocking buffer. For human monoclonal antibodies this was substituted with a 1 in 400 dilution of goat anti-human immunoglobulin (DAKO). The peroxidase

conjugated antibody was preabsorbed with 2μl of red cell membranes and 2μl of FCS immediately before dilution. Filters were given five, 5 min washes in PBS.
The peroxidase label was then detected using one of the following staining methods at room temperature.

2.2.4.9. Detection of Peroxidase Label.

2.2.4.9.1. 4 Chloro-1-naphthol. 60mg of 4 chloro-1-naphthol was dissolved in 20ml of methanol and added to 100ml of 0.1M Tris, pH 7.5. 60μl of 9% hydrogen peroxide was added and the solution poured over filters. The reaction was stopped by washing with tap water when colour developed or after 20 min.

2.2.4.9.2. Diaminobenzidine (DAB). 0.5ml of 5mgml^{-1} 3,3'diaminobenzidine was added to 25ml of PBS. 12.5μl of 9% hydrogen peroxidase were added and the solution poured over filters. The reactions were stopped by washing with tap water when the colour was well developed or after 20 min.

2.2.4.9.3. Chemiluminescent Stain. The chemiluminescent stain described by Laing (1986) was used. A stock solution containing 88mg luminol (Sigma), 200mg of methyllumbelliferone (Sigma) and 30ml of 1M Tris base was stored in 1.5ml aliquots at -20^{o}C. Immediately before use a 1.5ml aliquot of stock solution was thawed and added to 0.5ml of 1M Trizma hydrochloride (Sigma), 18ml of distilled, deionised water, 25μl of D-luciferin (10mgml^{-1}) and 5μl of 30% H_2O_2. Nitrocellulose filters were incubated in this solution in the dark for 5 min. Filters were then removed, placed in plastic bags and exposed to Fuji X-ray film in a cassette for 15 s to 15 min depending on the intensity of the stain.

2.2.4.10. Immunoprecipitation.
Immunoprecipitation was carried out as follows. 1ml of packed, washed red cells was incubated with varying amounts of hybridoma culture supernatant containing antibody (2-30ml), either for 1 h at room temperature or overnight at 4^{o}C with continuous mixing. Red cells were washed four times in PBS, then once in 0.1M sodium phosphate buffer, pH 7.3 at 4^{o}C. The cells were lysed in cold 5mM sodium phosphate buffer, pH 7.3 and centrifuged at 11,200xg for 30 min at 4^{o}C. This step was repeated twice. The remaining ghosts were allowed to warm to room temperature and a sample taken to run on the SDS-PAGE gel as a control. The ghosts were solubilised in Triton-PBS (PBS containing 1-5% Triton X100 and 2mM PMSF), for 15 min at room temperature. This was then centrifuged at 11,200xg for 1 h at room temperature. The

supernatant was decanted from the cytoskeleton and incubated with either 15-25mg Protein A Sepharose (Pharmacia), 200μl of RAM-Sepharose (see 2.4.10.1.) or 20μg of Protein G Sepharose (Pharmacia) for 30-90 min at room temperature. The Sepharose was washed four times in 1% Triton-PBS at 5,000xg for 5 min at room temperature, then once in distilled water. 100μl of SDS-PAGE dissolving buffer (2.2.4.5.2.) were added and samples boiled for 5 min. Samples were run on SDS-PAGE gels. The gels were silver stained (2.2.4.6.2.) or electroblotted and immunostained with monoclonal antibodies.

2.2.4.10.1. Preparation of Rabbit anti-Mouse Sepharose (RAM-Sepharose). Rabbit anti-mouse immunoglobulin (RAM) (Dako) was coupled to cyanogen bromide-Sepharose (CNBR-Sepharose)(Pharmacia) as follows. 600mg of CNBR-Sepharose was swollen with 20ml of 1mM HCl in a glass universal, centrifuged at 3,000xg for 30 s and supernatant removed. This was repeated twice. The Sepharose was washed once in coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3). 2ml of RAM and 2ml of coupling buffer were added and mixed either for 2 h at room temperature or overnight at 4^{o}C. The Sepharose was centrifuged as above and supernatant removed. It was then mixed with 20ml 0.2M glycine, pH 8.0 for 2 h at room temperature or overnight at 4^{o}C in order to block the remaining active groups. The Sepharose was washed again in coupling buffer, then acetate buffer (0.1M sodium acetate, 0.5M NaCl, pH 4). This was repeated, followed by a wash in coupling buffer and finally a wash in PBS. The Sepharose was resuspended to 100mgml^{-1} in PBS containing 0.02% sodium azide and stored at 4^{o}C.

2.2.4.11. Immunoprecipitation using ^{125}I-Labelled Red Cells.

Cells labelled as described below were used for immunoprecipitations carried out as in 2.2.4.10., and run on SDS-PAGE gels. Gels were then stained with Coomassie Brilliant Blue (2.2.4.6.1.) and dried on a Biorad gel drier for 4 h at 80^{o}C under vacuum. Dried gels were exposed to Kodak XOMAT X-Ray film in cassettes containing intensifying screens at -70^{o}C for 1 day to 5 weeks. Films were developed in a 1 in 10 dilution of phenisol (Ilford) and fixed in fixative (Ilford).

2.2.4.11.1. ^{125}I- Labelling of Red Cells Using Lactoperoxidase.

^{125}I labelling was carried out in a fume cupboard.

The following reagents were added to a glass universal: 1ml washed, packed red cells, 1μl of ^{125}I (100mCiml^{-1}), 13μl of 2.5mgml^{-1} lactoperoxidase (Sigma) in PBS, 20μl of a 1 in 100 dilution of glucose oxidase (Sigma) in PBS, 110μl of glucose (18mgml^{-1}) in PBS. This was incubated for 15 min at 37^{o}C. Cells were washed four

times in PBS. Samples of red cells were taken before and after washing in order to estimate the percentage incorporation of ^{125}I.

A modification of this method was used to label minor components of the red cell membrane. The quantities of reagents were increased to 3μl ^{125}I, 65μl lactoperoxidase (5mgml^{-1}), 20μl of a 1 in 20 dilution glucose oxidase and 110μl glucose (90mgml^{-1}). The incubation time was increased to 30 min at 37^{o}C.

2.2.4.12. Immunoprecipitation using Biotin-Labelled Red Cells.

Non-radioactive immunoprecipitation was carried out as described by Laing *et. al.* (1987). PBS without Ca^{2+} or Mg^{2+}, pH 7.3 or 8.5 (Gibco) was used and all solutions were made using distilled, deionised water. Red cells were washed twice with PBS, pH 7.3 at 900xg for 5 min and once with PBS adjusted to pH 8.5 with NaOH. A dilution of N-hydroxysuccinimidobiotin (NHS-Biotin) (Sigma) in DMSO (66.67mgml^{-1}) was freshly prepared and 75μl added to 1.75ml of the washed, packed red cells with 14ml of PBS, pH 8.5. This was incubated with occasional shaking at 37^{o}C for 30 min. Red cells were washed three times with PBS, pH 7.3 as described above. Immunoprecipitation was then carried out as in 2.2.4.10. except that 0.5ml of red cells were used instead of 1ml. Also, cells were lysed with 20mM Tris-HCl, pH 7.6 instead of 5mM sodium phosphate buffer and PBS without Ca^{2+} and Mg^{2+}, pH 7.3 was used.

Precipitates were run on SDS-PAGE gels and electroblotted. Nitrocellulose filters were blocked in 5% BSA in PBS, pH 7.3 overnight at 4^{o}C. Filters were incubated in 10ml of a 1 in 500 dilution of peroxidase conjugated extrAvidin (Sigma) in 5% BSA in PBS, pH 7.3 for 1 h in a 37^{o}C water bath. Filters were then given six 5 min washes with 0.05% Tween 20 in PBS, pH 7.3 and stained using the chemiluminescent detection described in 2.2.4.9.3.

2.2.4.13. Antibody Purification.

Antibodies were purified using Protein A Sepharose (Pharmacia) and high ionic strength buffers recommended by Pharmacia for the purification of IgG1 antibodies. Culture supernatant harvested from hybridomas grown in RPMI medium containing 5% FCS was used. The ionic strength of this was modified by addition of glycine to give a final concentation of 0.75M, NaCl to give a final concentration of 1.5M and the pH adjusted to 8.9 with 5M NaOH.

Sodium azide was added to give a concentration of 0.01%.

1.5g of Protein A Sepharose was swollen in 0.1M glycine/HCl, 0.01% sodium azide, pH 3.0 for 30 min and placed in a small column. The column was stored at 4^{o}C. Purification was carried out at room temperature. The column was regenerated with

30ml of regeneration buffer (100mM citric acid, 0.01% sodium azide, pH 3.0) and equilibrated with 50ml of binding buffer (1.5M glycine, 3M NaCl, 0.01% sodium azide, pH 8.9). 200ml of modified culture supernatant was then circulated through the column overnight. 5ml of binding buffer were then passed through the column. Antibody was eluted from the column with 30ml of elution buffer (100mM citric acid, 0.01% sodium azide, pH 5.0) and collected in 5ml fractions. The column was regenerated with 30ml of regeneration buffer.

The total protein present in the fractions was determined from its absorbance at 280nm using an extinction coefficient of 1.4 for 1mg of protein per ml. Fractions with a reasonable protein concentration were dialysed to PBS containing 0.01% sodium azide at $4^{o}C$ overnight. The antibody activity was assessed by haemagglutination. Antibody was concentrated to approximately $1mgml^{-1}$ using Centriprep concentrating tubes (Amicon), centrifuged at 1,500xg at $4^{o}C$. Concentrated antibody was stored at $-70^{o}C$. The amount of antibody remaining in the culture supernatant was assessed serologically and the supernatant reused two or three times until no antibody activity remained.

2.2.4.14. Red Cell Binding Assays.

Binding assays using ^{125}I-labelled, purified monoclonal antibodies were carried out as described by Gardner *et. al.*(1989) to determine the biological activity and the functional affinity constants of the antibodies and to determine the number of antigen binding sites per red cell. They were also used in competitive binding experiments.

The purified, concentrated antibodies were iodoinated as follows: 2 iodobeads (Pierce) and approximately 100μCi of ^{125}I (Amersham) were added to 1ml of purified concentrated antibody and incubated for 15 min at room temperature. The antibody was dialysed to two changes of PBS containing 0.01% sodium azide overnight. Samples were taken from each change of PBS in order to calculate the percentage incorporation of ^{125}I.

For the binding assays, antibody at various dilutions (see following sections for dilutions) was incubated together with washed red cells in a total volume of 150μl 3% BSA in PBS at $37^{o}C$ for 90 min. Reaction mixtures were transferred to 0.4ml microtubes (Sarstedt) containing 150μl of a 8:2 mixture of di-n-butyl phthalate and di-nonyl-phthalate (Sigma) and centrifuged for 1 min at 10,000xg. The tubes were frozen and cut just above the red cell pellet to separate the bound from the free antibody. These were counted on a LKB Compu-gamma counter.

2.2.4.14.1. Determination of the Biological Activity and Functional Affinity Constants of Antibodies. The biological activity of antibodies in labelled preparations was determined using binding assays (section 2.2.5.14), where a constant amount of antibody protein was incubated with at least four different volumes of red cells, ranging from 1μl to 50μl. The antibody concentration was then calculated using Scatchard analysis (Scatchard, 1949) (section 4.4.2.). The functional affinity constants of the antibodies were determined from similar experiments where the working dilutions of antibody had been adjusted to give a range of values for bound antibody below and above 50% at the end of the incubation period. The functional affinity was then determined using a Karush plot (Karush, 1962) (section 4.4.3.).

2.2.4.14.2. Calculation of Number of Antigen Binding Sites. Different amounts of antibody, ranging from 1μg to 50μg were incubated with 0.5, 1 or 2μl of red cells and treated as described above (section 2.2.4.14.). A duplicate dilution of red cells was used to determine the exact number of cells in the dilution using a Coulter Counter. Values of the maximum number of antibody molecules which could bind to one red cell were obtained by extrapolation of the Scatchard plot (section 4.4.4.).

2.2.4.15. Competitive Binding Assays.

Labelled and unlabelled purified antibodies were incubated simultaneously with red cells in order to determine whether binding of one antibody inhibited binding of the other. The volume of cells which would bind approximately 50% of 0.5μg of labelled antibody was determined (approximately 5μl packed cells). This amount of red cells, approximately 0.5μg of labelled antibody and varying amounts of unlabelled inhibiting antibody in about 5 to 10 fold molar excess, was incubated in a total volume of 150μl and treated as described above (section 2.2.4.14). The uptake of the labelled antibody in the presence of the inhibitor was compared to the uptake when no inhibitor was present and expressed as the percentage of labelled antibody bound. This was plotted as graphs of the concentration of inhibitor against the percentage of labelled antibody bound to cells (section 4.5).

2.2.5. Flow Cytometry.

Leucocyte, red cell and cell line samples were labelled by indirect immunofluorescence and analysed on a flow cytometer.

2.2.5.1. Preparation of Red Cells for Flow Cytometry.

Red cells were fixed with di-methyl-suberimidate (DMS) before incubating with antibody, to prevent agglutination. The cells were vortexed thoroughly at all stages of the procedure. 100μl of a 50% suspension of packed red cells in PBS was washed in 4ml 0.001% SDS in PBS. The red cell pellet was suspended with 400μl carbonate saline (1.05% Na_2CO_3, 0.87% NaCl, 0.004% Na_2EDTA, mixed with 0.084% $NaHCO_3$, 0.87% NaCl, 0.004% Na_2EDTA, to pH 10) and 500μl of a 3mgml^{-1} solution of DMS in carbonate saline. This was incubated at 37^{o}C for 30 min with occasional vortexing. The pellet was washed twice in PBS and resuspended in 1% DL lysine in PBS to 1 ml. Red cells were stored at 4^{o}C at this stage. The red cell suspension was diluted 1 in 10 in PBAST (0.5% BSA, 0.1% Tween 20 in PBS) and 25μl (approximately 10^6 cells) incubated with 50μl of monoclonal antibody for 1 h at room temperature. Red cells were washed in 3ml of PBAST and incubated with 25μl of a 1 in 25 dilution of fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse Ig (Dako) in PBAST for 30 min at room temperature. 300μl PBAST were added and samples analysed on a flow cytometer (section 2.2.5.5).

2.2.5.2. Preparation of Leucocytes for Flow Cytometry.

Blood samples were collected in sodium citrate and kept at room temperature for up to 24 h before use. 1 volume of blood was mixed with 2 volumes of 0.3% sodium citrate in PBS and 1 volume of 6% dextran and allowed to stand for approximately 1 h until the red cells had separated out. The supernatant containing the leucocytes was removed and the leucocytes washed twice in 0.3% sodium citrate in PBS, centrifuging at 500xg for 5 min. Remaining red cells were lysed by incubating with 50 ml of red cell lysing buffer (0.83% ammonium chloride and 0.1% $KHCO_3$) for 5 min on ice. Leucocytes were centrifuged and washed in 1% BSA in PBS. This lysis was repeated if necessary. Leucocytes were then resuspended in PBAS to give 2-5 x 10^6 cells per ml and stored on ice until labelled.

2.2.5.3. Preparation of Cell Line Samples for flow cytometry.

Cells were harvested as described in 2.2.1.3. and washed twice in PBS. Cells were then resuspended in 1% BSA in PBS to give 2-5 x 10^6 cells per ml and stored on ice until labelled.

2.2.5.4. Reaction of Leucocyte and Cell Line Samples with Antibodies for Flow Cytometry.

50μl of cell suspension (2-5 x 10^6 cells per ml) and 50μl of monoclonal antibody were incubated on ice for 1 h. Cells were washed once with 4ml 1% BSA in PBS, centrifuging at 500xg for 5 min. The cell pellet was incubated with 25μl of a 1 in 25 dilution of FITC conjugated rabbit anti-mouse Ig (Dako) in 1% BSA in PBS for 30 min on ice. 300μl 1% BSA in PBS was then added and samples analysed on a flow cytometer (sections 2.2.5.5.).

2.2.5.5. Analysis of Flow Cytometry Readings.

The flow cytometer recorded the light scatter, electronic volume and the intensity of the fluorescent signals emmited by each cell. This data was then analysed in various ways. Dot plots showing the amount of light scattered from the cells in a forward and sidewards manner were used to attribute the data to separate cell subpopulations by size. Histograms were plotted showing the amount of fluoresence emitted by the cells. The percentage of cells which had reacted with the monoclonal antibodies could be calculated from these histograms by comparison with the amount of immunofluoresence emitted by the unlabelled control samples.

CHAPTER 3.

PRODUCTION OF A MONOCLONAL ANTIBODY WITH ANTI-Wr^a SPECIFICITY.

A primary aim of this project was to produce monoclonal antibodies to low incidence red cell antigens to use as tools to investigate the nature and structural features of such antigens. The Wr^a antigen was the first antigen chosen for the reasons outlined in Section 1.6. Experiments which led to the production of the anti-Wr^a monoclonal antibody, BGU1-WR, are described in this chapter.

3.1. Murine Monoclonal Antibody Production.

A number of fusions were carried out as described in section 2.2.2.1., using the spleens of mice immunised with Wr(a+) red cells as outlined in Table 2.4. The early fusions produced only a low percentage of hybridomas that were secreting antibodies which detected red cell antigens by haemagglutination. Later fusions were designed to compare different immunisation protocols and screening techniques in order to attempt to increase this percentage, with the hope of increasing the chance of finding an antibody which defines the Wr^a antigen.

3.1.1. Screening of Initial Fusions using Haemagglutination Tests.

Hybridoma supernatants from the initial three fusions were screened with the Wr(a+b+) cells used for immunisation of the mice and also with Wr(a-b+) red cells using direct and indirect haemagglutination tests as described in section 2.2.3. Table 3.1. gives a summary of these serological results for the first 8 fusions. Fusions 1 and 3 resulted in only a low number of hybridomas and none of these produced antibodies which reacted with red cells by haemagglutination. This was not totally unexpected since previous experience in this laboratory had suggested that when a low yield of hybridomas was obtained very few secreted antibodies which reacted with the immunising agent. Presumably this is because a poor immune response results in fewer hybridomas.

Fusion 2 produced a higher number of hybridomas, 12% of which recognised red cell antigens as judged by haemagglutination tests. None of the supernatants which gave a positive reaction distinguished between Wr(a+) and Wr(a-) cells indicating that none were anti-Wr^a. All of these supernatants were tested against a panel of untreated and

enzyme treated red cells in order to provide hints of the specificities of the antibodies. None of the specificities were determined.

However, even in fusion 2, the number of hybridomas secreting antibodies which caused haemagglutination of red cells was at the lower end of the range of yields reported by other workers, 10% to 58% of hybridomas obtained by Parsons (1991) in similar experiments reacted with red cells.

TABLE 3.1.
SCREENING OF HYBRIDOMAS BY DIRECT AND INDIRECT HAEMAGGLUTINATION TECHNIQUES.

FUSION	TOTAL HYBRIDOMAS PRODUCED	TOTAL SCREENED	NUMBER POSITIVE		
			AGG	IAT	TOTAL
A.	**INITIAL IMMUNISATION WITH Wr(a+) CELLS.**				
1	25	23	0	0	0
2	133	124	3	12	15
3	37	35	0	0	0
4	130	116	11	12	23
B.	**COMPARISON OF IMMUNISATION WITH FRESH & FROZEN RED CELLS**				
5	66	66	0	4	4
6	71	71	3	2	5
C.	**COMPARISON OF DIFFERENT MOUSE STRAINS.**				
7	137	104	5	5	10
8	152	95	8	16	24

This table shows the total number of hybridomas produced in Fusions 1 to 8, the number which directly agglutinated red cells (AGG), reacted by indirect antiglobulin test (IAT) and the total number which reacted with red cells. These figures refer to haemagglutination tests carried out using red cells from the same individual as those used for immunisation of the mice for each fusion (see Table 2.4 for further details).

3.1.2. Experiments to Determine Whether Hybridomas were Secreting Mouse Immunoglobulins.

An ELISA assay was used to screen hybridoma supernatants from fusion 2 to check that those that did not react with red cells were indeed secreting mouse antibodies (section 2.2.3.3.). Sixteen supernatants were tested: 5 were known to contain mouse immunoglobulin since they reacted with red cells by haemagglutination: the remaining 11 were negative when tested by haemagglutination. Under the conditions used 4 of the 5 red cell reactive supernatants gave a clear positive result as did 7 of the 11 non-red cell reactive supernatants. Thus, although the assay was clearly not optimised, 7 of the 11 apparently negative supernatants were certainly secreting mouse immunoglobulins.

3.1.3. Experiments to Validate the Sensitivity of Screening.

In fusions 1 to 4 the ratio of supernatants which reacted with red cells by IAT as compared with direct agglutination was almost 1:1. This was not consistent with the results of other workers who found that only a low percentage of hybridomas produced antibodies which agglutinate red cells and a much higher percentage produced "incomplete antibodies" which react by IAT (Goodfellow and colleges, Parsons and colleges, unpublished observations). This, together with the fact that apparently non-red cell reactive hybridoma supernatants were secreting antibody (section 3.1.2.) suggested that a problem with the IAT stage of the screening procedure existed.

Numerous ways of improving the sensitivity of the IAT were investigated. Three different rabbit anti-mouse immunoglobulin reagents were used to test a number of the supernatants. All gave identical results as did a fourth reagent made up of a mixture of purified anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA. Altering the red cell concentration, incubation after the immunoglobulin reagent was added and increasing the amount of washing had no effect. The test performed in tubes also produced identical results to that obtained in microtitre plates.

Another possible explanation for the low yields of positive supernatants was that antibodies were being missed which bind to red cells but do not readily cause agglutination even in the presence of a second antibody. These could possibly be detected using a radioimmunobinding assay (RIA) which is probably more sensitive since bound antibody is directly detected and does not depend on secondary agglutination. RIA was therefore used as a primary screen for supernatants from fusions 7 and 8 in addition to haemagglutination techniques (see section 3.1.6).

3.1.4. Identification of Supernatants Containing Antibodies which Recognise Internal Red Cell Epitopes.

Since a number of culture supernatants which did not react with red cells by haemagglutination had been shown to contain mouse immunoglobulins, two different methods were used to investigate whether these antibodies recognised internal or cryptic red cell antigens.

The eleven culture supernatants which were shown to contain immunoglobulins in section 3.1.2., despite not reacting with red cells, were tested using a red cell membrane ELISA assay (section 2.2.4.3.1.). Two of these supernatants gave a positive reaction. Some of the control supernatants, which reacted with red cells by haemagglutination, gave a negative result with this ELISA test suggesting that it was not optimised.

A further seventeen supernatants from fusion 2, thirteen of which did not react by haemagglutination, were screened using electroblotting techniques (section 2.2.4.7.). Five of the supernatants recognised red cell membrane components by immunoblotting, three of which were negative serologically. One supernatant contained antibodies which recognised bands with an apparent M_r of 45,000, 74,000 and 90,000 possibly corresponding to the monomer, heterodimer and dimer of glycophorin A. Antibodies present in another two supernatants recognised bands with an apparent Mr of 210,000 possibly corresponding to spectrin. The remaining two supernatants recognised many bands and appeared to contain more than one antibody. The specificity of these antibodies was not investigated further.

It is possible that some of the antibodies which did not react with intact red cell but did react with red cell components by immunoblotting or ELISA recognise internal membrane epitopes. These antibodies may have been generated as a result of red cells lysing on entering the mouse. The Wr(a+) cells used for immunising the mice had been stored at -30^{o}C in freezing fluid (section 2.1.2.). Immediately before immunisation of mice for fusions 1 and 2 the cells were dialysed and suspended in sterile saline. This could possibly have increased the fragility of cells leading to increased lysis. Therefore the Wr(a+) cells used for immunising mice for fusions 3 and 4 were not dialysed but left in the freezing fluid for immunisation, a procedure used successfully by Parsons (1991). Fusion 4 produced approximately the same number of hybridomas as fusion 2, slightly more hybridomas produced antibodies which reacted with red cells by haemagglutination.

3.1.5. Fusions to Investigate Effect of Immunisations with Fresh and Frozen Red Cells.

Although a proportion of the supernatants contained antibodies which recognised internal or cryptic antigens a number which contained mouse immunoglobulins still did not recognise red cell antigens. Consideration was therefore also given to other antigens the mice could have been challenged with during the immunisation period. Since the freezing fluid used up to this point was non-sterile, several tests were carried out to determine whether microorganisms were present. Inoculation of the freezing fluid on agar plates, in L-broth and RPMI showed that it did indeed contain bacteria. Two comparison fusions, 5 and 6 were therefore set up to determine whether the non sterile freezing fluid might be responsible for the production of antibodies that were not against red cells: namely that antibodies to microorganisms had been produced. Fusion 5 used the spleen of a mouse immunised with red cells stored and injected in the original non-sterile freezing fluid and fusion 6 used that of a mouse immunised with fresh cells in sterile saline. In these two experiments Wr(a-) cells were used for the immunisation since fresh and frozen Wr(a+) cells from the same individual were not available. The results in Table 3.1. show that there was no difference between the fusions and that again a low percentage of the supernatants reacted with red cells.

3.1.6. Fusions to Investigate the Effect of Different Mouse Strains.

Fusions 7 and 8 were set up to determine whether changing the strain of mouse might improve the yield of antibodies due to a difference in immune response. Different mouse strains have different genetic make-ups and it is possible that one inbred line would recognise determinants that a different inbred line would not. Since other workers have had success with the inbred strain BALB/c I decided to use an independently bred variant of BALB/c rather than a different strain. Fusion 7 which acted as the control strain used the same inbred line of BALB/c mouse as previous fusions whereas fusion 8 used BALB/tk, a variant with tail kinks. Heterozygous female mice with normal tails were used. A radioimmune binding assay (RIA) (section 2.2.4.2.) was used in addition to haemaggluination tests as a screening procedure in these experiments.

More positive hybridomas were produced from the BALB/tk fusion indicating a slightly better immune response to human red cells in this mouse strain. More hybridomas were shown to be secreting antibodies which reacted with red cells by RIA than by the haemagglutination techniques used, as shown in Table 3.2. Although a small number of supernatants which agglutinated red cells strongly were negative by RIA (possibly because the cells were very closely packed and the binding sites for the ^{125}I-anti-mouse immunoglobulin were not available). It is not clear, however, that

this difference in sensitivity between the RIA and haemagglutination tests was real since, there were only enough of the Wr(a+b+) cells used to immunise the mice available for the RIA tests. The haemagglutination tests were carried out with Wr (a+b+) red cells from a second individual. Indeed supernatants which initially gave a positive reaction by RIA, but not by haemagglutination, were subsequently found not to react by RIA with the red cells of the second individual.

3.1.7. Identification of an Anti-Wr^a Antibody.

None of the positive supernatants from fusions 2 and 4 appeared to be anti-Wr^a and their specificities have not been determined, neither have those of the supernatants from fusions 5 and 6 which reacted with red cells.

The majority of the supernatants which reacted with red cells from fusions 7 and 8 did not appear to contain anti-Wr^a since they reacted with the Wr(a-b+) red cells used in the initial screening tests. One of these antibodies has subsequently been shown to have anti-N specificity (Letsu, personal communication). Some supernatants from fusions 7 and 8, however, hinted at possible anti-Wr^a specificity. Two of the supernatants from fusion 7 and nine from fusion 8 reacted only with the Wr(a+) cells in the initial RIA and haemagglutination screening tests. They were all tested with a further two Wr(a+) and two Wr(a-) cells by RIA. The results of these tests are shown in table 3.3. Both supernatants from fusion 7 and seven from fusion 8 were shown not to be anti-Wr^a since they did not react with all Wr(a+) cells. The specificity of these antibodies has not yet been determined. An eighth supernatant from fusion 8 (2F2) which had only reacted weakly with the immunising cells, did not react with any of the other cell samples tested. This supernatant was not investigated further.

The remaining supernatant from fusion 8 (2E5) which reacted with all three Wr(a+) samples but not with the Wr(a-) samples was tested further using haemagglutination tests. It was shown to react by IAT with seven different Wr(a+) samples and not with ten Wr(a-) samples. Using Fisher's Exact method for a two by two contingency table (Race and Sanger, 1975) it was calculated that there is a probability of 1 in 19,488 that this reaction pattern occurred by chance. Therefore, this supernatant which has been named BGU1-WR, contained anti-Wr^a.

TABLE 3.2
COMPARISON OF RIA AND HAEMAGGLUTINATION RESULTS OF FUSIONS 7 AND 8.

MOUSE	FUSION	TOTAL TESTED	HAEMAGGLUTINATION		RIA	TOTAL	
			AGG	IAT		No	%
BALB/c	7	137	5	5	17	21	15
BALB/tk	8	152	8	16	33	38	25

This table shows number of hybridomas found to react with red cells by direct agglutination (AGG), indirect antiglobulin test (IAT) and by radioimmunobinding assay (RIA). The total gives the number and percentage of hybridomas shown to be secreting antibodies which reacted with red cells by one or more of the tests used.

TABLE 3.3.
IDENTIFICATION OF ANTI-Wr^a.

FUSION	SUPERNATANT	INITIAL SCREEN		FURTHER SCREEN			
		Wr(a+)	Wr(a-)	Wr(a+)	Wr(a+)	Wr(a-)	Wr(a-)
7	4G8	+	-	-	-	+	-
7	2D11	+++	-	-	+	+	+
8	2A9	++	-	++	++	++	++
8	2C11	++	-	++	-	+	++
8	2E7	+	-	+	+	+++	+++
8	2C10	+++	-	+	++	++	-
8	2C8	+	-	-	-	-	+
8	2F8	+	-	+	+	+	++
8	3H7	++	-	+	-	++	+
8	2F2	+	-	-	-	-	-
8	2E5	++	-	++	++	-	-

This table shows the results of screening tests by RIA used to identify an anti-Wr^a antibody. The assay was carried out as described in section 2.2.4.2. The results come from assays performed on different occasions. The background reading for each plate was determined from the counts recorded from a red cell sample incubated with RPMI medium instead of hybridoma supernatant. The positive wells were scored as follows:-

+	-	3 x background reading
++	-	4 x background reading
+++	-	>5 x background reading

3.1.8. Comparison of BM Condimed Media and Feeder Cells.

In fusions 5, 6, 7 and 8 a comparison of the use of feeder cells and of their replacement with an enriched medium, BM condimed media (Boehringer Mannheim) was carried out. Fusions 5 and 6 were plated out into 24 well plates and fusions 7 and 8 in 96 well plates. Feeder cells were replaced with a 10% solution of this medium in RPMI in half the plates from each fusion. The rate of growth of cells in each medium was assessed by comparing the amount of hybridomas present after 16 days and the average number of days it took hybridomas to grow before supernatant was ready to be harvested. The results of these comparisons are shown in table 3.4. No difference in the length of time hybridomas took to produce supernatants ready to harvest was observed for cells growing with or without feeder cells. There was no difference in the number of hybridomas present on 24 well plates fed with the medium or in the presence of feeder cells. However, there was a significant decrease in the number of the cells in 96 well plates fed with the medium compared with those containing feeder cells. This suggests that this medium is not as effective as feeder cells when using the protocols followed in this laboratory.

TABLE 3.4.

COMPARISON OF FEEDER CELLS AND BM-CONDIMED H1 MEDIUM.

FUSION	No. WELLS WITH GROWTH AFTER 16 DAYS		AVERAGE No. DAYS BEFORE HARVESTING	
	FEEDER CELLS	CONDIMED MEDIUM	FEEDER CELLS	CONDIMED MEDIUM
5	28	23	36.1	33.5
6	31	27	34.1	33.7
7	96	41	63.6	60.2
8	100	52	61.9	63.7

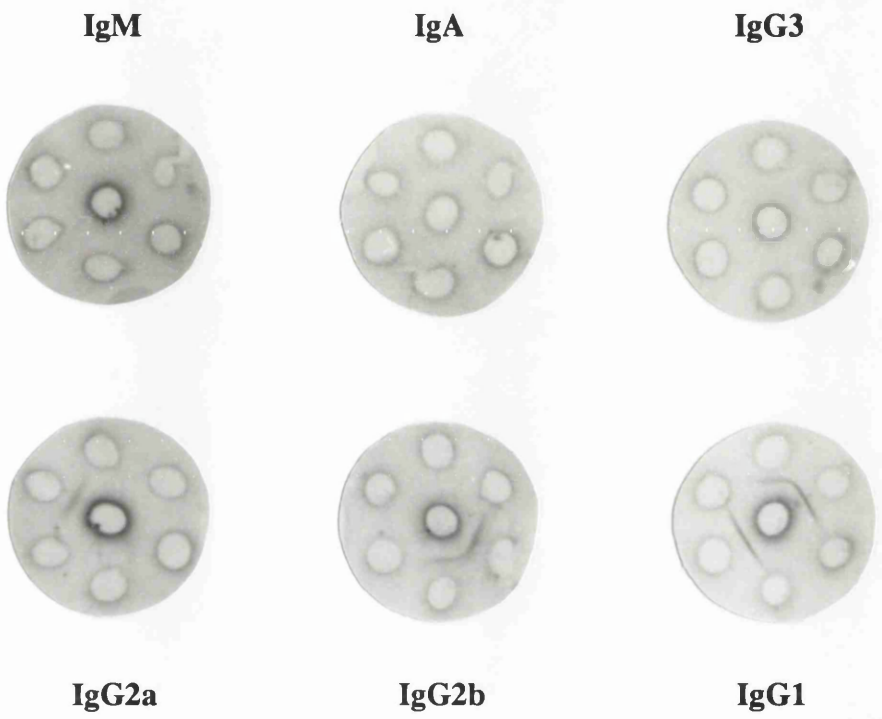
3.2. SUBCLONING BGU1-WR.

The BGU1-WR hybridoma line was grown up in 24 well plates. Cells from a well where the majority of cells had died but those which recovered still secreted antibody were selected for subcloning in soft agar (section 2.2.2.1.2.). Approximately fifty colonies were picked and grown up first in 96 well and then in 24 well plates. Forty eight of these were screened by haemagglutination and all were shown to be secreting anti-Wr^a. Therefore, the cell line was considered monoclonal. One subclone was selected and grown up for antibody production as described in section 2.2.1.4.

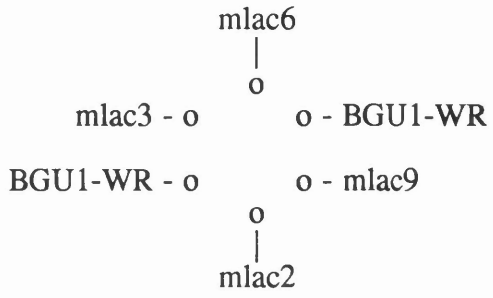
3.3. DETERMINATION OF THE IMMUNOGLOBULIN SUBCLASS OF BGU1-WR.

An Ouchterlony assay (section 2.2.4.1.) was used to determine the immunoglobulin subclass of BGU1-WR. The anti-lactase monoclonal antibodies mlac6, mlac3, mlac2 and mlac9 of IgG subclasses IgG1, IgG2a IgG2b and IgG2b respectively were used as controls. Plates using anti-mouse IgM, IgA, IgG1, IgG2a and IgG2b in the central well were made. Figure 3.1 shows the results of this experiment. Diffusion lines appeared between the expected wells on the relevant plates for the control antibodies. Lines appeared on the IgG1 plate for the BGU1-WR antibody showing that its immunoglobulin subclass is IgG1.
This result was confirmed in further experiments (results not shown) which also confirm that BRIC-14 the anti-Wr^b monoclonal antibody used in the following chapters is IgG2a as published (Gardner *et. al.*, 1989).

FIGURE 3.1.
OUCHTERLONY SHOWING IMMUNOGLOBULIN SUBCLASS OF BGU1-WR.



The central wells contained anti-IgG antibody as indicated on figure legend. The surrounding wells contained test monoclonal antibody supernatant as shown below:-



3.4. Further Attempts to Produce a Murine anti-Wr^a Monoclonal Antibody.

The experiments described in chapter 4 suggested that it would be useful to obtain a second anti-Wr^a monoclonal antibody therefore a further five fusions were carried out with the help of Miss Carole Green.

Since haemagglutination studies using BGU1-WR (section 4.1) have shown that papain treatment enhances the Wr^a antigen BALB-tk mice were immunised with papain treated Wr(a+) red cells which had been washed ten times in sterile saline after treatment. The immunisation protocols of mice used for fusions 1 to 8 had varied in both the number of boosts given and the length of time between boosts. There did not appear to be any correlation between these protocols and the number of hybridomas produced or the percentage of hybridomas which produced red cell-reactive antibodies. Therefore for fusions 9 to 13 a standard protocol was used where the initial immunisation was followed by one boost approximately two weeks later. Others workers (Daniels *et. al.*, 1983) have successfully used short immunisation protocols to produce anti-red cell monoclonal antibodies. Supernatants from the hybridomas were only screened using haemagglutination tests due to lack of time for further investigation. Table 3.5 gives a summary of the number of hybridomas produced and the percentage which reacted by haemagglutination from these fusions. The percentage of hybridomas which secreted antibodies was once again disappointingly low. Indeed in the most successful fusion (13) no antibodies which recognised red cells were detected. One possible explanation for this low yield is that antibodies have been made against the papain remaining in the red cell preparations. Further investigation of this was not possible due to lack of time. None of the supernatants from fusions 9 and 10 which reacted with red cells were anti-Wr^a and their actual specificities have not been determined.

TABLE 3.5.

FUSIONS FROM MICE IMMUNISED WITH PAPAIN TREATED CELLS.

FUSION	TOTAL HYBRIDOMAS PRODUCED	TOTAL SCREENED	NUMBER POSITIVE		
			AGG	IAT	TOTAL
9	7	7	0	1	1
10	75	64	1	13	14
11	75	43	0	0	0
12	5	5	0	0	0
13	119	119	0	0	0

This table shows the number of hybridomas which were produced in Fusions 9 to 13, the number which directly agglutinated red cells (AGG), the number which reacted with red cells by indirect antiglobulin test (IAT) and the total number which reacted with red cells.

3.5. Attempts to produce a Human Anti-Wr^a Monoclonal Antibody

During the course of this project attempts were also made to produce a human monoclonal antibody with anti-Wr^a specificity. Human monoclonal antibodies which recognise red cell antigens have been made successfully by other workers (Koskimies, 1980; Boylston *et. al.* 1980) as described in section 1.5. Anti-Wr^a often occurs in the serum of auto immune haemolytic aneamia (A.I.H.A.) patients or in that of healthy individuals (Section 1.4.1.). Attempts were made to produce cell lines secreting anti-Wr^a from the peripheral blood lymphocytes of six healthy donors with anti-Wr^a in their serum. The cells were transformed using Epstein-Barr virus as described in Section 2.2.2.2. and the cells were dispersed into 96 well plates. Lymphoblastoid cell lines were established from all the samples, over 60 clones from two of these samples, 14 from a third and less than 10 from the remaining three samples survived. Supernatants from these were screened by haemagglutination for the presence of anti-Wr^a as described in section 2.2.3. No clone was secreting anti-Wr^a. In retrospect this is not a surprising outcome as most reported human monoclonal antibodies are produced from recently boosted donors. Deliberate immunisation of donors with the Wr^a antigen was not possible.

Attempts were therefore also made to establish cells lines from the peripheral blood lymphocytes from four A.I.H.A. patients and the spleen of another. The rationale behind this was that as A.I.H.A. patients have a stimulated immune system and often produce anti-Wr^a they may recently have been boosted naturally. Unfortunately analysis of the serum of these A.I.H.A. patients showed that none were producing anti-Wr^a. Therefore these attempts to produce a human monoclonal anti-Wr^a antibody were also unsuccessful.

3.6. DISCUSSION.

The production of the BGU1-WR monoclonal antibody proved that murine monoclonal antibodies which recognise the low incidence red cell antigen Wr^a can be produced following immunisation with Wr(a+) red cells. The BGU1-WR antibody proved to be a good reagent for haemagglutination studies as described in section 4.1. It did not, however, prove to be a successful reagent for deducing the immunochemical nature of the Wr^a antigen. Attempts to produce another anti-Wr^a monoclonal antibody were unsuccessful.

In retrospect the selection of the Wr(a+) donor may also have been a contributing factor to the production of BGU1-WR in fusion 8, since absorption experiments (section 4.2.1) suggested that cells of this donor (S.W.) may have a relatively high number of Wr^a antigen sites. However, it is unlikely that the choice of Wr(a+b+) red cells used for immunisation of mice used for fusions 9 to 13 would have affected the chances of producing an anti-Wr^a monoclonal antibody since the major problem with these fusions was the failure to produce hybridomas secreting antibodies which recognised red cells (section 3.4.).

Hybridomas secreting antibodies with anti-Wr^b specificity are often produced following immunisation of mice with red cells (Anstee and Edwards, 1982; Ridgwell *et. al.*, 1983; Parsons, 1991). Some supernatants which reacted with red cells from fusions described in this thesis were tested with En(a-) red cells which lack the Wr^b antigen. No anti-Wr^b antibody was identified, although not all the supernatants were screened with En(a-) red cells since these cells were not always available.

The failure of these experiments to produce an anti-Wr^b antibody could, perhaps, be due to the fact that Wr(a+b+) red cells which have a lower level of the Wr^b antigen, were used to immunise mice for some of the fusions. But it may just reflect the emphasis of the screening or perhaps the particular strains of Balb/c mice used.

A disappointing yield of hybridomas producing anti-red cell antibodies was achieved during this study: The reasons for this were not obvious despite using several immunization protocols and trying several screening techniques. There is a slight hint from my experiments that BALB/tk mice, immunized with untreated cells give a better response than BALB/c mice. This would not have been related to the tk mutation but to the different genetic backgrounds of the strains. There was not sufficient time to further investigate this possibility.

CHAPTER 4.

COMPARISON OF THE PROPERTIES OF THE Wr^a AND Wr^b ANTIGENS.

This chapter describes a variety of immunochemical and serological studies designed to compare and characterise the Wr^a and Wr^b antigens using the anti-Wr^a monoclonal antibody, BGU1-WR, produced during the course of this work (Chapter 3), and BRIC-14, an anti-Wr^b antibody provided by Dr David Anstee. Studies to estimate the number of Wr^a and Wr^b antigen sites on red cells of different Wr phenotypes are also described.

4.1. HAEMAGGLUTINATION STUDIES.

4.1.1. Specificity of BGU1-WR.

The specificity of BGU1-WR as an anti-Wr^a was demonstrated as described in chapter 3, when it reacted with red cells of 7 known Wr(a+) individuals but not with those of 10 known Wr(a-) individuals. In further studies BGU1-WR was shown to react with all Wr(a+) cells tested (23 in total), including the Wr(a+b-) cells of donor M.Fr. (Adams *et. al.*, 1971). Dosage studies using these cells are described in section 4.1.2. BGU1-WR failed to react with many Wr(a-) cells including cells carrying the following low incidence antigens:- Dantu, He, Hil, Hop, Hut, M_1, M^g, Mit, Mt^a, Mur, M^v, Nob, Ny^a, Or, St^a, Vr, Vw, DANE, ERIK, Mut, C^w, E^w, Rh33, V, VS, Kp^a, Wk^a, Lu9, Lu14, Co^b, Yt^b, An^a, Bg^{a+b}, Bp^a, Dh^a, Di^a, Fr^a, Hg^a, Ls^a, ELO, Chr^a, Ol^a, Os^a, Rd, Re^a, Sc2, Sd^a, To^a, Wd^a, Wu, and SHIN. BGU1-WR also failed to react with Tn polyagglutinable cells.

Suggestions that the *WR* gene may encode a difference in the band 3 protein of Wr(a+) and Wr(a-) individuals (Wren and Issitt, 1988; Telen, 1987; Telen and Chasis, 1990) led me to test any red cell samples which carried an altered band 3 protein. Only one such sample became available during the course of study; an ovalocytic sample which carries a common point mutation at amino acid 56 (lysine to glutamate) and a unique 9 amino acid deletion of residues 400-408 (Schofield *et. al.*, 1992). These cells reacted normally with BRIC-14 but did not react with BGU1-WR. They were thus assumed to be Wr(a-b+). These findings were confirmed on a second sample of these cells. However, at this point some abnormality in the Wr^b antigen expression of these cells was suggested since they reacted less strongly than normal

cells with the anti-Wr^b monoclonal antibody, R7 and did not react with human anti-Wr^b from donor M.Fr. (Lomas, unpublished observations).

4.1.2. Dosage of Wr^a and Wr^b Antigens.

Preliminary attempts were made to compare the strength of Wr^a and Wr^b antigens on Wr(a+b-), Wr(a+b+) and Wr(a-b+) red cells by titration of the BGU1-WR and BRIC-14 antibodies. The titre of the BGU1-WR antibody with Wr(a+b+) and Wr(a+b-) red cells was the same (128). The BRIC-14 antibody in contrast had a titre of 128 with Wr(a+b+) red cells and 512 with Wr(a-b+) cells. Thus this experiment did not demonstate dosage of the Wr^a antigen and only suggested that dosage of the Wr^b antigen was possible. Further investigation of Wr^a and Wr^b antigen site number is described in Section 4.4.

4.1.3. Effect of Enzyme and Chemical Treatments on the Wr^a and Wr^b antigens.

Haemagglutination tests were used to investigate the effects of enzyme and chemical modification on the Wr^a and Wr^b antigens. Treatments were carried out as described in section 2.2.3.4. The titres of the BGU1-WR and BRIC-14 monoclonal antibodies with the treated cells and untreated were determined using simple direct agglutination tests (AGG) and indirect antiglobulin tests (IAT) as described in section 2.2.3. Table 4.1 shows the titres of BGU1-WR on treated and untreated Wr(a+b+) and Wr(a+b-) cells and Table 4.2 the titres of BRIC-14 on treated and untreated Wr(a+b+) and Wr(a-b+) cells from typical experiments.

Both the BGU1-WR and BRIC-14 antibodies did not react with untreated cells of any phenotype by direct agglutination but required the presence of a rabbit anti-mouse immunoglobulin reagent.

The effect of all the modifications on the Wr^a and Wr^b antigens is similar. Neither antigen is destroyed by proteinases, in fact direct agglutination is facilitated. Papain, pronase, bromelain and ficin treated cells are all agglutinated strongly by BGU1-WR and BRIC-14. Trypsin treated cells are also directly agglutinated by the antibodies but this enhancement is not as great. Direct agglutination of α-chymotrypsin treated cells is minimal. The titres of both antibodies on α-chymotrypsin treated cells determined by IAT is similar to that of untreated cells. Neuraminidase treatment with the receptor destroying enzyme from *Vibrio cholerae* did not destroy either antigen. Treatment with the thiol reagent 2-aminoethylisouronium bromide (AET) which destroys antigens such as Kell (Advani *et. al.*, 1982), did not affect the reactivity of the cells with the antibodies.

Both the monoclonal antibodies reacted normally with red cells heated to 45°C for 10 and 30 min (section 2.2.3.6). In a typical experiment BGU1-WR had a titre of 256 with cells treated for both lengths of time and BRIC-14 a titre of 2560 after 10 min.

and 1280 after 30 min. The monoclonal antibodies also bound to cells normally when incubation was carried out at pH 6.3, 7.3 and 8.3 as described in section 2.2.3.7.
The results above suggested that the Wr^a and Wr^b antigens are similar. They do not require intact disulphide bridges and do not require the sialic acid which is accessible to neuraminidase on intact cells for expression. The proteinase results suggest that if they are located on proteins, the antigens are located close to the membrane surface, as they were not destroyed by any of the treatments. This is in agreement with published data on Wr^b which suggests the antigen is located close to the membrane surface, associated with amino acids 56-72 of the unglycosylated region of glycophorin A (Ridgwell *et. al.*, 1983b; Dahr *et. al.*, 1986).

The BGU1-WR antibody did not react with Wr(a-b+) cells modified using any of the techniques described above. However, although the BRIC-14 antibody did not react with untreated Wr(a+b-) cells, it did react with very weakly after they had been enzyme treated. Table 4.3 shows that BRIC-14 has a titre of 1 in both agglutination and indirect antiglobulin tests with papain, trypsin, α-chymotrypsin and neuraminidase treated Wr(a+b-) red cells (other treatments were not investigated due to the limited supply of Wr(a+b-) cells). BRIC-14 did not, however, react with enzyme treated En(a-) cells which lack glycophorin A. Table 4.3 also shows the titres of other monoclonal anti-Wr^b antibodies with the enzyme treated Wr(a+b-) cells. LICRLON-R7 and 10-22 did not react with any of the cells. The fourth antibody, 4-21, which has been reported to have anti-Wr^b-like properties despite reacting with En(a-) cells (Telen and Chasis, 1990), did not react with untreated Wr(a+b-) cells but did react to varying degrees with the enzyme treated cells.
These results suggest that the Wr^b epitope detected by monoclonal antibodies is present at a very low level on Wr(a+b-) cells. This is in agreement with unpublished data which showed that monoclonal anti-Wr^b antibodies reacted weakly with fresh, untreated Wr(a+b-) red cells (Daniels, unpublished observations). The cells used in my experiments were older, which may explain why this low level of Wr^b expression was only detected after enzyme treatment. Three of the four monoclonal anti-Wr^b antibodies used here, reacted in different ways with the enzyme treated Wr(b-) cells, suggesting that they detect slightly different epitopes or have somewhat different binding properties.

TABLE 4.1.
HAEMAGGLUTINATION TITRES OF BGU1-WR ON UNTREATED, ENZYME TREATED AND CHEMICALLY MODIFIED CELLS.

	Wr(a+b+)		Wr(a+b-)	
	AGG	**IAT**	**AGG**	**IAT**
Untreated	0	128	0	128
α-chymotrypsin	8	256	nt	nt
Ficin	256	nt	nt	nt
Papain	128	nt	128	nt
Bromelain	1028	nt	nt	nt
Pronase	256	nt	nt	nt
Trypsin	32	256	16	128
Neuraminidase	0	512	nt	nt
AET	0	128	nt	nt

This Table shows haemagglutination titres of BGU1-WR culture supernatant determined as described in Section 2.2.3.8., taken as the highest dilution at which haemagglutination was visible. Several experiments were carried out with Wr(a+b+) cells and these results are from representative experiment (cells from donor L.C). The results with the Wr(a+b-) (donor M.Fr) are from a single experiment.

ABBREVIATIONS :-

AGG	-	Results from direct agglutination tests.
IAT	-	Results from indirect antiglobulin tests.
nt	-	Not tested.
AET	-	2-aminoethylisothioironium bromide

TABLE 4.2.
HAEMAGGLUTINATION TITRES OF BRIC-14 ON UNTREATED, ENZYME TREATED AND CHEMICALLY MODIFIED CELLS.

	Wr(a+b+)		Wr(a-b+)	
	AGG	IAT	AGG	IAT
Untreated	0	2560	0	2560
α-chymotrypsin	320	2560	320	2560
Ficin	1280	nt	2560	nt
Papain	640	nt	2560	nt
Bromelain	2560	nt	2560	nt
Pronase	1280	nt	2560	nt
Trypsin	640	nt	640	nt
Neuraminidase	0	2560	0	2560
AET	0	640	0	1280

This Table shows haemagglutination titres of BRIC-14 culture supernatant determined as described in Section 2.2.3.8., taken as the highest dilution at which haemagglutination was visible. Several experiments were carried out and these results are from a representative experiment (Wr(a+b+) donor L.C. and Wr(a-b+) donor S.R.). The abbreviations used are defined in the footnote to Table 4.1.

TABLE 4.3.
REACTION OF ANTI-Wr^b MONOCLONAL ANTIBODIES WITH Wr(a+b-) RED CELLS.

	BRIC-14	LICR-LON R7	10-22	4.21
UNTREATED	0	0	0	0
PAPAIN	1	0	0	16
TRYPSIN	1	0	0	8
NEURAMINIDASE	1	0	0	2
α-CHYMOTRYPSIN	1	0	0	2

This table shows the titres of monoclonal anti-Wr^b antibodies with Wr(a+b-) red cells (Donor M.Fr.). They represent the results of direct agglutination and IAT tests as identical titres were obtained using both techniques. Titres were determined as described in Section 2.2.3.8.

4.2. ABSORPTION STUDIES.

Since the first step towards characterising red cell antigens is to purify membranes, absorption studies were used to determine whether the Wr^a antigen remained on membranes made from Wr(a+) cells. These experiments would also determine whether absorption experiments provided a suitable method of examining the effect of various enzyme or chemical treatments on the Wr^a and Wr^b antigens present on membranes as opposed to intact cells.

4.2.1. Detection of Residual Antibody by Haemagglutination.

Membranes from Wr(a+) and Wr(a-) red cells were prepared as described in Section 2.2.4.4. and used to absorb BGU1-WR antibody. The level of antibody remaining in solution was then assessed by determining the haemagglutination titre. Initial titration experiments were carried out to determine the dilution of culture supernatant containing BGU1-WR at which absorption could most readily be detected. A 1 in 20 dilution of BGU1-WR was chosen. In a typical experiment the titre of BGU1-WR was reduced from 320 to 40 by absorption with Wr(a+b+) membranes (Donor S.W.). Absorption with Wr(a-b+) (donor S.R.) membranes did not reduce the titre of BGU1-WR from 320. The Wr^a antigen thus remains on membranes prepared from Wr(a+) cells and is not present on those from Wr(a-) cells.

In other experiments using Wr(a+b+) membranes made from the red cells of a different donor (R.D.) absorption of the BGU1-WR antibody was even more difficult to achieve. The titre was only reduced from 320 to 80 by absorption. This difference could be accounted for by person to person variation in the strength of Wr^a expression or it may simply have been that the cells from this donor were older than those from the first when membranes were made (ie. they had been stored for 4 weeks after bleeding instead of less than 2 weeks).

4.2.2.Detection of Residual Antibody by ELISA.

In a second series of experiments the residual antibody left in the BGU1-WR culture supernatant after absorption with red cell membranes was assessed using the ELISA technique described in Section 2.2.4.3.3. The anti-Wr^b monoclonal antibody, BRIC-14 was also available for use in these experiments. Red cell membrane samples from two different Wr(a+b+) individuals were used to absorb the antibodies and initial experiments determined the dilutions at which absorption was most apparent (1 in 20 for BGU1-WR and 1 in 50 for BRIC-14 using both membrane samples). Approximately 99% of BRIC-14 antibody was absorbed from the supernatant using

both membrane samples. One of the membrane samples (donor L.C.) absorbed approximately 95% of the BGU1-WR antibody whereas the other (donor I.W.) absorbed only about 70%.

The difference between the absorption of BGU1-WR with the two membrane samples again suggests variation in the number of Wr^a antigen sites between Wr(a+b+) individuals.

Absorption of BGU1-WR was also more difficult to acheive than absorption of BRIC-14 using intact Wr(a+b+) red cells.

In all experiments regardless of which membrane samples were used the BRIC-14 antibody was easier to absorb than BGU1-WR. This could be explained if BRIC-14 has a higher functional affinity constant than BGU1-WR. Alternatively these results suggest that there may be less Wr^a sites on Wr(a+b+) red cells than Wr^b sites. The functional affinity constants of BGU1-WR and BRIC-14 and the number of Wr^a and Wr^b antigen sites on cells were therefore investigated as described in Section 4.4.

4.3. Direct Binding of the BGU1-WR and BRIC-14 Antibodies to Red Cell Membranes.

Two different ELISA techniques to detect direct binding of antibodies to red cell membranes were also used to confirm that the Wr^a antigen remained on membranes prepared from Wr(a+b+) individuals.

4.3.1. Detection on previously purified Red Cell Membranes by ELISA.

Initial experiments were carried out using the ELISA method described in Section 2.2.4.3.1. in which prepared red cell membranes are bound to the ELISA plate. In these experiments GERO, an anti-Gerbich antibody (Daniels *et. al.*, 1983) was used as a positive control and consistently gave readings greater than three times the background absorbance on the plate (results not shown). BRIC-14 was not available at this time so a different monoclonal anti-Wr^b antibody, LICR-LON R7 was used. This reacted weakly with the membranes giving absorbance readings of approximately twice the background absorbance. The BGU1-WR antibody however, did not appear to react with red cell membranes in these experiments. Therefore a second ELISA technique was investigated.

4.3.2. Detection with Membranes Prepared *In Situ* on ELISA Plates.

This second technique (section 2.2.4.3.2.) does not require red cell membranes to be prepared in advance. Intact red cells are applied to the plate using poly-L-lysine and

are then lysed *in situ* by hypertonic lysis, leaving the membranes bound to the wells. The membranes are then glutaraldehyde fixed before continuing with the ELISA in the normal way.

This method improved the reaction for BGU1-WR with Wr(a+b+) cells giving an absorbance reading of approximately twice the background absorbance (results not shown). BRIC-14 reacted strongly with all cells consistently giving an absorbance reading of more than ten times the background absorbance. GERO however, did not react using this technique. Glutaraldehyde is known to destroy some red cell antigens, therefore in all subsequent experiments glutaraldehyde fixation was omitted. GERO then consistently gave a positive reaction showing that the Gerbich antigen recognised by GERO is destroyed by glutaraldehyde. The BGU1-WR antibody was shown always to react with Wr(a+b+) membranes although the strength of the reaction was variable.

Since papain treatment was shown to enhance the haemagglutination reaction of BGU1-WR (Section 4.1.3), an experiment was carried out using papain treated Wr(a+b+) red cells to try and enhance the reaction of BGU1-WR. The results of this experiment are shown in Table 4.4. The positive control antibody GERO, reacted strongly with the membranes from untreated cells but not with those from papain treated cells. Since the Gerbich antigen recognised by GERO is known to be destroyed by papain (Daniels *et. al.*, 1983), this showed that the papain treatment had been successful. BRIC-14, as in all previous experiments, reacted strongly with all the cells. The BGU1-WR antibody reacted strongly with the Wr(a+b+) membranes in this experiment whether or not they were from cells which had been papain treated. This confirmed that the Wr^a antigen remains on membranes prepared by hypertonic lysis from Wr(a+b+) but not from Wr(a-b+) cells.

It could be seen by eye that variable amounts of red cells and hence membranes appeared to bind to the ELISA plates in these experiments. This appeared to be a feature of the samples used, as reflected by the Wr(a-b+) sample (donor S.R.) in table 4.4. These cells were expected to react with the GERO antibody to the same extent as the other cell samples but gave a lower absorbance reading. The amount of background absorbance also varied from experiment to experiment. In further experiments it therefore proved difficult to consistently show that the BGU1-WR antibody reacted strongly with Wr(a+b+) cells. The reaction of the BRIC-14 antibody, however, was always easy to detect. This difference in reactivity between the BRIC-14 and BGU1-WR antibodies again suggested that BRIC-14 might have a higher affinity than BGU1-WR or that there are more Wr^b than Wr^a antigen sites on Wr(a+b+) red cells.

TABLE 4.4.
DIRECT BINDING OF BGU1-WR AND BRIC-14 MONOCLONAL ANTIBODIES TO RED CELL MEMBRANES.

	BGU1-WR	BRIC-14	GERO
UNTREATED CELLS			
Wr(a+b+) (B.L.)	0.597	1.537	1.369
Wr(a+b+) (R.D.)	0.476	1.817	0.948
Wr(a+b+) (I.P.)	0.481	1.479	1.068
Wr(a-b+) (S.R.)	0.021	0.807	0.623
PAPAIN TREATED CELLS			
Wr(a+b+) (B.L.)	0.226	1.009	-0.007
Wr(a+b+) (R.D.)	0.373	1.244	0.001
Wr(a+b+) (I.P.)	0.285	1.201	-0.010
Wr(a-b+) (S.R.)	0.026	1.018	0.064

Absorbance readings from an ELISA to detect direct binding of antibodies to red cell membranes prepared *in situ*. The figures are the mean of duplicate samples from one experiment with the background absorbance for the particular red cell sample subtracted. The background absorbance was determined from wells containing each cell sample incubated with RPMI 1640 medium containing 20% FCS instead of antibody containing culture supernatant.

4.4. Determination of Wr^a and Wr^b Antigen Sites on Red Cells.

The absorption (section 4.2.) and direct binding (section 4.3.) studies suggested that either BGU1-WR has a lower functional affinity constant (K) than BRIC-14 or that there are less Wr^a sites than Wr^b sites on Wr(a+b+) red cells. Gardner *et. al.* (1989) had previously determined that there are approximately 1,000,000 Wr^b sites on Wr(a-b+) red cells but no estimates of the number of Wr^a or Wr^b antigen sites on Wr(a+b+) red cells have been reported. Therefore the functional affinity constants of BGU1-WR and BRIC-14 and the number of Wr^a and Wr^b antigen sites on Wr(a+b+) red cells were determined in the following experiments. This work was carried out in collaboration with Brigitta Gardner of the Department of Haematology, St Mary's Hospital, London.

4.4.1. Purification and ^{125}I-labelling of BGU1-WR and BRIC-14.

The BGU1-WR and BRIC-14 antibodies were purified as described in Section 2.2.4.13. BGU1-WR was shown to be of immunoglobulin subclass IgG1 (Section 3.3.) which is known to only bind weakly to Protein A, therefore high ionic strength solutions were used to facilitate the binding of the antibody to the Protein A Sepharose. The same solutions were also used to purify BRIC-14, which was easier to purify than BGU1-WR and gave a higher antibody concentration in the eluted fractions. This higher yield is possibly because the BRIC-14 antibody belongs to the immunoglobulin subclass IgG2a which is known to bind to Protein A Sepharose more strongly than IgG1. The antibody containing fractions of both antibodies were shown to have retained antibody activity by haemagglutination tests. Pooled antibody-containing, eluted fractions were then concentrated and the final protein concentrations determined as described in section 2.2.4.14. The BGU1-WR sample had a concentration of 446 μgml^{-1} and that of BRIC-14, 499 μgml^{-1}.

The concentrated monoclonal antibodies were labelled with ^{125}I using Iodobeads as described in Section 2.2.4.13. Labelling of BRIC-14 was more successful than that of BGU1-WR. The BRIC-14 sample gave a percentage incorporation of 10.1% of the ^{125}I and had a specific activity of 33,000 counts per μg of protein. BGU1-WR on the other hand, gave a percentage incorporation 0.56% of the ^{125}I and a specific activity of only 2,477 counts per μg of protein. This specific activity, however, was high enough to use in the following experiments.

4.4.2. Determination of the Biological Activity of the ^{125}I-labelled Antibody Samples.

The biological activity of the ^{125}I-labelled antibody samples (i.e. the percentage of radioactivity representing active antibody molecules) was determined by Scatchard analysis (Scatchard, 1949).

Red cell binding assays were used to determine the maximum amount of antibody in the preparations which could bind to red cells; various concentrations of red cells were incubated with a constant antibody concentration as described in section 2.2.4.14.1. The counts associated with the red cells were used to calculate the amount of antibody bound to the cells using the specific activity of the antibodies. This was plotted against the fraction of bound antibody to free antigen. Figure 4.1. shows an example of such a plot for the BGU1-WR antibody.

The amount of free antigen present in each sample was determined by subtracting the amount of antibody bound specifically to the red cells from an estimate of the total amount of antigen present in each sample. This estimate was based on the number of antigen sites per red cell and the number of red cells in each sample. In the first instance an arbitrary figure for the number of antigen sites per red cell was used. In these experiments 500,000 was chosen as the number of Wr^a and Wr^b antigen sites on Wr(a+b+) red cells since Wr(a-b+) cells have been shown to have 1,000,000 Wr^b sites per cell (Gardner *et. al.*, 1989). It later became clear that this was an overestimate in both cases and the biological activity of the antibodies was recalculated using the more accurate antigen site number estimates given in section 4.4.4.

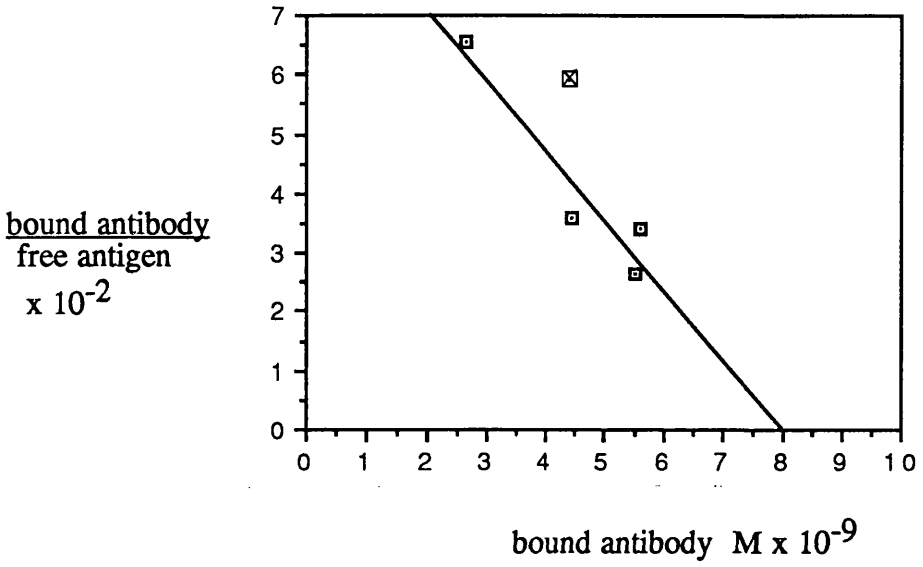
The maximum amount of antibody able to bind specifically to red cells in the samples was determined by extrapolation of the Scatchard plot to the base line. This was then expressed as a percentage of the total protein present in the sample (calculated from the absorbance of the sample, section 2.2.4.13.).

The example shown in figure 4.1 estimates that the maximum amount of BGU1-WR antibody able to bind to the cells is 8.0×10^{-9} M. The total protein concentration of this sample was 35.43×10^{-9} M, giving a percentage of active antibody of 22%. The mean value taken from three experiments using red cells from different Wr(a+b+) donors was 20%. This is lower than the expected figure and adjustments to the amount of BGU1-WR antibody used in site number and competitive binding assays (Sections 4.4.4 and 4.5) had to be made to account for this. This low percentage of active BGU1-WR antibody together with the fact that it was harder to purify than BRIC-14 (Section 4.4.1) suggest that BGU1-WR may not be a very stable antibody when used under these conditions.

The labelled BRIC-14 preparation was shown to contain approximately 55% active antibody which is well within the range obtained for purified ^{125}I-labelled monoclonal antibodies by this procedure (Gardner, Personal Communication).

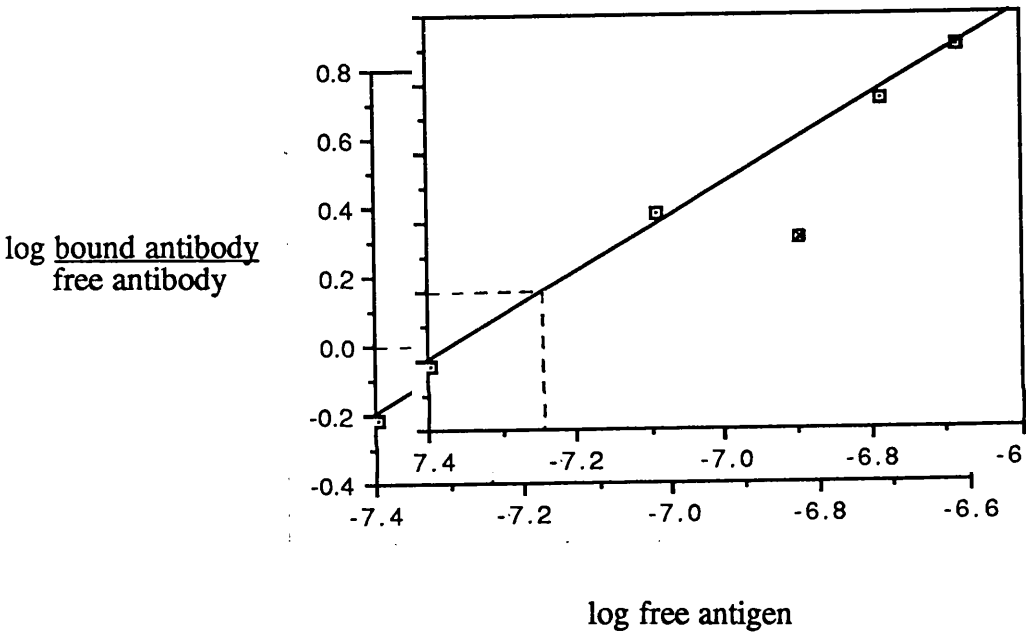
These active antibody values were taken into account in determining functional affinity constants (section 4.4.3) and the number of antigen binding sites (section 4.4.4.) of BGU1-WR and BRIC-14.

FIGURE 4.1.
SCATCHARD PLOT USED TO DETERMINE THE BIOLOGICAL ACTIVITY OF ^{125}I-LABELLED BGU1-WR ANTIBODY.



A Scatchard plot used to determine the maximum amount of BGU1-WR antibody able to bind specifically to red cells in ^{125}I-labelled samples. This was used to calculate the percentage of biologically active antibody in the samples. Figures were determined from a red cell binding assay where the red cell concentration was varied and the antibody concentration remained constant, as described in section 4.4.2.
Point x was not taken into account when extrapolating the line.

FIGURE 4.2.
KARUSH PLOT USED TO DETERMINE THE FUNCTIONAL AFFINITY CONSTANT OF THE BGU1-WR ANTIBODY.



A Karush plot used to determine the amount of free antigen present in a sample of BGU1-WR when the amounts of bound and free antibody were equal (i.e. when log bound antibody/free antibody = 0) This was then used to determine the functional affinity constant of BGU1-WR as described in section 4.4.3. Figures were determined from a red cell binding assay where the red cell concentration was varied and the antibody concentration remained constant, as described in section 4.4.2.
Point x was not taken into account when extrapolating the line.

4.4.3. Determination of the Functional Affinity Constants of BGU1-WR and BRIC-14.

The results of the red cell binding assays used above to determine the biological activity of the ^{125}I-labelled antibody preparations (section 4.4.2.), were also used to determine the functional affinity constants of the antibodies using Karush plots (Karush, 1962).

The following formula was used to determine the functional affinity constants (K):-

$$K = \frac{\text{bound antibody}}{\text{free antibody}} \times \frac{1}{\text{free antigen}}$$

The log of the free antigen (determined as described in section 4.4.2.) was plotted against the log of the fraction of the bound antibody/free antibody. The log of the free antigen when the amount of bound and free antibody was at equilibrium, was determined by extrapolation of the plot at the point where the log of bound/free antibody was 0. At this point the affinity constant could be determined using:-

$$K = \frac{1}{\text{free antigen}}$$

In the example shown in figure 4.2 the log of the free antigen at equilibrium is approximately -7.26, giving an affinity constant for the BGU1-WR antibody of 1.82×10^7. The BRIC-14 antibody was shown to have an affinity constant of 1.09×10^7. Both the BGU1-WR and BRIC-14 antibodies therefore have high affinity constants when compared to those of various monoclonal anti-glycophorin A antibodies which have been shown to range from less than 10^6 to 4×10^7 (Gardner *et. al.*, 1989). BRIC-14 has previously been reported to have an affinity constant of the same order of magnitude as that determined here (3.2×10^7) (Gardner *et. al.*, 1989).

4.4.4. Determination of the numbers of Wr^a and Wr^b sites per red cell.

Scatchard analysis (Scatchard, 1949) was used to determine the number of Wr^a and Wr^b antigen sites on red cells.

Binding assays were used to determine the maximum amount of antibody which could bind to a given number of red cells (a constant concentration of red cells was incubated with varying antibody concentrations as described in section 2.2.4.14.2).

The amount of antibody bound to the cells in each sample was determined from the number of counts associated with the red cells using the specific activity of the

antibody preparations. This was plotted against the fraction of bound/free antibody. An example of such a plot is shown in figure 4.3.

The amount of free antibody was determined by subtraction of the bound antibody from an estimate of the total antibody in each sample. This had been determined using the biological and specific activities of the labelled antibody preparations.

Extrapolation of the Scatchard plot to the base line gave the maximum amount of antibody able to bind to the cells. This was used in the following formula to give an estimation of antigen sites:-

$$\text{Sites per cell} = \frac{\text{Avogadro's number} \times \mu\text{g bound antibody}}{M_r \text{ of antibody} \times \text{number of cells}}$$

Where :-

Avogadro's number	=	6.02×10^{23}
M_r of antibody	=	1.6×10^{5}
Number of cells	=	determined from a representative red cell sample using a coulter counter (section 2.2.4.14.2.).

In the example given in Figure 4.3. the amount of bound antibody was 0.088×10^{-6} μg and the average number of cells in the samples was 4.9×10^{6}. The number of Wr^a antigen sites on these cells was therefore approximately 67,040. The mean of two experiments using these cells was 70,100.

Table 4.5. shows the estimates of the mean number of Wr^a and Wr^b antigen binding sites on Wr(a+b-), Wr(a+b+) and Wr(a-b+) red cells. The BGU1-WR antibody was shown to bind approximately 70,000 sites on three of the four Wr(a+b+) red cell samples and approximately 95,000 on the fourth. It is not clear how significant this difference is and whether or not it is a characteristic of this individual. It does not, however, appear to be a feature of the method of cell storage since there is no correlation between the values obtained and the way in which the samples had been stored (i.e. at 4^oC or -30^oC, see table 4.5. for further details).

The BRIC-14 antibody also gave variable values for the number of Wr^b antigen sites on these cells, giving estimates of about 140,000 on three individuals and 350,000 on the fourth.

The BGU1-WR antibody was estimated to bind approximately 70,000 sites on the Wr(a+b-) red cells (donor M.Fr.). Thus, these results do not demonstrate dosage of the Wr^a antigen. Whether or not there is dosage of the Wr^b antigen is also not clear from these experiments since the only Wr(a-b+) sample tested was estimated to have 484,000 Wr^b sites compared to 350,000, the highest estimate on Wr(a+b+) cells.

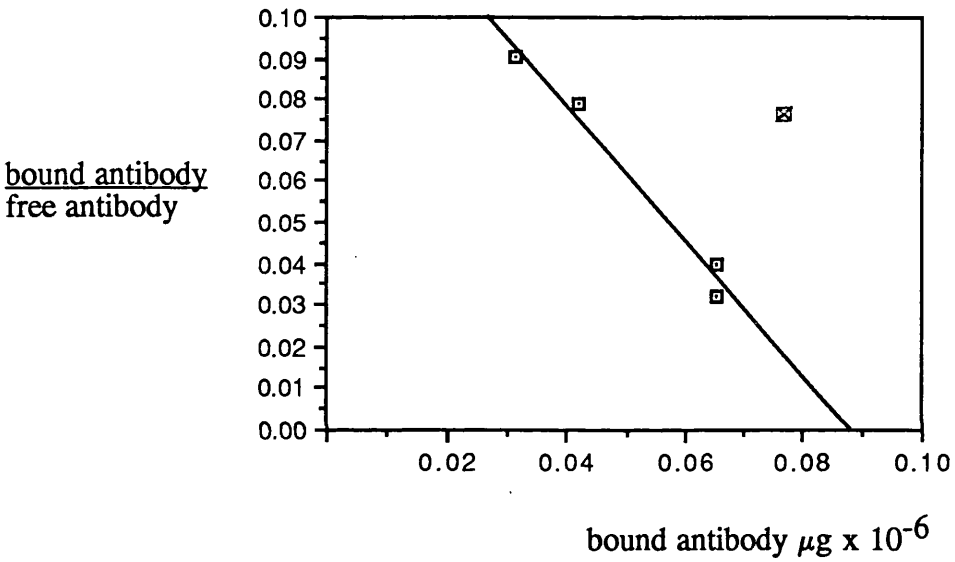
In a previous study (Gardner *et. al.*, 1989) the BRIC-14 antibody was shown to bind approximately 906,000 sites on Wr(a+b+) red cells. This is more consistant with the fact that there are known to be approximately 1,000,000 molecules of glycophorin A and band 3 on red cells. Since my estimate of the number of Wr^b antigen sites on Wr(a-b+) cells was only determined on the cells fom one individual it is possible that this was an underestimate. In this case dosage of the Wr^b antigen would be apparent.

The use of ^{125}I-labelled purified antibodies gave a more quantitative measure of the number of Wr^a and Wr^b antigen sites on red cells than other methods used in this project. However, the use of Fab fragments rather intact immunoglobulin has been shown to give better estimates. For example Gardner *et. al.* (1989) found that Fab fragments of some anti-glycophorin A monoclonal antibodies bound twice as many sites as IgG. These authors suggested that the smaller Fab fragments have access to both antigen sites on the glycophorin A dimer whereas the IgG only has access to one. Further experiments using Fab fragments of BGU1-WR and BRIC-14 would help to ensure that these site numbers are correct.

Gardner *et. al.* (1989) previously compared the use of anti-Wr^b IgG and Fab fragments to determine the number of Wr^b antigen sites per red cell. Four anti-Wr^b monoclonal antibodies including BRIC-14 were used. Experiments using IgG for all of these antibodies gave site number estimates of between 900,000 and 1,000,000. An estimate of the number of sites using fragments of one of these antibodies was similar. This suggested that the majority of the Wr^b antigen sites were accessible to BRIC-14 IgG. My experiments, where the the number of Wr^b sites on the cells of a Wr(a-b+) donor were estimated to be approximately 484,000, were carried out under the same conditions as those of Gardner *et. al.* (1989). It is therefore possible that the difference in site number estimate between these experiments and those in the published results are due to person to person variation.

The difference between the number of Wr^a and Wr^b antigen sites on Wr(a+b+) red cells is obvious. It is possible that use of Fab fragments could increase the number of Wr^a sites and not Wr^b sites and thus decrease this difference. The implications of this difference on the relationship of the antigens is discussed in section 6.3.

FIGURE 4.3.
SCATCHARD PLOT USED TO DETERMINE THE NUMBER OF Wr^a ANTIGEN SITES ON Wr(a+b-) RED CELLS.



Scatchard plot used to determine the maximum amount of BGU1-WR antibody which binds to Wr(a+b-) red cells (donor M.Fr.). This was then used to calculate the number of Wr^a antigen sites on these red cells (section 4.4.4.). Bound and free antigen values were determined from a binding assay in which a constant amount of red cells were incubated with varying antibody concentrations. Point x was not taken into account when extrapolating the plot.

TABLE 4.5.
Wr^a AND Wr^b ANTIGEN SITE NUMBERS.

DONOR	BGU1-WR SITES	BRIC-14 SITES
M.Fr. Wr(a+b-)	70,100	0
G.W. Wr(a+b+)	71,000	350,000
I.W. Wr(a+b+)	75,000	170,000
L.C. Wr(a+b+)	95,000	140,000
C. Wr(a+b+)	74,200	140,000
P.T. Wr(a-b+)	0	484,000

The number of antibody binding sites per red cell were determined as described in section 4.4.4. The red cell samples were either recently bled and stored at 4^oC (samples G.W. and C.) or had been stored at -30^oC and thawed as described in section 2.1.2. before use (samples M.Fr., I.W., L.C. and P.T.).

4.5. Competitive Binding Assays.

Competitive binding assays may help to determine the nature of the structure and relationship of the Wr^a and Wr^b antigens. Antibodies which are shown to inhibit each other are assumed to recognise identical or related epitopes depending on the extent of the inhibition. For example Gardner *et. al.* (1989) tested a panel of monoclonal anti-glycophorin A antibodies in competitive binding assays. Some were found to inhibit each other totally showing that they recognised identical or almost identical epitopes, whereas others showed partial inhibition suggesting they recognised related epitopes. Five of the monoclonal antibodies were shown to recognise a series of overlapping epitopes on a region of glycophorin A based on their inhibition pattern.

4.5.1. Inhibition of BGU1-WR and BRIC-14.

The inhibition of ^{125}I-labelled BGU1-WR and BRIC-14 by each other and BRIC-256 (an En^aFS-type murine monoclonal antibody which recognises an epitope located between amino acids 39 and 56 of glycophorin A [Anstee *et. al.*, 1982]) was investigated using the method described in Section 2.2.4.15. The ^{125}I-labelled BGU1-WR and BRIC-14 samples prepared as described in 4.4.1. were incubated with Wr(a+b+) red cells and various concentrations of the inhibiting antibody. The difference in the percentage purity of the two labelled antibodies meant that different quantities of labelled antibody were used; 1μl of ^{125}I-labelled BGU1-WR and 0.4 μl of labelled BRIC-14. The inhibiting antibodies were used at the following concentrations; BGU1-WR (1.5μg, 3μg, 6μg and 7.5μg); BRIC-14 (2.8μg, 5.6μg, 11.1μg and 13.9μg) and BRIC-256 (3μg, 6μg, 12μg and 15μg). The percentage of the total active labelled antibody present was then calculated and plotted against the concentration of the inhibitor as shown in Figure 4.4.

Figure 4.4.A shows the inhibition curves of BRIC-14. In the absence of an inhibitor 100% of ^{125}I-labelled BRIC-14 bound to the cells. This was reduced to 8% in the presence of an excess of unlabelled BRIC-14. The inhibition curve of BRIC-256 shows that binding of this antibody decreases the amount of labelled BRIC-14 antibody binding to about 65%. Binding of the BGU1-WR antibody, on the other hand, resulted in only a minimal decrease in BRIC-14 binding. This decrease is similar to that observed when two totally unrelated antibodies are used to inhibit each other (Gardner, personal communication).

Figure 4.4B shows the inhibition curves of BGU1-WR. 85% of the labelled BGU1-WR bound to cells in the absence of an inhibitor. The unlabelled BGU1-WR antibody clearly inhibited the labelled antibody. The other two antibodies BRIC-14 and BRIC-

256 did not appear to inhibit the BGU1-WR antibody significantly since the small decrease in BGU1-WR binding did not appear to depend on the concentration of the inhibitor.

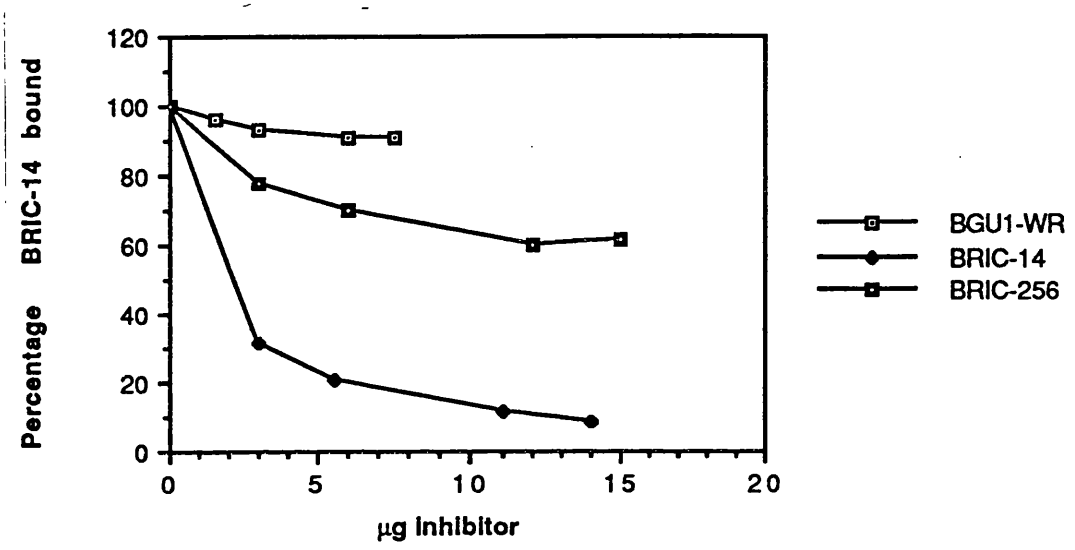
4.5.2. Discussion of Competitive Binding Experiments.

These competitive binding assays did not reveal signs that the BGU1-WR and BRIC-14 antibodies inhibit each other, suggesting that they are not competing for the same or neighbouring epitopes. This experiment, however, does not rule out the possibility that the Wr^a and Wr^b antigens are located on different allelic forms of a protein such as band 3. The Wr^a and Wr^b antigens may be formed by a difference in the amino acid sequence of the protein, resulting in the membrane of Wr(a+b+) cells containing a number of protein molecules which bind either anti-Wr^a or anti-Wr^b but not both antibodies.

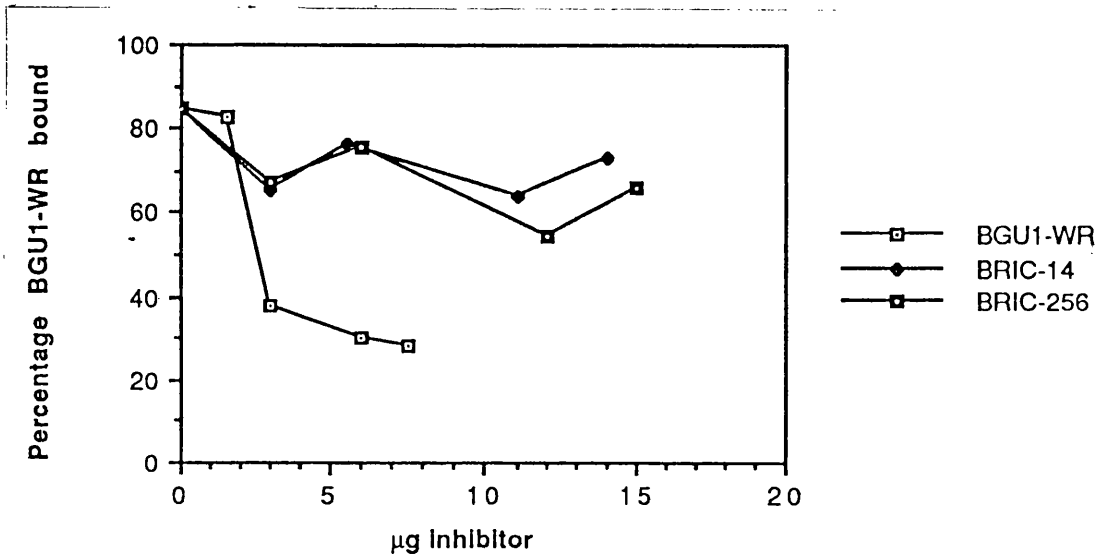
BRIC-14 was partially inhibited by the monoclonal anti-glycophorin A antibody, BRIC-256 showing that the Wr^b epitope is distinct from that recognised by BRIC-256. The partial inhibition is probably caused by binding of BRIC-256 altering the conformation of glycophorin A and thus decreasing the binding of BRIC-14. The same phenomenom was not observed for the BGU1-WR antibody.

FIGURE 4.4.
INHIBITION CURVES OF BGU1-WR AND BRIC-14.

A - Inhibition of BRIC-14.



B - Inhibition of BGU1-WR.



4.6. Immunoblotting Studies Using BGU1-WR and BRIC-14.

Immunoblotting experiments were carried out as described in Sections 2.2.4.6. to 2.2.4.9. using membranes made from Wr(a+b+) and Wr(a-b+) red cells. BRIC-14 was used for comparison throughout these studies and an anti-Gerbich monoclonal antibody, GERO, (Daniels *et. al.*, 1983) was used as a positive control.

Red cell membranes were subjected to electrophoresis under standard reducing conditions in which ß-mercaptoethanol is present in the loading buffer and were then electroblotted. A variety of blocking buffers were used to block the filters before immunostaining since some monoclonal antibodies are known to bind under some blocking conditions and not others (Swallow, personal communication). Detection of bound antibody was then carried out using a variety of staining methods. No specific bands were detected on immunoblots stained with BGU1-WR and BRIC-14 under any of the conditions outlined in Table 4.6. In all cases bands were detected on immunoblots stained with GERO showing that the detection systems were working.

Some blood group antigens such as the Kell antigens (Parsons *et. al.*, 1987) are known to be destroyed by ß-mercaptoethanol. Therefore membranes dissolved in non-reducing dissolving buffer were also tested with BGU1-WR and BRIC-14 under all of the blocking and staining conditions used above. No specific bands were detected using either antibody under any of these conditions.

The reactivity of BGU1-WR and BRIC-14 in haemagglutination tests, is enhanced by proteinase treatment of red cells (Section 4.1.3). Therefore membranes prepared from red cells treated with trypsin and α-chymotrypsin were electrophoresed under reducing and non-reducing conditions and electroblotted. Filters were then immunostained with BGU1-WR and BRIC-14 (blocked with 3% BSA and stained with 4-chloro-1-naphthol). No specific bands were detected with either antibody.

Immunoblotting experiments were also carried out on Wr(a+b-) membranes (donor M.Fr) and with BGU1-WR antibody purified and concentrated as described in Section 2.2.4.13. as well as the standard unconcentrated culture supernatant. No specific bands were detected when filters were blocked and stained under any of the conditions listed above.

In these experiments I was unable to show that the BGU1-WR antibody recognises a red cell membrane component either under reducing or non-reducing conditions. Similarly neither glycophorin A or any other red cell membrane component was detected by the BRIC-14 antibody.

TABLE 4.6.

Staining Methods and Blocking Conditions used for Immunoblots.

STAIN	BLOCKING CONDITIONS
4-chloro-1-naphthol	5% powdered milk in PBS
"	3% BSA in PBS;
diaminobenzidine	3% BSA in PBS
"	0.05% Tween-20 in PBS
"	0.15% Tween-20 in PBS
Chemiluminescent detection	5% BSA in PBS.

4.7. Immunoprecipitation Studies.

Immunoprecipitation experiments can be used to locate red cell antigens on particular red cell membrane components when the antigens are not detectable on the denatured SDS-treated electroblotted components. Gahmberg (1983) and Moore *et. al.* (1982) isolated red cell membrane components associated with the Rh blood group antigens using a modified immunoprecipitation technique. Intact red cells were radiolabelled and sensitised with antiserum before membranes were prepared and solubilised. The immune complexes were then isolated by absorption with protein A Sepharose. This immunoprecipitation technique is now often used in blood group antigen studies as the formation of immune complexes before membrane preparation and solubilisation maintains the integrity of the antigens. This approach enables the analysis of antigens which are dependent on an interaction between two or more membrane components. The following sections describe immunoprecipitation experiments similar to those used by Gahmberg (1983) and Moore *et. al.* (1982) using the monoclonal antibodies BGU1-WR and BRIC-14.

4.7.1. Immunostaining of Anti-Wr^a and Anti-Wr^b Immunoprecipitates With Anti-glycophorin A and anti-band 3.

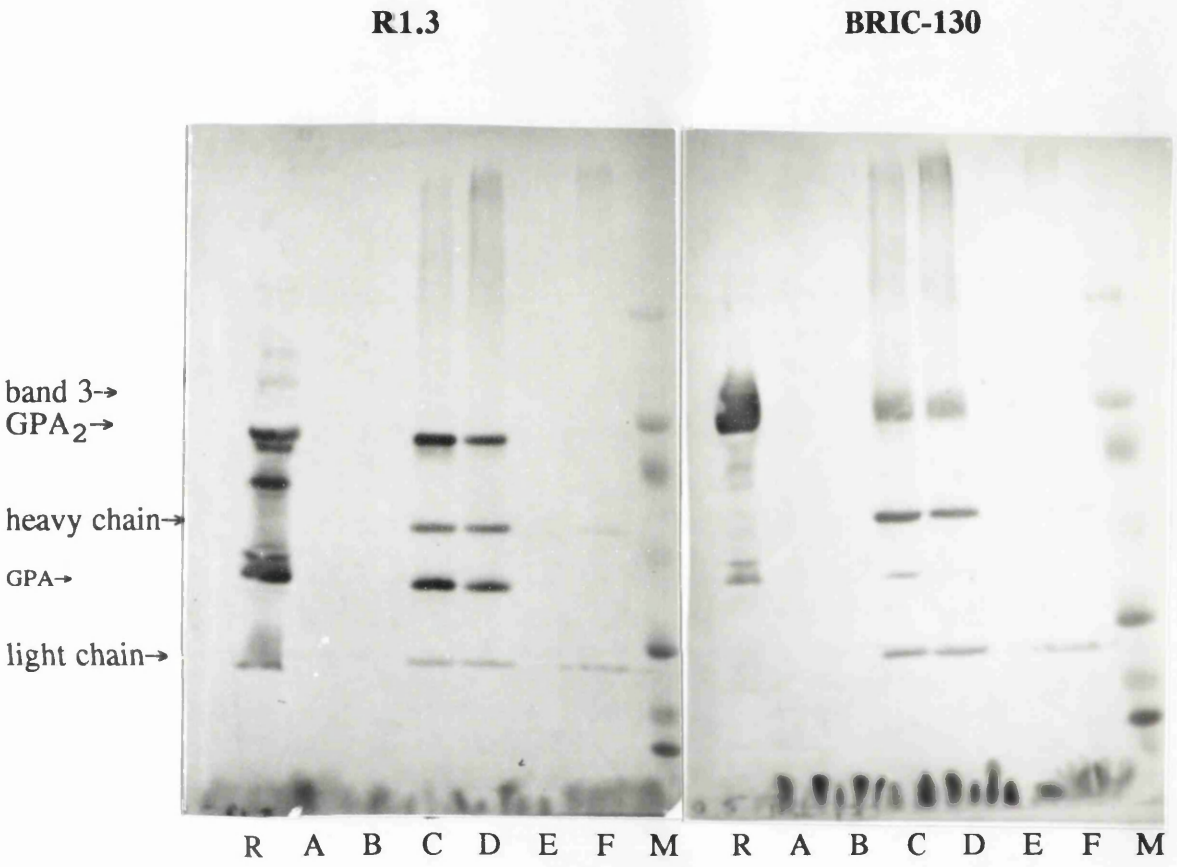
Previous studies have demonstrated that the anti-Wr^b monoclonal antibodies BRIC-14, BRIC-15 and BRIC-13 immunoprecipitate glycophorin A from periodate/NaB^3H_4-labelled normal red cells but not from Wr(b-) red cells (Ridgewell *et. al.*, 1983b) showing that the Wr^b antigen is associated with glycophorin A. Whilst work for this thesis was in progress Telen and Chasis (1990) reported that three monoclonal anti-Wr^b antibodies immunoprecipitated band 3 in addition to glycophorin A. This suggests that the Wr^b antigen may be dependent on an association between these two red cell membrane components (but see Section 4.7.4 and 6.1.3.). In the studies described in this thesis the BGU1-WR antibody was used to investigate whether Wr^a was similarly associated with glycophorin A or band 3.

Immunoprecipitation experiments using the BGU1-WR and Bric-14 monoclonal antibodies with Wr(a+b+) and Wr(a-b+) red cells were carried out as described in Section 2.2.4.10. Culture supernatant from the cell line P3/NS1/1-Ag4-1 (X63sec) was used as a negative control since it contains immunoglobulins which do not react with red cell membrane components. Initial experiments used 25ml of antibody supernatant to sensitise approximately 1 ml of packed red cells. Membranes were solubilised in 1% Triton X-100 before absorbing with RAM-Sepharose. RAM-Sepharose was used in place of Protein A-Sepharose as the BGU1-WR antibody was of

immunoglobulin subclass IgG1 which does not bind well to Protein A. Precipitates were run in triplicate on SDS-PAGE gels as described in Section 2.2.4.5. One set of samples were silver stained (Section 2.2.4.6.2.) and the other two electroblotted and immunostained (Sections 2.2.4.7. and 2.2.4.8.) with the monoclonal antibody R1.3 which recognises an epitope on both glycophorin A and glycophorin B and BRIC-130 an anti-band 3 monoclonal antibody.

Figure 4.5 shows immunoblots from this experiment. The BRIC-14 tracks immunostained with R1.3 antibody show bands with an apparent Mr of 42,000 and 85,000 which correspond to the glycophorin A monomer and dimer. Bands with an apparent M_r of 23,000 and 53,000 were also present. These correspond to antibody heavy and light chains which are detected by the peroxidase labelled Rabbit anti-mouse immunoglobulin used in the staining procedure. The BRIC-14 precipitates also show a diffuse band detectable with BRIC-130 with an apparent M_r of 90,000 to 100,000 which corresponds to band 3. The immunoblots also show that neither of the BGU1-WR precipitates contained band 3 or glycophorin A. Bands corresponding to the antibody heavy and light chains were present in the precipitate using Wr(a+b+) red cells but not in that from Wr(a-b+) cells. This shows that the BGU1-WR antibody had remained bound to the Wr^a antigen at least until the membranes were solubilised in Triton X100.

FIGURE 4.5.
IMMUNOSTAINS OF ANTI-Wr^a AND ANTI-Wr^b IMMUNOPRECIPITATES.



Immunoprecipitation was carried out as described in section 2.2.4.10. Tracks R contain red cell membranes and M rainbow molecular weight markers giving bands with apparent M_r of 14,300, 21,500, 30,000, 46,000, 69,000, 97,4000, 200,000. Tracks A [Wr(a-b+) red cells] and B [Wr(a+b+) red cells] contain X63 secretor immunoprecipitates; tracks C [Wr(a-b+) red cells] and D [Wr(a+b+) red cells] BRIC-14 immunoprecipitates and tracks E [Wr(a-b+) red cells] and F [Wr(a+b+) red cells] BGU1-WR monoclonal antibody immunoprecipitates.

The additional band present in tracks C and D immunostained with BRIC-130 appears to correspond to the glycophorin A monomer.

This experiment therefore demonstrated that the anti-Wr^b antibody BRIC-14 immunoprecipitates glycophorin A and band 3 whereas the anti-Wr^a does not immunoprecipitate either of these molecules under these conditions. The silver stained gel (not shown) did not reveal any additional bands in the immunoprecipitates.

Various detection procedures and immunoprecipitation conditions were then tried in an attempt to demonstrate the immunoprecipitation of band 3 and/or glycophorin A with BGU1-WR from Wr(a+b+) red cells:-

The precipitation was carried out as described above but was immunostained with a different monoclonal anti-band 3 (BRIC-132) and anti-glycophorin A (R18). The Bric-14 precipitates were again shown to contain band 3 and glycophorin A but the BGU1-WR precipitates were not.

Since Dahr *et. al.* (1986) had reported that the Wr^a antigen unlike the Wr^b antigen was not completely solubilised from the cytoskeleton until the Triton X-100 concentration was 2.5%, experiments were carried out in which the sensitised membranes were solublised with 2.5% Triton instead of 1%. The BRIC-14 precipitates again contained glycophorin A and band 3 but the BGU1-WR precipitates did not.

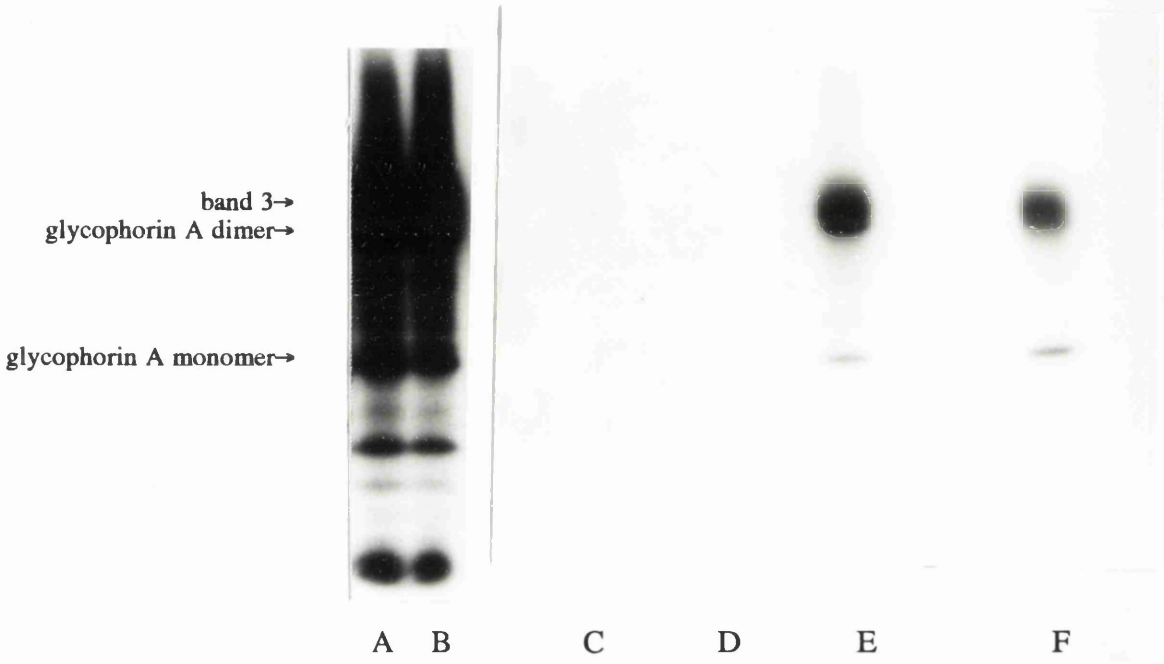
In other experiments Protein G Sepharose was used to absorb the immune complexes instead of RAM-Sepharose. Once again band 3 and glycophorin A were present in the BRIC-14 precipitates and not in those of BGU1-WR.

4.7.2. Immunoprecipitates From ^{125}I-labelled Red Cells.

The immunoprecipitation experiments described above show that BGU1-WR does not immunoprecipitate band 3 or glycophorin A. However they do not show whether the antibody immunoprecipitates other membrane components. No additional bands were detected in the immunoprecipitates by silver staining but the presence of bands corresponding to immunoglobulin heavy and light chains could have masked components with a similar apparent M_r. Immunoprecipitation was therefore carried out with red cells labelled with ^{125}I.

Wr(a+b+) and Wr(a-b+) red cells were labelled using the lactoperoxidase method described in Section 2.2.4.11.1. Immunoprecipitation was then carried out as in Section 2.2.4.11 with the BGU1-WR and BRIC-14 antibodies.

FIGURE 4.6.
ANTI-Wr^a AND ANTI-Wr^b IMMUNOPRECIPITATES FROM ^{125}I-LABELLED RED CELLS.



Red cells were labelled with ^{125}I as described in section 2.2.4.11.1. Tracks A, and B show samples of red cell membranes from labelled cells. Immunoprecipitation was carried out as described in section 2.2.4.11. Tracks E and F show immunoprecipitates using BRIC-14 antibody on Wr(a+b+) and Wr(a-b+) red cells respectively and tracks C and D immunoprecipitates using BGU1-WR antibody on Wr(a+b+) and Wr(a-b+) red cells respectively.

Precipitates were run on SDS-PAGE gels and the gels were subjected to autoradiography as described in Section 2.2.4.11. Figure 4.6 shows the results of such an experiment. The tracks containing the BRIC-14 precipitates show bands with an apparent M_r of 85,000 and 90,000 to 100,000 which correspond to the glycophorin A dimer and band 3. The BGU1-WR precipitate tracks contain no bands.

Cells were also ^{125}I-labelled using the modified lactoperoxidase technique in which higher proportions of ^{125}I, lactoperoxidase, glucose oxidase and glucose are used enable the labelling of minor red cell membrane components (Section 2.2.4.11.1.). Here again (results not shown) band 3 and glycophorin A were detected in the BRIC-14 precipitates but not in those of BGU1-WR.

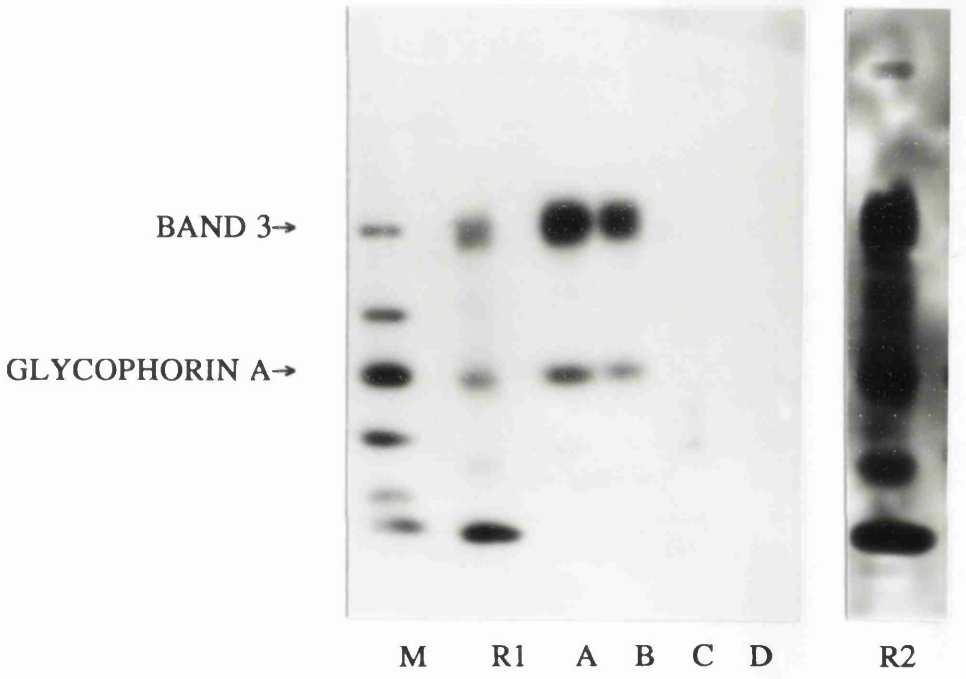
Since Moore *et. al.* (1982) had reported that Rh immunoprecipitates could only be detected when the SDS-PAGE dissolving buffer contained 8M urea, some precipitates were dissolved in reducing dissolving buffer containing 8M urea before running on SDS-PAGE gels. No bands could be seen in the tracks containing the BGU1-WR immunoprecipitates.

4.7.3. Immunoprecipitation Experiments Using Biotin-Extravidin Labelled Red Cells.

Biotin-extravidin labelling (Section 2.2.4.12.) was also used to label red cells for immunoprecipitation. The NHS-biotin labels primary amine groups whereas the lactoperoxidase method labels tyrosine residues with ^{125}I. It was therefore possible that the membrane components immunoprecipitated with BGU1-WR which could not be detected with one labelling method might be detected by the other. Experiments with these labelled red cells were carried out under a variety of conditions.

Sensitised red cell membranes were solubilised with 1%, 2.5% and 5% Triton-X100. Figure 4.7 shows BRIC-14 and BGU1-WR precipitates solubilised with 2.5% or 5% Triton X-100. Bands with an apparent M_r corresponding to glycophorin A and band 3 were identified in the tracks containing immunoprecipitates with the BRIC-14 antibody, none were found in any of the BGU1-WR precipitation tracks.

FIGURE 4.7.
ANTI-Wr^a AND ANTI-Wr^b IMMUNOPRECIPITATES SOLUBILISED AT DIFFERENT TRITON X-100 CONCENTRATIONS.



In this experiment red cells were labelled with NHS-Biotin and immunoprecipitated as described in section 2.2.4.12. Triton X-100 concentrations of 2.5% and 5% were used to solubilise the red cell membranes. Immunoprecipitates, labelled membrane samples and biotinylated markers were then detected using the chemiluminescent stain described in section 2.2.4.9.3.

Track M contains biotinylated markers (14,300, 20,100, 29,000, 39,800, 58,000 and 97,400); tracks R1 and R2 two different exposures of labelled red cell membrane samples; tracks A and B BRIC-14 antibody immunoprecipitates with Wr(a+b+) red cells solubilised with 2.5% and 5% Triton X-100 respectively and tracks C and D BGU1-WR antibody immunoprecipitates with Wr(a+b+) red cells solubilised with 2.5% and 5% Triton X-100 respectively.

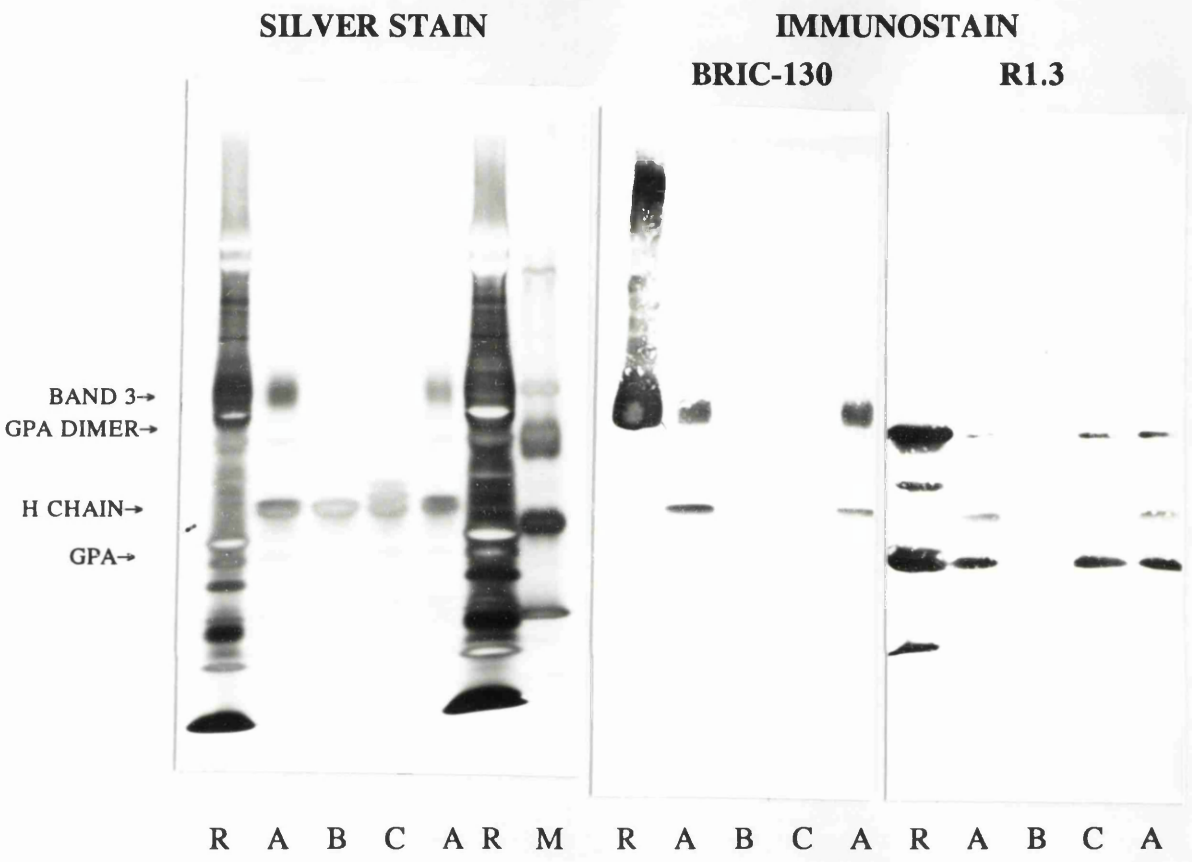
Various types of Sepharose were used to absorb the BGU1-WR immunocomplexes. Experiments using RAM-Sepharose or Protein G to absorb the BGU1-WR immunocomplex failed to reveal any bands after electrophoresis. Protein A Sepharose used together with a high salt high pH buffer recommended by Pharmacia-LKB to enable binding of IgG1 to protein A (0.725 M glycine [adjusted to pH 8.9 with NaOH]; 1.5M NaCl; 5% w/v Triton X-100) also failed to reveal any membrane component in the BGU1-WR immunoprecipitates. In comparison BRIC-14 immunoprecipitates prepared using all of the Sepharose reagents always contained bands corresponding to band 3 and glycophorin A .

Different SDS-PAGE dissolving buffers were also used in case the membrane components precipitated by BGU1-WR could not be detected after use of one or the other of them. No bands were detected in tracks containing BGU1-WR precipitates dissolved in non-reducing or reducing dissolving buffers or in reducing buffer containing 8M urea .

Finally Wr(a+b-) red cells from donor M.Fr. became available and were used in one immunoprecipitation experiment (membranes were dissolved in 2.5% Triton X-100 in PBS and immune complexes isolated by adsorption with protein G Sepharose) to see if the Wr^a related membrane component could be immunoprecipitated with BGU1-WR culture supernatant or with concentrated antibody from these cells. No bands were detected in the tracks containing this immunoprecipitate.

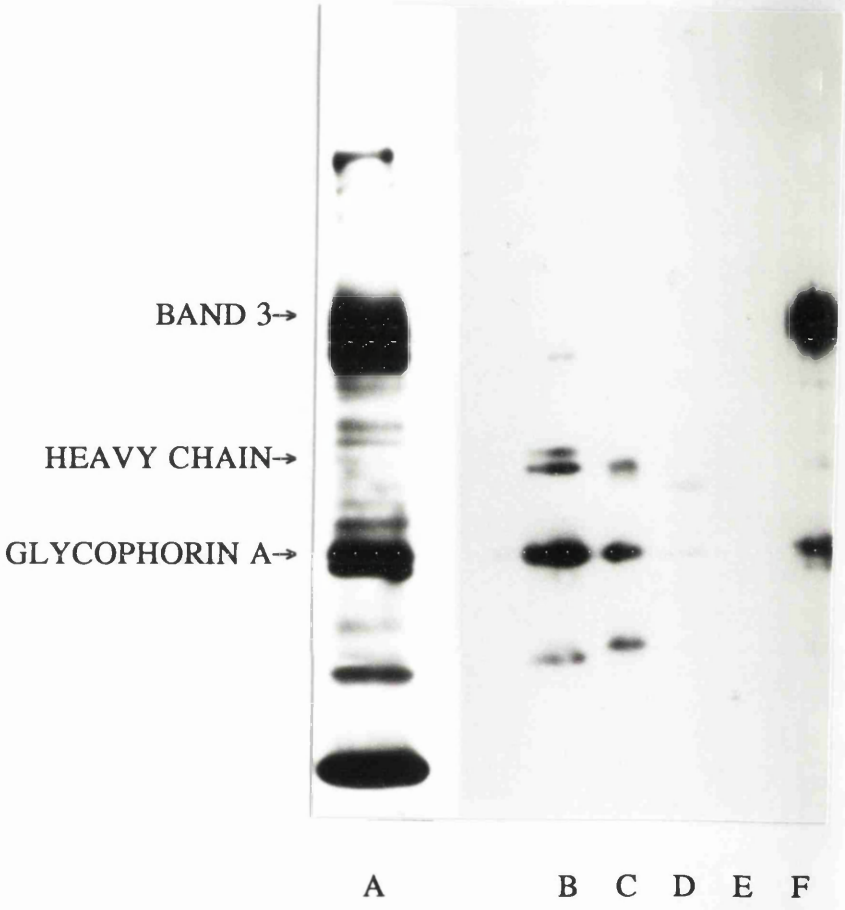
In conclusion all the experiments in Sections 4.7.1, 4.7.2. and 4.7.3 confirm that the anti-Wr^b monoclonal antibody BRIC-14 immunoprecipitates glycophorin A and band 3 under all conditions attempted. The anti-Wr^a monoclonal antibody, BGU1-WR, on the other hand, did not immunoprecipitate these or any other readily detectable membrane component from Wr(a+) or Wr(a-) red cells. This was despite the fact that the antibody always appeared to bind to the Sepharose reagents in immunoprecipitates using Wr(a+) cells but not from Wr(a-) cells, as judged by the presence of bands corresponding to IgG heavy and light chains detected in immunoblots.

FIGURE 4.8.
ANTI-GLYCOPHORIN A. ANTI-Wr^a AND ANTI-Wr^b IMMUNOPRECIPITATES.



Immunoprecipitation was carried out as described in section 2.2.4.10. After electrophoresis silver staining and immunoblotting were carried out as described in sections 2.2.4.6.2. and 2.2.4.8. Track M shows rainbow molecular weight markers (21,000, 46,000, 69,000, 97,400 and 200,000), track R red cell membrane samples, track A BRIC-14 (anti-Wr^b) immunoprecipitates, track B BGU1-WR (anti-Wr^a) immunoprecipitates and track C R18 (anti-glycophorin A) immunoprecipitates. Wr(a+b+) red cells were used for all immunoprecipitates.

FIGURE 4.9.
ANTI-GLYCOPHORIN A IMMUNOPRECIPITATES.



Immunoprecipitates were carried out using biotinylated Wr(a-b+) red cells (section 2.2.4.12.). After electrophoresis and electroblotting, the biotinylated components were detected using the chemiluminescent stain described in section 2.2.4.9.3. Track A is a labelled red cell membrane sample, tracks B, C and D are anti-glycophorin A immunoprecipitates (R18, R10 and R1.3 monoclonal antibodies respectively), track E an X63 secretor culture supernatant immunprecipitate and track F an anti-Wr^b immunoprecipitate (BRIC-14).

4.7.4. Further Investigatior of the Immunoprecipitation of Band 3 and Glycophorin A by BRIC-14.

The monoclonal antibody BRIC-14 was originally shown by Ridgewell *et. al.* (1983b) to precipitate glycophorin A. Band 3 was not detected. This could be because band 3 is not labelled very well by periodate/NaB^3H_4 the procedure used in these experiments. Alternatively immunoprecipitation of band 3 in my experiments could possibly be due to non-specific precipitation as has been reported for some Rh immunoprecipitates (Ridgewel *et. al.* 1983a). Therefore the following experiments were carried out to investigate whether the presence of band 3 in the precipitates was specific to anti-Wr^b monoclonal antibodies or was common in the precipitates of other monoclonal anti-glycophorin A antibodies under these conditions.

Figure 4.8 shows a silver stain and immunoblots, stained with the anti-band 3 monoclonal antibody BRIC-130 and the anti-glycophorin A and B monoclonal antibody R1.3, of BGU1-WR, BRIC-14 and R18 (anti-Glycophorin A) immunoprecipitates. These show that band 3 is only present in the anti-Wr^b immunoprecipitates.

Figure 4.9 shows results of an immunoprecipitation experiment using different monoclonal anti-Glycophorin A antibodies using the biotin-extravidin labelling method. These three monoclonal antibodies recognise epitopes at different positions on the glycophorin A molecule; R1.3, an epitope near the N-terminal of both glycophorins A and B; R10 an epitope in the En^aTS region and R18 an epitope in the En^aFS region. Glycophorin A, only, was immunoprecipitated by the these antibodies (the dimer and monomer are seen in the R18 precipitates and the monomer only in the R10 and R1.3 precipitates). Bric-14 on the other hand also precipitated band 3. Thus the monoclonal antibodies which recognise epitopes on the glycophorin A molecule further away from the membrane than the Wr^b epitope did not precipitate band 3. This is discussed further in Section 6.1.3.

In order to determine whether glycophorin A is coprecipitated with band 3 a preliminary experiment was also carried out on biotin-extravidin labelled red cells, using anti-band 3 monoclonal antibodies using. This experiment was carried out under slightly different conditions to the previous precipitations as the anti-band 3 monoclonal antibodies BRIC-130 and BRIC-132 recognise internal membrane epitopes. Therefore membranes were prepared before being sensitised. BRIC-14 immunoprecipitated band 3 and glycophorin A. Band 3, alone, was immunoprecipitated by BRIC-130 and BRIC-132. These results were surprising as Wainwright *et. al.* (1989) showed that BRIC-130 immunoprecipitated glycophorin

A in addition to band 3 from red cell membranes. It is interesting to note that in the immunostain shown in Figure 4.5. this monoclonal antibody appeared to have immunostained glycophorin A in addition to band 3.

The results from both the anti-glycophorin A and anti-band 3 immunoprecipitation experiments indicate that band 3 is immunoprecipitated specifically by BRIC-14. Whether this means that band 3 is genuinely part of the Wr^b epitope or is co-precipitated with glycophorin A is discussed in Section 6.1.3.

CHAPTER 5.

CELL AND TISSUE DISTRIBUTION OF THE Wr^a AND Wr^b ANTIGENS.

No studies on the expression of Wr^a on other tissues have previously been reported. The availability of the BGU1-WR monoclonal antibody now made such a study feasible.

Flow cytometry was the method chosen to look for the presence of the Wr^a antigen on other cells since it was the most convenient method available for detecting cell surface antigens. Immunoblotting or immunoprecipitation techniques for example, could not be used to detect the Wr^a antigen in cell extracts because the antigen carrying component had not been demonstrated in red cell membrane extracts. Flow cytometry has been used to show the presence of red cell antigens on other cells as illustrated by Spring *et. al.* (1988) who showed that an antigen located on the CDw44 glycoprotein recognised by the monoclonal antibody BRIC-35, is also expressed on granulocytes, T-lymphocytes, mononuclear blood leucocytes and HEL, HL60 and IM-9 cell lines.

Also, if the antigens were shown to be expressed on other human cells but not on those of rodents it might be possible to carry out gene mapping studies. Somatic cell hybrids could be used to map the *WR* gene to a particular chromosome or region of a chromosome using the BGU1-WR monoclonal antibody to recognise the antigen on the cell surface of the hybrids. The high frequency blood group antigen Ok^a was mapped to Chromosome 19 using this method (Williams *et. al.*, 1988).

The experiments in this chapter were carried out as described in section 2.2.5. in collaboration with Dr. Phil Judson of the South Western Regional Blood Transfusion Centre in Bristol.

5.1. Detection of the Wr^a and Wr^b Antigens on Red Cells by Flow Cytometry.

Initial experiments were carried out to ensure that binding of the BGU1-WR and BRIC-14 monoclonal antibodies to the Wr^a and Wr^b antigens respectively on red cells could be detected using the flow cytometry technique. DMS fixed red cells from four Wr(a+b+) and three Wr(a-b+) individuals were incubated with the monoclonal antibodies, followed by FITC-conjugated rabbit anti-mouse Ig. The cells were then examined on a flow cytometer and the amount of immunofluorescence emitted by 10,000 individual cells measured. This was logarithmically amplified and expressed as arbitrary units, directly proportional to the fluorescence intensity (peak channel numbers) (section 2.2.5.5.). The results were then plotted as histograms of these peak channel numbers against the frequency of cells in each channel. Examples of such histograms for the BGU1-WR and BRIC-14 antibodies with two different Wr(a+b+) red cell samples are shown in figure 5.1. It is interesting to note that the histograms for the BGU1-WR antibody are wider than those for BRIC-14 indicating that the level of Wr^a antigen expression varies more than that of Wr^b on the red cells.
The modal peak channel number (i.e. the peak channel with the highest cell frequency) was noted and converted to a linear value using the following formula:-

$$\text{linear value} = \text{Antilog} \frac{\text{Peak Channel No. x No decades log. scale}}{\text{Total number of peak channels}}$$

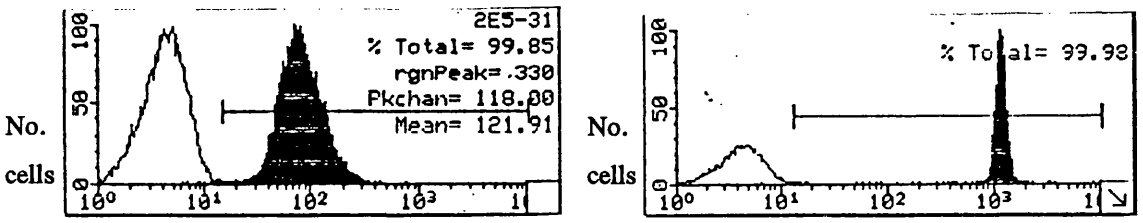
Where :- Number of decades on the logarithmic scale = 4
Total number of peak channels = 256

Table 5.1 shows the linear values for the modal peak channel numbers for the red cell samples tested with the BGU1-WR and BRIC-14 monoclonal antibodies and also BRIC-126 an anti-CD47 monoclonal antibody as a positive control and PBS as a negative control. These results show that binding of BRIC-14 to all cells tested and of BGU1-WR to the Wr(a+b+) cells could be detected by flow cytometry.

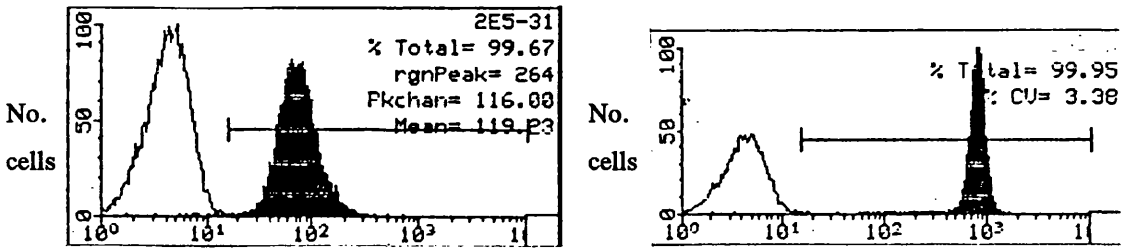
FIGURE 5.1.
DETECTION OF THE Wr^a AND Wr^b ANTIGENS ON RED CELLS BY FLOW CYTOMETRY.

BGU1-WR **BRIC-14**

(i) Wr(a+b+) donor C. **(ii) Wr(a+b+) donor C.**



(iii) Wr(a+b+) donor G.R. **(iv) Wr(a+b+) donor G.R.**



These histograms show the distribution of fluorescence emissions for Wr(a+b+) red cells reacted with the BGU1-WR and BRIC-14 monoclonal antibodies (right hand peaks) and with PBS (left hand peak). The histograms of the BGU1-WR results are based on the emissions from 10,000 red cell whereas the BRIC-14 results are from 5,000 cells, thus explaining the change in the shape of the PBS histograms.

TABLE 5.1

IMMUNOFLUORESCENCE EMISSIONS FROM RED CELLS TESTED WITH BGU1-WR AND BRIC-14 MONOCLONAL ANTIBODIES BY FLOW CYTOMETRY.

CELL PHENOTYPE	DONOR	-ve CONTROL PBS	+ve CONTROL BRIC-126	BGU1-WR	BRIC-14
CELLS STORED AT -30°C					
Wr(a+b+)	L.C.	3.68	77.04	88.17	1084.32
Wr(a+b+)	I.W.	4.57	119.71	162.53	777.37
Wr(a+b+)	G.R.	3.05	88.96	59.35	820.47
Wr(a+b+)	C.	5.19	86.60	71.69	1175.74
Wr(a-b+)	S.R.	4.00	62.64	4.26	777.37
Wr(a-b+)	P.T.	6.26	100.90	6.32	1186.37
Wr(a-b+)	C.G.	10.96	95.60	5.23	1286.40
CELLS STORED AT 4°C					
Wr(a+b+)	L.C.	5.65	92.22	254.83	620.82
Wr(a-b+)	S.R.	6.21	94.75	4.03	1074.61

This table shows immunofluorescence emissions, expressed as the linear value of the modal peak channel numbers, of Wr(a+b+) and Wr(a-b+) red cells treated with BGU1-WR and BRIC-14 monoclonal antibodies as described in section 2.2.5.1. Values for BRIC-126, an anti-CD47 monoclonal antibody used as a positive control and PBS as a negative control are also shown. Cell samples from all donors had been stored at -30°C and were thawed as described in section 2.1.2. Additional samples from donor L.C. and S.R. were also available from recent donations and were stored at 4°C until use.

TABLE 5.2
IMMUNOFLUORESCENCE EMISSIONS FROM RED CELLS TESTED WITH ANTI-GLYCOPHORIN A MONOCLONAL ANTIBODIES BY FLOW CYTOMETRY.

CELL PHENOTYPE	DONOR	BRIC-256	BRIC-127	R1.3
CELLS STORED AT -30°C				
Wr(a+b+)	L.C.	947.46	305.02	637.80
Wr(a+b+)	I.W.	626.43	220.67	598.89
Wr(a+b+)	G.R.	763.51	421.70	620.82
Wr(a+b+)	C.	842.91	327.81	777.37
Wr(a-b+)	S.R.	461.38	371.80	537.61
Wr(a-b+)	P.T.	716.92	324.88	598.89
Wr(a-b+)	C.G.	743.18	333.76	723.31
CELLS STORED AT 4°C.				
Wr(a+b+)	L.C.	620.82	410.47	542.47
Wr(a-b+)	S.R.	667.14	345.99	542.47

This table shows immunofluorescence emissions, expressed as the linear value of the modal peak channel numbers, of Wr(a+b+) and Wr(a-b+) red cells treated with the anti-glycophorin A monoclonal antibodies BRIC-256, BRIC-127 and R1.3 as described in section 2.2.5.1. The values for the negative (PBS) and positive (BRIC-126) control samples are shown in Table 5.1. Cell samples from all donors had been stored at -30°C and were thawed as described in section 2.1.2. Additional samples from donor L.C. and S.R. were also available from recent donations and were stored at 4°C until use.

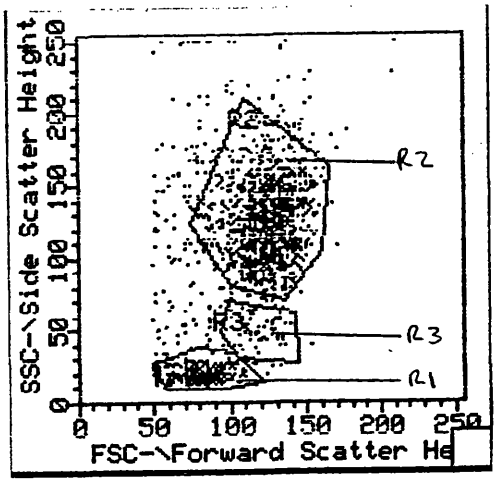
Flow cytometry has been successfully used by others to give a semi-quantitative estimate of the site density of various blood group antigens (Van Brockslaele *et. al.*, 1986; McHugh *et. al.*, 1987; Oien *et. al.*, 1988 and Nichloson *et. al.*, 1991). However examination of my results demonstrated that there is no significant difference for Wr^b between the two sets of values obtained from Wr(a+b+) and Wr(a-b+) red cells. The mean linear channel number for the Wr(a+b+) red cells with BRIC-14 is 964.44 whereas that for Wr(a-b+) cells is 1083.38, with some variation in the strength of reaction between individuals in each group. These results therefore do not lend support to the idea that there is more Wr^b antigen on Wr(a-b+) than on Wr(a+b+) red cells.

The binding of three anti-glycophorin A monoclonal antibodies to these red cells was also investigated; BRIC-256 (anti-En^aFS), BRIC-127 (anti-EnaTS-like) and R1.3 (which recognises an epitope near the N-terminus of both glycophorins A and B). The immunofluorescence emission values for these tests are shown in Table 5.2. The strength of these antibody reactions also varied between the cell samples and did not appear to be dependent on the Wr status of the cells.

The red cells used for all of the tests described above had been stored at -30°C and thawed as described in section 2.1.2. before use. Fresh red cell samples from two of the seven individuals, one Wr(a+b+) and one Wr(a-b+), were also available for testing. Therefore the expression of the antigens on these cells was examined to see whether storage of cells at -30°C might affect expression. The results shown in Table 5.1 are inconclusive. For example, the Wr^b expression on the Wr(a+b+) sample was lower on the cells which had been stored at -30°C whereas the expression was higher on the Wr(a-b+) sample stored in this way. The reaction of BGU1-WR was approximately three times stronger on the cells which had been stored at 4°C.

The variation in the strength of the antibody reaction shown in these experiments between red cells from different individuals of the same red cell phenotype is discussed in section 6.2.

FIGURE 5.2.
FLOW CYTOMETRY OF WHOLE CELL POPULATION OF PERIPHERAL BLOOD LEUCOCYTES.



This figure shows a dot plot of the forward and side scatter of a whole cell population of peripheral blood leucocytes showing the subpopulations of cells. Region 1 shows the lymphocytes, region 2 the monocytes and region 3 the granulocytes.

5.2. Expression of Wr^a and Wr^b antigens on peripheral blood leucocytes.

The experiments described above (section 5.1) confirmed that binding of the BGU1-WR and BRIC-14 monoclonal antibodies could be detected by flow cytometry. The expression of the Wr^a and Wr^b antigens on peripheral blood leucocytes was therefore examined with recently collected leucocytes from a Wr(a+b+) and a Wr(a-b+) donor. PBS was used as a negative control and the BRIC-126 monoclonal antibody, as a positive control. The leucocytes were prepared and incubated with the antibodies as described in section 2.2.5.2. and 2.2.5.4. before examination on a flow cytometer.

Figure 5.2 shows a dot plot of the forward and side scatter produced from the sample of labelled leucocytes from the Wr(a+b+) individual. Three separate populations of cells (lymphocytes, monocytes and granulocytes) can be identified by the positions of the cells on the plot which reflect the size and shape of the cells. The fluorescence emission from each group of cells was plotted as a histogram of channel numbers against the frequency of cells and the linear value of the modal peak channel number calculated as described in section 5.1. Figure 5.3 shows such histograms of the whole cell populations and Table 5.3 the mean linear peak channel number of the different subpopulations of cells in the two samples.

The mean linear peak channel number of cells labelled with PBS determines the background level of fluorescence. The immunofluoresence emissions for the lymphocytes from both donors (table 5.3) indicate that those from the Wr(a+b+) individual react more strongly with BRIC-126, the anti-CD47 monoclonal antibody, than those of the Wr(a-b+) individual. This is reflected in the histograms shown in figure 5.3 (ii) and (v). The second peak in that from the Wr(a+b+) individual represents the lymphocytes of this donor had a much higher mean peak channel number than the granulocytes or monocytes of this sample, thus these are represented by the larger first peak on the histogram.

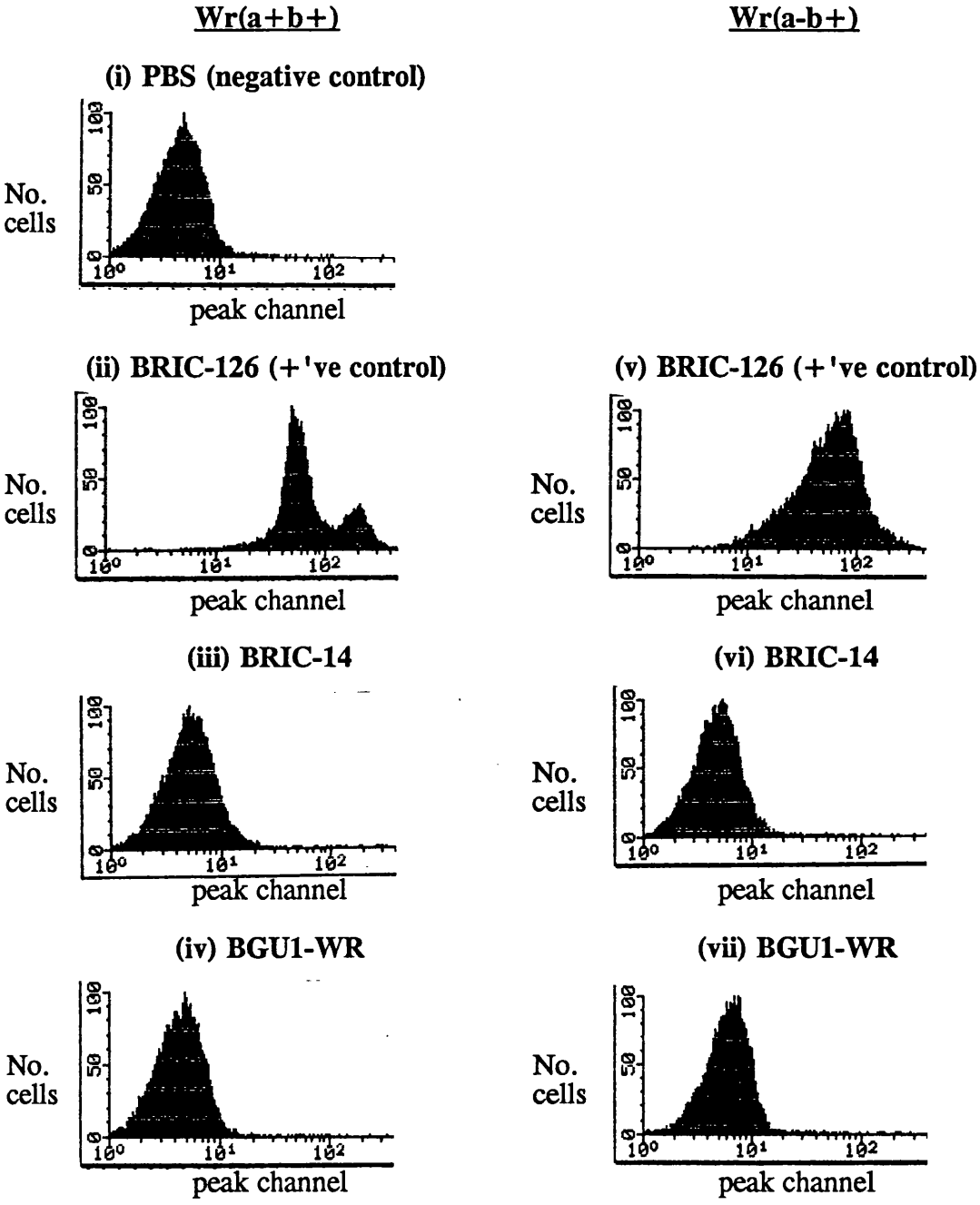
This discrepancy was probably caused by different rates of deterioration of the CD47 antigen on the two samples. Ideally leucocytes should be prepared for flow cytometry the same day as a peripheral blood sample is drawn. However due to the difficulty in obtaining fresh, unrefri gerated Wr(a+) samples both blood samples were stored at room temperature for at least 24 h before use.

None of the cell populations showed higher fluorescence emissions with the BGU1-WR monoclonal antibody than the PBS control suggesting that the Wr^a antigen is not carried on peripheral blood lymphocytes, granulocytes or monocytes from either the Wr(a+b+) or Wr(a-b+) donors.

Similarly, with BRIC-14 the anti-Wr^b monoclonal antibody neither of the whole cell, lymphocyte or granulocyte populations had a greater immunofluoresence than the PBS control. However, there was a small increase in the peak channel number of the monocyte populations from both donors; 12.86 from Wr(a+b+), 9.3 from Wr(a-b+) compared with the PBS control (5.23). This increase is probably not significant since a small amount of background binding can occur with monocytes due to nonspecific binding of antibodies to Fc receptors on the cells. On reflection this experiment might have been better controlled by using X63 secretor supernatant instead of PBS since it contains immunoglobulins.

FIGURE 5.3.

FLUORESCENCE HISTOGRAMS OF PERIPHERAL BLOOD LEUCOCYTES.



This figure shows histograms of the fluorescence, expressed as logarithmic peak channel values against the frequency of cells for whole cell populations of peripheral blood leucocytes reacted with the BGU1-WR, BRIC-14 and BRIC-126 monoclonal antibodies for flow cytometry

TABLE 5.3

IMMUNOFLUORESCENCE EMISSION FROM PERIPHERAL BLOOD LEUCOCYTES.

	WHOLE CELL POPULATION	LYMPHOCYTES	GRANULOCYTES	MONOCYTES
PBS				
-'VE CONT.	4.70	2.21	4.70	5.23
BRIC-126				
Wr(a+b+)	48.70	205.30	48.70	74.99
Wr(a-b+)	74.99	100.00	74.99	107.46
BGU1-WR				
Wr(a+b+)	4.87	4.50	4.87	5.42
Wr(a-b+)	6.98	4.87	7.50	6.98
BRIC-14				
Wr(a+b+)	5.23	3.92	5.23	12.86
Wr(a-b+)	5.42	3.65	5.23	9.30

This table shows the linear value of the modal peak channel numbers of peripheral blood leucocytes reacted with the monoclonal antibodies BGU1-WR, BRIC-14 and BRIC-126. The cell samples were separated by gating the different groups of cells shown on dot plots of the side and forward scatter of the cells similar to that shown in figure 5.2.

5.3. Expression of Wr^a and Wr^b on Human and Rodent Cell Lines.

The monoclonal antibodies BGU1-WR and BRIC-14 were used to test for the presence of the Wr^a and Wr^b antigens respectively on the following cell lines:- HEL and K562 (erythroleukaemic); HL60 and U937 (myeloid-like); MOLT-4 (T-ALL); RAJI and DAUDI (Burkitt's lymphoma), Henson, Wiseman and 6897, (EBV transformed lymphoblastic cell lines); Barker and Wiseman (skin fibroblasts); T47D and MCF7 (breast); HT29 (enterocyte); HEPG2 (hepatoma) and the rodent cell lines RAG (mouse), A23 (hamster) and FAZA (rat).

The antibody BRIC-126 which recognises CD47, a widely distributed human cell surface antigen, was used as a positive control and PBS as a negative control. BRIC-126 reacted with all human cell lines tested except HEPG2 which was not in very good condition. Not surprisingly, BRIC-126, which is a murine monoclonal antibody, did not react with any of the rodent cell lines.

BGU1-WR did not react with any of the cell lines tested including those of myeloid or erythroleukaemic origin. Histograms of the erythroleukaemic and myeloid-like cell line results are shown in Figures 5.4 and 5.5 and their mean linear peak channel numbers in Table 5.3. Results from the other cell lines are not shown.

BRIC-14 reacted weakly with the myeloid-like cell lines as shown in Figure 5.5. 96.6% of the U937 cells and 31% of the HL60 cells reacted more strongly than the negative control. When compared with the mean linear peak channel numbers, shown in Table 5.2, it can been seen that this increase in fluorescence is very low especially for the HL60 cells. Further work is needed to confirm this observation. Figure 5.4 and Table 5.4 show that BRIC-14 does not react with erythroleukaemic cell lines. The antibody was also not found to react with any of the other cell lines (results not shown). Therefore the Wr^b antigen does not appear to be expressed on any of the cell lines tested except possibly at a low level on the myeloid cell lines.

TABLE 5.4
IMMUNOFLUORESCENCE EMISSIONS FROM ERYTHROLEUKAEMIC AND MYELOID CELL LINES.

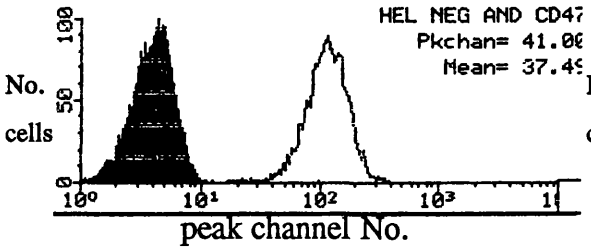
	K562	HEL	HL60	U937
PBS	4.87	4.37	2.46	3.65
BGU1-WR	4.87	3.65	2.84	3.40
BRIC-14	6.49	3.28	4.87	15.40

This table shows the linear value of the modal peak channel number of erythroleukaemic and myeloid-like cell lines with the BGU1-WR and BRIC-14 monoclonal antibodies.

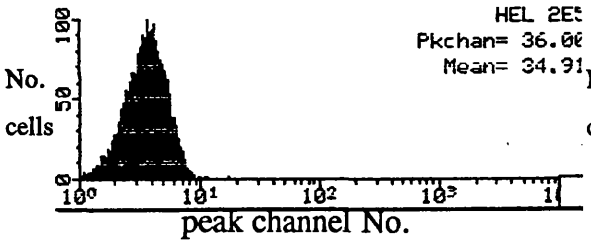
FIGURE 5.4.
FLUORESCENCE HISTOGRAMS OF ERYTHROLEUKAEMIC CELL LINES.

(i) HEL

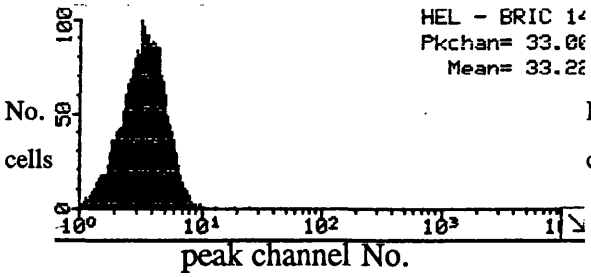
A. +'ve and -'ve control



B. BGU1-WR

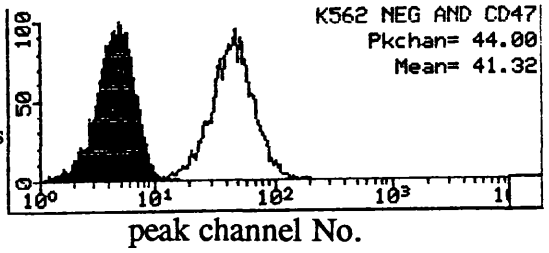


C. BRIC-14

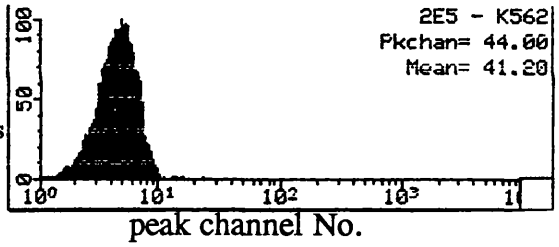


(ii) K562

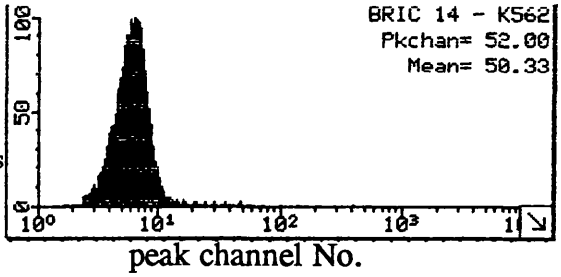
A. +'ve and -'ve control



B. BGU1-WR



C. BRIC-14

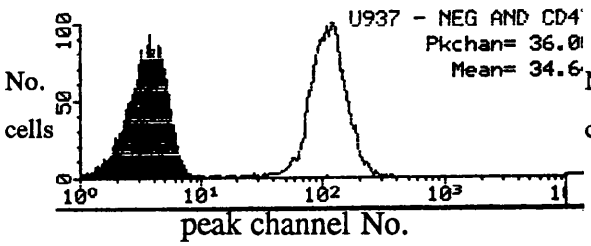


This figure shows histograms of fluorescence, expressed as logarithmic peak channel values against cell frequency for erythroleukaemic cell lines with BGU1-WR and BRIC-14 monoclonal antibodies.

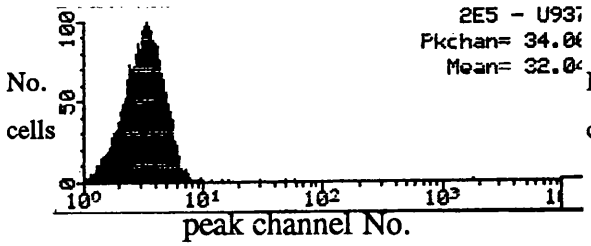
FIGURE 5.5.
FLUORESCENCE HISTOGRAMS OF MYELOID-LIKE CELL LINES.

(i) U937

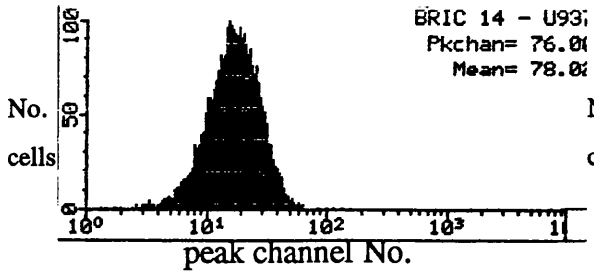
A. +'ve and -'ve control



B. BGU1-WR

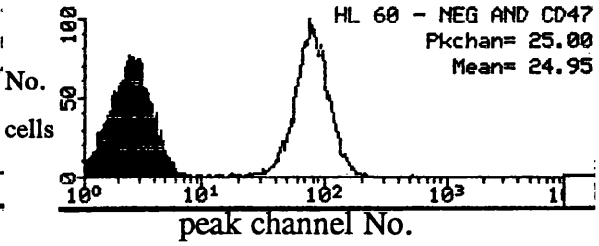


C. BRIC-14

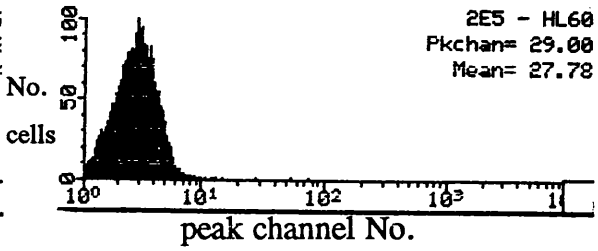


(ii) HL60

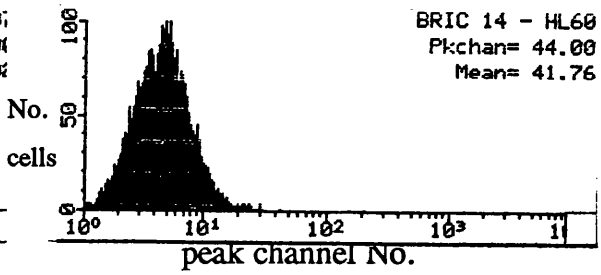
A. +'ve and -'ve control



B. BGU1-WR



C. BRIC-14



This figure shows histograms of fluorescence, expressed as logarithmic peak channel values against cell frequency for myeloid-like cell lines with BGU1-WR and BRIC-14 monoclonal antibodies.

5.4. Discussion of Tissue Distribution Results.

The results described above suggest that the Wr^a and Wr^b antigens are essentially erythrocyte specific.

The BRIC-14 results are in reasonable agreement with published data. Telen *et. al.* (1987) showed Wr^b not to be present on K562 or HEL cells using a monoclonal antibody, E6. Reardon *et. al.* (1985) showed that three different monoclonal anti-Wr^b antibodies did not react with HEL and K562 cell lines. Reardon *et. al.* (1985) also showed that a monoclonal anti-Wr^b did not react with peripheral blood monocytes, lymphocytes or granulocytes.

However, my results suggest that the Wr^b antigen may be expressed at a low level on the myeloid cell lines, HL60 and U937. This differs from the results of Reardon *et. al.* (1985) who reported that the monoclonal anti-Wr^b antibody MoAb-36 did not react with the HL60 cell line.

Since the Wr^b antigen is present on erythrocytes but not on peripheral blood leucocytes it might have been expected that the Wr^b antigen would be present on erythroleukaemic cell lines but not the less differentiated myeloid cells. However, although used as model systems, the cell lines do not accurately reflect their normal human counterparts. The erythroleukaemic cell line HEL, for example, has been shown to express platelet glycoproteins (Tabillo *et. al.*, 1984). Thus other monoclonal antibodies recognising Wr^b should be used to confirm the presence of the antigen since it is conceivable that the results are due to cross reactivity of BRIC-14 and that the BRIC-14 epitope occurs as part of another component expressed on the surface of this cell line.

The results with BGU1-WR clearly show that Wr^a is not expressed on peripheral blood lymphocytes, granulocytes or monocytes of Wr(a+b+) or Wr(a-b+) individuals. Neither is the antigen expressed on any of the cell lines tested. However, it is important to remember that the Wr^a status of the individuals from whom the cell lines were taken was unknown, but statistically most were likely to be from Wr(a-) individuals. Therefore the possibility that the Wr^a antigen could be present on such cell lines taken from individuals with a Wr(a+) red cell phenotype has not been eliminated.

The expression of antigens on various tissues can also be investigated using immunohistological methods. The tissue distribution of the Wr^a antigen could therefore be studied further on tissue sections using such techniques with the BGU1-WR monoclonal antibody.

CHAPTER 6.
DISCUSSION.

This thesis has described the production of the monoclonal antibody, BGU1-WR which recognises the low incidence red cell antigen Wr^a. This is the first report of an anti-Wr^a monoclonal antibody. The availability of this antibody enabled the investigation of the nature of the Wr^a antigen and its proposed antithetical relationship to the high incidence antigen Wr^b.

6.1. NATURE OF THE Wr^a AND Wr^b ANTIGENS.

6.1.1. Similarities Between the Wr^a and Wr^b Antigens.

Several experiments described in this thesis suggest similarities in the nature of the Wr^a and Wr^b antigens. Haemagglutination tests on red cells treated with various enzymes (section 4.1) suggest that, if both antigens are located on proteins, they are located close to the membrane surface and do not require the presence of intact disulphide bonds. The antigens are not dependent on the sialic acid residues accessible to *Vibrio cholerae* neuraminidase. Other antigens such as those of the Rh system would also react with enzyme treated cells in this way.

The experiments described in section 4.6. showed that under a range of conditions neither antigen was detectable on denatured SDS treated red cell membranes by immunoblotting. This suggested that the epitopes are probably discontinuous and the antigens may require an interaction between various red cell membrane components for expression as is the case with some Rh antigens (section 1.3.4.) which are known to be carried on proteins but are dependent on membrane lipid for antigen integrity or expression.

Wr^b, like the Rh antigens (Hughes-Jones *et. al.*, 1975; Plapp *et. al.*, 1980), has been reported to be phospholipid dependent. Reardon (1985) showed that Wr^b expression decreased after red cells were treated with phospholipase A_2. This suggests that the Wr^b antigen is dependent on an interaction with membrane components. Preliminary experiments to investigate the effect of phospholipase A_2 on the Wr^a antigen were carried out for this project. Red cell membranes treated with phospholipase A_2 were used to absorb BGU1-WR, the anti-Wr^a monoclonal antibody. Reduction of Wr^a antigen expression was suggested by these experiments, however, it was very difficult to demonstate convincingly due to the problems encountered when absorbing the BGU1-WR antibody with red cells membranes (section 4.2.). Reduction of Wr^b expression was observed in similar experiments using the anti-Wr^b

monoclonal antibody, BRIC-14. Unfortunately due to lack of time more quantitative experiments, which would have used ^{125}I-labelled anti-Wr^a to examine antigen expression on phosopholipase A_2 treated whole red cells, were not carried out.

6.1.2. Further Characterisation of the Wr^a Antigen.

Further attempts to characterise the nature of the Wr^a antigen proved difficult. The method used for the immunoprecipitation experiments described in section 4.7 enables the precipitation of membrane components which interact to form an antigen. The antibodies were incubated with intact red cells before the preparation and solubilization of membranes. This probably helps to maintain the structure of the antigen (Gahmberg, 1983; Moore *et. al.*, 1983). These experiments did not reveal any membrane components detected by the BGU1-WR monoclonal antibody. It is possible that this may have been due to a property of the antibody since not all monoclonal antibodies with the same specificities are equally efficient at recognising identical epitopes in various immunochemical techniques. For example, Ridgwell *et. al.* (1983b) found that BRIC-14 had the highest binding affinity of the monoclonal anti-Wr^b antibodies they tested and was thus more effective in immunoprecipitation experiments. Haemagglutination studies (section 4.1) had suggested that the BGU1-WR monoclonal antibody would be a good reagent for investigating the nature of the Wr^a antigen. The antibody reacted well with Wr(a+b+) red cells by haemagglutination. Also the functional affinity constant determined in section 4.4.3. was found to be of the same order of magnitude as that of BRIC-14. The relatively high binding affinity of BGU1-WR implies that the failure of experiments using this antibody to identify the membrane components associated with the Wr^a antigen is a consequence of the quantity or the elusive nature of the Wr^a antigen rather than the properties of the antibody.

The site number of approximately 70,000 on Wr(a+b+) which is less than that of the Wr^b antigen on the same cells. However immunoprecipitation of red cell antigens with a much lower site number has been achieved, for example Moore *et. al.* (1982) successfully immunoprecipitated the Duffy glycoprotein which is known to have approximately 12,200 molecules per cell (Nichols *et. al.*, 1987). It is therefore unlikely that a site number of 70,000 antigen sites per cell is the single reason why immunoprecipitation experiments failed to reveal the nature of the Wr^a antigen.

The relatively low number of Wr^a antigen sites may however, be the reason why the absorption experiments attempted with the BGU1-WR monoclonal antibody were not informative This was unfortunate as several experiments could have been carried out using such techniques to try to give an indication of the properties of the antigen. For example, Dahr *et. al.* (1986) used absorption tests to demonstrate the effect of

various enzyme and chemical treatments of red cell membranes on the Wr^b antigen. Similar experiments might have revealed further similarities or differences between the Wr^a and Wr^b antigens.

Dahr *et. al.* (1986) also reported that the Wr^a antigen appeared to differ from Wr^b in that it was not solubilised to the same extent by Triton X-100. Wr^b appeared to be completely solubilised in 0.5% Triton X-100 and all antigen activity was present in the supernatant. Wr^a, on the other hand, was not solubilised until the Triton X-100 concentration was increased to 2% and in this case little antigen activity was detected in the supernatant. This suggests that the Wr^a antigen is unstable. This may account for the inability of the BGU1-WR to immunoprecipitate the membrane components associated with the Wr^a antigen.

Further experiments which may help to reveal the nature of the Wr^a antigen would include competitive binding assays with intact red cells such as those carried out in section 4.5. In preliminary experiments the anti-Wr^a monoclonal antibody, BGU1-WR was not inhibited by the anti-Wr^b or anti-glycophorin A monoclonal antibodies tested. However experiments using a wider range of anti-red cell antibodies might provide clues to the membrane components associated with the Wr^a antigen.

Another approach would be to analyse the glycosphingolipid components from red cell membranes from Wr(a+) and Wr(a-) individuals using the BGU1-WR monoclonal antibody in immunoblotting experiments similar to those described by Towbin *et. al.* (1984). These authors separated the lipids in membrane preparations by thin layer chromatography, then blotted and stained them with the monoclonal antibodies in a similar way to protein immunoblotting. Membrane lipids from a Wr(a+b+) individual were immunostained with the BGU1-WR and BRIC-14 monoclonal antibodies in a preliminary experiment by Dr. Alison Petty. No bands were detected. This suggests that the Wr^a antigen is not carried exclusively by the membrane lipids detected using this method but it does not rule out the formation of the antigen by interaction of the lipid and other membrane components.

6.1.2.1. Association of the Wr^a antigen with the Sd^a antigen.

A carbohydrate antigen which might possibly be involved with the Wr^a and Wr^b antigens is Sd^a. One family was reported which suggested that the gene encoding the Wr^a antigen is linked to that encoding the Sd^a antigen (Lewis *et. al.*, 1973).

Sd^a is a high incidence ubiquitous antigen whose level of expression on red cells is very variable. Sd^a is dependent on an oligosaccharide with N-acetylgalactosamine, ß-linked to galactose at its non-reducing end (Soh *et. al.*, 1980; Donald *et. al.*, 1982; Cartron *et. al.*, 1982).

Some individuals have red cells with an exceptionally high level of Sd^a activity (Cad family) and are termed Sd(a++). Biochemical analysis of such cells has also shown that the glycophorins A and B have an increase in their apparent M_r of 3,000 and 2,000 respectively, compared to those of normal cells. This was shown to be due to the addition of an N-acetylgalactosamine residue ß-linked to most of the disialotetrasaccharides carried by the glycophorins (Cartron and Blanchard, 1982). Cad red cells have also been shown to have a reduced level of the major ganglioside in normal cells, sialosylparagloboside. They also have an unusual ganglioside with a lower mobility which represents sialosylparagloboside with an additional terminal ß-N-acetylgalactosamine residue (Blanchard *et. al.*, 1985).
Whether there is any relationship between the level of expression of the carbohydrate antigen Sd^a and the Wr^a and Wr^b antigens should perhaps be investigated.

6.1.3. Further Characterisation of the Wr^b Antigen.

The immunoprecipitation experiments described in section 4.7. showed without doubt that BRIC-14, the anti-Wr^b monoclonal antibody immunoprecipitated glycophorin A and band 3. In an early report Ridgwell *et. al.* (1983b) showed that BRIC-14 and two other anti-Wr^b monoclonal antibodies immunoprecipitated glycophorin A but band 3 was not observed in the precipitates. My results, however, which showed the immunoprecipitation of both glycophorin A and band 3, are in agreement with those of Telen and Chasis (1990). These authors carried out their immunoprecipitation experiments with red cells labelled with ^{125}I using Iodo-Gen. These authors also used a different immunoprecipitation protocol in that red cell membranes were prepared and solubilised in Tris-buffered saline containing 1 mmol EDTA, 0.1% gelatin and either 1% deoxycholate or 1% Nonidet-P40 before incubation with the antibodies. The experiments described in section 4.7. were similar to those of Ridgwell *et. al.* (1983b) since intact red cells were incubated with the antibodies before membranes were prepared and solubilised in PBS containing Triton X-100 but were detected by immunoblotting. Telen and Chasis (1990) identified band 3 and glycophorin A in the anti-Wr^b immunoprecipitates on the basis of their apparent M_r from the SDS-PAGE gels. My results (section 4.7.1.) confirm that these components are indeed glycophorin A and band 3 since they are immunostained by the anti-glycophorin A and anti-band 3 monoclonal antibodies.
One possible explanation for the fact that Ridgwell *et. al.* (1983b) did not appear to immunoprecipitate band 3 is that they used sodium metaperiodate and NaB^3H_4 to label the red cells. This labelling method selectively labels sialic acid containing glycoproteins (Gahmberg and Andersson, 1977) and hence does not effectively label band 3.

However, since non-specific background precipitation of band 3 is known to occur using red cell immunoprecipitation experiments such as these (Ridgwell *et. al.*, 1983a) it was important to show that the immunopreciptation of band 3 was specific for the anti-Wr^b monoclonal antibody. The experiments in section 4.7.4 helped to confirm this since band 3 was not present in the immunoprecipitates of the glycophorin A monoclonal antibodies R1.3, R10 and R18.

Although my experiments show that band 3 as well as glycophorin A are specifically immunoprecipitated by anti-Wr^b they do not indicate whether part of the band 3 molecule helps to form the Wr^b epitope or whether the molecule is co-precipitated. Band 3 and glycophorin A are assumed to be associated in the red cell membrane since Nigg *et. al.* (1980) demonstated that binding of a divalent anti-glycophorin A serum prevented rotational diffusion of band 3 by cross-linking the glycophorin. Also an anti-band 3 monoclonal antibody BRIC-130 which binds to the cytoplasmic C-terminal region of the protein has been shown to immunoprecipitate glycophorin A in addition to band 3 (Wainwright *et. al.*, 1990), presumably by stabilising the association between the molecules. This association of band 3 and glycophorin A also appears to be stabilised to different extents by anti-glycophorin A reagents. This probably depends on the position at which the ligands bind to the glycophorin A molecule. In a preliminary paper Chasis *et. al.* (1985) showed that binding of anti-glycophorin A ligands increased the membrane rigidity probably due to an association with the cytoskeletal proteins spectrin and band 2.1 via its association with band 3. In a second paper Chasis *et. al.* (1988) showed that the closer to the membrane a glycophorin A ligand bound, the greater the membrane rigidity. It is therefore possible that since the Wr^b epitope within glycophorin A is close to the membrane (Ridgwell *et. al.*, 1983b., Dahr *et. al.*, 1986) binding of anti-Wr^b increases the membrane rigidity enough to cause the co-precipitation of the band 3 molecule. Other anti-glycophorin A monoclonal antibodies, such as those used in the experiments described in section 4.7.4., bind the glycophorin A molecule further away from the membrane surface and may therefore not increase the rigidity of the membrane enough to cause this co-precipitation of band 3.

Other indirect evidence that indicates that Wr^b antigen expression is dependent on band 3 in addition to glycophorin A comes from the cell line K562. This and the other erythroleukaemic cell line HEL, used in section 5.3, have been shown to express glycophorin A (Gahmberg *et. al.*, 1984; Gahmberg *et. al.*, 1979). If the expression of Wr^b is dependent only on glycophorin A the expression of Wr^b might be expected on these cells. The fact that my tissue distribution results (section 5.3), like those of Reardon *et. al.* (1985) and Telen *et. al.* (1987), did not demonstrate that the Wr^b antigen is expressed on this cell line supports the idea that

another membrane component, perhaps band 3, in addition to glycophorin A is required for Wr^b expression. The K562 cell line has also been shown to not express band 3 (Gahmberg *et. al*, 1979) thus providing further evidence that this membrane component is required for Wr^b antigen expression.

Another hint that band 3 is required for Wr^b expression is given by the ovalocytic cells. Section 4.1.1 describes experiments which show that although BRIC-14 reacted normally with the ovalocytic cells described by Schofield *et. al.* (1992), other anti-Wr^b reagents did not. These cells carry a variant band 3 molecule which may account for this decreased expression of some Wr^b epitopes.

Association of the Wr^b, and possibly the Wr^a antigen, with band 3 may provide an explanation for the deterioration of the antigens on old cells (section 6.2.2.). Band 3 is modified in aging cells, a breakdown product with an apparent M_r of 62,000 can be seen in SDS-PAGE analysis of old cells (Kay *et. al.,* 1991). This modification produces the senescent cell antigen on an external portion of the transmembrane region of the protein which then promotes cell destruction. This modification of band 3 could conceivably affect the Wr^a and Wr^b antigens. However, the immunofluoresence histograms (figure 5.1) show that Wr^b expression on the red cells is fairly uniform since there is not much spread in the amount of fluoresence em itted by the cells. This suggests that there is not a decrease in the am ount of Wr^b antigen present on older cells. The histograms of the immunofluoresence em itted by red cells reacted with the anti-Wr^a monoclonal antibody, BGU1-WR on the other hand show a wider spread. A decrease of the Wr^a antigen on older red cells is therefore conceivable. This could be tested by separating old red cells from young red cells by density gradient centrifugation and looking at the Wr^a and Wr^b antigens on the two sets of cells.

6.2. QUANTITATIVE ANALYSIS OF THE Wr^a AND Wr^b ANTIGENS.

The proposed antithetical relationship of the Wr^a and Wr^b antigens was based on semi-quantitative studies of antigen dosage. The original Wr(a+b-) donor M.Fr. (Adams *et. al.*, 1971) was so classified since the antibody which recognised a high incidence antigen in her serum usually gave a double-dose score with Wr(a-) and a single-dose score with Wr(a+) red cells from unrelated individuals in haemagglutination tests. The antibody was therefore assumed to be anti-Wr^b. Also her red cells usually reacted as if they carried a double-dose of the Wr^a antigen when tested with a number of anti-Wr^a reagents. The proposita's family, although small, supported this theory. Out of the family members tested her only child and three of her four sibs were Wr(a+) as were six out of seven children of her sibs. The cells of all of these individuals reacted with the anti-Wr^b antibody in her serum and were thus all assumed to be Wr(a+b+). Further evidence supporting this allelic relationship was provided by Wren and Issitt (1988) who used an enzyme linked antiglobulin test (ELAT) to show that on average Wr(a+b+) red cells bind a little over half the human polyclonal anti-Wr^b bound by Wr(a-b+) cells. They also showed that Wr(a+b+) red cells bind about half the amount of human polyclonal anti-Wr^a bound by Wr(a+b-) cells. These experiments were carried out on a large number of red cell samples; 24 Wr(a+b+) samples were compared to 23 Wr(a-b+) comparable control samples. The experiments described in this thesis show very little evidence of dosage of the Wr^a and Wr^b antigens. This is possibly a reflection of this particular monoclonal antibody or the techniques used.. Alternatively the failure to demonstate dosage may have been due to large person to person variation in relation to the small numbers of samples tested in my experiments; or the deterioration of the Wr^a or Wr^b antigens as described in the next sections.

6.2.1. Person to Person Variation.

Absorption studies and red cell membrane direct binding studies using the BGU1-WR antibody (sections 4.2 and 4.3) suggested that there is person to person variation in the number of Wr^a antigen sites between Wr(a+b+) individuals. The somewhat preliminary results of the immunofluorescence experiment shown in Table 5.1. tend to support this observation and also indicate that there may be variation in the number of Wr^b antigen sites on the cells. Further evidence for the variation of antigen site numbers between individuals was provided by the quantitative estimates of antigens carried out using ^{125}I-labelled antibodies (section 4.4.4). Table 4.5. records the observed number of Wr^a antigen sites on Wr(a+b+) red cells. These range from

71,000 to 95,000. The number of Wr^b sites on the same cells was approximately 140,000 on 3 samples and 350,000 on the fourth.

This variation in antigen site number appears to be in agreement with the data of Wren and Issitt (1988). They also showed that the amount of anti-Wr^b which bound to Wr(a+b+) and Wr(a-b+) red cells varied from person to person. The average amount of anti-Wr^b which bound to Wr(a+b+) red cells was a little over half the average amount which bound Wr(a-b+) cells. However red cells from a Wr(a+b+) individual with a particularly high expression of Wr^b could bind more anti-Wr^b than cells from a Wr(a-b+) individual with a low expression of Wr^b.

Variation in the number of antigen sites between individuals may also explain why the number of Wr^b antigen sites on the only Wr(a-b+) red cells tested in section 4.4.4. was found to be 484,000 whereas Gardner *et. al.* (1989) reported that there were 1,000,000 sites on such cells.

These experiments illustrate the need for dosage experiments to be based on average reactions of large number of red cells samples.

6.2.2. Deterioration of the Wr^a and Wr^b antigens.

It has previously been noted that the Wr^a antigen deteriorates on stored red cells (Green, unpublished observations). Therefore Wr(a+b+) red cells were always used in the best possible condition and Wr(a-b+) red cells of a similar age were used as controls. However the only Wr(a+b-) sample (donor M.Fr.) available for use during this study arrived in the laboratory in poor condition without a comparable control sample, two weeks after being collected in the U.S.A. The results of haemagglutination (section 4.1) and site number (section 4.4.4.) studies must therefore be interpreted with caution since the cell samples were not comparable.

6.3. The Relationship of the Wr^a and Wr^b antigens.

The results described in this thesis do not provide evidence to support an allelic relationship between the Wr^a and Wr^b antigens. They do, however, support the fact that the antigens are phenotypically related.

Allelic relationships might be due to sequence variations in the gene encoding the structural proteins which carry blood group antigens. For example, amino acid changes in the glycophorin A molecule result in the formation of the M and N blood group antigens (section 1.3.3.1.). Alternativelty allelic variation may occur in a biosynthetic enzyme, as is the case with the A, B and O transferases in the ABO blood group system (section 1.3.6.1.).

Non-allelic, phenotypic relationships can be explained if the antigens are produced by the action of enzymes encoded by different genes, at different stages of a biosynthetic pathway. For example, as the result of glycosylation, such as occurs in the conversion of H antigen to A and B (section 1.3.6.1.).

The Wr^b antigen has been shown, without doubt, to be associated with glycophorin A, both by other workers (Ridgwell *et. al.*, 1983b; Dahr *et. al.*, 1986; Telen and Chasis, 1989) and by the experiments described in this thesis. This was the original observation which cast doubt on the presumed allelic relationship of the Wr^a and Wr^b antigens since the Wr^a antigen had been shown by genetic studies to not be part of the MNS blood group system and hence was not encoded by the *GYPA* gene. Analysis of the MN groups of the individuals whose red cells reacted with the BGU1-WR anti-Wr^a monoclonal antibody showed the expected distribution of MN phenotypes. Furthermore, the amino acid sequence of glycophorin A from Wr(a+b-), Wr(a+b+) and Wr(a-b+) individuals has been shown to be identical (Dahr *et. al.*, 1986) thus also ruling out the possibility that the Wr^a and Wr^b antigens are controlled by alleles of the *GYPA* gene.

The proposed relationship between the Wr^a and Wr^b individuals was based on evidence from one individual (M.Fr.) (Adams *et. al.*, 1971). It is possible that the alloantibody in this individual's serum is not antithetical to anti-Wr^a and the high incidence antigen recognised by M.Fr.'s antibody may be totally unrelated to the Wr^a antigen present on M.Fr's cells. However, a second individual (ascertained through the antibody in her serum) whose red cells total lack the same antigen has also been shown to be Wr(a+) (Schutt, H., Daniels, G.L., Tippett, P. and Dahr, W. , unpublished observations). It seems unlikely that these two unrelated individuals, who both had normal glycophorin A, would express these two rare phenotypes by chance.

Therefore, this second individual confirms that a relationship between the Wr^a and Wr^b antigens exists.

That the Wr^b antigen is mainly dependent on the helical region of the glycophorin A molecule (amino acids 62-72) was first proposed by Ridgwell *et. al.* (1983b). This proposal is supported by the observation that an anti-Wr^b monoclonal antibody, MAB-145 was shown to be inhibited by a synthetic peptide representing amino acids 65-70 of the glycophorin A molecule (Reardon, 1989). Other monoclonal anti-Wr^b monoclonal antibodies were not inhibited suggesting that the configuration of the molecule is important for some Wr^b epitopes. This region of glycophorin A is also associated with the En^aFR antigen, the ficin resistant portion of the molecule to which some En(a-) individuals produce antibodies. Little distinction can be made between this antigen and Wr^b except that Wr^b but not En^aFR has been shown to be missing from the red cells of the two Wr(a+b-) individuals. Individuals who lack the En^aFR antigen also lack Wr^b and make anti-Wr^b. This region of glycophorin A is not glycosylated. It is therefore unlikely that the Wr^a and Wr^b antigens could be formed by postranslational modification of this region by glycosyltransferases encoded by either allelic or non-allelic genes.
Much evidence supports the idea that the orientation of the glycophorin A molecule in the red cell membrane is important for Wr^b antigen expression and that membrane lipid is in someway involved. Other workers (Dahr *et. al.*, 1986) could only inhibit human anti-Wr^b when glycophorin A extracts were incorporated into liposomes. The Wr^b antigen has also been shown to be phospholipid dependent (Reardon, 1985).

Experiments described in this thesis have confirmed that the Wr^b antigen is also associated with band 3. The relationship between the Wr^a and Wr^b antigens could be explained if both the antigenic determinants were dependent on the amino acid sequence of band 3 at the point at which it interacts with glycophorin A. The Wr phenotype could then be explained by amino acid changes in the band 3 molecule. However, certain observations described in this thesis do not provide strong support for this theory. For example, the fact that the Wr^b epitope recognised by some monoclonal antibodies is present on Wr(a+b-) cells would be difficult to explain since all the band 3 molecules would be the same on these cells. Also the number of Wr^a antigen sites on Wr(a+b-) cells might be expected to be related to the number of band 3 molecules present on cells (approximately 1,000,000) the estimate of 70,000 sites on such cells (section 4.4.4.) therefore does not support this model.

An alternative model can be envisaged where the configuration of glycophorin A maintains the Wr^b antigen. The band 3 and glycophorin A molecules have been shown to be associated in the red cell membrane (Nigg *et. al.*, 1980). It is conceivable that the orientation of one molecule depends on the orientation of the other. The Wr^b antigen could therefore be located on glycophorin A but be dependent on the band 3 interaction to maintain the configuration of the molecule. An alteration in the band 3 amino acid sequence could thus alter the orientation of band 3 and hence affect the interaction with glycophorin A and thus mask the Wr^b antigen. The cells of Wr(a+b+) individuals heterozygous for this change in the band 3 sequence would therefore have red cells where some of the Wr^b antigen was masked and some was not. The majority of the Wr^b antigen on the red cells of Wr(a+) homozygous individuals would be masked or possibly be detected at a low level. Other evidence exists to suggest that the level of Wr^b expression is affected by the orientation of glycophorin A has been provided by a study by Tweedy *et. al.* (1987) who showed that Wr^b expression increased on cells of individuals treated with the hemorrheologic agent, pentoxifylline, which increases the red cell membrane deformability.

The amino acid change in the band 3 molecule postulated above could also lead to the production of the Wr^a antigen which would only be dependent on the band 3 molecule. However, the immunoprecipitation evidence in this thesis (section 4.7) does not lend support to this suggestion since immunoprecipitates prepared from Wr(a+) red cells with the BGU1-WR, anti-Wr^a monoclonal antibody, did not appear to contain band 3. However, the heavy and light chains from the BGU1-WR monoclonal antibody were detected on immunoblots of immunoprecipitates from Wr(a+b+) cells and not from Wr(a-b+) red cells which suggest that solubilisation of the red cell membranes in Triton-X100 caused the antigen-antibody complexes to disassociate. This is possibly more likely with a red cell membrane component like band 3 which is known to traverse the lipid bilayer many times (Tanner *et. al.*, 1988a).

The number of Wr^a and Wr^b antigen sites, however, also make this model hard to envisage. Experiments described in this thesis suggested that there are only approximately 70,000 Wr^a antigen sites on Wr(a+b-) red cells whereas there are known to be approximately 1,000,000 band 3 molecules per cell (Low, 1986). It is possible that a change to the nucleotide sequence of the band 3 gene could also result in there being less band 3 molecules per red cell. Alternatively the lower Wr^a antigen site number could be provided by alternative splicing of the mRNA from an altered band 3 gene which might result in an altered protein. However, band 3 is the anion transport protein and it seems reasonable to assume that such a dramatic reduction in the number of band 3 would effect the ability of the red cells to transport anions. The

Wr(a+b-) red cells of donor M.Fr appear to be normal, although studies which have looked specifically at anion transport in these cells have not been reported.
A further possible explanation for the lower number of Wr^a antigen sites could be related to glycosylation. The glycosylation of band 3 molecules has been shown to be heterogeneous. Some band 3 molecules in the red cell membrane are glycosylated to a greater extent than others (Markowitz and Marchesi, 1981). This heterogeneity in glycosylation of the single glycan moiety on band 3 is due to variation in the number of repeating N-acetyllactosamine units present on each lactosaminoglycan (Tsuji *et. al.*, 1980; Fukuda *et. al.*, 1984) The Wr^a antigen could thus conceivably be masked on some band 3 molecules and not others. It is also interesting to note that The band 3 present on the red cells of En(a-) red cells which lack glycophorin A, is known to be more heavily glycosylated than that of normal red cells (Gahmberg *et. al.*, 1976). This suggests that the band 3 and glycophorin A proteins interact at some stage in biosynthesis or processing which restricts the accessibility of the band 3 molecule to glycosyl transferases. Investigation of the effect of removing oligosaccharides using endoglycosidases on Wr^a expression would shed light on this hypothesis.

The possibility that the Wr^a antigen is formed by an amino acid change in the band 3 molecule could be tested by DNA sequence analysis of the band 3 gene from a Wr(a+b-) individual following PCR amplification. The external sequences of the band 3 molecule and the region which interacts with glycophorin A would be the obvious candidate regions to examine.

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