C3b RECEPTORS (CR1) ON PERIPHERAL HUMAN BLOOD CELLS

by

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COMPLEMENT NOMENCLATURE

The components of the classical and the terminal sequence are denoted by the letter C followed by a number, e.g. C1, C4, C2, C3, C5, C6, C7, C8 and C9. The components of the alternative pathway are termed factors and each is represented by a letter, e.g. factor B, factor $\bar{D}$ and Properdin. These are abbreviated to B, $\bar{D}$ and P. The control proteins are referred to by their trivial names, $C\bar{1}$-inhibitor ($C\bar{1}$-INH), C4 binding protein (C4BP), C3b inactivator (C3b INA) and βIH globulin (βIH). The latter two proteins have now been assigned the symbols I and H. The abbreviated forms of the control proteins are given in brackets.

Enzymatically active forms of complement components have a bar over the symbols, e.g. $C\bar{1}$, $C\overline{42}$, $\bar{D}$, Cleavage fragments are suffixed by lower cased letters, e.g. C4a, C4b, C4c, C4d. The polypeptide chains of the components are denoted by Greek letter, α being assigned to the largest then β then γ, e.g. C4α, C4β, C4γ. Throughout the text the abbreviated symbols for the complement components will be used.

ABBREVIATIONS

VLDL	very low density lipoprotein
SLE	systemic lupus erythematosus
RA	rheumatoid arthritis
CR	complement receptors
CR1	C3b receptor
CR2	C3d-g receptor
CR3	iC3b receptor
PMNs	polymorphonuclear leucocytes
SRBC	sheep red blood cells
EA	antibody coated SRBCs
EAC	antibody and complement coated SRBCs
IA	immune adherence
EBV	Epstein-Barr virus
LFA	lymphocyte function associated antigen
NP-40	Nonidet P-40
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
gp	glycoprotein
F	fast migrating
S	slow migrating
DAF-S	decay accelerating factor-Stroma
BSA	bovine serum albumin
RF	rheumatoid factor
ARA	American Rheumatism Association
IAHA	immune-adherence haemagglutination
H	high
M	medium
L	low
CIC	circulating immune complexes
TEMED	N, N, N', N'-Tetramethylethylene diamine
DEAE	diethylaminoethyl
R.T.	room temperature
PEG	polyethylene glycol
hrs	hours
mins	minutes
D^2	diameter squared
SDS	sodium dodecyl sulphate

Abbreviations (contd)...

v/v	volume for volume
w/v	weight for volume
E	erythrocytes
cpm	counts per minute
NHS	normal human serum
PMSF	phenyl methyl sulfonyl fluoride
RIA	radioimmunoassay
BHK	baby hamster kidney cells
DE52	diethylaminoethyl cellulose
NRS	normal rabbit serum
MCC tubes	microcapped centrifuge tubes
cf	correction factor
r	linear regression coefficient
SBTI	soya bean trypsin inhibitor
EACA	ε -amino-n-caproic acid
DTBSP	dithiobissucinyl propionate
ANF	antinuclear factor
AHG	aggregated human IgG
SAS	saturated ammonium sulphate

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"Away down the river,

A hundred miles or more,

Other little children,

Shall bring my boats ashore."

Robert Louis Stevenson

PUBLICATIONS

Some of the work performed in this thesis has been published in the following journals:

Naama, J.K., Holme, E., Hamilton, E.& Whaley, K. (1985) Prevention of immune precipitation by purified components of the alternative pathway. Clinical and Experimental Immunology, 60, 169-177.

Holme, E., Fyfe, A., Zoma, A., Veitch, J., Hunter J. & Whaley, K. (1986) Decreased C3b receptors (CR1) on erythrocytes from patients with systemic lupus erythematosus. Clinical and Experimental Immunology, 63, 41-48.

SUMMARY

CR1 is the receptor for the activated third component of complement C3b. It is present on the cell membranes of human erythrocytes, lymphocytes, polymorphonuclear leucocytes, mononuclear phagocytes, mast cells, B lymphocytes, some T lymphocytes and kidney podocytes.

Purification of CR1 from human erythrocytes using cation exchange and affinity chromatography revealed a single protein with a molecular weight of 230000 daltons. This protein was used to raise a polyclonal antiserum in rabbits, which was then utilised in the development of a radioimmunoassay to quantitate the number of CR1 on human peripheral blood cells. This assay was used successfully to quantitate CR1 levels on erythrocytes. However, it could not be adapted to assess CR1 levels on monocytes, lymphocytes or polymorphonuclear leucocytes.

CR1 levels have previously been reported to be reduced on erythrocytes from patients with systemic lupus erythematosus (SLE). However, there has been no agreement in the literature as to whether the reduced erythrocyte CR1 levels observed are acquired as a consequence of the pathological process of the disease or are an inherited defect. The aim of the experiments performed in this thesis was to establish whether CR1 levels are inherited or acquired. To examine this erythrocyte CR1 levels were studied in normal individuals and SLE patients. In addition, patients with rheumatoid arthritis (RA) were investigated. Serial studies were performed on normal individuals, SLE and RA patients to see whether there were

temporal changes in CR1 levels. CR1 levels were also assessed in normal families and in families where one or more individuals had SLE to ascertain whether receptor levels were inherited.

CR1 levels were quantitated using a radioimmunoassay, which measured the amount of ^{125}I-F(ab')$_2$ anti-CR1 binding to the erythrocytes. From this the number of binding sites per erythrocyte for the F(ab')$_2$ anti-CR1 moiety was calculated (CR1 sites/erythrocyte).

Seventy normal erythrocyte specimens were studied; the mean was 3320 CR1 sites/erythrocyte (range 0 to 21692 CR1 sites/erythrocyte) whilst the mean for the SLE patients was 1541 (n=41, range 0 to 15766 CR1 sites/erythrocyte), significantly lower ($p < 0.001$) than the normals. Likewise the mean (1410 CR1 sites/erythrocyte, n=25) for the RA patients was also significantly lower ($p < 0.05$). A striking observation was that many of the SLE patients had no receptors (27% as opposed to 1.5% in the normals and 0% in the RA group). When the SLE patients were further subdivided into those who were in an active disease phase or an inactive disease phase, the majority of patients with zero receptors were in an active disease state. When analysed further the mean CR1 level for the patients with active disease (356 CR1 sites/erythrocyte) was significantly lower ($p < 0.001$) than those with inactive disease (2428 CR1 sites/erythrocyte). These levels were not related to any of the serological parameters studied. Serial studies on SLE patients showed that in some individuals the CR1 levels varied with disease activity, decreasing during exacerbations and increasing during remission.

Family studies showed a clustering of low, medium or high CR1 levels in individual families. However, where a member of a family had SLE their CR1 levels were not akin to their families suggesting acquisition and not inheritance. Studies of two sets of identical twins showed that they did not have identical CR1 suggesting that they had acquired different levels..

Two main conclusions may be drawn from these studies; firstly, genetic factors probably do influence CR1 levels as supported by the clustering of CR1 levels in the families studied and secondly, in some patients with SLE the CR1 levels were acquired, as indicated by the change in CR1 levels with disease activity and the lower levels found in patients with disease exacerbations.

Two possible explanations could account for the acquisition of reduced receptor levels, receptor removal or receptor blockade. In support of the latter it was observed that large opsonized immune complexes (formed with thyroglobulin or DNA, with specific antiserum) bound to and totally or partially blocked the erythrocyte CR1. Thus in vivo blockade of CR1 by circulating immune complexes could account for the reduced CR1 levels seen in some SLE patients when their disease is in exacerbation.

C H A P T E R 1

INTRODUCTION

1.1 The Complement System

The immune system, as it is known today is complex. The original simple concept, that the body's immune system was a self protective mechanism against disease, is still the central tenet, but the multitude of means employed to fulfil this role is bewildering. Importantly, the number of diseases associated with a failure of one or more aspects of the immune system is also enormous.

The complement system is an integral part of the immune system. Composed of fourteen distinct proteins together with six known direct regulatory factors and a growing number of membrane receptors for a variety of these proteins, the complement system is itself complex.

The presence of complement in serum was discovered before the turn of the century. Many decades later with the introduction of new biochemical methods of protein separation, the complement system was demonstrated to be a cascade of self-assembling plasma proteins, the activation of which, causes a series of molecular interactions ending in the formation of a terminal membrane attack complex, together with by-products exhibiting a variety of biological activities.

The complement system can be divided into three pathways; the classical, the alternative and the membrane attack. These are schematically represented in Fig. 1. The proteins of the classical (C1q, C1r, C1s, C4 and C2, Table 1) and the alternative (B, $\overline{D}$, C3b and P, Table 2) pathways are concerned with the generation of enzymes which cleave C3 and C5. Whilst the proteins of the membrane attack pathway (C5b, C6, C7, C8 and C9, Table 3) form a multimolecular complex which brings about complement-mediated cytolysis. There are in addition, a group of regulatory proteins (Table 4) which control the activation and turnover of the complement system.

Fig 1. Schematic representation of the complement system

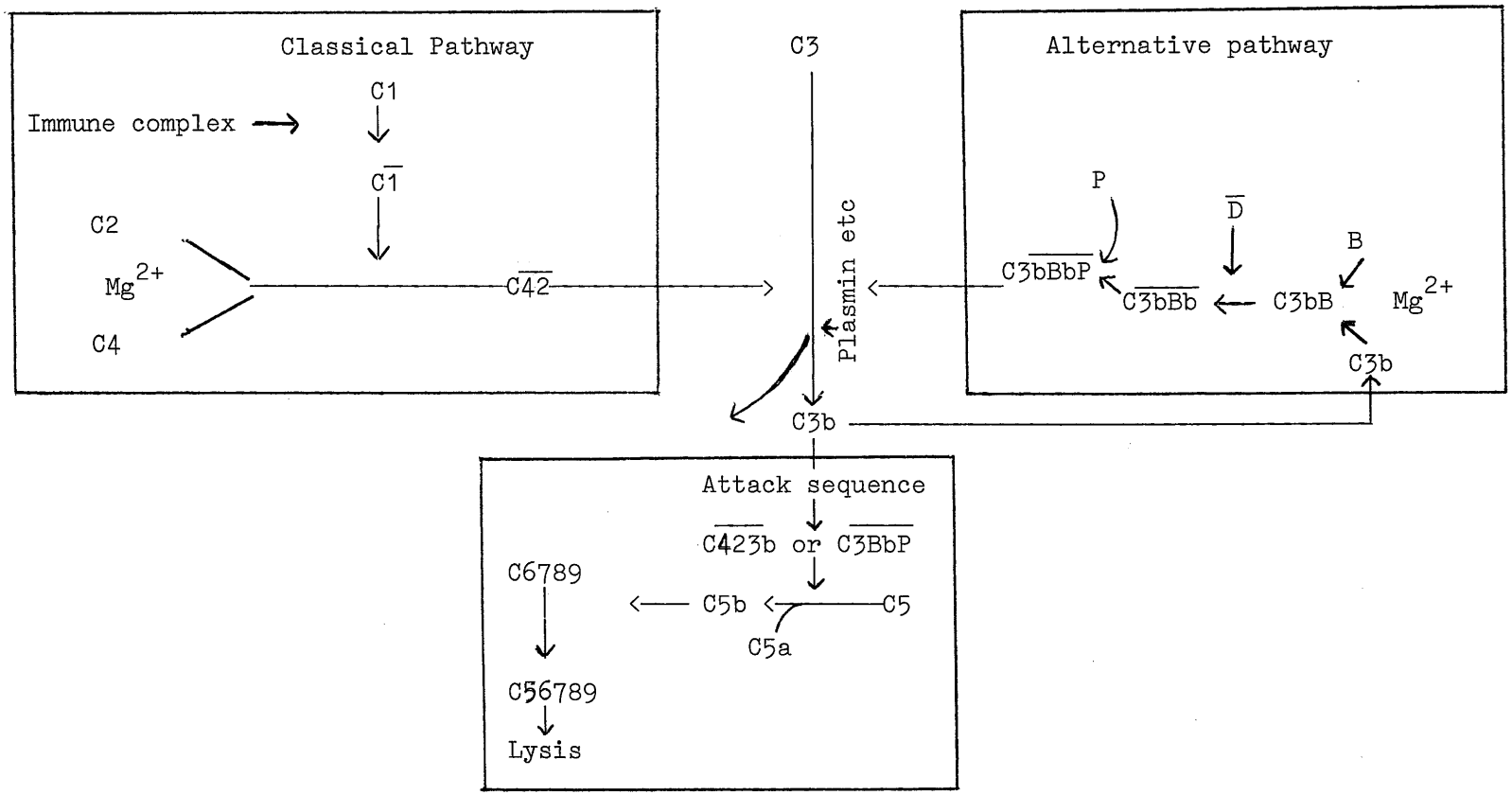


Table 1. Proteins of the classical pathway.

Component	Molecular weight	Serum concentration ug/ml	Polypeptide chain structure	Cleavage products	Biological activity
C1q	400,000	250	18 (6x3)	-	Binds to immune complexes
C1r	90,000	110	1	H & L chains	Cleaves C1s
C1s	90,000	80	1	H & L chains	Cleaves C4 and C2
C4	204,000	430	3	C4a	Anaphyltoxic
				C4b	Forms part of C3 C5 convertase opsonin
C2	100,000	20	1	C2a	Forms part of C3 and C5 convertase
				C2b	?

Figure 1. SCHEMATIC REPRESENTATION OF THE COMPLEMENT SYSTEM.

Table 2. Proteins of the alternative pathway.

Component	Molecular weight	Serum concentration ug/ml	Polypeptide chain structure	Cleavage products	Biological activity
C3b	181,000	?	2	C3bi	? ligand for CR3
				C3c	?
				C3d	?
				C3e	Mobilises PMN from bone marrow
Factor B (B)	93,000	150	1	Ba	? chemotactic
				Bb	Part of C3 and C5 convertase
Factor D ($\overline{D}$)	25,000	2	1	-	Cleaves B
Properdin (P)	22,000	30	4	-	Stabilises $\overline{C3bBb}$

Table 3. Proteins of the membrane attack pathway.

Component	Molecular weight	Serum concentration ug/ml	Polypeptide chain structure	Cleavage products	Biological activity
C5	185,000	75	2	C5a	Chemotactic anaphylatoxin
				C5b	Part of membrane attack complex
C6	128,000	60	1	-	Part of membrane attack complex
C7	121,000	60	1	-	
C8	153,000	80	3	-	
C9	79,000	50	1	-	

Table 4. Regulatory proteins of the complement system

Complement	Molecular weight	Serum concentration ug/ml	Polypeptide chain structure	Cleavage products	Biological activities
$C\overline{1}$-INH	105,000 90,000	180	1	-	Inhibits certain serine proteases including $C\overline{1}r$ and $C\overline{1}s$
C4 BP	540,000 590,000	?	8	-	1) Binds to C4b to accelerate decay of C4b2a 2) With Factor I cleaves C4b to C4c and C4d
Factor I	90,000	50	2	-	Degrades C4b and C3b together with cofactors C4 binding protein and Factor H.
Factor H	150,000	300	1	-	1) Binds to C3b to accelerate decay of $C\overline{3bBb}$ and $C\overline{3bBbP}$ 2) Acts as a cofactor and together with Factor I cleaves C3b to iC3b
S protein	88,000	150	1	-	Binds to C3b-9 preventing membrane insertion
Anaphylotoxin inactivator	300,000	?	8	-	Removes terminal arginine from C3a and C5a

Activation of the classical pathway is antibody-dependent. The antigen to which the antibody is directed can be soluble or cellular. But in humans, for C1 activation the antibody which combines with the antigen must be IgM or one of the following IgG subclasses, IgG_1, IgG_2 , IgG_3 (Augener et al, 1971) with IgG_3 being the most potent activator followed by IgG_1, then IgG_2 (Ishizaka et al, 1970).

The first component of the classical pathway C1 is a macromolecular complex composed of three peptides C1q, C1r and C1s, the stability and reactivity of which is maintained by the continual presence of calcium ions.

The C1q moiety provides the classical pathway recognition unit for the immunoglobulins. The binding site for C1q is located within the $C\gamma 2$ domain of IgG or the $C\mu 4$ domain of IgM. The conversion of C1 to $\overline{C1}$ after the interaction of C1q with the appropriate immunoglobulin, involves the other two sub-components C1r and C1s. C1q after interaction with antibody undergoes a critical conformational change which in turn induces the proenzyme C1r to convert to the active form $\overline{C1}r$, which sequentially cleaves C1s to $\overline{C1}s$. $\overline{C1}s$ is a serine protease and cleaves its two natural substrates C4 and C2.

C4 is cleaved by $\overline{C1}s$ to C4a and C4b (Schreiber and Müller-Eberhard, 1974). C4a is released into the fluid phase, leaving the major product C4b which possesses a labile binding site in the α' chain. This binding site depends upon the presence of an internal thiolester group in the α chain and upon activation, a reactive acyl group is transferred from the thiol to a hydroxyl or an amino group on the acceptor molecule to form an ester or an amide bond (Campbell, Gagnon and Porter, 1981). By this means C4b can bind covalently to the antigen or the antibody of the immune complex.

This covalent binding of C4b localises further complement activation. Most of the C4b which is formed fails to bind as the binding site is extremely short-lived. This lability is due to the reaction with water, and so most C4b becomes inactive C4b which plays no further role in the activation of the classical pathway. Similarly C2 which can bind reversibly to bound or fluid phase C4b is cleaved by $\overline{C1}s$. Cleavage of C2 yields C2a and C2b, these remain attached and bind to C4b via the C2b moiety although the enzymatic site resides in C2a (Nagasawa and Stroud, 1977). The formed complex $\overline{C4b2a}$ with the enzymatic activity located within the C2a moiety is the classical pathway C3 convertase, cleaving C3 to C3a and C3b. This convertase is however unstable having a very short half-life at 37°C. After the formation of the classical pathway C3 convertase the presence of C1 or antibody are no longer required for the completion of the classical pathway.

The regulation of the classical pathway, preventing excessive or unnecessary turnover is controlled by three proteins; $\overline{C1}$-INH, C4BP and I.

$\overline{C1}$-INH stoichiometrically inhibits the action of a number of serine proteases which are present in plasma, but with respect to the classical pathway, $\overline{C1}$- INH binds near or at the active enzymatic site of $\overline{C1}r$ and $\overline{C1}s$ (Ziccardi and Cooper, 1976; Harpel and Cooper, 1975) thereby limiting C4 and C2 cleavage. Binding of $\overline{C1}$-INH to $\overline{C1}$ dissociates the molecule leaving $\overline{C1}q$ bound to antibody and releasing a $\overline{C1}r$:$\overline{C1}s$:$\overline{C1}$-INH complex into the fluid phase, the molar ratio being 1:1:2 (Sim, Arlaud and Colomb, 1979; Ziccardi and Cooper, 1979).

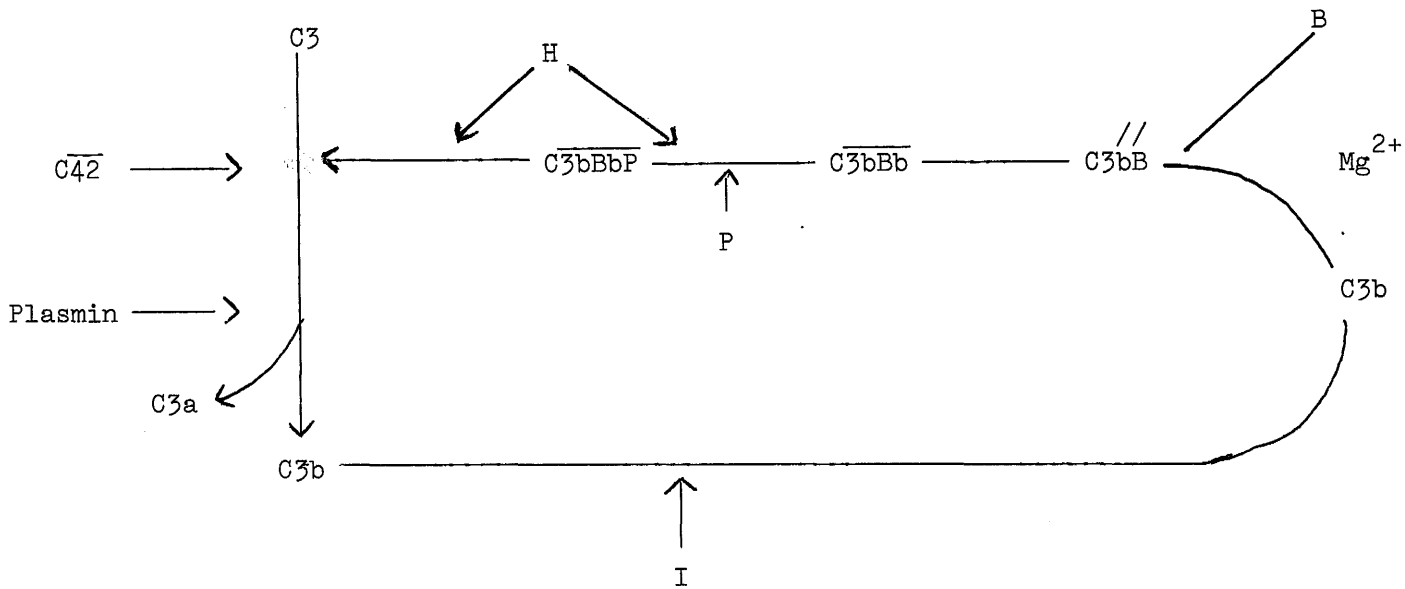
C4BP alone can accelerate the decay of the C3 convertase by

displacing C2 (Gigli, Fujita and Nussenzweig, 1980), or can act as a cofactor to I facilitating the cleavage of the C4b α' chain. In this way the formation of the convertase is regulated and the stability of the preformed convertase is decreased. Both of these actions will limit the breakdown of C3.

Activation of the alternative pathway unlike the classical pathway is not confined to immune complexes comprised of certain immunoglobulin classes and subclasses. In fact a diverse range of agents can mediate activation of this pathway including yeast cell walls, endotoxins, the surfaces of certain intact bacteria and fungi to name but a few. Clearly, this pathway may provide a first line defence against certain organisms in the absence of specific antibodies, as well as mediating inflammation and tissue injury in the absence of infection. Thus the alternative pathway has been implicated as the original primitive complement system forming a natural and non-specific defence mechanism.

The manner of activation of the alternative pathway is probably still not completely understood. There is no molecule which initiates the turnover of the alternative pathway. The proteins C3b, B, $\overline{D}$ and P are involved in the assembly of the alternative pathway C3 and C5 convertases, and their turnover is controlled by I and H. The convertase formation depends upon the complexing of C3b with B in the presence of magnesium ions. $\overline{D}$ then cleaves B to Ba and Bb, Ba is released into the fluid phase and Bb remains complexed to C3b, giving $\overline{C3bBb}$. This convertase is said to be unstable because of the rapid temperature-dependent dissociation of Bb. However, the enzyme is stabilized by the binding of P to C3b (Fig. 2). The resulting enzyme, $\overline{C3bBbP}$ is termed the P-stabilized alternative pathway C3 convertase, which cleaves C3 at the same site as $\overline{C4b2a}$, to form C3a and C3b. C3b as well as being a constituent of the alternative pathway C3 convertase, is also a product

Fig 2. Alternative pathway activation.



A // above a symbol indicates that the component has undergone a conformational change. A bar above a symbol indicates that the component is in its activated form. (From Whaley and Ferguson, 1981)

Fig. 2. ALTERNATIVE PATHWAY ACTIVATION.

of the enzyme on its substrate. Thus a positive-feedback loop (the alternative pathway amplification loop) is formed, which in the absence of regulatory mechanisms would continue to be activated until the supply of C3 or B was exhausted. Limitation of turnover depends upon the presence of two control proteins, the enzyme I and its cofactor H. I, in the presence of the cofactor H, cleaves the α' chain of C3b to form iC3b; this can no longer bind B and therefore stops convertase formation. In addition H alone can compete with B (Conrad, Carlo and Ruddy, 1978) for the binding site on C3b (Whaley and Ruddy, 1976a) and accelerate the decay of $\overline{C3bBb}$ (Whaley and Ruddy 1976b; Weiler et al, 1976) and this re-exposes C3b to the proteolytic activities of I. Spontaneous hydrolysis of the thiolester bond of C3 occurs, which converts C3 to "C3b-like " C3 (Pangburn and Müller-Eberhard, 1980). C3b-like C3 has the capacity to bind B before acquiring susceptibility to the regulating actions of H. Thus low-grade fluid phase turnover of the alternative pathway occurs, but C3b which is formed is rapidly degraded by H and I.

The ability of certain surfaces to activate the alternative pathway is a result of the protection they confer on C3b from the controlling effects of I and H. Thus uncontrolled assembly of $\overline{C3bBbP}$ on the cell surface can occur (Fearon and Austen, 1977a, b). Consequently microorganisms, for example, can be covered with large quantities of C3b and be phagocytosed or lysed via the terminal pathway. Thus alternate pathway activators shift the low-grade C3b turnover to rapid solid-phase turnover, therefore amplifying the whole system.

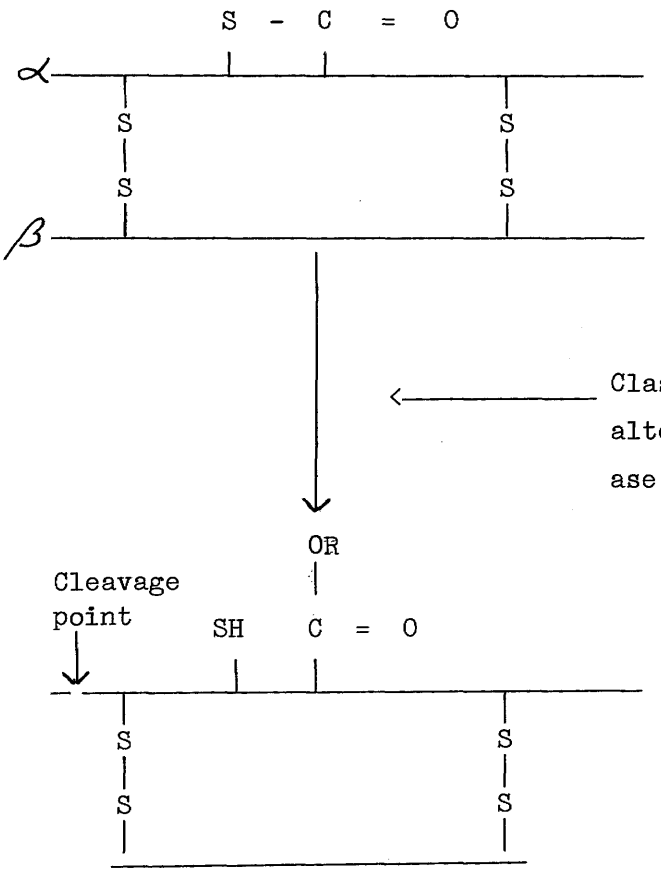
It is apparent that the carbohydrate composition of cell surfaces affects their ability to activate the alternative pathway. For example, surfaces low in sialic acid, e.g. zymosan, rabbit

erythrocytes and neuraminidase treated sheep erythrocytes activate the alternative pathway in human serum (Fearon, 1978; Pangburn and Müller-Eberhard, 1978). These surfaces reduce the affinity of H for C3b therefore favouring the binding of B to C3b (Kazatchkine, Fearon and Austen, 1979). Other factors can also affect convertase formation. Nephritic factor is an autoantibody found in the serum of some patients with mesangiocapillary glomerulonephritis. This autoantibody binds to $\overline{C3bBb}$ and stabilizes the enzyme (Daha, Austen and Fearon, 1978), and this becomes resistant to decay-dissociation by H (Weiler et al, 1976). Cobra venom factor is isolated from the saliva of two snakes Naja naja and Naja Naje, in mammalian serum this causes exhaustive activation of the alternative pathway by complexing with B in the presence of $\overline{D}$. This forms a stable convertase which is unsusceptible to H mediated decay-dissociation (Nagaki, Iida and Okerbo, 1978).

It is clear that activation of both the classical and alternative pathways leads to the cleavage of C3. C3 constitutes the bulk protein of the complement system, with normal serum concentrations ranging from 768 µg/ml to 1700 µg/ml. It has a molecular weight of 190,000 daltons and is composed of two polypeptide chains designated α (110,000 daltons) and β (75,000 daltons) which are held together by disulphide bridges plus non-covalent forces (Fig. 3, Table 5).

The cleavage of C3 to C3a and C3b by the C3 convertase is the major event of the complement system. Both convertases cleave C3 at the same point, between arginine 77 and serine 78 at the N-terminus of the α chain. The first 77 aminoacids of the α chain constitute C3a which is released into the fluid phase and is a potent anaphylotoxin. C3b consists of the remaining α' chain and an intact β chain and as a result of the cleavage a labile

Fig. 3. SCHEMATIC REPRESENTATION OF C3 AND CLEAVAGE TO C3a and C3b.



R = labile binding site on C3b

Table 5. Characteristics of C3 and breakdown fragments.

Component	Molecular weight	Serum concentrations ug/ml	Polypeptide chain structure	Cleavage products	Biological activity
C3	190,000	1300	2	C3a	Anaphylotoxin
				C3b	1) Part of classical and alternative pathway C3 and C5 convertase 2) Opsonin 3) Solubilization of complexes
				C3c	?
				C3d	?
				C3e	Mobilization of PMNs from bone marrow

thiolester bond is exposed in the α' chain. This allows C3b to bind to cell surfaces or immune complexes. However, the labile binding site is short-lived, with an approximated half-life of 50 milliseconds, after which the C3b decays, probably because of the interaction of the thiolester with water to form inactive fluid-phase C3b and conformational changes to a more stable molecular configuration. A consequence of this is that only about 10% of the available C3b binds to targets, the remainder becoming inactive fluid-phase C3b. This form, despite being unable to take any further part in the complement cascade sequence, still retains its ability to bind to C3 receptors, although Arnaout et al (1981) present experimental evidence indicating that monomeric C3b does not bind to C3 receptors. This molecular form can, however, enter into the alternative pathway feedback cycle.

C3b which does bind close to the alternative or classical C3 convertases alters the specificities of these enzymes from C3 to C5. The classical pathway C5 convertase ($\overline{C4b2a3b}$) still has the active enzymatic site in C2a, and likewise the alternative pathway C5 convertase ($\overline{C3bBbPC3b}$) enzymatic site resides still in Bb, however they have altered molecular specificities cleaving C5 to C5a and C5b instead of C3. This change in specificity occurs because C3b acts as a binding site for C5 and presents to the enzyme for cleavage. C5 cleavage allows the commencement of the terminal attack pathway.

The terminal attack pathway, unlike the classical or alternative pathway, has no proteolytic steps, but is a sequential series of self-assembling proteins. C5 is very similar to C3 consisting of two polypeptide chains linked by disulphide bridges and non-covalent forces (Nilsson, Tomar and Taylor, 1972; Tack, Morris and Prahl, 1979). On cleavage C5a, a potent chemotaxin and anaphylotoxin, is released from the N-terminus of the α chain (Fernandez

and Hugli, 1978), leaving C5b composed of the remaining α' chain and an intact β chain. C5b now contains a newly exposed labile binding site in the α' chain, allowing it to bind to cell membranes. The half-life of this labile binding site is approximately the same as that of C3b, but about 4% of C5b becomes membrane associated (Cooper and Müller-Eberhard, 1970) compared to 10% of C3b. Once membrane bound the functional activity of C5b decays and this is inhibited by its further interaction with C6. The binding of C6 to C5b ($\overline{C5b6}$) imparts stability and this complex has the capacity to travel considerable distances, consequently allowing binding to bystander cells. C6 is thought to bind C5b by adsorption and probably as a result of a conformational change in C5b, C7 can bind to give an equimolar trimolecular complex $\overline{C5b67}$ (Podack et al, 1978). The configuration of this complex is essential for the binding of C8, which is thought to bind to all three components C5b, C6 and C7 at the centre of the triangular shaped complex (Kolb et al, 1972). C8 binds on a one-to-one basis with the trimolecular complex to give a tetrahedral complex. It is via C8 that several molecules of C9 can bind to the C5b-8 complex. This complex $\overline{C5b6789}$ has a molecular weight of 995,000 daltons (Kolb and Müller-Eberhard, 1975) and in a dimeric form it is inserted into the lipid bilayer of the cell. In fact, as the complex forms it attains a greater ability to insert into the cell membrane, however it only maintains this for about 0.1 seconds, after which the binding site is lost. Molecular re-arrangement may account for this, or two serum proteins, S protein and very low density lipoprotein (VLDL) can compete with the labile binding site and prevent complex insertion (Lint, Behrends and Gewurz, 1977; Podack, Kolb and Müller-Eberhard, 1978).

The insertion of the terminal complex $\overline{C5b6789}$ into the cell membrane is as a dimer and this forms the characteristic 'holes' seen in electron micrograms. These holes have a diameter of 10 μm

and appear as hollow cylinders projecting outwards from the cell membrane and into the lipid bilayer. The formed channel allows the passage of water and electrolytes finally bringing about osmotic lysis of the cell. The nature of the channel is still not fully resolved, leaking of cell contents maybe through the centre of the channel or the complex may cause such gross distortion of the lipid bilayer that the contents leak around the periphery of the complex. Whatever the exact mechanism, the membrane attack pathway results in the cytolysis of nucleated and unnucleated mammalian cells, bacteria,platelets, mycoplasmae and viruses, in addition the mobility of $\overline{C5b6}$ can result in the complexes forming on bystander cells and hence causing inappropriate lysis.

1.2. Biological activities of complement.

The lytic effect of the terminal membrane attack complex is now well established. However,during the turnover of the pathway, a variety of fragments are produced which have biological activities of their own, and these will be discussed in turn.

C4a, C3a and C5a are anaphylotoxins; ranked in order,C5a is most potent, then C3a then C4a. Both C3a and C5a act on mast cells and basophils causing histamine release, C3a also releases serotonin from platelets (Johnson, Hugli and Müller-Eberhard, 1975). C3a and C5a can induce smooth muscle contractions independent of histamine release, and similarly such an action on endothelial cells of the post-capillary venules would open up gap-junctions and increase vascular permeability. These effects are inhibited by the inactivation of the anaphylotoxin by carboxypeptidase N, which cleaves the carboxy terminal arginine residue (Damerau, Grunefeld and Vogt, 1978), to give C4a des arg and C3a des arg and C5a des arg.

C5a and C5a des arg are potent chemotactic agents attracting neutrophils, eosinophils and monocytes (Fernandez et al, 1978; Damerau et al, 1978). The activity of C5 des arg seems to require some serum cofactor for the expression of its activity (Perez et al, 1980).

C3e causes leukocytosis, although this is usually preceded by an initial phase of leucopenia. C3e probably causes this effect by mobilizing leucocytes from the bone marrow (McCall et al, 1974; Rother, 1972).

The role of C3b as an opsonin is well known. C3b-coated immune complexes or particles adhere to C3 receptors on phagocytic cell membranes and together with the interaction of IgG with the Fcγ receptors, these complexes can be phagocytosed, facilitating their removal from the circulation.

Complement also plays a vital role in the solubilization of immune complexes within the circulation. The solubilization phenomenon was first described in 1975 by Miller and Nussenzweig. This process alters the physical and biological properties of complexes probably by disrupting the original antigen-antibody lattice (Takahashi, Tack and Nussenzweig, 1977), thus making the immune complexes more soluble.

The effects of the complement system are multiple and its importance is stressed and clearly demonstrated in patients with complement deficiencies. There are reported deficiencies of all complement components and Schifferli and Peters (1983) recently reviewed the literature. It is clear that complement deficiencies lead to an increase in infections and defects particularly of the classical pathway and C3 is associated with immune complex diseases (Table 6). A possible reason for this is the failure of the complement system to prevent immune complex precipitation, and rather than be removed by the fixed mononuclear phagocytic system

Table 6. Human disorders associated with complement deficiencies.

Component	Disorder
C1q	SLE or similar syndrome, hypogammaglobulinaemia.
C1r	Renal disease, SLE or similar syndrome, recurrent infections, rheumatoid disease.
C1s	SLE
C4	SLE
C2	Arthralgia, SLE or similar syndrome, nephritis, susceptibility to infections.
C3	Recurrent infections with pyogenic bacteria.
C5	SLE, recurrent infections, recurrent gonococcal infections.
C6	Recurrent gonococcal and meningococcal infections, Raynaud's phenomenon.
C7	Recurrent gonococcal and meningococcal infections, Raynaud's phenomenon, glomerulonephritis.
C8	Recurrent gonococcal infections, SLE
$C\bar{I}$-INH	Hereditary angioedema.
I	Repeated infections, recurrent infections with pyogenic infections.

(From Cooper, 1984)

they become trapped in extravascular spaces and hence initiate tissue damage. Therefore in the immune complex diseases, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) low complement levels may contribute significantly to the disease process. Low haemolytic complement levels are found in patients with SLE and RA, however, many have normal complement levels. Thus, this implies that some other facet of the complement system may be at fault in these patients. Recently, increasing importance has been placed on the role of complement receptors (CR) in the immune system. For example, a defect in the C3b receptor (CRI) on phagocytes, could have a drastic effect on the removal of immune complexes from the circulation and this may increase the propensity to immune complex diseases.

1.3. Membrane receptors.

The nature of the plasma membrane of mammalian cells is such that it is impermeable to molecules of high molecular weight, each cell therefore becomes an isolated specialized chemical environment. To enable communication between cellular compartments, mechanisms are present which allow extra-cellular macromolecules to be presented to the cell interior. One such mechanism is fluid phase pinocytosis, where uptake is non-selective, linear and solely dependent upon the extra-cellular macromolecular concentration. Slightly more selective is the process of endocytosis, where fluid is entrapped by a fold in the plasma membrane; this can then be expelled from the cell or taken into the cell as a cytoplasmic vesicle. However, Kaplan (1981) suggests that this is probably a mechanism for termination (i.e. expulsion of cell contents) rather than initiation (i.e. internalization of extra-cellular fluids) of information transfer.

To overcome the lack of specificity of information transfer imparted by these two mechanisms, cells have developed plasma membrane receptors. Such receptors have the capacity to form high affinity complexes with specific extra-cellular ligands, allowing the ligand to impart information to the cell via the receptor. In this way a wide variety of ligands such as hormones, neurotransmitters, chemotactic agents, viruses and drugs can elicit their biological activities.

The discovery of a diverse range of receptors on circulating immune effector cells has been of great interest, in particular their possible involvement in abnormal immune responses. Circulating immune effector cells have been shown to have receptors for the Fc portion of immunoglobulins, interleukines, leukotrienes etc. To date, receptors have been described for five complement components; C1q, C4, C3, C5 and H (Ross, 1980; Ross, 1982; Arnaout and Colten, 1984; Fearon, 1984). It is plausible that these receptors play a vital role in linking the humoral arm of the immune system with the cellular effector mechanisms of the immune system. The distribution and characteristics of these receptors are summarized in Tables 7 and 8.

At present the best characterized complement receptors are CR1, CR2 and CR3 (Table 8). Unlike conventional nomenclature where the receptor is named after the ligand, the multi-ligand specificity of these receptors precludes this, thus they have been designated complement receptors (CR) and are numbered in their order of discovery. The subject of this thesis is CR1, the receptor for the ligand C3b; however, a brief discourse about CR2 and CR3 the receptors for C3d-g and iC3b respectively is warranted. It is fair to point out that early studies on these receptors were dogged with problems, consequently this has led to many conflicting experimental results in earlier papers. Two of the main reasons for this were the lack of knowledge of the physiological breakdown

Table 7 The specificity and distribution of receptors for C1q, C5 and H.

Receptor	Ligand	Distribution
C1q	C1q	B lymphocytes, monocytes, neutrophils.
H receptor	H	B lymphocytes, monocytes, neutrophils
C5a receptor	C5a	Mast cells, granulocytes, monocyte macrophages, platelets.

Table 8. The specificity and distribution of receptors for C3 fragments.

Receptor	Ligand	Distribution
CR1	C3b, iC3b, C4b	Erythrocytes, granulocytes, monocytes, macrophages, B lymphocytes, some T lymphocytes, glomerular podocytes, mast cells.
CR2	C3d-g, C3d, iC3b, C3b (weak)	B lymphocytes
CR3	iC3b, C3d-g?	Neutrophils, monocytes, macrophages, large granular lymphocytes

of C3 and the insensitivity of the assay techniques used to investigate the receptors.

Before introducing CR1 and describing in brief CR2 and CR3 an appreciation of the C3 cleavage is necessary.

1.4. C3 and the generation of C3 breakdown fragments.

The series of proteolytic cleavages leading to the formation of a variety of C3 breakdown fragments, some of which are ligands for the complement receptors CR1, CR2 and CR3, is illustrated in Fig. 4.

As previously described, C3 is cleaved by either the classical or alternative pathway C3 convertase yielding C3a and C3b. C3a is released into the fluid phase and the labile thiolester bond located in the α' chain of C3b then becomes exposed. This thiolester bond can a) undergo spontaneous hydrolysis rendering it non-functional or b) allow the binding of the C3b molecule to a cell surface or immune complex bearing the C3 convertase.

C3b, whether bound to an immune complex or a cell surface, is next converted to the haemolytically inactive form iC3b by the actions of I. I is a serine protease and together with the co-factor H cleaves the α' chain of C3b at two sites. Released into the fluid phase is a small fragment C3f (3,000 daltons) and remaining covalently bound to the cell or immune complex are the two α' chain fragments (68,000 and 43,000 daltons) attached to an intact β chain, this comprises iC3b. This cleavage can also be mediated by I but with CR1 as the cofactor (Medof et al, 1982; Ross et al, 1982; Medicus, Melamed and Arnaout, 1983).

Further cleavage of iC3b by I requires CR1 as an obligate cofactor (Medof et al, 1982; Medicus and Arnaout, 1982; Ross et al, 1982; Medicus et al, 1983) as no known fluid phase counterpart

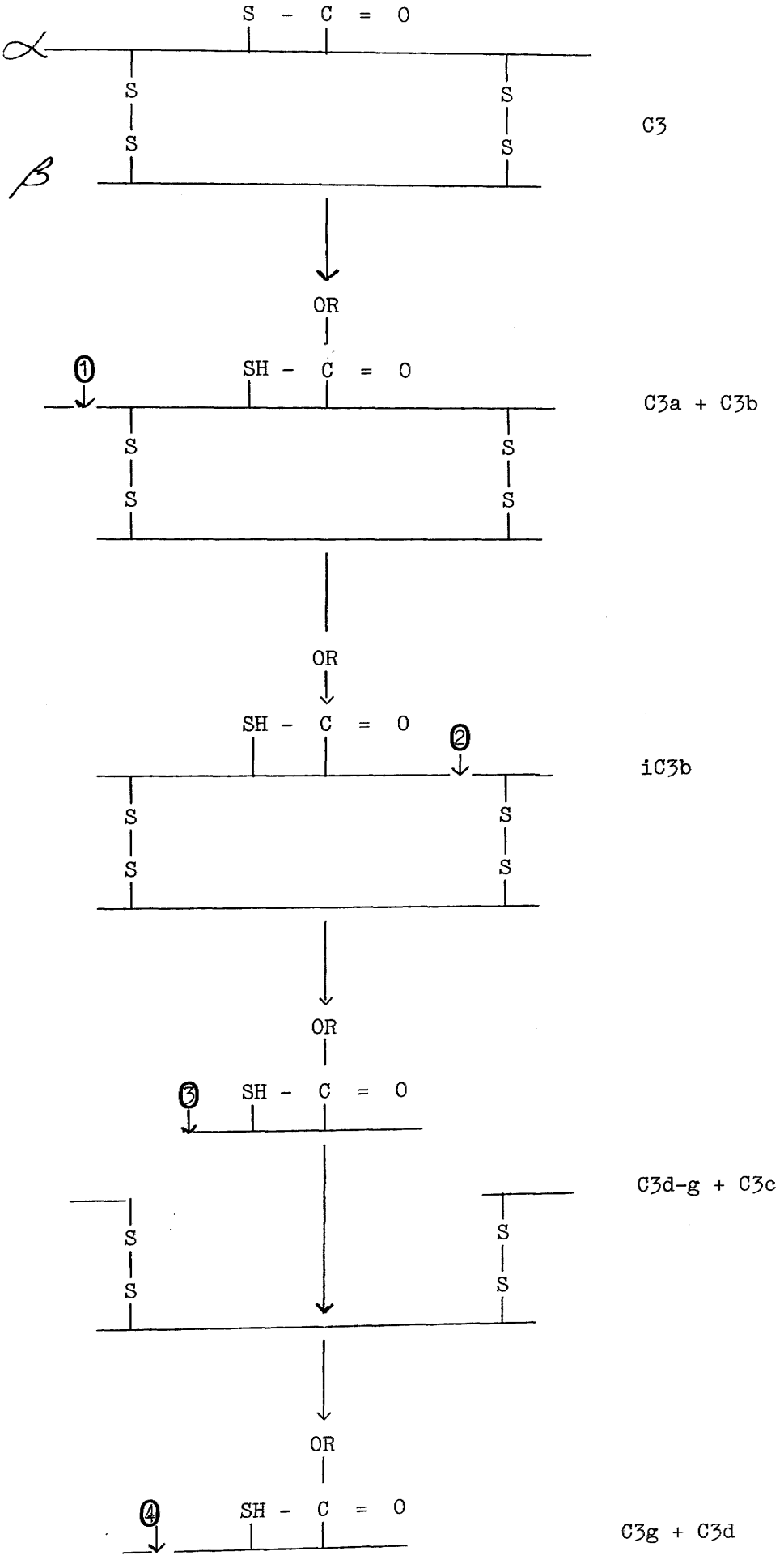
Fig. 4. THE SEQUENTIAL CLEAVAGE OF C3 :

(1) cleavage by the C3 convertase,

(2) cleavage by I and H or I and CR1,

(3) cleavage by I and CR1.

(4) cleavage by non-specific proteases.


S - C = O
α
S
S
S
S
β
C3
OR
①
SH - C = O
C3a + C3b
OR
SH - C = O
②
iC3b
OR
③
SH - C = O
C3d-g + C3c
OR
④
SH - C = O
C3g + C3d

has yet been discovered. This results in the cleavage of the α' chain at a third site generating C3d-g (42,000 daltons) which remains covalently attached to the cell surface or immune complex whilst C3c (140,000 daltons) is released into the fluid phase. The *in vivo* sequence of proteolytic cleavages probably terminates in the formation of C3c and C3d-g (Ross et al, 1982; Lachmann , Pangburn and Oldroyd, 1982; Medicus et al, 1983). Further cleavage of C3d-g can however be brought about *in vitro* using non-specific proteases such as trypsin, plasmin and leukocyte elastases, which split C3d-g to C3d (31,000 daltons) and C3g (8,000 daltons) (Davis, Harrison and Lachmann, 1983).

Finally C3e (10,000 daltons) is probably derived from C3c (Gherbrehiwet and Müller-Eberhard, 1979) by an as yet unidentified plasma enzyme. C3d-K is derived from proteolysis of iC3b by kallikrein. Its N-terminus aminoacid sequence overlaps the C-terminal sequence of C3e (Meuth et al, 1983). Thus the C3 breakdown fragments C3b, iC3b and C3d-g covalently attached to an immune complex or cell surface are the primary ligands for the C3 receptors CR1, CR3 and CR2 respectively.

1.5. Complement receptors.

The first description of complement receptors was by Duke and Wallace in 1930; they observed the adherence of human erythrocytes to trypanosomes in the presence of antibody and complement. In 1968 the presence and properties of complement receptors were further examined by Lay and Nussenzweig. They used rosetting assays to study the complement receptors on mouse macrophages, polymorphonuclear leukocytes (PMNs) and lymphocytes. This technique utilises as indicator cells sheep red blood cells (SRBC) coated with antibody (IgM or IgG, EA) and complement (EAC), and it is this technique

which has been most frequently used to detect complement receptors. However, at the time when Lay and Nussenzweig (1968) performed these experiments the exact nature of the complement components on the indicator cells was unknown. They demonstrated first the necessity to use IgM to make EACs as EAs made with IgG formed spontaneous rosettes with the cells under study, and they consequently showed rosetting of EACs (made with IgM) with peritoneal macrophages, PMNs, some monocytes, 10 to 25% of lymph node cells but not thymus cells. By using C5 deficient mouse serum they concluded that the first four components of the classical pathway were required for rosette formation, with rosetting being minimal at $4^{o}C$. (The observation of reduced ligand binding at $4^{o}C$ can probably be attributed to reduced receptor levels as Fearon and Collins (1983) have shown PMNs to have seven-fold more receptors at $37^{o}C$ than at $4^{o}C$.) By performing the experiments in the presence or absence of divalent cations, they suggested there were two types of receptors, with receptors on monocytes, macrophages and PMNs requiring divalent cations for EAC binding but not those on lymphocytes.

In 1953 Nelson documented the complement receptor on human erythrocytes. He was studying the phagocytosis of the virulent spirochaete Treponema pallidum, which had been sensitized with syphilitic serum. In control preparations where the spirochaete suspension was incubated with erythrocytes alone as opposed to whole blood, he observed that instead of remaining in suspension, the spirochaetes within five minutes adhered to the erythrocytes. As lysis of the erythrocytes returned the spirochaetes into suspension he presumed they had adhered to the cell membrane and as the reaction was heat sensitive he assumed it to be mediated by complement. He called this phenomenon immune adherence (IA), a term still commonly used.

Pursuing this work further Nelson (1956) showed using complement

sensitized starch granules the dependency of IA on the classical pathway components and particularly C3.

An important step towards the elucidation of the complement receptor ligands was finding that C4 and C3 complexed to indicator cells were in the cleaved forms C4b and C3b (Dalmasso and Müller-Eberhard, 1967). With this knowledge, Ross et al (1973) made indicator cells using as a complement source purified human complement components (EAChu) or C5 deficient mouse serum (EACmo) and compared their ability to form rosettes with human erythrocytes or lymphoblastoid cell lines. The EAChu demonstrated enhanced binding to the erythrocytes, while the EACmo bound more effectively to the lymphoblastoid cells. Theoretically both types of indicator cells bore C3b, however the observed differential binding did not support this supposition. Further use of crude antiserum preparations raised against the two cell types in a rosette inhibition assay indicated the presence of two different complement receptors. Firstly CR1, the erythrocyte IA receptor binding C3b (EAChu) and secondly CR2, the receptor on lymphoid cells for C3d (now known to be iC3b, EACmo), formed by the actions of I (in the C5 deficient mouse serum) on C3b. The ability of EAChu treated with I to behave in a similar manner to EACmo confirmed this proposal.

Ross et al (1973) cites CR2 as binding C3d, based on the assumption that I mediated cleavage of C3b yielded C3c and C3d. However, in 1977, Pangburn, Schreiber and Müller-Eberhard elucidated the cleavage pattern of C3b. They showed that I together with the cofactor H cleaved C3b to an intermediate form iC3b and that further <u>in vitro</u> cleavage of iC3b to C3c and C3d required the action of enzymes such as plasmin or trypsin. Thus prior to 1977 all references to CR2 binding C3d are actually referring to iC3b binding to a third receptor CR3.

1.6 CR1

CR1 is now known to bind C3b, C4b (Bokisch and Sobel, 1974; Cooper, 1969; Ross and Polley, 1975) and iC3b (Ross and Polley, 1975; although in this paper it is cited as C3d because of the unknown cleavage pattern of C3).

The cellular distribution of CR1 on cells other than erythrocytes has been evaluated using the rosetting technique. Lay and Nussenzweig (1968) demonstrated rosetting with mouse peritoneal macrophages, PMNs, monocytes and a proportion of lymph node derived lymphocytes but not thymocytes. The presence of CR1 on human monocytes and PMNs was also demonstrated in 1968 (Gigli and Nelson; Huber et al) and in 1973 Ross et al showed the identity of the lymphocyte CR1 with the erythrocyte IA receptor. However neither Ross et al (1973) nor Bianco, Patrick and Nussenzweig (1970) could detect CR1 on T lymphocytes. More recently using an indirect immunofluorescence technique with an anti-CR1 antiserum Wilson, Tedder and Fearon (1983) have identified a population of T lymphocytes (approximately 14% of the total T lymphocyte population) expressing CR1. CR1 has also been detected on mast cells (Sher and McIntyre 1977) and human kidney glomerular cells (Gelfand, Frank and Green, 1975; Fischer, Appay and Kazatchkine, 1984).

1.7. CR2.

In present classification schemes of complement receptors (Arnaout and Colten, 1984; Fearon, 1984) CR2 binds the ligand C3d but like CR1, CR2 can bind more than one ligand, if the assay conditions are specifically adjusted or sufficiently sensitive to

demonstrate this. Lambris and Ross (1982) have analysed the ligand specificity and distribution of CR2 with C3 coated fluorescent microspheres,a technique more sensitive than rosetting assays. This has shown CR2 to be restricted to B lymphocytes but having the ability to bind C3d, C3d-g and iC3b. They could not demonstrate the binding of C3b, although it has since been shown that even this ligand will bind to CR2, albeit rather weakly (Fearon, 1984; Weis, Tedder and Fearon, 1984).

Various workers have examined the molecular structure of CR2 on B cells and lymphoblastoid cell lines. In 1981 Lambris, Dobson and Ross isolated from the culture medium of a Raji cell line a glycoprotein of molecular weight 72,000 daltons. This glycoprotein was presumed to be CR2 (shed from the cell during membrane turnover) because of its ability to bind EAC3d and not EAC3b.

Likewise in 1981 Nadler et al using a monoclonal antibody identified a human lymphocytic antigen designated B_2. The expression of B_2 was confined to B lymphocytes and by surface labelling followed by immunoprecipitation the B_2 antigen with a molecular weight of 140,000 daltons was isolated.

In 1983 Iida, Nadler and Nussenzweig linked the B_2 antigen (Nadler et al 1981) with CR2 based on their dual presence on B cells and distribution similarities on lymphoblastoid cell lines. Additional evidence of identity was attained with rosette inhibition assays where the anti-B_2 monoclonal antibody inhibited EAC3d rosetting with B lymphocytes.

Weis et al (1984) have also identified and purified a membrane protein of molecular weight 145,000 daltons from B lymphocytes and lymphoblastoid cell lines using a monoclonal antibody HB-5. They also presume this antigen to be synonymous with CR2, as the

purified antigen can bind EC3bi, EC3d, EC3d-g and they also cite the very weak binding of EC3b. When the monoclonal antibody is cross-linked with a second antibody rosetting is inhibited.

The relationship between the three putative CR2 molecules described by Nadler et al (1984), Lambris et al (1981) and Weis et al (1984) is unclear. It seems probable that the antigens with molecular weights of 145,000 and 140,000 daltons are the same, with the molecular weight variation due to the different experimental techniques. However, their relationship to the 72,000 dalton glycoprotein described by Lambris et al (1981) is not clear, but it is possibly a result of proteolysis of the complete molecule.

The functional attributes of CR2 have caused much speculation. The restriction of CR2 to B lymphocytes implies a possible role in B lymphocyte activities. Lambris et al (1982) looked at the effects of anti-CR2 antiserum ($F(ab')_2$ and Fab'), EAC3d and C3d on the B lymphocyte response to mitogens, antigens and allogenic cells. B lymphocyte proliferation in response to allogenic cells, pokeweed mitogen and tetanus toxoid was inhibited by these ligands. However they did not affect the response to concanavalin A or phytohaemagglutinin. These data imply that binding of CR2 specific ligands in some way regulate B lymphyocyte responses.

The strong expression of B_2 (CR2) on B lymphocytes particularly in the germinal centres of lymphoid follicles has led Iida et al (1983) to propose a possible role for CR2 in antigen retention and development of B cell memory which is known to be antibody and C3 dependent.

A number of studies have suggested a relation between the Epstein-Barr Virus (EBV) receptor site on lymphocytes and CR2. By using double immunofluorescence and capping experiments,

Yefenof et al (1976) demonstrated that both these receptors stained and redistributed as one unit within the lymphocyte membrane, suggesting they were closely linked or the same (Yefenof et al, 1976; Klein et al, 1978). More recently the EBV receptor on lymphocytes has been purified (150,000 daltons) and as a result of inhibition assays performed with specific receptor antisera it was concluded that CR2 is probably not the binding site for EBV (Simmons et al, 1983; Hutt-Fletcher et al, 1983).

As yet there is no clear role for CR2, but the use of specific monoclonal antibodies may resolve this in the future.

1.8. CR3.

CR3 unlike CR1 and CR2 binds only one ligand iC3b (Arnaout and Colten, 1984; Fearon, 1984) although there is some evidence to support C3d-g binding (Ross et al, 1983). The ability of iC3b to bind to CR1 and CR2 as well as CR3 has caused considerable difficulties in studying CR3-iC3b interactions, however the binding is now distinguishable by its divalent cation dependency (Ross et al, 1983) and by utilizing monoclonal antibodies to the receptors.

CR3 is found on monocytes, macrophages, PMNs (Carlo et al 1979; Ross and Rabellino, 1979; Ross et al, 1983) and large granular lymphocytes (Nocera et al, 1982). Like CR2 the structural characteristics of CR3 have been examined using monoclonal antibodies.

Beller, Springer and Schreiber (1982) raised a rat monoclonal antibody to a mouse macrophage antigen designated MacI. This antigen was composed of two non-covalently linked polypeptide chains (α 170,000 and β 95,000 daltons) and found additionally on mouse PMNs and natural killer cells, as well as cross-reacting with the counterpart human cells. As a result of the monoclonal antibody's ability to selectively inhibit EAC3bi rosetting with murine

macrophages and human PMNs as well as the distribution similarities of MacI and CR3, MacI was thought to be CR3. However Beller et al (1982) have not excluded the possibility that MacI could be distinct but intimately associated with CR3 or perhaps has a CR3 promoting function.

The antigen MacI seems to be synonymous with the antigen recognised by OKM10 (Wright et al, 1983). OKM10,like anti-MacI, can block EAC3bi binding to human monocytes and macrophages and immunoprecipitates two polypeptides (185,000 and 105,000 daltons) retaining their iC3b binding activity.

Arnaout et al (1983a) have also detected a monocyte-granulocyte glycoprotein termed Mo1 using a monoclonal antibody. Like anti-MacI and OKM10, anti-Mo1 inhibits EAC3bi rosetting to granulocytes and immunoprecipitates two polypeptides with molecular weights of 155,000 and 94,000 daltons.

Three groups, Beller et al (1982), Wright et al (1983) and Arnaout et al (1983a), have detected and isolated two surface membrane polypeptides of similar molecular weights, iC3b binding properties and cellular distribution. Thus it seems probable that the surface antigens recognised by anti-MacI, OKM10 and anti-Mo1 are the same and synonymous with CR3.

The functional role of CR3 like CR2 has aroused much speculation and as yet there seems to be no clear indications about its relation to cellular functions in the cells on which it is found. Work on patients with a putative CR3 molecule defect has shed some light on this area. Arnaout et al (1982) have investigated a young male patient who suffered from recurrent pyogenic infections. They found this boy to have defective receptor PMN functions, such as phagocytosis of immunoglobulin coated particles, superoxide generation and reduced de-granulation induced by opsonized zymosan.

The only defect found was a 50% reduction in the levels of the 150,000 dalton polypeptide chain and they proposed this to be causing the defective cellular functions and thus its possible role in triggering normal neutrophil functions.

Arnaout et al (1985) has also studied Mo1 levels on neutrophils with a view to understanding the mechanism causing haemodialysis-induced granulocytopenia. Eight patients were studied and after 15 minutes of haemodialysis there was a five-fold increase in the mean cell-surface expression of Mo1. This coincided with the maximal drop in neutrophils and the peak in the complement activation products C3a des arg and C5a des arg. From these data they suggested that Mo1 may provide a mechanism for initiating leucoaggregation, sequestration of granulocytes and neutropenia during haemodialysis.

Interestingly it has also been shown that the β subunit of Mo1 is shared with another surface structure; lymphocyte function associated antigen (LFA-1). By performing functional studies on lymphocytes it has been shown that LFA-1 is involved in promoting adherence between LFA-1 positive cells and targets (Arnaout and Colten, 1984).

The relationship between CR3 and LFA-1 is unclear, their role in affecting cellular immune functions has been touched on, and further investigation will be required to determine their possible association for example with phagocytic defects.

1.9. The structure of CRI.

CRI was first isolated from the membranes of pooled human erythrocytes in 1979 by Fearon. Prior to this the physiochemical characterization of CRI was limited to the demonstration of its

trypsin sensitivity (Lay and Nussenzweig, 1968; Huber et al, 1968) which suggested it was a protein.

Fearon (1979) initially set about purifying an alternative pathway C3 convertase inhibitory protein. This was based on the observation that human erythrocytes did not activate the alternative pathway, even after the removal of the membrane-associated sialic acid residues which promote high affinity binding of H to C3b inhibiting $\overline{C3bBb}$ formation. This inhibitory protein was intrinsic to the cell membrane and by solubilizing the membranes with a non-ionic detergent Nonidet P-40 (NP-40) followed by chromatographic procedures (anion exchange, gel filtration and affinity chromatography) a protein displaying the observed inhibitory effect on the alternative pathway C3 convertase was purified to homogenicity. On sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) both reduced and non-reduced, the protein presented as a single band with a molecular weight of 205,000 daltons. However prior to affinity chromatography the molecular weight was 1 to 1.2×10^6 daltons, thus indicating its possible existence in the cell membrane as an oligomer.

This inhibitory protein could impair the function of $\overline{C3bBbP}$ by displaying Bb and promoting I mediated cleavage of C3b and binding reversibly to Sepharose-C3 and Sepharose-lentil lectin, thus indicating it to be a glycoprotein (gp) with an affinity for C3. These data all suggested its possible identity with C3 and this was further investigated by Fearon (1980) using rosetting assays with EC3b, EC3bi and EC3d, and rosette inhibition-assays with an anti-gp 205 antiserum raised in rabbits. $F(ab')_2$ anti-gp 205 only inhibited EC3b rosetting with human erythrocytes, PMNs, B lymphocytes and monocytes in a dose-dependent fashion. In addition this specific antiserum could be used to immunoprecipitate the gp 205 from the previously mentioned cell types, thus giving

direct evidence for the identity of gp 205 with CR1.

A similar result was obtained by Dobson, Lambris and Ross (1981) who also isolated CR1 from human erythrocytes. The isolated protein consisted of a single band with a molecular weight of 195,000 daltons and bound EAC14b and EC3b.

Mussel et al (1982) and Gerdes and Stein (1980) have attempted to isolate human erythrocyte CR1 by using 2M KBr to solubilize the cell membranes. Gerdes and Stein (1980) purified three bands of molecular weights 1 to 1.3×10^6, 80,000 and 60,000 daltons, the latter two bands being reduced to 38,000 and 18,000 daltons. These bands were immunoprecipitated from the cell lysates by a crude anti-CR1 antiserum (Gerdes, Klatt and Stein, 1980) and they bear no relation to the CR1 protein isolated by Fearon (1979) or Dobson et al (1981).

Mussel et al (1982) purified a band of molecular weight 55,000 to 60,000 daltons from C3-sepharose treated 2M KBr lysates. This material did not agglutinate $EAC14^{oxy}23b$, but inhibited erythrocytes coated with C3b from binding to human erythrocytes. They designated this monovalent CR1 and concluded that it represented the head region of CR1.

Neither of these results attained by Mussel et al (1982) or Gerdes and Stein (1980) have been repeated, and their true relation to CR1 is unknown nor been investigated further.

Recently more careful studies of the structure of CR1 in individual donors have been carried out and this has led to some very interesting discoveries.

Dykman et al (1983a) first examined CR1 on the erythrocytes of individual donors, using cell surface-labelling with ^{125}I, then cell lysis with NP-40 followed by affinity chromatography (Sepharose-C3 or Sepharose-C4) or immunoprecipitation (monoclonal anti-human CR1) SDS-PAGE and finally autoradiography. With this combination

of techniques they demonstrated that the structure of CR1 within the population is polymorphic, expressing itself in three patterns, types A, B and C. In a group of 33 normal people, on non-reduced gels, 70% had a major band of molecular weight 190,000 daltons (type A), 33% had a major band of 220,000 daltons (type B) and 27% expressed both bands (type C). In all three types a minor band, with a molecular weight 15,000 daltons larger than the major band was also seen. Further studies of the three receptor patterns within four families provided evidence to support the transmission of the CR1 structural phenotypes by two codominant alleles designed A and B, coding respectively for the 190,000 and 220,000 dalton CR1 proteins.

Similar polymorphisms exist in the CR1 molecules purified from leucocytes. By examining the CR1 profiles on the erythrocytes and leucocytes of three donors, Dykman et al (1983b) found that within an individual donor the CR1 phenotype on the erythrocytes and leucocytes was identical. However two cell specific differences in CR1 were found; 1) a 5,000 dalton increase in the molecular weight of CR1 on PMNs and 2) minor bands were only found on erythrocytes.

Wong, Wilson and Fearon (1983) have also examined CR1 polymorphism on human erythrocytes and leucocytes using similar techniques to Dykman et al (1983a, b). Likewise they have found two major bands but with a molecular weight of 250,000 (F=fast migrating) and 260,000 (S=slow migrating) daltons on reduced gels. In a study of 111 unrelated individuals the following phenotype frequencies were found: 64.9% were homozygous for F, 1.8% were homozygous for S and 33.3% heterozygotes. In addition a study of 15 families gave further evidence to support the inheritance of F and S by two autosomal codominant alleles.

Dykman et al (1983a, b) and Wong et al (1983) are obviously

both studying CR1, with the differences in molecular weights probably arising from the different analytical systems used. This may also account for the discrepancy in the molecular weights of CR1 as observed by these two groups and the 205,000 daltons CR1 molecule isolated by Fearon (1979). Both groups' data on the observed frequencies of the CR1 phenotypes within the population do not differ significantly from those predicted by the Hardy-Weinberg equilibrium for two codominant alleles, and this is further supported by family studies. Both groups also observed that the ratio of the two bands in heterozygotes was variable but characteristic for an individual. The major difference between the two groups was the inability of Wong et al (1983) to demonstrate the minor band found on erythrocyte membranes and the increased molecular weight of CR1 derived from PMNs . However, the published gels of Wong et al (1983) have a heavy background staining which may have prevented the identification of these minor molecular differences.

Further investigations by Dykman's group have led to the discovery of two additional CR1 phenotypes (C and D), based on their structural and ligand binding characteristics, cellular distribution and familial transmission. In 1984 Dykman, Hatch and Atkinson described a third band with a molecular weight of 160,000 daltons (C) which co-existed with A or B. In a study of 104 unrelated, normal individuals, the observed phenotype frequencies were AA 69.2%, AB 25%, BB 3.8%, AC 2.9% and CC 0%. These results were compatible with the Hardy-Weinberg equilibrium for three codominant alleles at a single locus. In their most recent paper Dykman et al (1985) describe a fourth structural variant (D) with a molecular weight of 250,000 daltons. This was found in only one person out of a total of 200 studied and existed in the heterozygous state with A (190,000 daltons).

Thus to date four alleles coding for structural variants of CR1 have been described. Both groups point out that structural polymorphisms apparent by molecular weight differences are very unusual, the more normal case being a difference in net charge as is seen with the polymorphic complement components, for example C2 (Alper, 1976; Meo et al, 1977), C4 (Teisberg et al, 1976), C4 (Azen and Smithies,1968), C5 (Hobart, vaz Guedes and Lachmann, 1981) and B (Alper, Boenisch and Watson, 1972). Nor is this difference in molecular weight small, the extreme being 90,000 daltons between C and D; this cannot be accounted for by post-transcriptional modification. Wong et al (1983) also rule out the possibility of N-linked carbohydrate differences as the cause. Although O-linked carbohydrate differences have not been ruled out, it seems more likely that differences in the primary peptide sequences are giving rise to the polymorphism of CR1.

With such vast differences in the molecular weights of CR1, it poses the question as to whether the polymorphisms are beneficial or arise solely as a result of non-advantageous mutations. There is no apparent linkage of the CR1 phenotype with HLA (Hatch et al, 1984), CR1 numbers (Wong et al, 1983; Medof, Iida and Nussenzweig, 1983), functional ability to bind dimeric C3b (Wong et al, 1983) or the ability to serve as a cofactor to I (Medof et al, 1983). However, the ability of the different phenotypes to mediate such functions as phagocytosis and adsorptive endocytosis by PMNs and monocytes has not been investigated.

1.10. The functions of CR1.

The number of CR1 receptors on immune effector cells have been quantitated using ^{125}I-labelled dimeric C3b (Arnaout et al, 1981) or ^{125}I-labelled polyclonal F(ab')$_2$ anti-CR1 (Fearon, 1980)

and the results are expressed in Table 9. Clearly the cellular distribution and density of CR1 on the different cell types poses the question: What is the function of CR1?

Many functions of CR1 are cited in the literature. Generally these functions can be divided into two categories, those which influence an individual cell population or those affecting all cells bearing CR1. This division is broad but allows a clearer discussion of the universal role of CR1 as a potent inhibitor of complement turnover and its individual effects on the cellular function of kidney podocytes, lymphocytes, phagocytic cells and erythrocytes within the immune system.

Obviously if CR1 plays a role in the induction and/or regulation of the immune system, it must be sensitive to modulations in the levels of the ligands; C3b, iC3b and C4b. There has been controversy as to whether C3 binds to CR1. This would be detrimental to the sensitivity of CR1, as the receptor would be continually swamped with C3 due to its high serum concentration. It is now apparent that native C3 does not bind to CR1 (Berger et al, 1981; Schreiber, Pangburn and Müller-Eberhard, 1981; Berger and Fleisher, 1983) and only binds after cleavage of the internal thiolester bond of the α chain (Dixit et al, 1982; Berger et al, 1981; Schreiber et al, 1981). Nor does monomeric C3b bind under normal physiological conditions, with the receptor having a higher affinity for dimeric or oligomeric C3b (Arnaout et al, 1983b). This is contrary to the results obtained by workers such as Tack, Segal and Schechter (1978), Newman and Johnston (1979) and Sim and Sim (1981), who all report C3 binding to CR1. It seems likely that the C3 preparations used in these experiments were either contaminated with C3b-like haemolytically inactive C3 or the C3 may have been cleaved by cell associated

Table 9. Density of CR1 on human immune effector cells as determined by ^{125}I-dimer C3b (Arnaout et al, 1981) or ^{125}I-F(ab')$_2$ anti-CR1 (Fearon, 1980).

	C3b dimer	F(ab')$_2$ anti-CR1
Erythrocytes	360	950
PMNs	21,000	57,000
B cells	20,000	21,000
Monocytes	30,000	48,000

proteases released during the time course of the experiments (Frade and Strominger, 1980). However this does not explain the results attained by Sim and Sim (1981) who showed the binding of $5x10^5$ ^{125}I-labelled C3 molecules to human lymphocytes; the reasons for this are unclear.

1.11. CR1, a potent inhibitor of complement turnover.

There is now strong evidence to support the proposal that CR1 on all immune effector cells, functions as a potent *in vivo* inhibitor of complement turnover. This inhibitory activity was first described by Fearon (1979) who demonstrated the ability of purified CR1 to accelerate the decay of the properdin stabilized alternative pathway C3 convertase, in a dose-dependent fashion. CR1 mediates this activity by displacing Bb from the convertase by competing for the C3b binding site. Normally this role is performed by the plasma protein H. H, as well as functioning independently, acts as a cofactor to the serine protease I, together they control the alternative pathway C3 convertase by cleaving C3b to the inactive form iC3b. Fearon (1979) has further shown the ability of CR1 to replace H as a cofactor in this cleavage step. However, H cannot normally act as a cofactor to I to bring about the further cleavage of iC3b to C3c and C3d-g. It is evident that C3c and C3d-g are the terminal cleavage products of C3, as the erythrocytes from patients with cold-agglutinating disease bear C3d-g on their surface and not iC3b or C3d (Lachmann, 1981). Ross et al (1982) have demonstrated the ability of H and I to cleave iC3b but only in low ionic strength buffer (4mS at room temperature) not physiological buffer strength (12mS at room temperature). It is now clear that purified CR1 or membrane bound CR1

can act as a cofactor to I to bring about the cleavage of iC3b to C3d-g and C3c (Medof et al, 1982; Ross et al, 1982; Medicus et al, 1983).

C4b is also a ligand for CR1 and this led Iida and Nussenzweig (1981) to examine the possible controlling influences of CR1 on the classical pathway enzymes containing C4b, namely $\overline{C4b2a}$ and $\overline{C4b2a3b}$, the C3 and C5 convertases. As with the alternative pathway purified CR1 can decay-dissociate the classical pathway C3 convertase by competing with C2a for the C4b binding site. This function is also mediated by C4BP and like C4BP, CR1 can act as a cofactor to I to bring about further cleavage of C4b (Iida and Nussenzweig, 1981; Gigli and Fearon, 1981). The ability of CR1 to inhibit the C5 convertase is probably similar to its effects on both C3 convertases, but in this case the CR1 competes with C5 for the binding site on the C3b molecule in the C3 convertase (Iida and Nussenzweig, 1981).

CR1, whether in a purified form or as a constituent of a cell membrane, can perform these regulatory functions (summarized in Table 10 along with the plasma proteins, where applicable, whose functions CR1 mimics).

Clearly CR1 in vitro has the potential to inhibit all the amplifying enzymes of the complement system and researchers in this field have speculated about its in vivo role.

It is postulated that CR1 operates as an inhibitory protein at two levels. Firstly, CR1 may prevent inappropriate self lysis by complement. Such situations may arise due to deposition of C3b on self cells as a result of the low spontaneous turnover of C3 by the alternative pathway or during periods of inflammation. For example, C3b or C4b bearing substrates may interact with phagocytes via CR1. Subsequently this may lead to the activation and deposition of complement components on or in close proximity to host

Table 10. Summary of CR1's inhibitory activities on the complement system

(From Iida and Nussenzweig, 1981)

Inhibition of haemolytic activity of:	CR1	C4-bp	Factor H
Classical pathway C3 convertase	Yes	Yes	No
Alternative pathway C3 convertase	Yes	No	Yes
C5 convertase	Yes		
Cofactor activity for the cleavage of fluid phase: C3b	Yes	Yes	Yes
iC3b	Yes	No	No
C4b	Yes	Yes	No

cells which may lead eventually to self lysis. The ability of some immune effector cells to produce complement components may aggravate this situation further. Therefore a strong local concentration of inhibitor, namely CR1, would be vital to stop excess tissue damage especially during infections or situations of high complement turnover. Secondly, CR1 can interact with C3b or C4b bearing substrates and bring about C3b and C4b degradation so they can no longer take part in complement activation and this will also generate the ligands for CR2 and CR3.

These inhibitory roles described for CR1 are very much simplified and in vivo the situation is probably more complex. For example, it has been shown that larger quantities of CR1 are required for I mediated cleavage of iC3b as opposed to C3b (Medicus et al, 1983). This may be of physiological importance, allowing the preferential formation of iC3b as opposed to C3d-g bearing substrates which will interact with CR3 on phagocytic cells and eventually be phagocytosed. Another possible subtlety has been described by Medof and Nussenzweig (1984), who found that the inhibitory effects of CR1 on C4b or C3b bearing substrates was modulated by C3b or C4b in the vicinity. They showed that C4b enhanced CR1 and I mediated cleavage of C3b and vice versa. Clearly the situation is complex.

It must also be pointed out that CR1 is not the only cell surface constituent with a controlling influence on the classical pathway C3 convertase. A membrane protein called Decay Accelerating Factor-Stroma (DAF-S) has been purified from human erythrocytes. Although it is structurally and antigenically distinct from CR1, it also accelerates the decay of the classical pathway C3 convertase. However, this action of DAF-S is more potent than that of CR1 (Nicholson-Weller et al, 1982; Iida and Nussenzweig, 1983).

DAF-S therefore may also have a role in controlling *in vivo* complement activation and turnover.

1.12. The function of CR1 on kidney podocytes.

There is very little known about the function of CR1 on kidney podocytes. Obviously it may play a part in protecting the cells from damage due to complement activation.

Recently Fischer et al (1984) has implicated a role for the receptor in the adsorptive endocytosis of soluble ligands bearing C3b, because of experimental findings which show the internalization of $F(ab')_2$ anti-CR1 by kidney podocytes. However, there is no other known work to support this.

1.13. The function of CR1 on lymphocytes.

As yet there is no concrete evidence to support a particular function for CR1 on lymphocytes, particularly B cells. Results obtained from experiments performed on animals implicate its role in humoral immune responses; for example, lymphokine production, antibody synthesis and generation of B cell memory. Some of the data is supported by *in vitro* studies on human lymphocytes.

Sandberg, Wahl and Mergenhagen (1975) and Koopman et al (1976) have examined the production of a macrophage chemotactic lymphokine by guinea-pig spleen cells in response to C3b stimulation. This could not be reproduced using other C3 fragments and C3b aggregates were thought to be cross-linking the receptors and consequently eliciting the lymphokine release.

The effects of C3b on antibody production has also been

examined. There seems to be general agreement from the results attained in animals, that mitogens and T-dependent antigens can activate B cells in the absence of complement (Pepys, 1976). However, T-dependent antigens require C3 to initiate antibody production. This has been demonstrated in mice depleted of C3 by cobra venom factor and anti-C3 antiserum. Primary immunization of these mice with a T-dependent antigen induces only low levels of IgM, and this production could be attributed to CR1 negative B cells (Lewis et al, 1976; Lewis, Ranken and Goodman, 1977). Using an in vitro human system Lobo and Burger (1982) enhanced by 78% the differentiation of adult B cells into plasmacytoid cells by their incubation with EAC14b3b as opposed to EAs. In a murine system, Daha, Bloem and Ballieux (1984) induced IgM, IgG and IgA antibody synthesis in a lymphocyte culture treated with $F(ab')_2$ anti-CR1 and sub-optimal doses of pokeweed mitogen. For an optimal response cross-linking of CR1 was required as seen by the diminished antibody synthesis with monovalent Fab' anti-CR1. However, these results could not be repeated with C3b or C3b bound to fragments of sheep erythrocyte membranes and this does question their validity. In contrast, Berger and Fleisher (1983) found C3b to inhibit antibody production. Nariuchi, Kakiuchi and Ueno (1985) support this finding and, in addition, found cross-linked C3b enhanced antibody production.

From these results one can propose a role for CR1 in controlling antibody production. With antibody production being increased when there are circulating C3b-coated antigen-antibody complexes and decreased when there is C3b alone in the circulation. It must be stressed that in all the aforementioned experiments the presence of T cells was obligatory.

Finally, both Pepys (1976) and Klaus and Humphrey (1977)

have demonstrated the inability of C3 depleted mice to localize immune complexes, aggregated IgG or T-dependent and independent antigens in lymphoid follicles. Lymphoid follicles are predominantly B cell areas with dendritic reticular cells. This cellular combination allows the trapping of immune complexes via the interaction of IgG Fc and C3b with the cellular Fcγ receptors and CR1, or in the case of T-independent antigens which activate the alternative pathway by the interaction of C3b with CR1. The close contact between B cells and dendritic reticular cells in this area suggests that they play an important role in the initiation of B cell memory and antibody responses, based on the inability of C3 depleted mice to elicit these two functions. However, with respect to antibody responses it now seems that the observed reduction in response was mediated by C3a and possibly other α chain fragments generated as a result of C3 activation by cobra venom factor and it was these fragments which suppressed antibody production (Weiler et al, 1982).

1.14. The function of CR1 on phagocytic cells.

Classically the role of CR1 in the phagocytic responses of human monocytes and neutrophils was one of enhancing the adherence of these cells to C3b-bearing particles. Research has shown that C3b-bearing complexes bind to phagocytic cells, but do not elicit phagocytosis unless accompanied by IgG. Together the interaction of IgG and C3b with Fcγ receptors and CR1 respectively, stimulates ingestion and accompanying biological reactions such as the release of superoxide ions, β-glucuronidase and lysozyme (Huber et al, 1968; Ehlenberger and Nussenzweig, 1977; Newman and Johnston, 1979). These results suggested that CR1 and Fcγ receptors have separate but complementary functions in

the phagocytic process.

However, there is now accumulating experimental evidence from in vivo and in vitro studies to support the notion that under certain circumstances CR1 alone can mediate phagocytosis. Wright, Craigmyle and Silverstein (1983) proposed the existence of CR1 on phagocytic cells in two states, inactive and active, where cells with CR1 in an inactive state cannot phagocytose via CR1, whereas in an active state binding to CR1 induces phagocytosis. Griffin (1980) has put forward the hypothesis that once macrophages have phagocytosed an immune complex, they then contact T cells which release an 'activating factor' (Griffin and Griffin, 1979). This factor, in turn, enhances CR1 mediated phagocytosis by macrophages. Such activation of macrophages can be demonstrated in vivo. Mouse resident peritoneal macrophages cannot phagocytose complexes when CR1 alone is engaged, whilst thioglycollate-elicited peritoneal macrophages are able to perform this function (Griffin and Mullinax, 1981). Other agents which have been shown to augment phagocytosis are the oligopeptides tuftsin and angiotensin II (Foris, Füst and Medgyesi, 1983), $F(ab')_2$ anti-CR1 and multimeric C3b (Fearon, Kaneko and Thompson, 1981). There are obvious physiological advantages in being able to phagocytose via CR1 alone. These have been highlighted by Griffin (1980). He stresses that when there is a large antigen load on the body (e.g. during an infection) there will consequently be high levels of circulating antibody and immune complexes. These may bind to the phagocytic cell Fcγ receptors and either block the receptor or saturate the phagocytic process. Thus the selective maintenance of another receptor, i.e. CR1, which could mediate phagocytosis in addition to Fcγ receptors would be advantageous.

An interesting feature which may relate to this last point is the ability of phagocytic cells to greatly increase the

number of CR1 on their cell surface. Fearon and Collins (1983) found at 37°C PMNs to have 38,000 CR1 and this could be increased 8- to 10-fold with the chemotactic agent C5a des arg or f-met-leu-phe. Similar results have been cited by Kay, Glass and Salter (1979) using N-formyl-methionyl peptides, casein and supernatants from cultured C. parvum or human lymphocytes (Glass and Kay, 1980), and this enhancement is also reversible. Increased CR1 numbers on phagocytic cells is not an in vitro artefact. Lee, Hakim and Fearon (1984) have examined CR1 levels on the neutrophils of patients undergoing haemodialysis. In these patients the haemodialysis membrane causes complement activation. For example, after 10 minutes (mins) of haemodialysis complement activation has increased by 225% and after 120 mins 160%. There are corresponding changes in CR1 levels, a 127% increase in 10 mins and 296% increase in 120 mins.

Thus phagocytic cells have an internal CR1 pool which may possibly allow for: a) increased phagocytosis, b) increased adhesion to opsonised particles or c) concentrated source of complement turnover inhibitor. Therefore up-regulating CR1 may be a means of augmenting the capacity to respond to C3b opsonised material.

1.15. The function of CR1 on erythrocytes.

Cells of the circulatory system are traditionally and conveniently colour coded; the red cells transporting oxygen to the tissues, the white cells performing immunological functions. Yet the presence of CR1 on human erythrocytes poses the possibility that erythrocytes are, in some way, involved in immune responses.

This was first suggested by Nelson (1953). He demonstrated that adherence of sensitized bacteria to erythrocytes led to enhanced phagocytosis of the bacteria by the leucocytes. Thus he concluded that the erythrocytes were acting as 'carriers' enabling immune complexes to be presented to phagocytic cells and subsequently removing them from the circulation.

These early experimental findings which proposed a role for erythrocytes in the immune response have now been supported by recent experimental evidence. Brown and Nelson (1973) examining the phenomenon of erythrocyte spherocytosis, argued that the loss of their characteristic biconcave shape was due to a reduction in cell surface area and volume. They noticed that EACs which interacted with monocytes or macrophages became contracted and spherical, with the erythrocyte membrane becoming indented by cytoplasmic processes from the mononuclear cells. They further examined this process in vivo by injecting rabbits with EACs. These animals were subsequently killed at various time intervals and the liver sections were examined using electron microscopy. This showed the binding of the EACs to the Kupffer cells within two minutes of injection with accompanying cell membrane distortion, with pieces being pinched off. Subsequently these erythrocytes were released back into the circulation.

In 1982 Medof and Oger using an in vitro system demonstrated the preferential binding of serum coated ^{125}I-labelled bovine serum albumin (BSA) anti-BSA complexes to the erythrocytes in whole human blood, with greater than 80% of the bound immune complexes being found on the erythrocytes. They confirmed the binding to be via CR1 as it did not occur in heat-inactivated serum. Furthermore, the complexes could not bind to non-primate erythrocytes which do not possess CR1. Binding was also

dependent upon the ratio of serum to immune complex. By using diluted serum or serum from a hypocomplementemic patient binding was delayed and sustained. However, using undiluted serum maximal binding occurred within 2 to 4 mins and decreased thereafter. The reason for the immune complex dissociation with increases in serum concentration could be due to one or all of the following: a) cleavage of C3b due to increased concentrations of I and H, b) solubilization of the immune complexes or c) competition for the receptor by free C3b and C3b breakdown products.

A similar type of experiment has been performed *in vivo* using baboons (Cornacoff et al, 1983). Using preformed ^{125}I-labelled BSA-anti-BSA complexes opsonized with baboon serum, they examined their fate after infusion into the baboons. Within 60 to 180 seconds the infused immune complexes had bound to the erythrocytes and after 5 minutes 73% were removed from the circulation. These were removed primarily during passage through the liver and, to a lesser extent, the spleen. The erythrocytes themselves did not become trapped but remained in the circulation. However, when antigen and antibody were infused separately they could not detect binding of the labelled BSA anti-BSA complexes to the baboon erythrocytes. The possible reasons which they gave for this observation were that either the binding occurred but was too rapid to record, or alternatively, the immune complexes which formed were too small to bind to the erythrocyte. It had been shown previously that the preformed complexes used were between 10S and 28S, which suggests that size may be a critical factor in determining whether a complex will bind to erythrocyte CR1 *in vivo*.

Thus these experiments demonstrate the ability of immune complexes to bind preferentially to erythrocytes via CR1 and consequently resulting in their removal from the circulation.

However, the question which still remains to be answered is "What is the significance of the binding of immune complexes to CR1 on human erythrocytes?"

Researchers in this field have put forward various proposals in an attempt to answer this question. It has been calculated that 95% of CR1 in the circulatory system is present on the surface of erythrocytes and because of the high red cell to white cell ratio, a circulating opsonized immune complex is 500 to 1,000 times more likely to bind to CR1 on an erythrocyte than a white cell (Siegel, Liu and Gleicher, 1981). Thus erythrocytes may serve as a high capacity buffer competing with other CR1 bearing cells for circulating opsonized immune complexes. This would prevent unnecessary activation of CR1 on leucocytes and enable the erythrocytes to 'carry' the complexes to the mononuclear phagocytic system for phagocytosis. Erythrocyte CR1 may function by preferentially binding large complexes, therefore preventing their deposition in organs, tissues and blood vessels where they could produce tissue injury or prevent further complement activation in the blood. Finally, erythrocyte CR1 may act as a large ubiquitous and readily available pool of complement turnover inhibitor.

All this tends to support the view that erythrocyte CR1 is important in the handling and influencing the fate of immune complexes in the circulation. It is therefore possible to conceive that a breakdown or defect in this mechanism may cause abnormal handling of immune complexes, which may lead to increased immune complex loads and consequently aberrant immune responses. The relationship between erythrocyte CR1 levels and immune complex diseases will be discussed in the next section.

1.16 CR1 levels in immune complex diseases

It is now widely accepted that the deposition, or the formation of antigen-antibody complexes in tissues produces disease. In 1977 the World Health Organization proposed a means of classification in which immune complex diseases were divided into three groups based on the nature and origin of the antigens involved. To briefly summarize, the first group includes diseases in which the major antigens of the immune complex are endogenous in origin. The second group is comprised of antigens which are exogenous and in the third group, the nature of the antigens is unknown.

In this thesis two groups of patients with immune complex diseases were studied and both diseases fit into the first group as described above. They are systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).

SLE is a chronic systemic inflammatory disease which follows a pattern of alternating exacerbations and remissions. During periods of exacerbation there is multi-organ involvement with the disease affecting many parts of the body including the skin, lungs, central nervous system, joints, muscles and frequently the kidneys. The antigen-antibody complexes present in the glomeruli of SLE patients are believed to contain DNA and anti-DNA antibodies (Koffler, Schur and Kunkel, 1967; Milgrom, Campbell and Andres, 1976). This is supported by the observation that circulating complexes contain DNA and anti-DNA antibodies (Bluestone et al, 1970; Winfield, Koffler and Kunkel, 1975). Hypocomplementaemia also often occurs in patients with active SLE (Schur, 1975) and using immunofluorescence the deposition of complement components within the glomerular and skin lesions of SLE patients has been demonstrated (Rothfield et al, 1972).

RA is a chronic recurrent systemic inflammatory disease primarily involving the joints. This disease characteristically begins in the small joints of the hands and feet and progresses in a centripetal and symmetrical fashion. Extra-articular manifestations include vasculitis, atrophy of the skin and muscles, subcutaneous nodules, lymphoadenopathy, splenomegaly and leucopenia. The serum and synovial fluid of RA patients contain rheumatoid factors (RF) which are antibodies directed against the Fc piece of IgG (Egeland and Munthe, 1983) and these can be of any immunoglobulin class with IgM being the type detected in conventional agglutination assays. Complexes containing IgG-rheumatoid factor (self-associated IgG) are present in the synovial fluids of patients with RA (Hannestad and Melbye, 1967; Winchester, Agnello and Kunkel, 1970; Munthe and Natvig, 1971; Male, Roitt and Hay, 1979). Similar complexes have also been detected in the sera of RA patients with systemic vasculitis (Stage and Mannik, 1971, Theofilopoulos et al, 1974). It is unknown whether all the IgG rheumatoid factor-IgG complexes represent the vast majority of immune complexes in RA, as it is possible that some of the IgG exists as antibody to an unknown antigen. In RA patients complement activation does occur in the synovial fluid (Ruddy and Austen, 1973) and in the serum of those patients with systemic vasculitis (Schur, 1975).

Very little is known about the stimuli which causes the autoantibody production, namely anti-DNA antibodies in SLE and anti-IgG Fc antibodies in RA, nor why this production is not successfully controlled by the immune system. There is now growing evidence which also suggests that the mechanism for handling the formed immune complexes within the circulation and mononuclear phagocytic system of these patients is in some way inefficient. As mentioned

previously it is probable that CR1 on erythrocytes are important in the handling of immune complexes within the circulation. This assumption has led researchers to look for CR1 defects on the erythrocytes from patients with SLE or RA.

Prior to the commencement of this project, four studies examining erythrocyte CR1 levels predominantly in SLE, had been performed.

Miyakawa et al (1981) examined CR1 levels on the erythrocytes from SLE patients. The 56 patients studied all satisfied the diagnostic criteria proposed by the American Rheumatism Association (ARA), all had anti-nuclear antibodies in their sera and all were receiving maintenance dosages of steroids. The assay used to examine the erythrocyte CR1 levels was a modified IA reaction called immune-adherence haemagglutination (IAHA) where test erythrocytes were incubated in a microtitre plate with doubling dilutions of aggregated human gamma-globulin and complement. No agglutination represented no receptors on the erythrocytes.

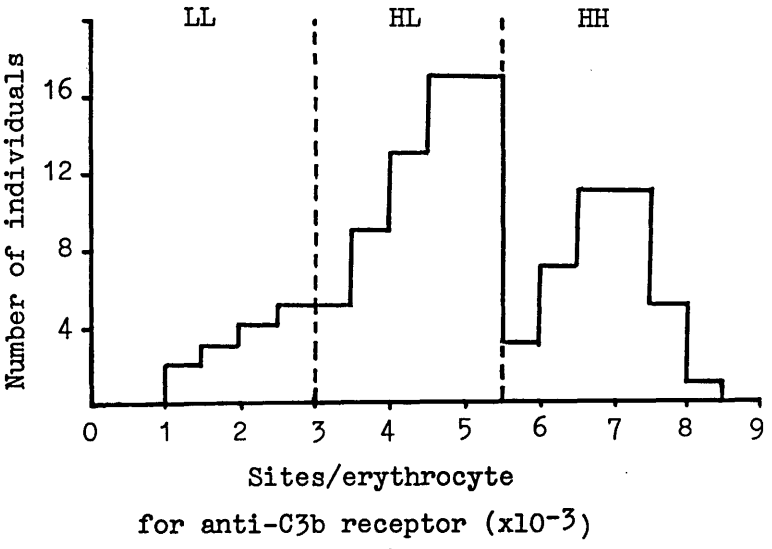
Those showing agglutination had receptors and the higher the titre the more CR1 per erythrocyte. With this technique Miyakawa et al (1981) showed 66% of their SLE patients to be IAHA negative compared to 4% of the controls. The low CR1 levels in the SLE patients were not due to the steroid therapy, nor had the receptors been blocked with immune complexes as most of the patients were in remission at the time of the study. It was therefore proposed that the observed low CR1 levels were an intrinsic defect. This view was further supported by serial measurements of IAHA titres in individual patients. These results showed that the IAHA titre remained virtually unchanged during

periods of exacerbation and remission of disease. The examination of the relatives of SLE patients showed a higher frequency of low CR1 levels than in normal families. Thus they concluded that low erythrocyte CR1 levels are an inherited defect rather than a secondary manifestation of the disease.

Wilson et al (1982) examined erythrocyte CR1 levels in 38 SLE patients (all of whom fulfilled ARA criteria, Cohen et al, 1971) and 113 normals. The receptor numbers were initially quantified using both ^{125}I-labelled dimeric C3b and $F(ab')_2$ anti-CR1, with the former being used to determine if antigenically reactive receptors were also functionally reactive. This was found to be the case with approximately 9 $F(ab')_2$ anti-CR1 molecules binding per C3b dimer; thus the remaining experiments were performed using $F(ab')_2$ anti-CR1 and the results expressed as the number of antigenic sites per erythrocyte (sites per erythrocyte). The mean number of sites per erythrocyte in 113 normals was 5014 ± 155 compared to 2804 ± 241 in the SLE patients. The difference was significant, persistent with time and was not due to steroid therapy, autoantibodies to CR1 or blockage by immune complexes. By constructing a histogram of the normal CR1 values (Fig. 5) they demonstrated a bimodal distribution of CR1 numbers. One group represented 34% of the population with a range of 5500 to 8500 sites per erythrocyte, the second group represented 54% with a range of 3000 to 5499 sites per erythrocyte. This second group had a shoulder extending into the lower range and represented 12% of the population (1000-2999 sites per erythrocyte). They proposed that a genetic basis may account for the observed trimodal distribution, with two codominant alleles determining high (H) and low (L) receptor numbers. Therefore HH would represent group one, HL group two and LL group three. By calculating

Fig. 5. HISTOGRAM SHOWING THE FREQUENCY DISTRIBUTION OF THE NUMBER OF BINDING SITES ON ERYTHROCYTES FOR ^{125}I-F(ab')$_2$ ANTI-CR1 AMONG NORMAL SUBJECTS.

(FROM WILSON ET AL, 1982)


LL
HL
HH
Number of individuals
16
12
8
4
0
1
2
3
4
5
6
7
8
9
Sites/erythrocyte
for anti-C3b receptor (x10-3)

the Hardy-Weinberg equilibrium for two codominant alleles the expected values did not differ significantly from the observed frequencies. This was also supported by the pattern of inheritance of receptor numbers in normal and SLE families; with the SLE families having a three to four times increase in the prevalence of the LL phenotype. Thus Wilson et al (1982) proposed that the CR1 phenotypes were genetically determined, but they stress that the wide variations in numbers in each group suggests the existence of modifying factors.

Iida, Mornaghi and Nussenzweig (1982) examined, using ^{125}I-labelled monoclonal anti-CR1, the CR1 numbers on the erythrocytes of 34 SLE patients, 10 RA patients and 52 normals. The normals had 1410±620 receptors per cell and there were no differences with sex, age or blood groups. The SLE patients had 600±307 receptors per cell and the RA patients 903±417 receptors per cell. Both these values were significantly lower than the mean for the normals. This could not be attributed to the steroid therapy, but there was a correlation between low haemolytic C4 levels, high immune complex levels and low CR1 numbers. They showed that despite the stability of CR1 numbers in normals with time, the CR1 numbers in two out of four SLE patients increased during a remission in their disease. These results also showed that low CR1 numbers are not confined to the disease SLE and that the deficiency is reversible which tends to support the idea the low CR1 numbers are acquired according to the disease process.

Inada et al (1982) using a modified IAHA showed in four SLE patients that erythrocyte CR1 levels were not constant with time and correlated inversely with circulating immune complex (CIC) levels, that is, as CIC levels increased receptor numbers dropped and vice versa. They suggest that CR1 levels in SLE are not

inherited but related to disease activity, particularly CIC levels, and they indicate that such CIC may even be blocking receptor expression.

These results from geographically widespread areas all find a significant decrease in the erythrocyte CIC levels in SLE patients, and in one study RA patients, compared to normal controls. However, the interpretation of the data by each group differs. Iida et al (1982) and Inada et al (1982) favour the acquisition of low erythrocyte CR1 levels secondary to the pathophysiological manifestations of the disease; contrary to this Miyakawa et al (1981) and Wilson et al (1982) favour the inheritance of CR1 levels.

In view of these results the aim of the proposed study was to examine whether CR1 numbers on human peripheral blood cells are inherited or acquired. This was to be investigated by quantifying the CR1 numbers on the blood cells from normal individuals and patients with SLE and RA. If CR1 numbers appeared to be acquired the possible reasons for this would be investigated.

CHAPTER 2

MATERIALS AND GENERAL METHODS

INTRODUCTION

The chapter describes all materials and general methods used, with subsequent chapters describing in detail more specific methods employed.

2.1 MATERIALS

With the exception of those listed below chemicals were of Analar quality from British Drug Houses Ltd., Poole, Dorset.

Materials obtained from Sigma Chemical Co. Ltd. Fancy Road, Poole, Dorset, were:

- Bovine serum albumin
- ε-amino-n-caproic acid
- Thyroglobulin
- Calf thymus DNA
- Porcine pepsin
- Trypsin
- Soya bean trypsin inhibitor
- Folin and Ciocalteu's phenol reagent
- Dextran
- Bromophenyl blue
- High molecular weight markers (SDS-6)
- Ammonium persulphate
- N, N, N', N'-Tetramethylethylenediamine (TEMED)
- 2-mercaptoethanol
- Phenylmethylsulfonyl fluoride
- L-tyrosine
- Dowex 100-200 mesh chloride form
- Caprylic acid
- Zymosan
- α-methyl-D-mannoside
- Ethanolamine
- Triethanolamine
- Sodium dodecyl sulphate (lauryl sulfate)

Materials obtained from Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Central Milton Keynes, were:

Diethylaminoethyl (DEAE) Sephacel
Sephadex G150 Superfine
Sephadex G200 Superfine
CNBr-activated Sepharose 4B
Lentil lectin Sepharose 4B
Ficoll 400

Materials obtained from the Scottish Antibody Production Unit were:

Normal sheep serum
Sheep anti-Human IgG antiserum
Donkey anti-rabbit IgG antiserum
Sheep anti-whole rabbit serum antiserum

Materials obtained from Cappel Laboratories, Daux Road, Billingshurst, Sussex, were:

Goat anti-rabbit IgG
Sheep anti-mouse IgG
FITC conjugated swine anti-goat IgG
FITC conjugated rabbit anti-sheep IgG
Sheep anti-rabbit IgG ($F(ab')_2$ fragment specific)

Materials obtained from Gibco Europe Ltd., Trident House, PO Box 35, Renfrew Road, Paisley, were:

Foetal calf serum
L-glutamine
Penicillin
Fungazone
Streptomycin

Materials obtained from Flow Laboratories, Irvine, Ayrshire, were:

RPMI-1640 medium without sodium bicarbonate, without glutamine with Hepes

Hanks buffered salt solution

Materials obtained from Aldrich Chemical Co. Ltd., Old Brickyard, New Road, Gillingham, Dorset, were:

Dimethyl sulphoxide

Benzamidine hydrochloride hydrate

Materials obtained from Bio-Rad Laboratories Ltd., Caxton Way, Watford, Hertfordshire, were:

Silver staining kit

Hydroxylapatite (Bio-Gel HT)

Biorex 70 100-200 mesh sodium free

Materials obtained from Berhing Diagnostics, Hoechst House, Salisbury Road, Hounslow, were:

Rabbit anti-human C3

Rabbit anti-human C4

Rabbit anti-human C5

Materials obtained from Difco Laboratories Ltd., PO Box 14B, Central Avenue, East Molesey, Surrey, were:

Freund's complete adjuvant

Freund's incomplete adjuvant

Materials obtained from Amicon Ltd., Upper Mill, Stonehouse, Glos. were:

YM30 and PM30 filtration membranes
Pellicon cassette

Sources of the following materials were:

Mouse monoclonal anti-CR1: Dako Ltd., 22 The Arcade, The Octagon, High Wycombe, Bucks

Dimethyl suberimidate: Pierce and Warriner, 44 Upper Northgate, Chester

Dithiobissuccinyl propionate: was a kind gift of Dr. Coggins, Department of Biochemistry, University of Glasgow

Millex-GS 0.22μm filter units: Millipore UK Ltd., Millipore Giysem, 11-15 Peterborough Road, Harrow, Middlesex

Antrypol and Mannitol, Hypaque and preservative free sodium heparin: were supplied by the Pharmacy, Western Infirmary, Glasgow

Methanol: May and Baker Ltd., Liverpool Road, Barton Moss, Eccles, Manchester

Diethylaminoethyl (DE52): Whatman Ltd., Springfield Mill, Maidstone, Kent

Ethanol: James Burrough, 60 Montford Place, London

Anti-human factor I (KAF C36 inactivator): Seward Laboratory, Norse Road, Bedford

Goat anti-human β IH globulin (factor H): Atlantic Antibodies, American Hospital Supply (UK) Ltd., Station Road, Didcot, Oxfordshire.

Rabbit anti-human P: was a kind gift of Prof. K. Whaley, Department of Pathology, Glasgow University

E11, mouse monoclonal anti-human CR1: was a kind gift of Dr. N. Hogg, Imperial Cancer Research Fund, Lincoln's Inn Fields, London

2.2 BUFFERS

Deionised water was used to make up all buffers and for methods employing water.

Veronal buffered saline (5 x VBS) pH 7.4

85g NaCl

3.75g Barbitone sodium

5.75g Barbitone

These were added together, dissolved in 1 litre of hot water and then made up to a final volume of 2 litres with water.

GVB^{++} pH 7.4

200 ml 5 x VBS

5 ml 0.03M $CaCl_2$

10 ml 0.1M $MgCl_2$

10 ml 10% gelatin

Made up to 1 litre with water

GVB^{--} pH 7.4

200 ml 5 x VBS

10 ml 10% gelatin

Made up to 1 litre with water

Isotonic dextrose containing cations ($D5W^{++}$) pH 5.5

50 g D-glucose

5 ml 0.03M $CaCl_2$

10 ml 0.1M $MgCl_2$

Made up to 1 litre with water

Isotonic 0.086M EDTA pH 7.2-7.6

197.7 ml of 0.3M NaOH was added to 500 ml of 0.12M EDTA to give a final pH between 7.2 and 7.6

0.04M EDTA GVB^{--}

230 ml of isotonic 0.086M EDTA pH 7.4 was made up to 500 ml with GVB^{--}

0.01M EDTA GVB^{--}

57.5 ml of isotonic 0.086M EDTA pH 7.4 was made up to 500 ml with GVB^{--}

D50S

50g D-glucose

5.95g NaCl

Made up to 100 ml with water

Mannitol GVB^{++}

9 ml 20% mannitol

3 ml GVB^{++}

Phosphate buffered saline (PBS) 150 mM

8.0g NaCl

1.21g K_2HPO_4

0.34g KH_2PO_4

Mg EGTA

38.04g EGTA was made up to 500ml in distilled water, concentrated NaOH was added until the pH was 7.4. The final volume was made up to 1 litre with distilled water.
To 10ml of the 0.1M EGTA, 7ml of $MgCl_2$ (0.1M) was added to give 0.1M Mg EGTA

2.3 Measurement of pH and conductivity.

pH: The pH of samples and column fractions were measured using a Pye-Unicam pH meter, Model 292.

Conductivity: The conductivity of samples and column fractions was measured at $0^{o}C$ using a conductivity meter model CDM3, Radiometer, Copenhagen.

2.4 Measurement of protein

Absorbance at 280 nm: The absorbance at 280 nm of samples and column fractions was read using a Shimadzu UV-120-02 spectrophotometer, with the buffer in which they were suspended being used to zero the spectrophotometer.

Folin assay: Protein was measured according to the method of Lowry et al (1951), using Folin and Ciocalteu's phenol reagent and known concentrations of bovine serum albumin (BSA) as standards.

The method employed is as follows:

Reagent A: 2g Na_2CO_3 Plus 20mg NaK tartarate made up to 100 ml with 0.1M NaOH

Reagent B: 0.15% $CuSO_4.5H_2O$

Reagent C: 5ml of A plus 0.1ml B made freshly on day of use.

Reagent D: Commercial Folin and Ciocalteu's phenol reagent diluted with an equal volume of water.

Into a series of test tubes 2ml of C was placed, to this a volume of the sample to be tested for protein was added, the volume being dependent upon the estimated protein concentration, i.e. small

volumes (10µl) for high protein concentrations and vice versa. To an additional 5 tubes, 5µl, 10µl, 25µl, 50µl and 75µl of BSA (1mg/ml) was added, this gives the standard protein curve. The negative control was 100µl of buffer. To all tubes 0.2ml of reagent D was added. The tubes were mixed thoroughly and left for 30 mins at room temperature (R.T.). The optical density of the suspensions was measured at 700nm. A standard curve was constructed from the readings for the known quantities of BSA used in the assay. From this curve the protein content of the unknown samples were determined.

2.5 Concentration of protein samples.

Sucrose concentration: Protein samples were placed in visking tubing sacks which had been boiled thrice in deionised water plus 0.001M EDTA. These sacks were covered in sucrose and left until the required degree of concentration was achieved.

Freeze drying: Protein samples were freeze dried using an Edwards freeze drier.

Vacuum dialysis: Protein samples were concentrated by vacuum dialysis using visking tubing, treated as described above.

Ultrafiltration: Samples were concentrated using an Amicon with YM30 or PM30 membranes, these membranes exclude all molecules smaller than 30000 daltons.

2.6 Preparation of glass plates for methods employing agarose.

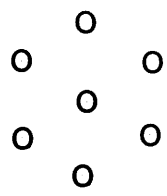
Glass plates were washed and cleaned with ethanol before use. Plates were poured on a level table and allowed to cool before the wells were punched. The size of plates used varied, 9.6ml of agarose were used for 10 x 10cm glass plates, 3ml of agarose for glass slides and 3.3ml of agarose for 50mm plastic petri dishes.

2.7 Double immunodiffusion (Ouchterlony technique, Ouchterlony, 1958)

Buffer: 5 litre of deionised water, 9.21g barbitone, 51.54g sodium barbitone and 5g sodium azide.

Agarose: 5g of agarose was added to 500ml of hot buffer and when dissolved, 15g of polyethylene glycol (PEG, molecular weight 4000) was added.

This technique was employed to identify antigens or test antiserum specificity, using known antiserum or antigens. Two or 5mm holes were punched in agarose gel on microscope slides in a pattern consisting of a central well surrounded by 6 equally spaced peripheral wells (as shown below). A distance of 10mm separated the inner



and outer wells. The inner well was filled to the brim with antiserum (or antigen) and the outer wells with antigen (or antiserum),

or doubling dilutions of a given sample. Plates were left overnight at R.T. in a humidified chamber to allow the antibody and antigen to diffuse towards each other. When the diffusion was complete the presence or absence of precipitation lines (indicating the specific interaction of antibody and antigen) was recorded. For large antigens or antiserum containing IgM the plates were allowed to develop for up to 48 hours (hrs).

This technique is sensitive (picking up to 1µg of protein), however its application is limited to precipitating antibody-antigen reactions. In general it was used as a rapid means of identifying proteins and screening antisera, but not for any quantitative analysis.

2.8 Radial immunodiffusion (Mancini technique, Mancini, Carbonara and Heremans,1965)

Buffer (a): As described in 2.7

Agarose(a): As described in 2.7

Agarose(b): 20 ml of 5 x VBS and 10 ml of 0.1M EDTA were heated together and 1.5g agarose was added, when dissolved the final volume was made up to 100ml with water.

This technique was used to quantitate antigen concentrations. Agarose(a) was used for non-complement components and agarose(b) for complement components. The appropriate agarose contained a monospecific antiserum at a dilution suitable for the determination of the antigen concentration. The radial immunodiffusion was performed in plastic petri dishes (diameter 50mm) covered with

3.3ml of agarose containing the appropriate antiserum dilution. Two mm wells were punched and filled to the brim with antigen or doubling dilutions of the test antigen together with doubling dilutions of a known standard antigen concentration. The plates were left at R.T. for 48hrs in a humidified chamber. The diameter of the precipitin rings were measured and a standard curve was plotted using the diameter squared (D^2) of the known standards against their antigen concentration (Fig. 6). From the standard curve the concentration of the unknown antigen was determined, this technique is reputed to be sensitive to 1-3µg/ml of antigen (or antibody). This, however, may be limited by the concentration of antibody (or antigen) in the gel.

2.9 Immunoelectrophoresis (IEP, Graber and Williams 1953)

Buffer (a): As described in 2.7

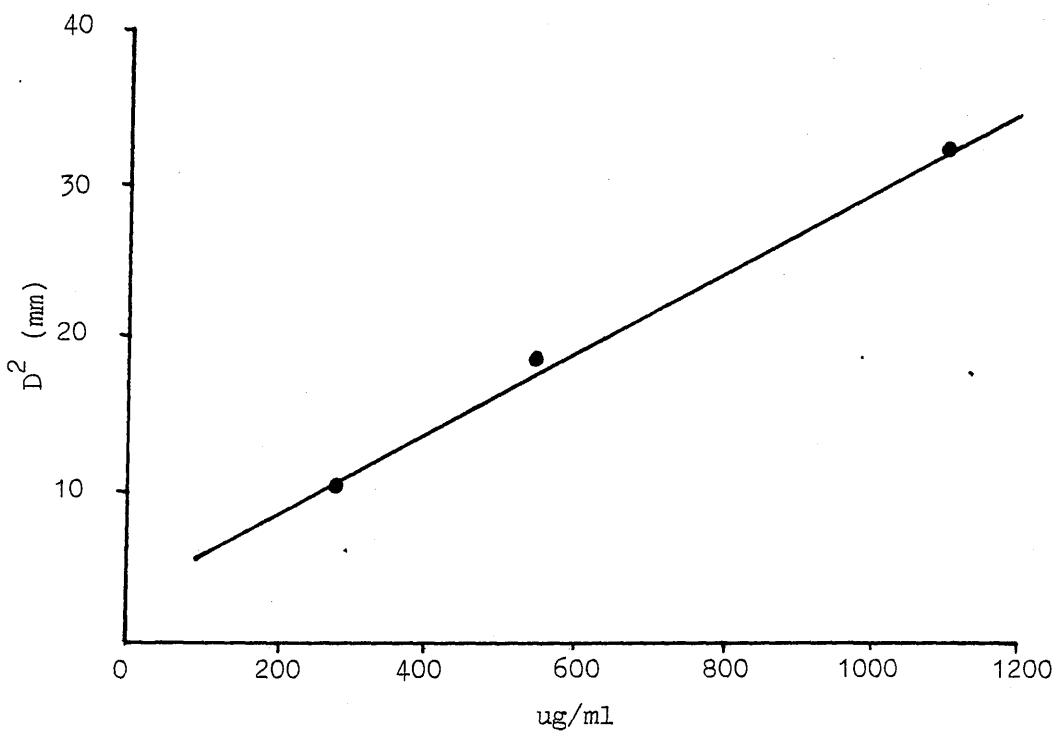
Agarose (a): As described in 2.7

Buffer (c): 17.0g of barbitone was dissolved in 500ml of water, 23.5 ml of 1M HCl was added to give a pH of 8.4. Finally the volume was made up to 2 litres with water. This was used as the IEP tank buffer.

Agarose (c): 1.5g of agarose was added to 100ml of buffer containing 4.5g sodium barbitone, 11.65ml of 0.086M EDTA, pH to 8.4 with approximately 32.5ml of 0.1M HCl with the remaining volume being made up with water.

IEP combines electrophoretic separation, diffusion and immune precipitation of proteins. In most cases it was used to identify proteins and their cleavage products. Glass plates were covered with agarose (agarose (a)) for electrophoresis of non-complement proteins

FIG. 6 Example of a standard curve for a Mancini plate


40
30
20
10
D^2 (mm)
0
200
400
600
800
1000
1200
ug/ml

and agarose(c) for electrophoresis of complement proteins) and a series of wells (2mm) and troughs were cut in the agar using a template. The wells were filled to the brim with the test antigen and the plate was subjected to electrophoresis for 2 to 3 hrs at 40mA. The extent of the electrophoresis was monitored by the passage of bromophenyl blue across the plate. After electrophoresis the appropriate antiserum was placed in the troughs and both antigen and antibody were allowed to diffuse at R.T. overnight in a humidified chamber. The resultant precipitation arcs between antigen and antibody were recorded.

2.10 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) phosphate buffer (tube gels)

Protein samples were electrophoresed in polyacrylamide tube gels in the presence of 0.1% SDS according to the method of Weber, Pringle and Osborn (1972). Four and 5% gels were made up from stock solutions as follows:

Stock	4% gel	5% gel
30g acrylamide, 0.8g bis-acrylamide in 100ml water	10 ml	10 ml
1M phosphate buffer pH 6.5	7.5 ml	6.0 ml
Water	56.7 ml	43.4 ml
10% SDS	0.75ml	0.60ml
TEMED	40µl	30µl
Ammonium persulphate	75mg	60mg

The first four solutions were mixed together then the TEMED and ammonium persulphate were added, after the ammonium persulphate had dissolved the gels were immediately poured. The gels were cast in

glass tubes (0.5cm x 10cm), overlaid with water and left overnight to set. Before use the tube gels were pre-electrophoresed for 30 mins at 2mA per tube in tank buffer (100ml 1M sodium phosphate buffer pH 6.5 and 10ml 10% SDS made up to 1 litre with water). Protein samples were run reduced or non-reduced in sample buffer (0.5ml 10% SDS, 0.05ml 2-mercaptoethanol, 1.0ml glycerol, 0.05ml 1M sodium phosphate buffer, pH 6.5, 0.1ml 0.5% bromophenyl blue (tracking dye) made up to 5ml with water, for non-reduced sample buffer the 2-mercaptoethanol was excluded). Reduced samples were boiled for 2 mins prior to electrophoresis. The samples were layered onto the tube gel surface and electrophoresed at 6mA per tube at a constant current setting, the electrophoresis was stopped when the tracking dye (marking the buffer front) was about 1cm from the tube base. The gels were then removed and stained for 1hr at 37^{o}C with 0.1% Page blue G90 in methanol/acetic acid/water 5/1/4 (v/v/v). The gels were then destained with methanol/acetic acid/water 1/1/8 (v/v/v). Together with the protein samples reduced Sigma and BDH molecular weight markers (2.1) were run and used to form a standard curve from which the molecular weights of the protein samples could be determined (Fig. 7). This technique can pick up approximately 1µg of protein.

2.11 SDS-PAGE in slab gels.

Samples were electrophoresed in 5% polyacrylamide gels according to the method of Laemmli (1970). The following stock solutions were made up and used in the preparation of both the separating and stacking gels as follows:

FIG. 7 Standard curve for Sigma molecular weight markers (reduced)

Stock solution	Separating gel 5%	Stacking gel 3%
Acrylamide 30%/bis-acrylamide both (w/v)	10 ml	1.8 ml
0.75M Tris Hcl pH 6.8	-	3 ml
0.75M Tris Hcl pH 8.8	30 ml	-
10% SDS	0.6ml	0.18ml
Ammonium persulphate	60 mg	18 mg
TEMED	30 µl	9 µl
Water	19.4ml	13 ml

Both gels were prepared by mixing the above ingredients and the gels were poured immediately. Slab gels were poured between glass plates (20 x 20cm) separated by 1.0mm spacers, the separating gel was overlaid with water until set, this was removed and the stacking gel poured, a comb forming lanes was then inserted and the gel allowed to set. The electrophoresis buffer contained 10ml of 10% SDS, 33ml of 0.75M Tris and 220ml of 0.86M glycine made up to 1 litre with water. Samples were run reduced by boiling in an equal volume of reducing sample buffer (30ml of 10% SDS, 8.5ml of 0.75M Tris-Hcl, pH 6.8, 10ml glycerol and 5ml 2-mercaptoethanol made up to 100ml with water) for 2 mins. The samples were carefully layered on top of the gel and electrophoresed for approximately 2.5hrs at 35mA, at a constant current setting or until the tracking dye had reached the bottom of the gel. The gel was stained as described in 10.7. The electrophoretic mobilities for each protein were calculated and the system was calibrated using reduced Sigma high molecular weight markers as described in 2.10. The staining of gels with Page blue G90 allows 0.2µg of protein to be detected, however if gels are silver stained (see below) the sensitivity is increased 10-50 fold.

2.12 Silver staining.

Slab gels were stained using a silver staining kit. The following protocol was followed. The slab gel was:

1) fixed for 30 mins in 100ml of buffer containing 40% methanol and 10% glacial acetic acid,
2) fixed for 15 mins in 100ml of buffer containing 10% methanol and 5% glacial acetic acid,
3) fixed for 15 mins in 100ml of buffer containing 10% ethanol and 5% glacial acetic acid,
4) 200ml of oxidiser (20ml concentrate plus 180ml water) was added for 5 mins at R.T.,
5) gel was washed thrice with 400ml of water, 5 mins for each wash,
6) 200ml of silver reagent (20ml concentrate plus 180ml water) was added for 20 mins at R.T.,
7) the gel was then washed in 400ml of water for 20 mins.
8) 200ml of developer (1 bottle in 3.6 litres of distilled water) was added for 30 seconds, the developer was removed and the cycle repeated twice,
9) the reaction was stopped by adding 400mls of 5% glacial acetic acid for 5 mins,
10) the gel was finally stored in water.

The protein bands stain dark brown.

2.13 Separation of serum from rabbit blood.

Rabbit blood was collected into glass universal containers, heated to 37°C for 30 mins (to allow clot formation) followed by a further 30 mins at 4°C (to allow clot retraction). The blood was then spun and the serum decanted. The serum was further heat-inactivated

at 56°C for 30 mins to destroy the complement components. The serum was aliquot ed and stored at -20°C until required.

2.14 Standardization of erythrocytes (E)

Suspensions of sheep or human E's were standardized using the following method. A 100µl sample of E s was removed from a known volume and added to 2.9ml of water. The optical density of the lysate was measured using a wavelength of 541nm (OD_{541}) and 414nm (OD_{414}). An OD reading of 0.370 at OD_{541} corresponds to an E concentration of $1x10^9$ cells/ml, likewise 0.185 at OD_{541} equals $5x10^8$ cells/ml and 0.327 at OD_{414} equals $1x10^8$ cells/ml. The volume of the E suspension can be adjusted to the required cell concentration using the following formula:

$$\text{Volume of E required} = \text{Initial volume} \times \frac{\text{OD measured}}{x}$$

x = 0.327 at OD_{414} gives $1x10^8$ cells/ml

x = 0.185 at OD_{541} gives $5x10^8$ cells/ml

x = 0.370 at OD_{541} gives $1x10^9$ cells/ml

2.15 Radioiodination of proteins

Radiolabelled proteins were used for most of the work described in this thesis. The following method was used to label proteins with ^{125}I and is a modification of the method described by McConahey and Dixon (1966).

One ml of protein (1mg/ml) in PBS was incubated in a glass bijoux bottle with 10µl of freshly made Chloramine T (5mg/ml in

PBS) and 37 MBq of carrier-free ^{125}I for 70 seconds at R.T. Then 50µl of L-tyrosine (0.5mg/ml in 0.01M NaOH) was added to stop the reaction. At this stage a 10µl sample was taken enabling the determination of labelling efficiency and total isotope input.

Protein-bound ^{125}I was separated from free isotope by passage over a 0.5cm x 8cm Dowex (100-200 mesh) column equilibrated with PBS, collecting 0.5ml fractions. A 10µl sample from each fraction was added to 0.99ml of PBS and these were counted in an automatic gamma counter (Packard, Model 300C) to determine the distribution of radioactivity. The tubes containing the radiation peak were pooled. This contains the radiolabelled protein, the free iodine remains bound to the column. The quantity of radiolabelled protein was determined by precipitation in 10% trichloracetic acid. This was performed by adding 10µl of the pooled radiolabelled protein to 200µl of BSA (10%) and 1ml of TCA (20%). After mixing the tubes were spun at 10000g for 5 mins and the counts in the supernatant and pellet were measured. The percentage precipitable radiolabelled protein is equal to the counts per minute (cpm) in the pellet divided by the cpm in the pellet and supernatant times 100. The protein content of the pool was determined by measuring the absorbancy at 280nm (2.4).

C H A P T E R 3

PURIFICATION OF CR1 FROM HUMAN ERYTHROCYTES AND THE PRODUCTION OF ANTISERUM TO CR1.

INTRODUCTION

CR1 was originally purified from human erythrocytes in 1979 by Fearon, who lysed washed human erythrocytes in hypotonic buffer and then solubilized the washed membranes with the non-ionic detergent NP-40. Soluble CR1 was purified by sequential ion exchange, gel filtration and affinity chromatography. Prior to the commencement of my project, this purification method had been successfully repeated in this laboratory by Dr. W. S. Kilpatrick who also raised a rabbit anti-CR1 antiserum. The purification procedure was repeated twice unsuccessfully during the course of this project; with modifications to the method a third attempt was fruitful and this method will be described.

The antiserum used throughout the project was raised by Dr. W. S. Kilpatrick. This antiserum was monospecific giving a single precipitin line when tested against purified CR1 by double immunodiffusion. Its specificity for CR1 was confirmed by its ability to inhibit the agglutination of C3b coated erythrocytes by CR1, to agglutinate human lymphocytes, monocytes and PMNs which bear CR1 and its inability to agglutinate trypsin treated human erythrocytes which no longer bear CR1.

METHODS

3.1 Column screening procedure

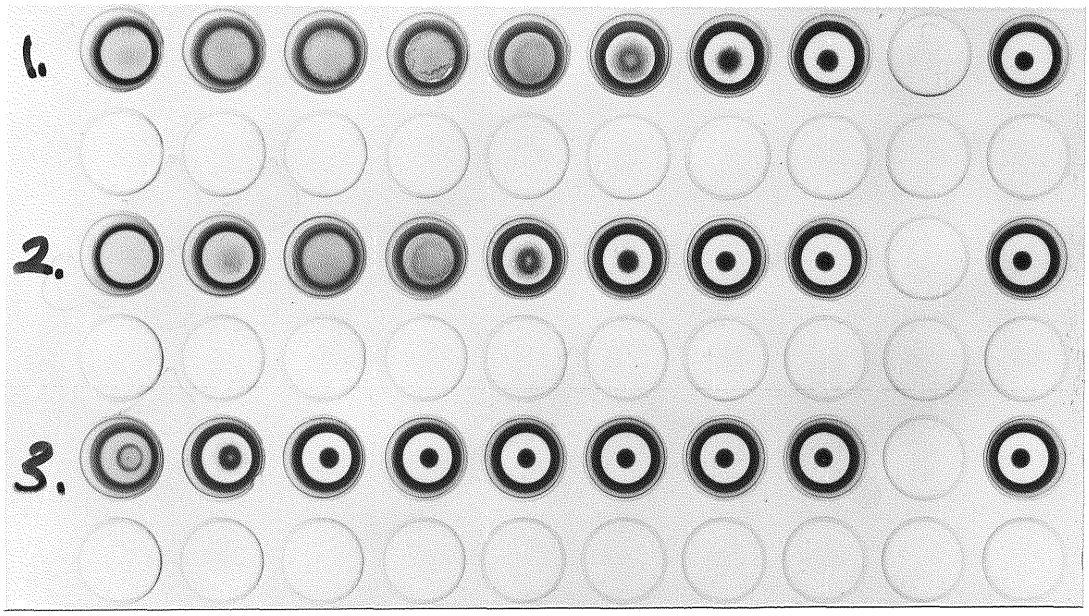
The presence of CR1 in the starting material and column fractions were screened for using the haemagglutination of EAC43b (3.3); fractions were screened for protein using the Folin assay (2.4), pH (2.3) and conductivity (2.3).

3.2 Preparation of EAC43b (Lachmann & Hobart, 1979)

Treatment of human serum with zymosan: 100mg of zymosan was suspended in 10ml of isotonic VBS and boiled for 30 mins. The suspension was then washed thrice in isotonic VBS and finally resuspended in 10ml. One ml of the zymosan suspension was then spun down, the supernatant removed and the pellet was resuspended in 10ml of normal human serum (NHS). This was incubated for 1 hr at $37^{o}C$ in a shaking water bath. The suspension was then centrifuged for 5 mins at $2^{o}C$ at 1000g. The supernatant was decanted, the serum is now depleted of C3 by approximately 50% and this is called R3.

Sheep red blood cells coated with rabbit IgG anti-SRBC were kindly given by Mrs. J. Veitch and these were standardized to $2x10^{8}$/ml in GVB^{++} (2.14), 20ml was put into a round bottomed glass flask and warmed to $37^{o}C$. To this was added 1ml of warmed human R3 followed 75 seconds later by 200µl of warmed Antrypol (100mg/ml). This mixture was incubated for a further 2 mins at $37^{o}C$ in a shaking water bath, then 20ml of ice cold GVB^{++} was added and the cells were washed thrice in ice cold GVB^{++}.

PLATE 1. Haemagglutination of EAC43b by purified CR1. Far righthand wells are the controls.


1.
2.
3.

After the final spin the cell pellet was resuspended in 5ml of ice cold 0.01M EDTA in GVB^{--} and incubated for 2 hrs at $37^{o}C$ in a shaking water bath to allow the decay of the terminal complement components. Finally the cells were washed thrice in GVB^{++} and standardized to $1x10^{8}$/ml (2.14), the cells were stored at $4^{o}C$ until required.

3.3. Haemagglutination of EAC43b.

Fifty µl of GVB^{++} was put into the wells of a round bottomed micro-titre plate, and an additional 50µl was put into the first well; to this 5µl of the sample to be tested for CR1 was added. This was doubly diluted across 7 wells, then 50µl of EAC43b ($1x10^{8}$/ml) were added to each well and to an additional well containing buffer alone which acted as the control. The plate was gently shaken and left at R.T. for 2 to 4 hrs or until the cells in the control well formed a firm button in the well bottom. The degree of agglutination was then determined, expressing the haemagglutination titre as the last dilution at which there was positive agglutination. The result is expressed as the titre or the reciprocal titre (Plate 1).

3.4 Preparation of erythrocyte membranes.

Wash buffer: Isotonic saline 150mM containing 10mM benzamidine, 0.5mM phenyl methyl sulfonyl fluoride (PMSF) in dimethyl sulphoxide, 0.02% (w/v) sodium azide and 0.5mM isotonic EDTA.

Lysis buffer: 5mM sodium phosphate, 0.5mM isotonic EDTA

pH 7.8 containing 1mM benzamidine and 0.5mM PMSF.

Solubilization buffer: 5mM sodium phosphate, 0.5mM isotonic EDTA, 150mM NaCl, 1% NP-40, pH 7.5 containing 10mM benzamidine, 0.5mM PMSF and 0.02% (w/v) sodium azide.

Six packs of fresh blood and 12 packs of expired blood were pooled and washed thrice in wash buffer ensuring the complete removal of the buffy coat layer. The erythrocyte pellets were then resuspended after the final wash in lysis buffer and the final volume was made up to 7 litres with lysis buffer. This was left overnight at 4°C, the cell membranes were then washed with 100 litres of lysis buffer in a Pellicon cassette (Amicon) or until the membranes were free of haemoglobin. The Pellicon cassette is a positive pressure dialysis unit, and contains a membrane which has an exclusion limit of 2000000 daltons, therefore molecules larger than 2000000 daltons (i.e. CR1) are retained in the system recycled and continually washed free of haemoglobin. Once washed the membranes were concentrated using the Pellicon cassette, then made up to 1.3 litre with solubilization buffer. The membranes were solubilized overnight at 4°C with continuous stirring. The solubilized membranes were then spun at 48,200g for 30 mins. at 4°C in a Beckman ultracentrifuge model L2-65B to free the solution of insoluble material. The supernatant containing the solubilized CR1 was removed and to this sufficient ice-cold water containing 10mM benzamidine, 0.5mM isotonic EDTA, 0.02% sodium azide(w/v) and 0.5mM PMSF was added to lower the conductivity of the solution to the conductivity of the buffer used for the Biorex cation exchange column (5 to 6 mS at 0°C).

3.5 Biorex cation exchange column

Biorex 70 is a weakly acidic carboxylic cation exchanger binding positively charged ions, the extent of binding being dependent upon the degree of charge. The material to be applied to the column should be dialysed into the starting buffer at a pH above the isoelectric point therefore giving the molecules a net positive charge. These will bind to the Biorex and can be eluted by increasing the salt concentration as the Na^+ ions competitively displace the positively charged bound proteins.

Buffer: 50mM sodium phosphate, 20mM NaCl, 0.2% NP-40 pH 7.2, containing 10mM benzamidine, 0.5mM isotonic EDTA, 0.02% (w/v) sodium azide and 0.5mM PMSF.

Column: Biorex 70 mesh size 100-200 was extensively equilibrated with buffer until the final pH was 7.2. and a conductivity of 5 to 6 mS at $0^{o}C$, after degassing the Biorex 70 was poured into a 5x30cm column. Two column volumes of buffer were run through before sample application.

The solubilized erythrocyte membranes (1800ml) were applied to the column with a flow rate of 80ml per hour, collecting 20ml fractions. Once the starting material had been washed through with buffer until the protein content of the fractions was less than 0.01mg/ml, a 5 litre 0.3M linear salt gradient was applied to elute the CR1 from the column. The column fractions were screened as described in 3.1. The tubes showing positive haemagglutination were pooled and concentrated using ultrafiltration (2.5) with a PM30 membrane. To this concentrated pool a four-fold volume of ice cold water containing

10mM benzamidine, 0.5mM isotonic EDTA, 0.02% sodium azide (w/v) and 0.5mM PMSF was added. This pool was applied to the C3-sepharose affinity chromatography column.

3.6 C3-sepharose affinity chromatography column.

A C3-sepharose affinity chromatography column was used to bind and immobilise all C3b-binding material from the Biorex 70 pool, while the non-C3b-binding material was washed straight through the column. C3b-binding material was then specifically eluted with NaCl and NP-40. As C3b is the natural ligand for CR1, it should bind specifically and be eluted with the NaCl and NP-40.

Buffer: 10mM sodium phosphate pH 7.5 containing 10mM benzamidine, 0.5mM isotonic EDTA, 0.02% sodium azide (w/v) and 0.5mM PMSF.

Column: C3-sepharose was prepared as follows:

CNBr-activated Sepharose 4B (2g) was washed 4 times in 0.001M HCl and the pellet was then resuspended in 2ml of human C3 at 4mg/ml (purified as described in Chapter 6) in 0.1M $NaHCO_3$ plus 0.15M NaCl. This was incubated overnight at 4°C with continuous mixing. The suspension was then spun for 5 mins at 1000g, the supernatant was removed and its protein content established by measuring the absorbancy at 280nm (2.4). This enabled the calculation of how much C3 had bound to the sepharose beads, this was found to be 99%. The sepharose beads were then incubated with a 10-fold volume of 1M ethanolamine pH 8.0 for 2 hrs at R.T. with mixing, to inactivate any uncoupled sites. The beads were then washed with 0.1M acetate

buffer pH 4.0 containing 1.0M NaCl followed by 0.1M borate buffer pH 8.0 containing 1.0M NaCl, this washing cycle was repeated thrice. The conjugated sepharose beads were then resuspended in buffer, and 5ml of the C3-sepharose was poured into a 5ml syringe barrel, the end of which had been blocked with glass wool.

The diluted pool from the Biorex column (3.5) was applied to the C3-sepharose column collecting 15ml fractions. A step-wise elution scheme was used to elute CR1. During the wash steps 5ml fractions were collected and for the elution steps 2ml fractions were collected. The flow-rate was variable as the column was small and not attached to a pump. The elution scheme was as follows: 50ml of buffer, 50ml of buffer containing 0.1% NP-40, 50ml of buffer, 50ml of buffer containing 200mM NaCl, 50ml of buffer and finally 50ml of buffer containing 0.1% NP-40 and 200mM NaCl. The column fractions were screened as described in 3.1, the tubes containing CR1 activity as determined by haemagglutination were pooled and dialysed into the starting buffer for the lentil lectin-sepharose column.

3.7 Lentil lectin-sepharose affinity chromatography column.

Lentil lectin binds glycoproteins and this material bound to sepharose was used to specifically bind proteins containing glycoprotein eluted from the C3-sepharose column. CR1 contains glycoprotein and therefore should bind specifically to the lentil lectin-sepharose. Bound material was competitively eluted using α-methyl-D-mannoside.

Buffer: 10mM Tris/HCl, 200mM NaCl, 0.1% NP-40, 0.7mM $CaCl_2$, 0.7mM $MgCl_2$, 0.7mM $MnCl_2$ pH 7.4 containing 10mM

benzamidine, 0.5mM isotonic EDTA, 0.02% sodium azide (w/v) and 0.5mM PMSF.

Column: 1ml of lentil lectin-sepharose in a plugged glass pipette.

The dialysed pool from the C3-sepharose column was applied to the lentil lectin-sepharose column, the column was then washed with buffer (20ml) and the CR1 was then eluted using buffer containing 200mM α-methyl-D-mannoside. Two ml fractions were collected and these were screened as described in 3.1. The tubes containing CR1 activity as determined by haemagglutination were concentrated by ultrafiltration using a PM-30 membrane (2.5). The protein content and the haemagglutination titre of the concentrated pool were re-determined. The purity of the CR1 was tested using SPS-PAGE in 5% slab gels (2.11) with silver staining (2.12).

3.8 Production of anti-CR1 antiserum.

Using both pool 1 and pool 2 of purified CR1, 700ul of the protein was made up to 2ml with PBS. An equal volume of Freund's complete adjuvant was added and the mixture was emulsified and injected subcutaneously into rabbits. This was repeated a further 3 times at 3 to 4 week intervals, substituting Freund's incomplete for Freund's complete adjuvant. A further 2 boosts were given at 3 weekly intervals intraperitoneally. The rabbits were then bled during intervals over a week (2 weeks after the last boost) and exsanguinated at the end of this period.

The antiserum specificity was tested using double immuno-

diffusion (2.7).

3.9 Anti-CR1 antiserum produced by Dr. W.S.Kilpatrick.

The anti-CR1 antiserum used for the experiments documented in this thesis was raised prior to the initiation of this project by Dr. W. S. Kilpatrick. The following briefly outlines the nature of the antigen and the immunization schedule used. 0.5ml of CR1 purified from human erythrocytes (protein concentration unknown) was emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly and subcutaneously into stock rabbits. After two boosts at 14 day intervals with 0.5ml of CR1 emulsified in an equal volume of Freund's incomplete adjuvant, the rabbits were bled at intervals during the following 7 days with exsanguination at the end of this period.

The specificity of the antiserum was tested using double immunodiffusion (2.7), inhibition of lymphocyte and neutrophil rosette formation and agglutination of human erythrocytes (Fearon, 1979).

For all experiments using anti-CR1 the serum from only one rabbit was used.

RESULTS.

A total of 18 packs of blood, 12 expired and 6 fresh, were used for the isolation of CR1 from the erythrocyte membranes. After washing in saline the cells were lysed in 7 litres of lysis buffer giving erythrocyte ghost membranes and the released cellular contents. The ghost membranes were washed free of haemoglobin using positive pressure dialysis in a Pellicon cassette. This apparatus allows the continuous and rapid washing of the membranes and within 24 hrs using 100 litres of lysis buffer the membranes were free of haemoglobin. Using the Pellicon the membranes were then concentrated and re-suspended in 1.3 litre of solubilizing buffer. After overnight stirring at $4^{o}C$ the membranes dissociate because of the presence of detergent in the solubilization buffer. At this stage the membranes had a haemagglutination titre of 1/250. The protein content was not tested because of the high density of cellular material present which would make an accurate determination difficult. The insoluble material was removed by ultracentrifugation. The supernatant containing the soluble CR1 had a protein content of 1.55mg/ml (in a volume of 1.3 l) and a haemagglutination titre of 1/250; therefore no CR1 activity had been lost with the removal of the insoluble material. By adding 500ml of ice cold water the ionic strength of this pool was lowered to 5.5mS (at $0^{o}C$), the conductivity of the starting buffer for the Biorex column.

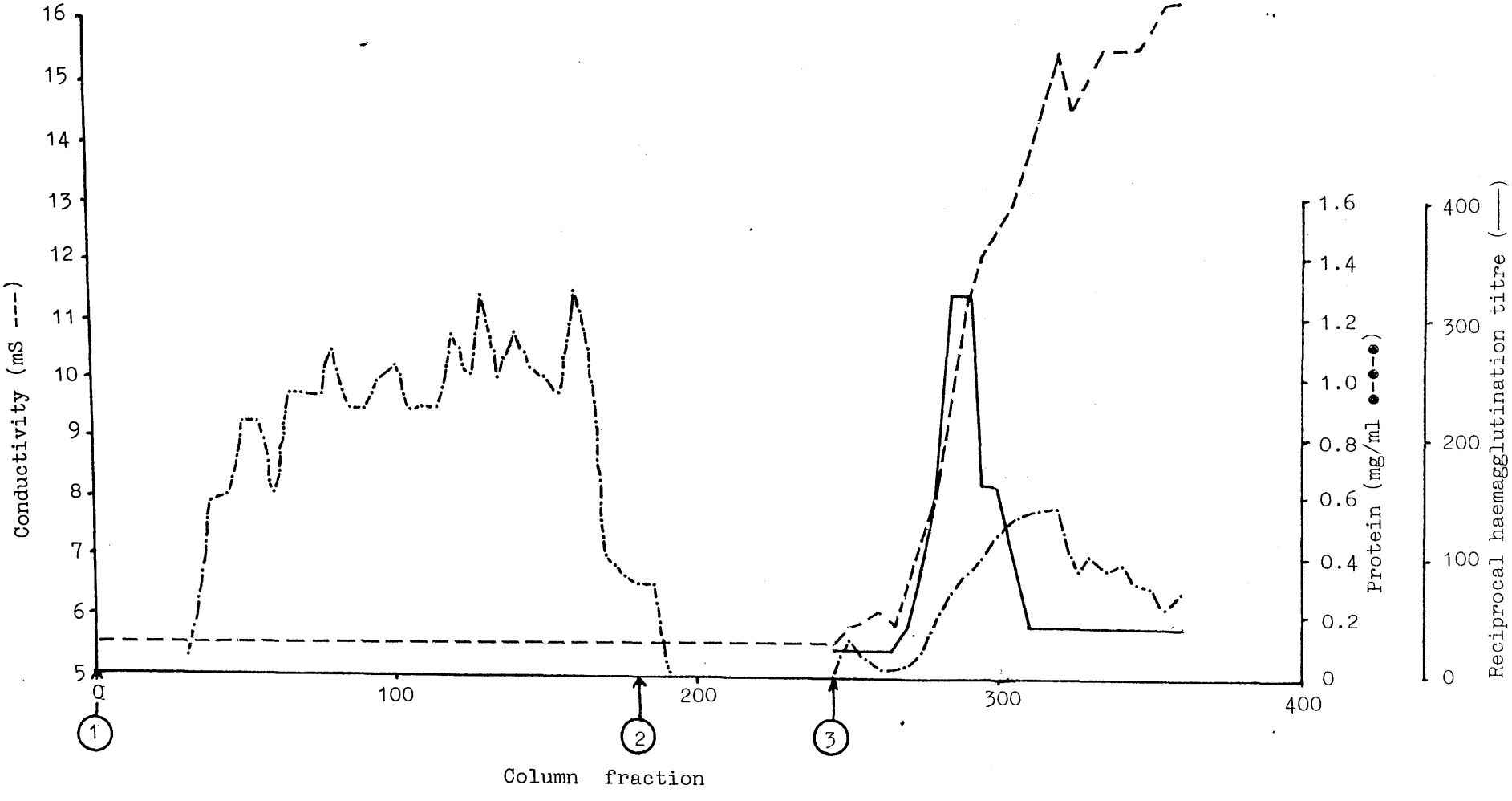
The starting material (1800ml) was applied to the Biorex-70 cation exchange column (Fig. 8). A large amount of protein did not bind to the column and was eluted in the first 200 fractions; however, none of these fractions showed positive haemagglutination. The column was then washed with 600ml of buffer and the salt

FIG. 8. Elution profile from Biorex-70 100-200 mesh column, showing conductivity (mS at $0^{o}C$ ---), protein (mg/ml o-o-) and reciprocal haemagglutination titre (———).

(1) Application of sample,

(2) Application of wash buffer,

(3) Application of 600mM linear salt gradient.


Conductivity (mS ---)
16
15
14
13
12
11
10
9
8
7
6
5
0
100
200
300
400
Column fraction
1
2
3
Protein (mg/ml ●-●-●)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Reciprocal haemagglutination titre (——)
400
300
200
100
0

gradient was applied. Immediately the gradient was applied a small amount of weakly bound protein was eluted (fractions 240-255), this had no CR1 activity. CR1 was eluted between a conductivity of 6 to 14mS (at 0°C) from fraction 265 to 310, the CR1 containing fractions eluted immediately prior to the major protein peak. Fractions 265 to 310 were pooled to give a volume of 550ml. This volume was then reduced to 234ml by ultrafiltration using an Amicon flow cell (2.5) with a PM30 membrane. The protein content of the concentrated pool was 0.71mg/ml and the haemagglutination titre was 1/640. One hundred and thirty four ml of the pool was frozen at -70°C for further purification at a later date, the remaining 100ml was mixed with 400ml of ice-cold water to lower the conductivity to 2.8mS (at 0°C).

This 500ml pool was applied to a 5ml C3-sepharose column. The column profile (Fig. 9) shows most of the proteins to have passed straight through the column or to have been eluted with the first wash or the first elution buffer containing NP-40 applied at fraction 38 (Fig 9,[3]) after which no detectable protein was eluted. CR1 with a haemagglutination titre of 1/20 was eluted between fractions 5 to 29 and 51 to 63. However the majority of the CR1 was eluted with the column buffer containing 0.1% NP-40 and 200mM NaCl (Fig 9, [7]) with a peak haemagglutination titre of 1/1280. Fractions 92 to 104 were pooled (45ml), the pool had a haemagglutination titre of 1/320 but had no detectable protein. This pool was dialysed into the starting buffer for the lentil lectin Sepharose affinity chromatography column.

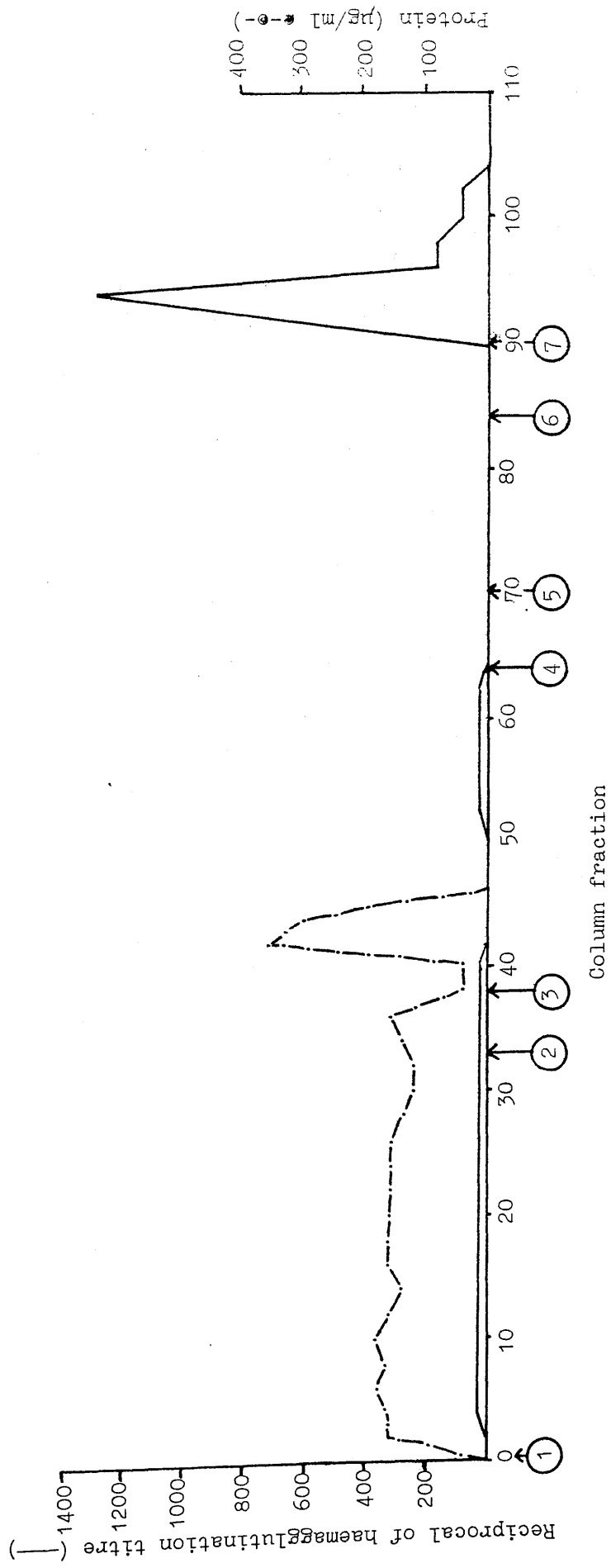

Reciprocal of haemagglutination titre (——)
1400
1200
1000
800
600
400
200
Protein (μg/ml ●-●-)
400
300
200
100
0
10
20
30
40
50
60
70
80
90
100
110
Column fraction
1
2
3
4
5
6
7

FIG. 9 Elution profile of CR1 from C3-sepharose 4B affinity chromatography column.

[1] Application of sample,

[2] Application of wash buffer,

[3] Application of wash buffer, plus 0.1 % NP-40,

[4] Application of wash,

[5] Application of wash buffer plus 200mM NaCl,

[6] Application of wash buffer,

[7] Application of wash buffer plus 0.1% NP-40 and 200mM NaCl

All of the 45ml pool was applied to the 1ml lentil-lectin-Sepharose column (Fig. 10). The column was screened for protein using the Folin assay but none was detected. CR1 was eluted with the elution buffer containing ∝-methyl-D-mannoside in two distinct peaks, both having a haemagglutination titre of 1/1280. Therefore two pools were made, pool 1 consisting of fractions 23 to 25 and pool 2 fractions 26 to 31. Pool 1 had a volume of 4ml and pool 2 was 8ml, these were concentrated using ultrafiltration to 2ml and 2.5ml respectively. The protein content and the haemagglutination titres of the two pools are given below.

	Pool 1	Pool 2
Protein ug/ml	48	32
Haemagglutination titre	1/1280	1/1280

The purified protein from pools 1 and 2 were anlysed by SDS-PAGE using 5% slab gels (2.11). The purity was tested by silver staining the gel (2.12), as this method has a high sensitivity, detecting very low concentrations of contaminants. A single protein band was detected (Plate 2), this had a reduced molecular weight of 230000 which is consistent with that described by Fearon (1979). A summary of the CR1 purification is given in Table 11.

Antiserum was successfully raised against the purified CR1 and showed positive reactivity against CR1 when tested by double immunodiffusion (3.8, Plate 3).

FIG. 10 Elution profile of CR1 from lentil-lectin-Sepharose 4B affinity chromatography column.

(1) Application of sample,

(2) Application of wash buffer,

(3) Application of wash buffer plus 200mM α-methyl-D-mannoside.

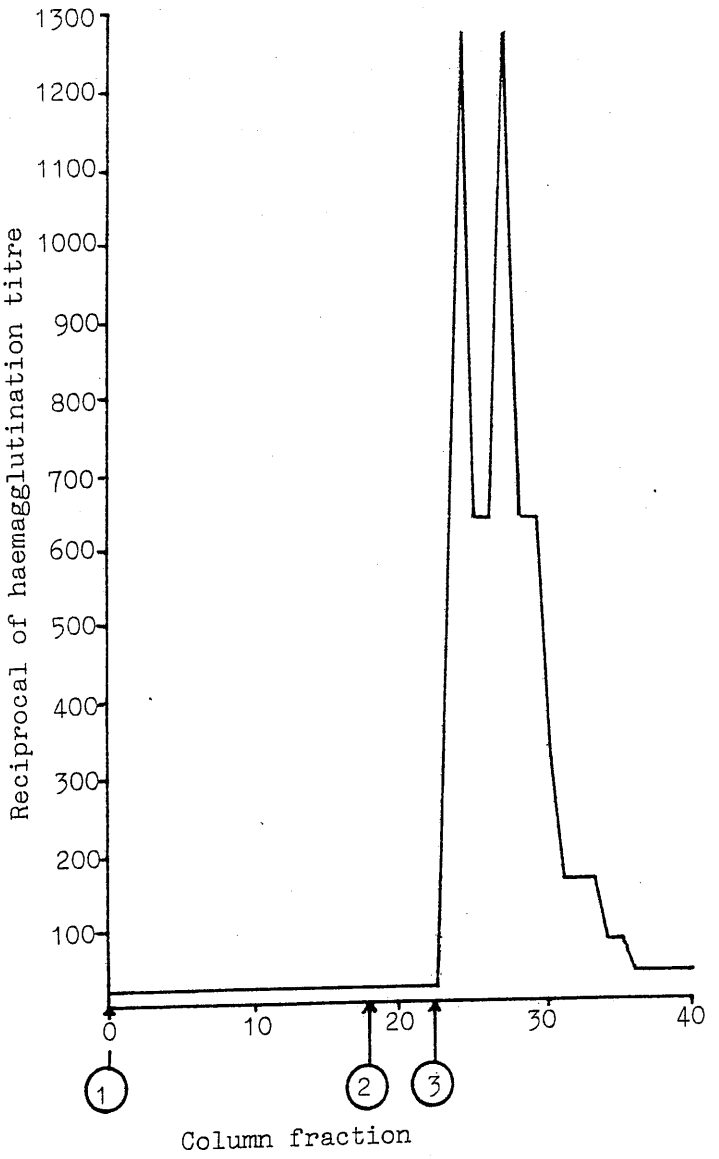

1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
Reciprocal of haemagglutination titre
0
10
20
30
40
1
2
3
Column fraction

TABLE 11. Summary of CR1 purification profile

	Protein content	Haemagglutination titre	Volume
Lysed membranes	N.T.	N.T.	7000 ml
Solubilized membranes	N.T.	1/256	1300 ml
Biorex column S.M.	1.55 mg/ml	1/256	1800 ml
C3-Sepharose S.M.	710 ug/ml	1/640	234 ml*
Lentil lectin-Sepharose S.M.	N.D.	1/320	45 ml
Purified CR1 pool 1	48 ug/ml	1/1280	2 ml
pool 2	32 ug/ml	1/1280	2.5 ml

N.T. - not tested

N.D. - not detectable

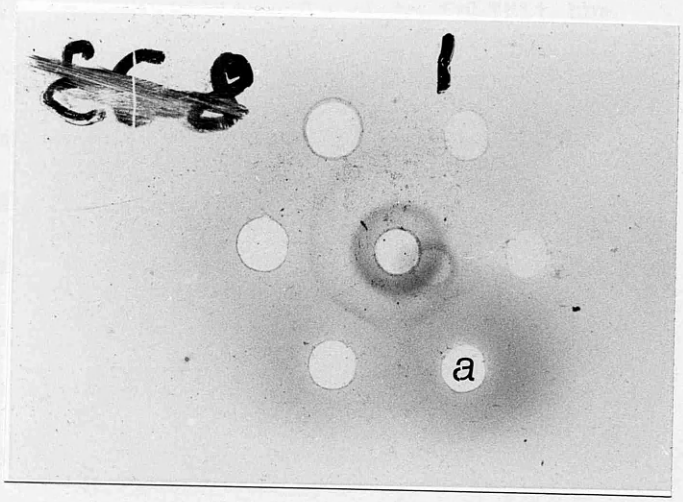
S.M. - starting material

* - only 100ml further purified.

PLATE 2 Purified CR1 (track a = pool 1, track b = pool 2) run reduced on a 5% SDS-PAGE Laemmli slab gel, silver stained (track c = reduced Sigma molecular weight markers)


a
b
c

PLATE 3 Double immunodiffusion plate showing positive reaction between pure CR1 (centre well) and doubling dilutions of rabbit anti-CR1 antiserum (a = neat, doubly diluted in a clock-wise direction)


a

DISCUSSION

Prior to this successful purification of CR1, I had made two previous unsuccessful attempts following precisely the method described by Fearon (1979). There were 3 main problems with this method which probably lead to the loss of CR1 during purification. Firstly, we were informed by Dr. R. Sim (personal communication) that CR1 is exquisitely sensitive to degradation, however, in Fearon's method there was only one enzyme inhibitor used, PMSF and only at the stage when the cells were being washed free of haemoglobin. Secondly, the column was screened for CR1 using an assay which quantified the ability of CR1 to inhibit the activity of the alternative pathway C3 convertase ($C3b\overline{BbP}$). This assay was time consuming, and whilst controls gave repeatable results the samples did not always do so; hence it was very difficult to establish exactly where CR1 was being eluted. Thirdly, the time scale of the purification was in excess of 4 weeks, resulting from a) the use of the ultracentrifuge to wash the membrances free of haemoglobin which could take up to 10 days, and b) the inability to get consistency between the assays used to screen for CR1. Therefore the combination of CR1's sensitivity to proteolysis, the variability in screening the column for CR1 and the time taken for purification probably accounts for the lack of success of this method on the two previous attempts. These difficulties were overcome by introducing the following modifications to the method: a) proteolytic inhibitors, e.g. benzamidine, PMSF, sodium azide and EDTA, were included in all the buffers throughout the purification, b) the haemagglutination of EAC43b was used to screen for CR1; this assay was quick, sensitive and reproducible, c) membranes were washed rapidly

by pressure dialysis in a Pellicon cassette. This procedure took only 12 hours whereas 10 days were required to wash the membranes by ultracentrifugation.

Approximately $1x10^3$ washed human erythrocytes were used for the purification of CR1, this is equivalent to the amount used by Fearon (1979). The cells were then lysed using a hypotonic buffer, washed free of haemoglobin and the cleaned membranes were then solubilized using the non-ionic detergent NP-40 releasing the CR1 into the soluble phase. This solution had a haemagglutination titre of 1/256 and this was not reduced when the insoluble material was removed by ultracentrifugation. The soluble material in the supernatant was applied to the Biorex anion exchange column after the conductivity had been reduced to 5.5 mS (at 0^oC) using ice cold water.

A total of 2015mg of protein was applied to the Biorex column (in 1800ml) with a haemagglutination titre of 1/256. CR1 was eluted with the salt gradient between 6 and 14 mS (at 0^oC), this was not mirrored by the protein profile (Fig. 8). This pool (550ml) was concentrated by ultrafiltration to 234ml (haemagglutination titre 1/640) and had a total protein content of 166mg (0.71mg/ml), therefore 8% of the applied protein was recovered in this pool. Only 100ml of this pool was processed further, the remainder being stored at -70^oC for use at a later date.

In the original paper (Fearon, 1979) the next purification step was the passage of the pool over a Bio-Gel A-5M gel filtration column. This step was omitted because on the two previous attempts it was found that it did not contribute significantly to the purification of CR1. Furthermore it brought about significant dilution of the CR1 pool.

The 100ml pool was then applied to a C3-Sepharose followed by a lentil-lectin affinity chromatography column, with CR1 binding to its natural ligand C3b linked to the Sepharose and being eluted with NP-40 and a high salt buffer; and with the lentil-lectin Sepharose colum CR1 would bind via its carbohydrate moieties using α-methyl-D-mannoside for elution.

The majority of CR1 was eluted from the C3-Sepharose column using NP-40 and 200mM NaCl, however this contained no detectable protein, with all the protein passing straight through the column or being eluted with 0.1% NP-40. Some C3 binding protein was eluted using NP-40 alone, this protein agglutinated EAC43b at a very low titre. This maybe CR1 or perhaps another protein exhibiting C3 binding properties. The fractions containing CR1, as determined by haemagglutination were pooled (Fig 9). This pool (45ml) contained no detectable protein and had a haemagglutination titre of 1/320; this was then applied to the lentil-lectin Sepharose column after dialysis into the starting buffer. CR1 was eluted in two peaks both with a haemagglutination titre of 1/1280. It was possible that these two peaks represented two of the polymorphic variants of CR1, therefore two pools were made. As for the C3-Sepharose column, no detectable protein was found in the column fractions containing CR1. These fractions were concentrated, pool 1 contained 48ug/ml and pool 2 32ug/ml, in total 176ug. However, if the whole 234ml had been processed the recovery would have been approximately 412ug and this represents 0.02% of the starting material applied to the Biorex column. Fearon's (1979) recovery from approximately the same quantity of erythrocytes was 150ug of CR1; therefore the modifications to this method increased the yield nearly two-fold.

The silver staining of the slab gel shows the CR1 to be

pure (Plate 2) and a single band of molecular weight 230000. There was no obvious molecular difference between the CR1 of pools 1 and 2 from the lentil-lectin Sepharose column. However, minor differences may have existed between the CR1 of those two pools, for example, carbohydrate composition. Such minor differences may not have been picked up by SDS-PAGE. Both of these pools had a final haemagglutination titre of 1/1280.

C H A P T E R 4

METHOD FOR QUANTITATING THE NUMBER OF ANTIGENIC CR1 SITES PER CELL USING A RADIOIMMUNOASSAY.

INTRODUCTION

The number of CR1 antigenic sites per erythrocyte have previously been quantitated by Wilson et al (1982) and Iida et al (1982) using a radioimmunoassay (RIA). The latter group used a radiolabelled monoclonal antibody directed against the receptor whilst the former group used a polyclonal antiserum. As the anti-CR1 antiserum used in this project was polyclonal (3.9) the assay system used was modified from the method described by Wilson et al (1982). Basically the assay quantitates the amount of ^{125}I-labelled F(ab')$_2$anti-CR1 bound to the cell surface and from this, using Scatchard plot analysis (Scatchard, 1949) the number of receptor sites for the F(ab')$_2$ anti-CR1 molecules per cell can be calculated. However, as the antiserum is polyclonal two controls were introduced into the system. Firstly a batch of cells were pre-incubated with an excess of F(ab')$_2$ derived from normal rabbit serum IgG (NRS F(ab')$_2$) prior to incubation with labelled F(ab')$_2$ anti-CR1. The excess of NRS F(ab')$_2$ blocks non-specific binding sites and this allows the total binding of the label to the cells to be measured. Secondly a batch of cells were incubated with an excess of unlabelled F(ab')$_2$ anti-CR1 prior to the addition of ^{125}I-F(ab')$_2$ anti-CR1. The excess of cold F(ab')$_2$ anti-CR1 blocks all specific binding sites and therefore any binding of the label to the cells is non-specific. The specific binding of the ^{125}I-F(ab')$_2$ anti-CR1 is equal to the total, minus the non-specific, and it is this value which is used for Scatchard plot analysis.

The F(ab')$_2$ moieties of both the NRS and anti-CR1 antiserum were used to stop the interaction of the Fc portion with Fc γ receptors of leucocytes.

This assay was initially established on erythrocytes as they

were easily isolated in a pure form and in large numbers. Using erythrocytes the interassay variation was established and the effect of storage of blood at $4^{o}C$ on the CR1 numbers. This was necessary as it seemed likely that patient samples taken in afternoon clinics, would require to be stored for assaying the next day.

Lay and Nussenzweig (1968) and Huber et al (1968) established the trypsin sensitivity of CR1. We examined the effect trypsin had on CR1 levels investigating whether there was a dose dependent removal of CR1 by different amounts of trypsin and whether the assay could pick this up.

Once the RIA conditions were established using erythrocytes, the assay system was then extended to quantitate CR1 levels on all the peripheral blood cells. Fearon (1980) did single point determinations of CR1 numbers on erythrocytes, B cells, PMNs and monocytes using a polyclonal $F(ab')_2$ anti-CR1 and found that they had 950, 21,000, 57,000 and 48,000 sites per cell respectively. However the study was not extended to examine a larger sample of the population. In 1982 Iida et al examined CR1 numbers in the lysates of peripheral blood erythrocytes, mononuclear cells and PMNs using a monoclonal antibody to CR1, and found them to have respectively 1,400, 38,000 and 140,000 CR1 per cell. However the number of individuals studied was not disclosed.

The aim was thus to investigate this area further by examining CR1 levels on intact cells which is perhaps more akin to the <u>in vivo</u> situation, and to look at the distribution of CR1 levels on the peripheral blood cells.

The protocol used to separate the cell populations from whole blood involved several steps. The blood was firstly spun over a Ficoll-Hypaque gradient. This gave two cellular pools, a pool containing erythrocytes and PMNs which were separated further

using dextran sedimentation, and a pool of lymphocytes and monocytes. These were separated using tissue culture flasks coated with the micro-exudate from baby hamster kidney cells (BHK flasks), the monocytes adhere to the micro-exudate within a 45 minute period, leaving free in suspension the lymphocytes which are decanted. The monocytes are then 'stripped off' using EDTA. This method of lymphocyte-monocyte separation was used in preference to the more conventional technique of allowing monocytes to adhere to tissue culture plates, as this procedure takes up to two hours, inhibiting the completion of the RIAs on the same day as the blood was sampled.

All assays were performed on ice to stop internalization of the $F(ab')_2$ fragments by the leucocytes. EDTA was present in the media during the RIA to stop cell clumping.

Peripheral blood samples from 10 people were studied, 8 were normal and 2 were RA patients (criteria for RA patients is given in 7.3).

MATERIALS AND METHODS

4.1 Normal rabbit serum (NRS)

NRS was obtained by bleeding stock rabbits from the marginal ear vein as required. The serum was separated from the blood as described in 2.13.

4.2. Rabbit anti-human erythrocyte CR1

Anti-CR1 was prepared as described in 3.9 and the serum was separated from the blood as described in 2.13.

4.3 Purification of IgG from rabbit serum

Diethylaminoethyl cellulose (DE52) is an anion exchanger binding negatively charged ions, the extent of binding being dependent upon the degree of charge. The material to be applied to the column should be dialysed into the column buffer (pH 7.6, 0.8 to 1mS at 4°C), under these conditions IgG should not bind to the column and be washed straight through, with the remaining proteins being retained.

Column: 2.7 x 9.0cm column (60ml syringe barrel) containing DE52 extensively equilibrated with buffer.

Buffer: 0.01M sodium phosphate buffer pH 7.6 (0.8 to 1mS at 4°C).

A 10 to 15ml serum sample dialysed into 0.01M sodium phosphate buffer pH 7.6 was applied to the column and washed through with buffer.

Five ml fractions were collected, the protein concentration of each tube was measured using the absorbancy at 280nm (2.4). The elution profile from such a column is shown in Fig. 11. The tubes containing the protein peak were pooled and concentrated using sucrose or freeze drying (2.5). The purity of the IgG was tested using immunoelectrophoresis (2.9) with sheep anti-whole rabbit serum antiserum. A single precipitin arc indicated pure IgG. The IgG was also run on 5% SDS-PAGE in phosphate buffer (2.10) and presented as a single band with a molecular weight of 150,000 daltons. Approximately 50 to 75mg of IgG was recovered per 10ml of serum applied.

The IgG was aliquoted and stored at -20°C until required.

C1q and P may be eluted under these conditions, these were not detected on the gel and if present would not interfere with the RIA.

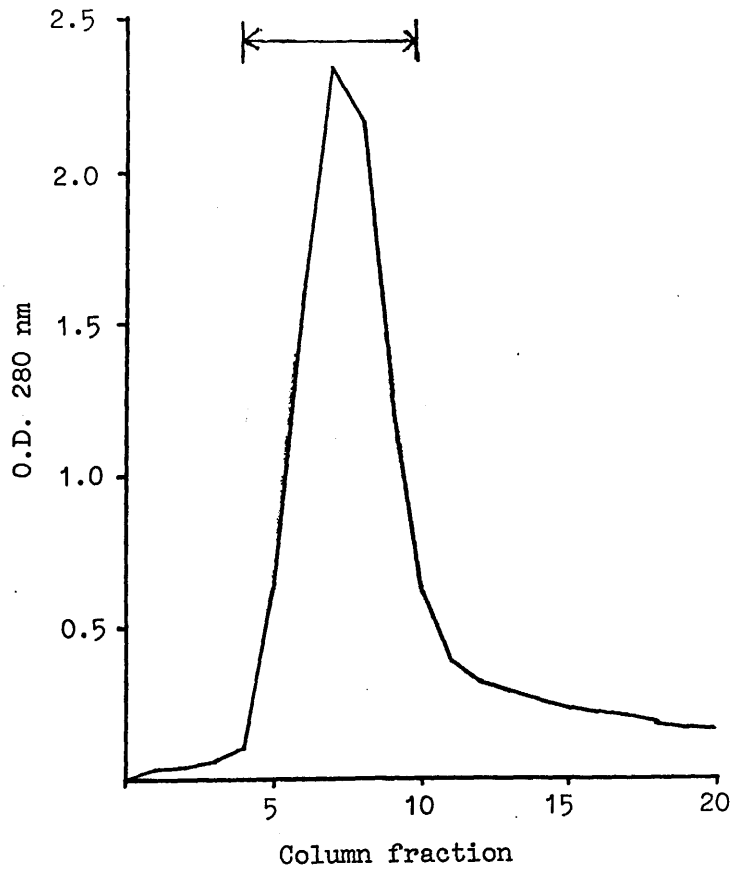
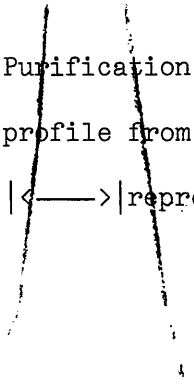

2.5
2.0
1.5
1.0
0.5
O.D. 280 nm
5
10
15
20
Column fraction

FIG.11 Purification of rabbit IgG, elution profile from a DE52 column, |<——>| represents fractions pooled.



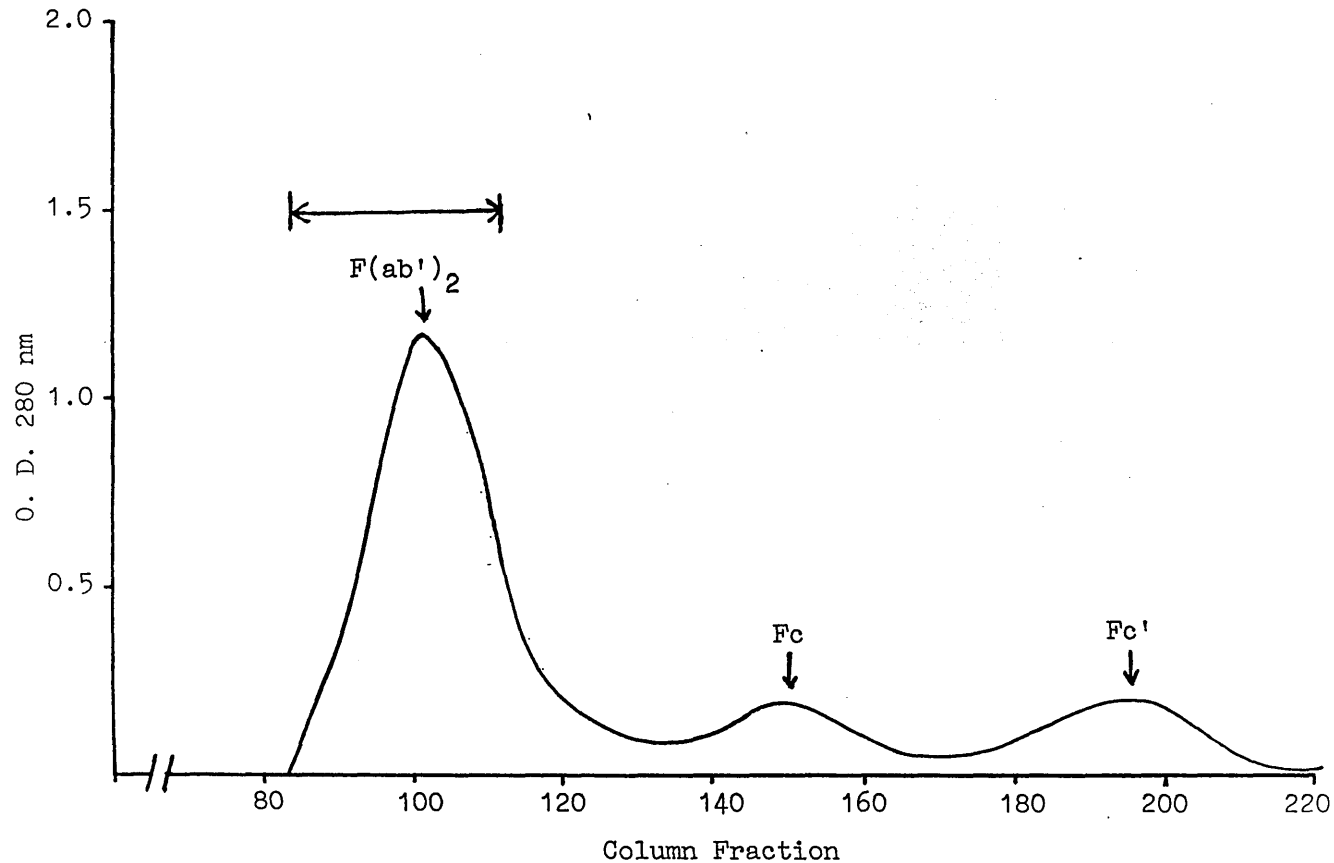

2.0
1.5
1.0
0.5
O. D. 280 nm
F(ab')2
Fc
Fc'
80
100
120
140
160
180
200
220
Column Fraction

FIG 12 Elution profile from a Sephadex G150 superfine column, showing the separation of $F(ab')_2$, Fc and Fc' fragments, |<——>| represents fractions pooled

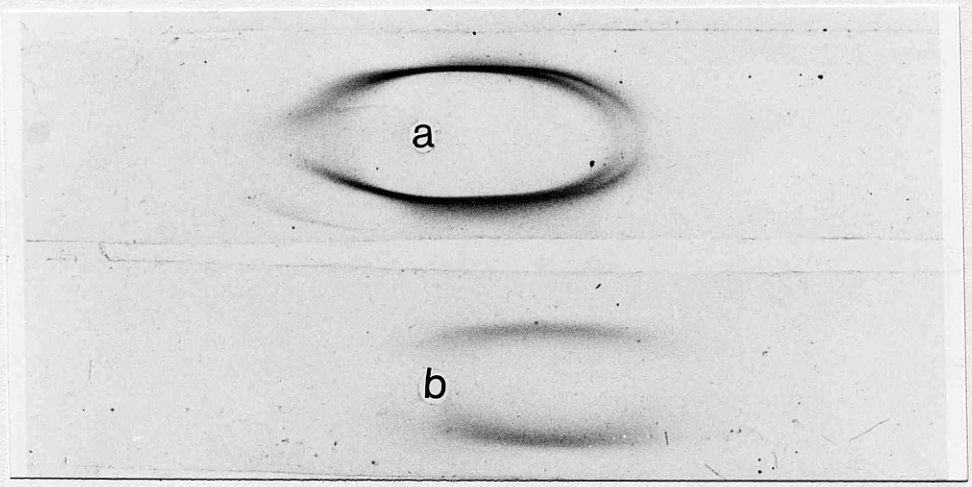
4.4 Preparation of $F(ab')_2$ fragments from rabbit IgG

IgG was purified from NRS (4.1) and anti-CR1 antiserum (4.2) according to the method described in 4.3. Aliquots of the rabbit IgG were dialysed overnight at 4°C into 5 litres of 0.1M sodium acetate. After dialysis the pH of the IgG pool (approximately 5ml, variable protein concentration) was adjusted to 4.5 with 0.1M acetic acid, warmed to 37°C and then incubated overnight at 37°C with 1mg of porcine pepsin. The digest was then spun at 4,500g for 10 mins to precipitate any insoluble material. The supernatant was decanted and the pH was adjusted to 7.4 using solid Tris. The digest was then concentrated using sucrose or freeze drying (2.5) prior to gel filtration or applied directly to the gel filtration column.

Gel filtration was used to separate $F(ab')_2$, Fc and any undigested IgG. A 3 to 5ml sample of digested IgG was applied to a 1.5x90cm Sephadex G150 superfine column equilibrated with PBS. The flow rate was 10 to 12ml per hour and 1ml fractions were collected. These were screened for protein by measuring the absorbancy at 280 nm (2.4). The peaks obtained were screened for IgG, $F(ab')_2$ and Fc by double immunodiffusion (2.7) and immunoelectrophoresis (2.9) using a 1/10 dilution of sheep anti-rabbit $F(ab')_2$ specific antiserum to precipitate $F(ab')_2$, and also 5% SDS-PAGE tube gels (2.10).

The conversion of IgG to $F(ab')_2$ was visualized by immunoelectrophoresis, with the $F(ab')_2$ fragments being more electronegative than intact IgG therefore travelling further towards the anode (Plate 4). Double immunodiffusion was used as a first line screen of the peaks obtained from the gel filtration column to distinguish the pool containing Fc (which showed no reaction with sheep anti-rabbit $F(ab')_2$ antiserum) and those containing $F(ab')_2$ or undigested IgG. The latter two pools were distinguished

PLATE 4. Immunoelectrophoresis plate showing the difference in electrophoretic mobility between rabbit IgG (well a) and rabbit $F(ab')_2$ (well b). The trough contained a 1/10 dilution of sheep anti-rabbit $F(ab')_2$.


a
b

by immunoelectrophoresis and by SDS-PAGE gels, with intact IgG having a molecular weight of 150,000 daltons and $F(ab')_2$ fragments 100,000 daltons in a non-reduced form. The elution profile for a Sephadex G150 superfine gel filtration column is shown in Fig. 12.

The retained pools of NRS $F(ab')_2$ and $F(ab')_2$ anti-CR1 were stored in aliquots at -20°C until required.

4.5 Preparation of human erythrocytes

10ml of venous blood was mixed with 20µl of preservative free sodium heparin in a glass universal container. The blood was then washed three times in RPMI 1640 containing 1% BSA (RPMI-BSA) ensuring the complete removal of the buffy coat layer. The erythrocytes were standardized to 2.5×10^8/ml (2.14)

4.6 Radiolabelling $F(ab')_2$ anti-CR1

$F(ab')_2$ anti-CR1 was radiolabelled with carrier-free ^{125}I as described in 2.15.

4.7 RIA to determine the number of antigenic sites per erythrocytes for $F(ab')_2$ anti-CR1

200µl of erythrocytes at 2.5×10^8/ml were added to 36 (12 tubes in triplicate) microcapped centrifuge tubes (MCC tubes) To tubes 1 to 6, 12.5µg of NRS $F(ab')_2$ was added. To tubes 7 to 12,

12.5ug of $F(ab')_2$ anti-CR1 was added. The tube contents were mixed thoroughly and then incubated for one hour at 4°C on a Matburn mixer. Increasing concentrations of ^{125}I-$F(ab')_2$ anti-CR1 were then added to tubes 1 to 6 and 7 to 12 as outlined in the table below:-

Tube number	1 & 7	2 & 8	3 & 9	4 & 10	5 & 11	6 & 12
^{125}I-$F(ab')_2$ anti-CR1 added (µg)	0.25	0.5	1.0	2.0	3.0	4.0

The tubes were inverted by hand and incubated for one hour at 4°C on a Matburn mixer, after which the contents were layered onto 300µl of separating fluid (6:4 dibutyl phthalate: dinonyl phthalate) in MCC tubes and spun at 8,000g for 2 mins. This separates the erythrocytes from the fluid in which they were suspended. The cells carrying bound ^{125}I-$F(ab')_2$ anti-CR1 were pelleted to the tube bottom and the unbound isotope remained on top of the separating fluid. The supernatants were then removed from each tube and discarded. The base of the tube containing the cell pellet was cut off and placed in a glass test tube. These tubes were then counted in a Packard gamma-counter for one minute to determine the amount of radioactivity bound to the cell pellet (counts per minute, cpm). Six empty tubes were included in each run to determine the machine background and 2 tubes containing 10µl of ^{125}I-$F(ab')_2$ anti-CR1 were included to calculate the input of isotope into each tube.

4.8 Numerical calculations

The machine background was subtracted from all cpm values. The triplicate values attained for bound cpm in tubes 1 to 12 were averaged and the standard errors calculated. If however any of the triplicate values diverged by greater than 10% from one another they were not included in the calculation. The mean bound cpm for tubes 1 to 6 and 7 to 12 were plotted together with the standard errors against the input of $^{125}I\text{-}F(ab')_2$ anti-CR1 expressed as cpm. The line drawn through cpm values attained from tubes 1 to 6 represented the total binding of the isotope to the erythrocytes, whilst the line through the cpm values from tubes 7 to 12 represented the non-specific binding of the isotope to the cells. From these two curves the specific binding curve for $^{125}I\text{-}F(ab')_2$ anti-CR1 was obtained by subtracting the non-specific binding values from the total binding values over a range of input values. Using the specifically bound cpm at given input values, a Scatchard plot was drawn, plotting specifically bound cpm versus bound/free cpm. A line was drawn through these points using linear regression analysis and the intercept of the line with the Y axis was calculated. The value for the point of intercept was then multiplied by the correction factor (cf). This factor accounts for the decay of radiation, the number of cells in the assay tubes and converts the Y intercept value obtained in cpm into the number of $F(ab')_2$ anti-CR1 molecules per cell, utilizing 6.03×10^{12}, the number of $F(ab')_2$ molecules in 1μg (derived from Avagadro's number)

$$cf = \left(\frac{6.03 \times 10^{12}}{\text{(input cpm for 1μg of } {}^{125}I\text{-}F(ab')_2 \text{ anti-CR1)}}\right) \Big/ \text{Number of cells per tube}$$

4.9 Trypsinization of erythrocytes

Erythrocytes were washed, standardized to 2.5×10^8/ml (4.5) and incubated for one hour at 37°C with 1mg, 0.01mg and 0.0001mg of trypsin per ml of erythrocytes. After the incubation the cells were put directly onto ice and washed thrice with ice cold RPMI-BSA. The cells were then re-standardized to the required cellular concentration.

4.10 Media used for isolation of peripheral blood cells

Hanks buffered salt solution (HBSS): One packet of powdered HBSS was dissolved in 80ml of water, 5ml of 7.5% sodium bi-carbonate was added and made up to a final volume of 100ml with water. 50ml of the solution was filtered through a sterile Millex-GS 0.22µm filter unit into a sterile bottle containing 450ml of sterile water.

RPMI-culture media (RPMI-CM): RPMI 1640 was supplemented with penicillin (10,000 units/ml), streptomycin (10,000 mcg), fungazone (25 mcg) and 2mM L-glutamine.

RPMI-foetal calf serum (RPMI-FCS): As for RPMI-CM but supplemented with 10% FCS which had been heat-inactivated for 2 hours at 56°C.

EDTA-PBS : 25ml of 20 times normal strength PBS was added to 25ml of water and 65ml of 0.086M isotonic EDTA pH 7.2 to 7.4. This was filtered as described for HBSS preparation and added to 450ml of sterile water.

Ficoll-Hypaque: 25ml of 33.9% Hypaque and 60ml of 9% Ficoll were mixed together to give Ficoll-Hypaque with a specific gravity of 1.077.

4.11 Cell Separation

40ml of venous blood was aliquotted into 2 sterile universals containing 20µl of preservative-free sodium heparin. The 20ml aliquots were then mixed with 15ml of RPMI-CM and layered onto 15ml Ficoll-Hypaque in a sterile Falcon tube. The tubes were then spun at R.T. for 45 mins at 400g. After centrifugation the upper layer containing diluted plasma was decanted and retained. The white interfacial band containing the monocytes and lymphocytes was harvested and treated as described in 4.13. The remaining Ficoll-Hypaque was discarded and the pellet composed of erythrocytes and PMNs was treated as described in 4.12.

4.12 Separation of erythrocytes and PMNs

The pellet from the Ficoll-Hypaque separation was mixed with an equal volume of retained plasma. This was cooled for 30 mins at $4^{\circ}C$ and then mixed with 6% dextran (w/v) in PBS in a ratio of 400µl dextran to 1ml of blood. This was placed in a measuring cylinder and left at $37^{\circ}C$ for 30 mins or until the erythrocytes had sedimented below 50% of the starting volume. The top 50% which contained the PMNs contaminated with erythrocytes was removed and placed in sterile universal container. The contaminating erythrocytes were removed by repeated water shocking, returning the solution to isotonicity each time

by adding an equivalent amount of 2N saline as water. The PMNs and erythrocytes were finally washed and resuspended in RPMI-BSA plus 0.01M isotonic EDTA pH 7.2 to 7.4 (RPMI-BSA-EDTA).

4.13 Separation of monocytes and lymphocytes

Four BHK flasks (flasks in which baby hamster kidney cells have been cultured and then removed leaving the flasks coated with a micro-exudate to which monocytes preferentially adhere leaving the lymphocytes in suspension, kindly given by Mrs. L. Jones) were washed twice with HBSS before use. The white interfacial layer from the Ficoll-Hypaque separation was made up to 48ml with RPMI-FCS and 12ml aliquots were added to each BHK flask.

Each flask was gassed with a mixture of 5% CO_2 in air for 30 seconds and incubated at 37°C for 45 mins. The supernatant containing the non-adherent lymphocytes was decanted into a sterile universal container and washed in RPMI-BSA-EDTA. The flasks containing the adherent monocytes were then washed thrice with warmed RPMI with gentle swirling to remove any loosely bound cells. The platelets were then removed by adding per flask 5ml of 1:1 EDTA-PBS to RPMI-FCS for 30 seconds. After decanting this mixture, a second aliquot of 5ml was added to the flasks, which were then incubated for 15 mins at 37°C, in order to remove the monocytes from the micro-exudate. These were then washed in RPMI-BSA-EDTA.

4.14 Determination of CR1 sites/cell on human peripheral blood cells

Erythrocytes were standardized to $2.5x10^8$/ml in RPMI-BSA-EDTA (2.14). Leucocytes were counted in an improved Neubauer counting chamber (depth = 0.1mm 1/400mm^2) and adjusted to the following in RPMI-BSA-EDTA; monocytes $1x10^6$/ml, lymphocytes $5x10^6$/ml and PMNs $1x10^6$/ml. The RIA was performed as described in 4.7 but with the following modifications; a) all stages other than the separation of the cells from fluid were performed on ice, b) all points were performed in duplicate and c) only 5 input values of $F(ab')_2$ anti-CR1 were used 0.25, 0.5, 1.0, 2.0 and 3.0µg of protein.

RESULTS

Rabbit NRS IgG and rabbit IgG anti-CR1 were purified from their respective sera using anion exchange chromatography (4.3). The IgG was further cleaved using pepsin to obtain $F(ab')_2$ and Fc fragments and occasionally undigested IgG and Fc' fragments. These moieties were separated by gel filtration using Sephadex G150 superfine (4.4). The purity of the retained NRS $F(ab')_2$ and $F(ab')_2$ anti-CR1 was tested using double immunodiffusion, immunoelectrophoresis and SDS-PAGE phosphate buffer tube gels (4.4). Once purified these $F(ab')_2$ fragments were used to develop the RIA for quantitating the number of CR1 sites per erythrocyte for the ^{125}I-$F(ab')_2$ anti-CR1 molecule. The method used was based loosely on that described by Wilson et al (1982). As an example of how the receptor levels were calculated, the results of a RIA performed on the erythrocytes of a 30 year old

normal male are given. Table 12 shows the raw data in cpms. Columns a, b and c represent the triplicate determinations for tubes 1 to 6 (representing total binding of ^{125}I-F(ab')$_2$ anti-CR1 to the erythrocytes) and tubes 7 to 12 (representing non-specific binding of ^{125}I-F(ab')$_2$ anti-CR1 to the erythrocytes). The mean of these values, the mean minus the machine background, standard deviation and standard errors are tabled. The numbers asterisked were not used in further calculations as they deviated from the remaining two numbers by greater than 10%. However, this rule was not always possible to follow, for example tube 5, the triplicate values were 6746, 8428 and 9729 . These clearly differ by greater than 10%, but as no two numbers are close in their value, the mean of the three values was taken. These results were then plotted as a graph (Fig. 13). The Y-axis represents the amount of ^{125}I-F(ab')$_2$ anti-CR1 bound to the erythrocyte pellet and the X-axis the input of radiation expressed as cpms and μg of protein. The total binding curve (tubes 1 to 6) is concave to the abscissa indicating that the binding sites for the ^{125}I-F(ab')$_2$ anti-CR1 are saturable. The non-specific binding curve (tubes 7 to 12) however is linear indicating non-saturable binding sites. The specific binding curve attained by subtracting the non-specific binding values from the total binding values is also concave indicating saturable binding sites for the radiolabelled ligand.

By using the values for the specifically bound cpm at input values of 2000×10^2 to 4000×10^2 (Table 13), the free cpm can be calculated and a Scatchard plot drawn, plotting specifically bound cpm versus specifically bound divided by free cpm. The Scatchard plot derived from the figures in Table 13 is shown in Fig. 14. This graph is not normally drawn, with the intercept

TABLE 12 Raw data from RIA

in cpms

Tube number	a	b	c	Mean	Mean-B	SD	SE
1	1446	1373	1094*	1410	1080	52	37
2	2354	2292	2356	2332	2002	33	19
3	4264	4360	4235	4286	3956	65	38
4	5884	5988	6236	6036	5706	181	104
5	6746	8428	9729	8301	7971	1496	863
6	9712	9054	7521*	9383	9053	465	329
7	1217	1146	1004	1122	792	108	63
8	1655	1206*	1866	1761	1431	149	106
9	3085	2080	2665	2610	2280	505	291
10	3576	3807	3540	3641	3311	145	84
11	7151*	4502	3595	4049	3719	641	454
12	5915	4690	4160	4922	4592	900	520

B = mean-machine background

SD = Standard deviation

SE = Standard error

* = value not included in calculation

FIG. 13. Binding curves for ^{125}I-$F(ab')_2$ anti-CR1 to normal human erythrocytes from a 30 year old male

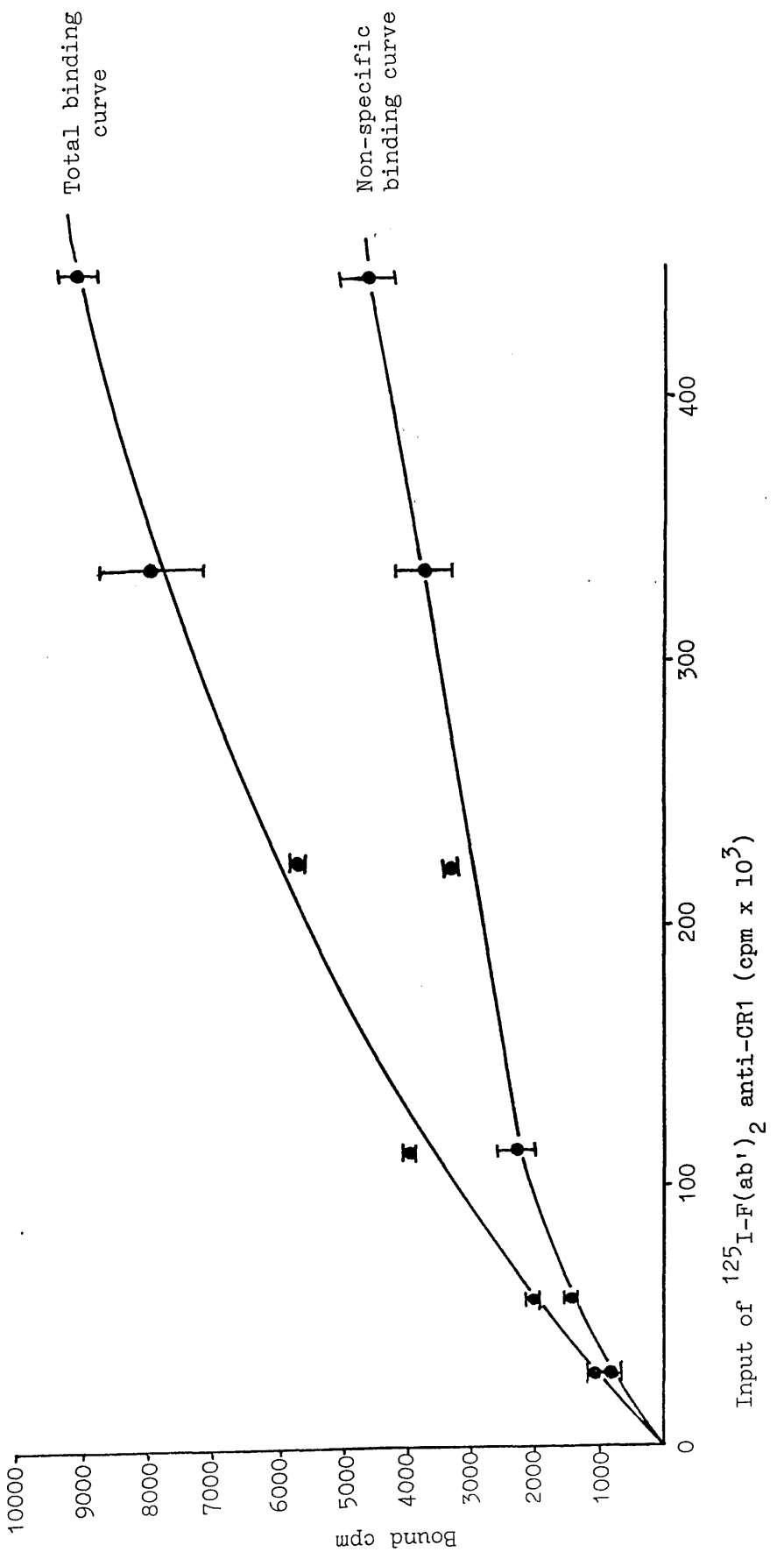

Total binding curve
Non-specific binding curve
Bound cpm
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
100
200
300
400
Input of ^{125}I-F(ab')$_2$ anti-CR1 (cpm x 10^3)

TABLE 13. Data used to construct a Scatchard plot

Input x 10^2	TB	NSB	SB	Free	SB/Free
2000	5600	2800	2800	197200	0.01420
2500	6550	3150	3400	246600	0.01379
3000	7300	3500	3800	296200	0.01283
3500	8000	3900	4100	435900	0.01185
4000	8600	4250	4350	395650	0.01099

TB = total bound cpm

NSB = non-specifically bound cpm

SB = specifically bound cpm

Free = free cpm

FIG. 14 Scatchard plot

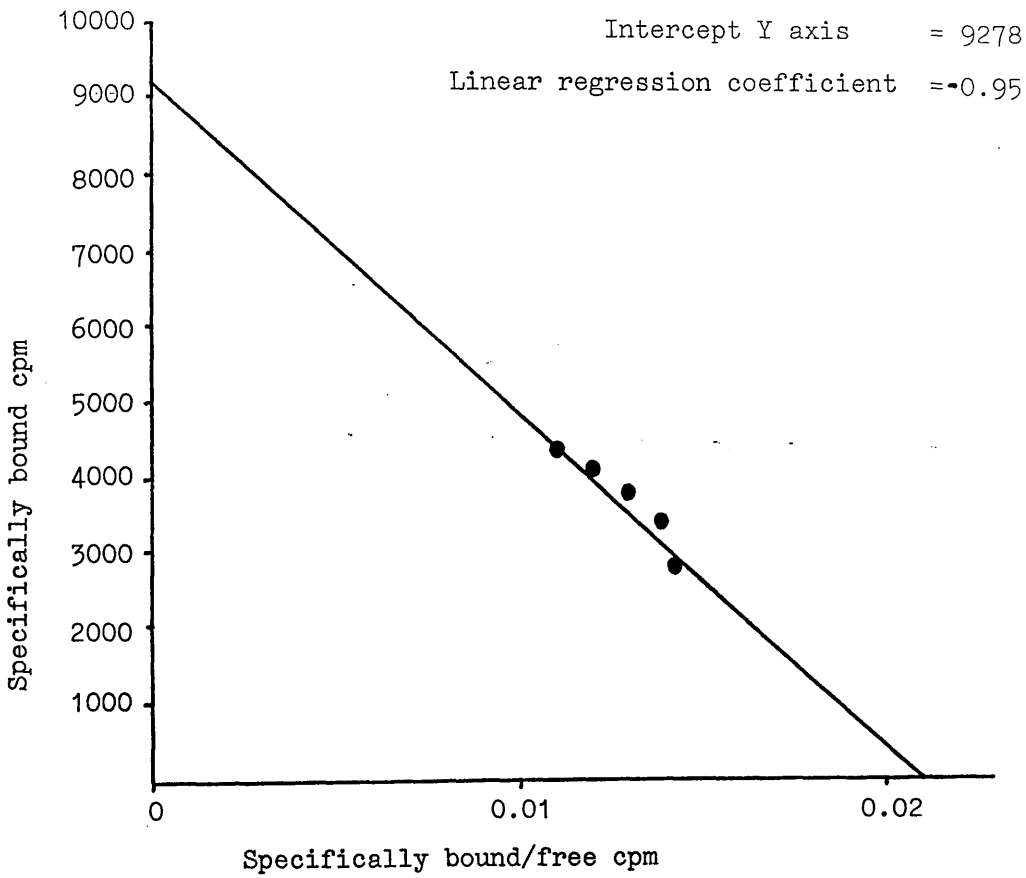
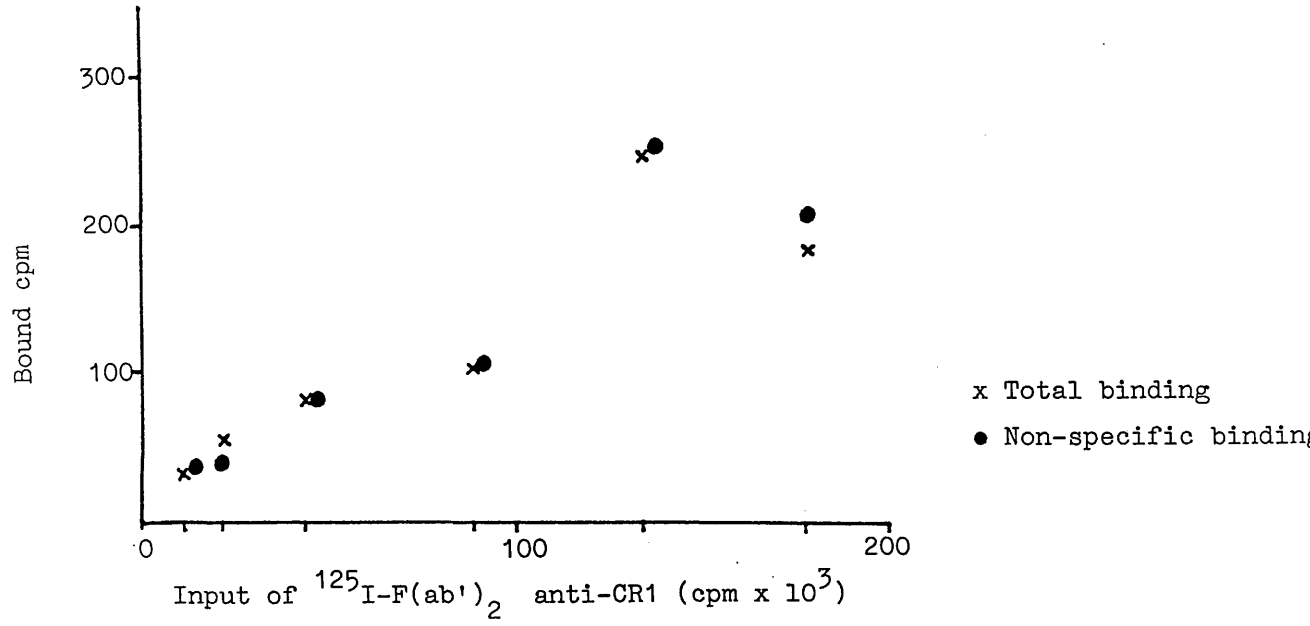

Intercept Y axis = 9278
Linear regression coefficient =-0.95
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
Specifically bound cpm
0
0.01
0.02
Specifically bound/free cpm

FIG. 15 Binding curves for ^{125}I-F(ab')$_2$ anti-CR1 to normal erythrocytes treated with 1mg/ml trypsin

x Total binding curve values

. Non-specific binding curve values (slight displaced to right where values overlap)


300
200
100
Bound cpm
0
100
200
Input of ^{125}I-F(ab')$_2$ anti-CR1 (cpm x 10^3)
x Total binding
● Non-specific binding

with the Y-axis being obtained by linear regression analysis. In this assay the intercept with the Y-axis is at 9278 and the cf (4.8) for this assay was 1.06; therefore there were 9834 sites per erythrocyte for the ^{125}I-F(ab')$_2$ anti-CR1 molecule (CR1 sites/cell). Assays which gave a Scatchard plot with a linear regress coefficient (r) of less than -0.8 were repeated.

The interassay variation was assessed by performing the RIA on a single normal blood sample twice on a given day; it was found to be approximately 10%.(e.g. 5064 and 5520, 1260 and 1316). The effects of storage of the erythrocytes at 4^{o}C in RPMI-BSA was also tested; in a single study it was found to have no effect on CR1 numbers within a 24hr storage period (see below).

	CR1 sites/erythrocyte
Pre-storage	9389
Post-storage	9734

The effects of storage periods greater than 24 hrs were not tested and this would require further investigation.

The effects of trypsin on CR1 numbers was examined by incubating erythrocytes with 3 doses of trypsin (1mg/ml, 0.01mg/ml and 0.0001mg/ml) for 1 hour at 37^{o}C (4.9); the excess trypsin was removed by repeated washings and the receptor levels were quantitated pre- and post- incubation (4.7). The effects of trypsin on the erythrocytes from 5 normal individuals were studied. In all cases, 1mg/ml of trypsin brought about total removal of the receptors (Fig. 15), with the total binding curve and the non-specific binding curve for ^{125}I-F(ab')$_2$ anti-CR1 to the trypsinized erythrocytes overlapping, making it impossible

to derive a specific binding curve. The lowest dose of trypsin (0.0001 mg/ml) did not remove all the receptors, as the total binding curve and non-specific binding curve for the ^{125}I-$F(ab')_2$ anti-CR1 no longer overlapped, and therefore a specific binding curve could be obtained. However large errors did not permit the determination of CR1 levels. This may have been caused by the alteration of the cell surface charge by trypsin, which may have influenced the binding of the $F(ab')_2$ moieties.

The RIA was adapted to determine the number of CR1 sites/cell on lymphocytes, monocytes and PMNs. The method of cell separation employed Ficoll-Hypaque to separate the pool of lymphocytes and monocytes from the pool of erythrocytes and PMNs (4.11). The monocytes and lymphocytes were further purified using flasks coated with the micro-exudate from baby hamster kidney cells (4.13). PMNs and erythrocytes were separated using dextran sedimentation (4.12). The combination of these techniques enabled the complete separation of lymphocytes, monocytes, PMNs and erythrocytes within 3 to 4 hours, and generally yielded sufficient cells to use at the stated cellular concentrations (4.14). When there were insufficient cells, the cellular concentration was decreased and the cf value (4.8) adjusted to accommodate for the change.

The RIA was performed on the peripheral blood cells of 10 individuals (Table 14), two of whom were RA patients. Out of the 34 assays performed 24 failed to give a result. In 20/24 assays the total binding and non-specific binding curves overlapped (LO, Fig. 16), consequently a specific binding curve could not be attained. The curves (Fig. 16) were similar to those found

when erythrocytes were treated with sufficiently high doses of trypsin (1mg/ml) to remove their receptors (Fig. 15).

In 4 assays the errors between the duplicate determinations were greater than 10%, a further 6 samples were not tested.

Some results were obtained from blood samples 2, 3, 4, 5 and RA patients 1 and 2 (Table 14). The CR1 sites/erythrocyte varied from 494 to 1594 with the lowest values being found in the RA patients. The two lymphocyte measurements of CR1 sites were very close; 1037 and 1076. There was one monocyte measurement of 889 CR1 sites, and 3 determinations of CR1 sites/PMNs, the results being 93,000, 10,000 and 6,327. Unfortunately there was no successful measurement of CR1 sites on the lymphocytes, monocytes, PMNs and erythrocytes from a single blood sample.

DISCUSSION

The result of a single RIA to determine the number of CR1 sites/erythrocyte has been given as an example, the result being 9605 CR1 sites/erythrocyte. This can be compared with the single determination of 950 CR1 sites/erythrocyte by Fearon (1980), the average of 113 normals of 5014±155 CR1 sites/erythrocyte by Wilson et al (1982) and the average of 52 normals of 1410±610 receptors by Iida et al (1982). The latter two groups looked at a large population sample; I have performed such a study the results of which will be discussed in a later chapter.

It must be pointed out that the CR1 numbers per cell determined by different groups will not correlate. This is a result of using a specific antiserum to quantitate the number of receptor sites. For example, with a monoclonal anti-CR1 antibody only one antibody will bind per receptor, whilst with a polyclonal

TABLE 14 CR1 sites/cell on the peripheral blood cells from 8 normals and 2 RA patients

Cell type sample	Erythrocytes	Lymphocytes	Monocytes	PMNs
1	NT	LO	NT	LO
2	1594	1037	NT	LO
3	LE	LO	889	93000
4	705	1076	LO	10000
5	LO	LO	NT	6327
6	LE	LO	LO	LO
7	LE	LO	LO	LO
8	LE	LO	LO	LO
R.A.1	632	LO	NT	NT
R.A.2	494	LO	LO	LO

NT = not tested

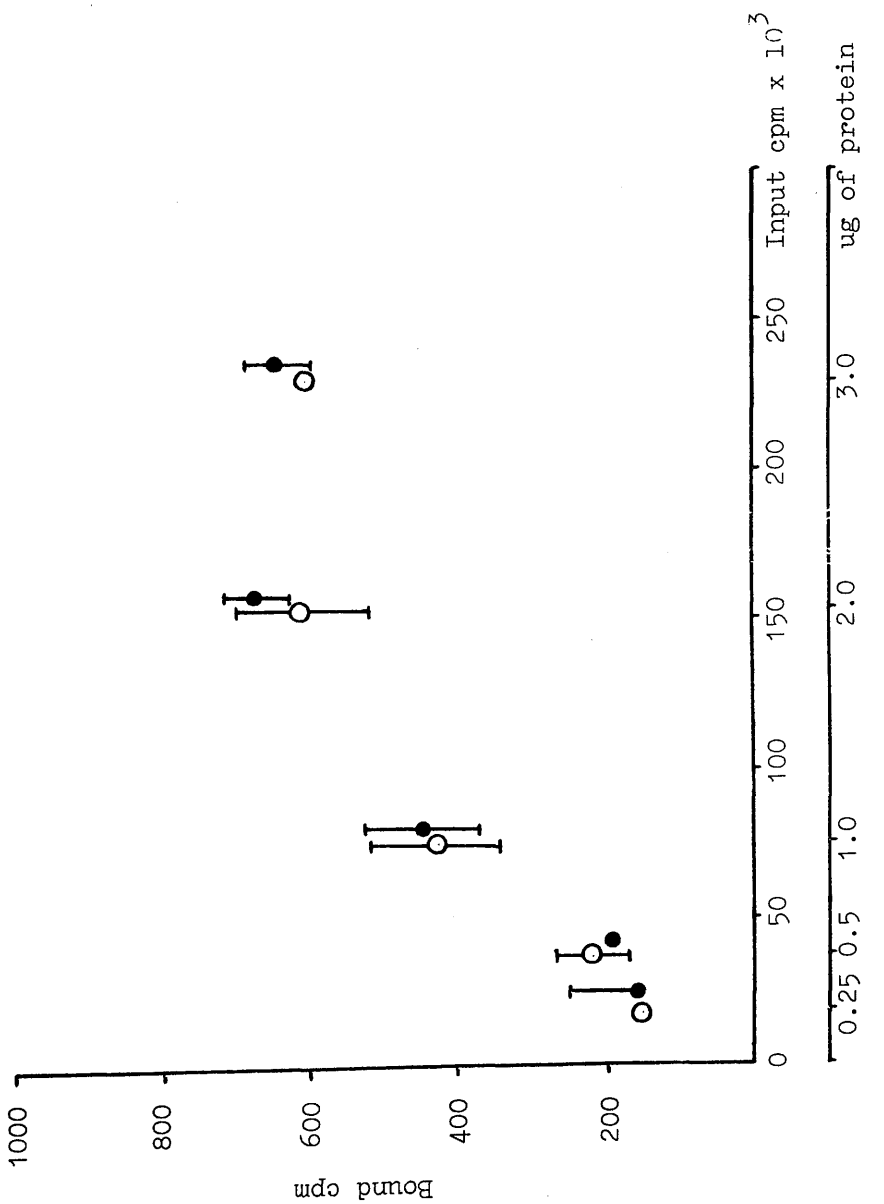
LO = lines overlapping

LE = large errors

FIG. 16 Binding of ^{125}I-F(ab')$_2$ anti-CR1 to lymphocytes of a normal individuals.

x Total binding curve values

• Non-specific binding curve values (slightly displaced to right where values overlap)


Bound cpm
1000
800
600
400
200
0
50
100
150
200
250
Input cpm x 10^3
0.25
0.5
1.0
2.0
3.0
ug of protein

antiserum more than one antibody molecule may bind to each receptor and the number of molecules binding will vary with different batches of antiserum.

As a consequence of this it is difficult to establish the exact number of CR1/cell. However when one uses the same antiserum for a complete study, then comparisons of the levels between individuals and groups of individuals are valid.

The interassay variation was found to be approximately 10% and 24 hrs storage periods at $4^{o}C$ in RPMI-BSA had no effect on the receptor levels. Longer storage periods were not tested, but such a study has been performed by Minota et al (1984). CR1 sites/cell were determined using a RIA with a monoclonal anti-CR1 antibody and the erythrocyte surface area was determined by measuring the level of wheat germ agglutinin (WGA) binding sites using a RIA (WGA binds to glycophorin a major component of erythrocyte membranes). This allowed them to standardize the number of CR1 sites in a given surface area of erythrocyte. They looked at these 3 parameters in 124 individuals over 8 days (Table 15). They found that CR1 levels declined when erythrocytes were stored for 8 days at $4^{o}C$ in Alsever's solution. As a result of these findings they suggested that erythrocyte CR1 levels should be measured within 24 hours of sampling. However, there are several criticisms of these experiments: a) there was no mention of the interassay variation for either assay used, b) the percentage drop in CR1 sites/cell was the same on day 1 as day 3 (Table 15), c) the number of WGA binding sites/cell varied in both upward and downward directions over the 8 day storage period. For example, the value on day 8 is 10% higher than day 0; if it were the day 0 value the standardized CR1 sites would be 8.6 rather than the stated $7.8x10^{4}$ (Table 15). On the basis of these experiments it was

TABLE 15. Decrease in CR1 during the storage of erythrocytes at $4^{o}C$ (From Minota et al, 1984)

Days stored at 4°C	CR1 sites/ cell	Lectin (WGA) binding sites/ cells x 10^5	Standardized CR1 sites x 10^4
0	829	7.7	10.7
1	705	8.1	8.7
2	663	7.8	8.5
3	707	8.2	8.6
4	687	7.7	8.9
5	643	8.4	7.6
6	645	8.1	7.9
7	660	8.5	7.8

difficult to determine whether CR sites/cell varied with storage. In addition Minota et al (1984) stored their erythrocytes in Alsever's and the storage media used may affect the stability of the receptor. In order to minimise the loss of receptors, especially from patients whose erythrocytes may already have low levels, the erythrocytes were assayed within 24 hours of receiving the blood, as this was shown to have no effect on receptor levels under the conditions used.

CR1 was sensitive to trypsin and 1mg/ml removed CR1 from all 5 samples of erythrocytes tested. This treatment caused the total binding and non-specific binding curves to overlap (Fig. 15). Therefore any samples of erythrocytes tested which gave such a graph were said to have no receptors.

For the experiments on the peripheral blood cells only duplicate point determinations were made; this was due to a) the large number of assays being performed at the same time and b) the demand for antiserum making it impossible to perform triplicate determinations. Consequently this may have contributed to the large errors found in some of the assays, particularly those performed on the erythrocytes which had given consistently good results during the development of the RIA.

Clearly it was the assays which were performed on the leucocytes which gave overlapping lines and prevented the calculation of the receptor levels. Erythrocytes treated with high doses of trypsin gave similar results. However, it cannot be assumed that leucocytes have no CR1, as this phenomenon was associated with the leucocyte RIAs it was thought possible that it was perhaps some property of the leucocyte which gave rise to the observed results. The following factors, therefore may have influenced the outcome of the assay on peripheral blood cells:

a) Leucocytes have a more involuted and fluctuating cell surface

compared with erythrocytes and perhaps the purification and assay techniques perturb the cell membrane in such a manner that the anti-CR1 antiserum could no longer gain access to the receptor. By studying fluorescent probes it has been shown that the distribution of CR1 within the membrane of leucocytes is as punctuated discrete clusters (Fearon et al, 1981, Abrahamson and Fearon 1983, Jack and Fearon, 1984) and that their motion was non-random within the plane of the membrane. This is unlike, for example, the HLA antigens which move freely and diffusely within the membrane (Petty et al, 1980, Hafeman et al, 1982). It is therefore possible that membrane folds for example may sterically hinder the access of specific antibodies to the clustered receptors. Another possibility is that the receptors become endocytosed. However this seems unlikely as Abrahamson and Fearon (1983) have shown that this process does not occur at $0^{o}C$ to $4^{o}C$, the temperature at which these assays were performed.

b) The antiserum used in the RIA was raised in rabbits against human erythrocyte derived CR1. It is therefore conceivable that antigenic differences between CR1 on erythrocytes and leucocytes may not enable the antiserum to cross-react. However structural studies on CR1 from human erythrocytes and leucocytes by Dykman et al (1983b) showed that the CR1 from these two cell types was identical although the CR1 from the PMNs was 5000 daltons heavier. The fact that six assays on leucocytes did work also seems to suggest that the antiserum does interact with the leucocyte CR1 and therefore is not the causal factor.

c) CR1 is known to be trypsin sensitive (Lay and Nussenzweig 1968, Huber et al 1968) and its effects on CR1 numbers on erythrocytes has already been discussed. It is thus possible that enzymes released from the leucocytes during the purification procedure

(performed at R.T.) may have cleaved the receptor from the cell surface.

Although the former two possibilities discussed should not be completely dismissed, it seems more likely that it is the latter mechanism which is responsible for the poor RIA results. This is further supported by recent work in our laboratory, where CR1 numbers have been successfully measured on human PMNs; however this was possible only if enzyme inhibitors such as soya bean trypsin inhibitor (SBTI), pepstatin A, leupeptin and EDTA were present in the media throughout the purification of the cells and during the RIA.

The results obtained for the CR1 sites/erythrocyte compared favourably with those found by Fearon (1980, 950 CR1 sites/erythrocyte) and Iida et al (1982, 1400 CR1 sites/erythrocyte) and CR1 levels on erythrocytes will be discussed more fully in Chapter 7. The results for the leucocytes however compared less favourably. For example, Fearon (1980) found B lymphocytes to have 21,000 CR1 sites/cell compared with 1037 and 1076 in this study, monocytes to have 48,000 CR1 sites/cell compared to 889 and PMNs to have 57,000 CR1 sites/cell with 6327, 10,000 and 93,000 being found in this study. The differences observed cannot be due to temperature as his assays were also performed at $4^{\circ}C$. However as the biggest discrepancies are between the lymphocytes and monocytes, this may be as a result of the purification procedure. Fearon (1980) used the panning technique on plastic petri dishes to separate the two cell populations as opposed to the BHK flask method used in my experiments. My technique may have resulted in the altered expression of the receptor in the cell membrane. The results also greatly differ from those found by Iida et al (1982). However, they were using cellular lysates and therefore it was probably

not correct to make a direct comparison as lysis may have exposed internal pools of CR1 (Fearon and Collins, 1983, Kay, Glass and Salter, 1979, Glass and Kay, 1980, Lee, Hakim and Fearon, 1984).

Unlike the RIA performed on the leucocytes being unsuccessful, the RIA performed on the erythrocytes was successful. The assay conditions were successfully determined and the ability to reproduce the assay was good. This assay was used to examine erythrocyte CR1 levels and these results are discussed in Chapter 7.

C H A P T E R 5

VISUALIZATION OF CR1 ON ERYTHROCYTES USING INDIRECT IMMUNOFLUORESCENCE.

INTRODUCTION

Immunofluorescence is a technique which has been widely used to visualize the location and distribution of cellular components. Several groups (Petty et al 1981, Fearon et al 1981, Hafeman et al 1982, Abrahamson and Fearon 1983, Jack and Fearon 1984) have looked at CR1 on human leucocytes using immunofluorescence, but such studies have not been performed on human erythrocytes. It was decided to examine erythrocyte CR1 using indirect immunofluorescence to investigate the possibility that this procedure might be useful in the study of erythrocyte CR1 in immune complex diseases.

In addition to performing immunofluorescence on normal human erythrocytes, the technique was used to examine normal erythrocytes which had been treated with different doses of trypsin (1mg/ml, 0.01mg/ml and 0.0001mg/ml),to enable the visualization of dose dependent loss of CR1 brought about by the trypsin (discussed in Chapter 4). The effects of trypsin on the pattern and intensity of fluorescent staining was compared with the quantitative determination of CR1 numbers by RIA (4.7) and the semi-quantitative determination of CR1 levels by haemagglutination of EAC43b.

MATERIALS AND METHODS

5.1 Polyclonal $F(ab')_2$ anti-CR1

This was obtained as described in Chapter 4.

5.2 Monoclonal anti-CR1 antibody

The commercial IgG, anti-CR1 monoclonal antibody was purified from the culture fluid in which it was supplied using an affinity chromatography column composed of sheep anti-mouse IgG antiserum conjugated to CNBr-activated Sepharose 4B. This was prepared as follows:

CNBr-activated Sepharose 4B (1g) was washed 4 times in 0.001M HCl and the pellet was then resuspended in 1ml of sheep anti-mouse IgG antiserum (10mg/ml) in 0.1M $NaHCO_3$ plus 0.15M NaCl. This was incubated overnight at 4°C with continuous mixing. The suspension was then spun for 5 mins at 1000g, the supernatant was removed and its protein content established by measuring the absorbancy at 280nm (2.4). This enabled the calculation of how much sheep anti-mouse IgG antiserum had bound to the Sepharose beads; this was found to be 90%. The Sepharose beads were then incubated with a 10-fold volume of 1M ethanolamine pH 8.0 for 2 hrs at R.T. with mixing, to inactivate any uncoupled sites. The beads were then washed with 0.1M acetate buffer pH 4.0 containing 1.0M NaCl followed by 0.1M borate buffer pH 8.0 containing 1.0M NaCl; this washing cycle was repeated three times. The conjugated Sepharose beads were then stored in VBS plus 0.01M EDTA at 4°C until required.

A glass pipette plugged with glass wool acted as the column. This was filled with 2ml of the conjugated Sepharose 4B and washed extensively with PBS. As it was probable that some of the conjugated antiserum may bind the substrate irreversibly because of high affinity binding reactions, it was decided to saturate these with normal mouse IgG prior to the application of the monoclonal as the conventional technique of dropping the pH may not dissociate high affinity bonds. The column was therefore run collecting

1ml fractions as follows: 1ml of normal mouse IgG (3mg/ml, kindly given by Miss A. Fyfe) was applied to the column and washed through until the absorbancy at OD_{280} dropped to 0.001, the bound material was then eluted with 0.1M glycine-HCl pH 2.5 (tubes containing 0.1M glycine-HCl pH 2.5 were returned to pH 7 using solid Tris). The column was then washed with PBS then 2ml of the monoclonal antibody (total protein 4mg/ml) was applied, washed through with PBS then eluted with 0.1M glycine-HCl, pH 2.5. The protein peak was then dialysed into PBS, then concentrated by freeze drying (2.5). The protein content was then measured using the absorbancy at 280nm (2.4).

5.3 Absorption of second and third layer antiserum with human erythrocytes

Fresh human erythrocytes were washed with PBS containing 1% BSA (PBS-BSA) and standardized to $1x10^9$/ml (2.14). To 1ml of packed erythrocytes, 1ml of antiserum was added, the erythrocytes were resuspended and left at R.T. for 1 hr with occasional mixing. Finally the cells were spun down and the absorbed antiserum (supernatant) was removed and stored at -20^oC until required.

5.4 Immunofluorescence using polyclonal $F(ab')_2$ anti-CR1

Normal erythrocytes were washed in PBS-BSA and standardized to $2x10^8$/ml (2.14). 50µl was added to LP2 tubes and to this 50µl of the polyclonal $F(ab')_2$ anti-CR1 (1mg/ml) was added. After mixing, the tubes were incubated at R.T. for 30 mins. The cells were then washed three times with PBS-BSA and then 50µl of 1/10 dilution

of goat anti-rabbit IgG was added and incubated in the dark for 30 min at R.T. The cells were then washed three times in PBS-BSA, then 50µl of 1/10 dilution of FITC conjugated swine anti-goat IgG was added and incubated in the dark for 30 mins at R.T. After a final wash in PBS-BSA the cells were resuspended in 100µl of PBS-BSA. For the control NRS $F(ab')_2$ was used instead of $F(ab')_2$ anti-CR1.

5.5 Immunofluorescence using monoclonal antibody to CR1

The method used was similar to that described in 5.4 with the following modifications:

a) erythrocytes were standardized to $1x10^8$/ml,

b) 20µl was added at all stages rather than 50µl,

c) second layer antiserum was absorbed sheep anti-mouse IgG,

d) third layer antiserum was FITC conjugated absorbed rabbit anti-sheep IgG.

For the control normal mouse IgG was used instead of the monoclonal antibody.

5.6 Microscopy and photography

The microscope used was a Leitz Orthoplan with a high pressure mercury lamp, HBO 220, light source and a Ploem illuminator. This system is designed to allow only light below a wavelength of 490nm to reach the mounted cells and only light above 520nm to reach the eye piece, since the peak of FITC absorption is at 490nm and the peak emission at 520nm. The cells were viewed in an improved Neubauer counting chamber (depth = 0.1mm 1/400mm^2) with an X40 objective. Photographs

were taken using Kodacolor VR 1000 ASA colour print film with a 90 second exposure time.

5.7 Trypsinization of erythrocytes

Normal human erythrocytes were trypsinized as described in 4.9.

5.8 Preparation of EAC43b

EAC43b were prepared as described in 3.5

5.9 Haemagglutination of EAC43b

Into the wells of a round bottomed microtitre plate was placed 50ul of GVB^{++}. Test human erythrocytes (50µl, $1x10^9$/ml) were added to the first well and doubly diluted across 7 wells. Then, to each well, 50µl of EAC43b ($1x10^8$/ml) in GVB^{++} were added. The plate was gently shaken and left for 2 to 4 hrs at R.T. Two controls were included in the plate, one containing 50µl of GVB^{++} and 50µl of EAC43b ($1x10^8$/ml) and one containing 50µl of GVB^{++} and 50µl of test human erythrocytes ($1x10^9$/ml). These control wells should show no agglutination, if they did the test was repeated.

RESULTS

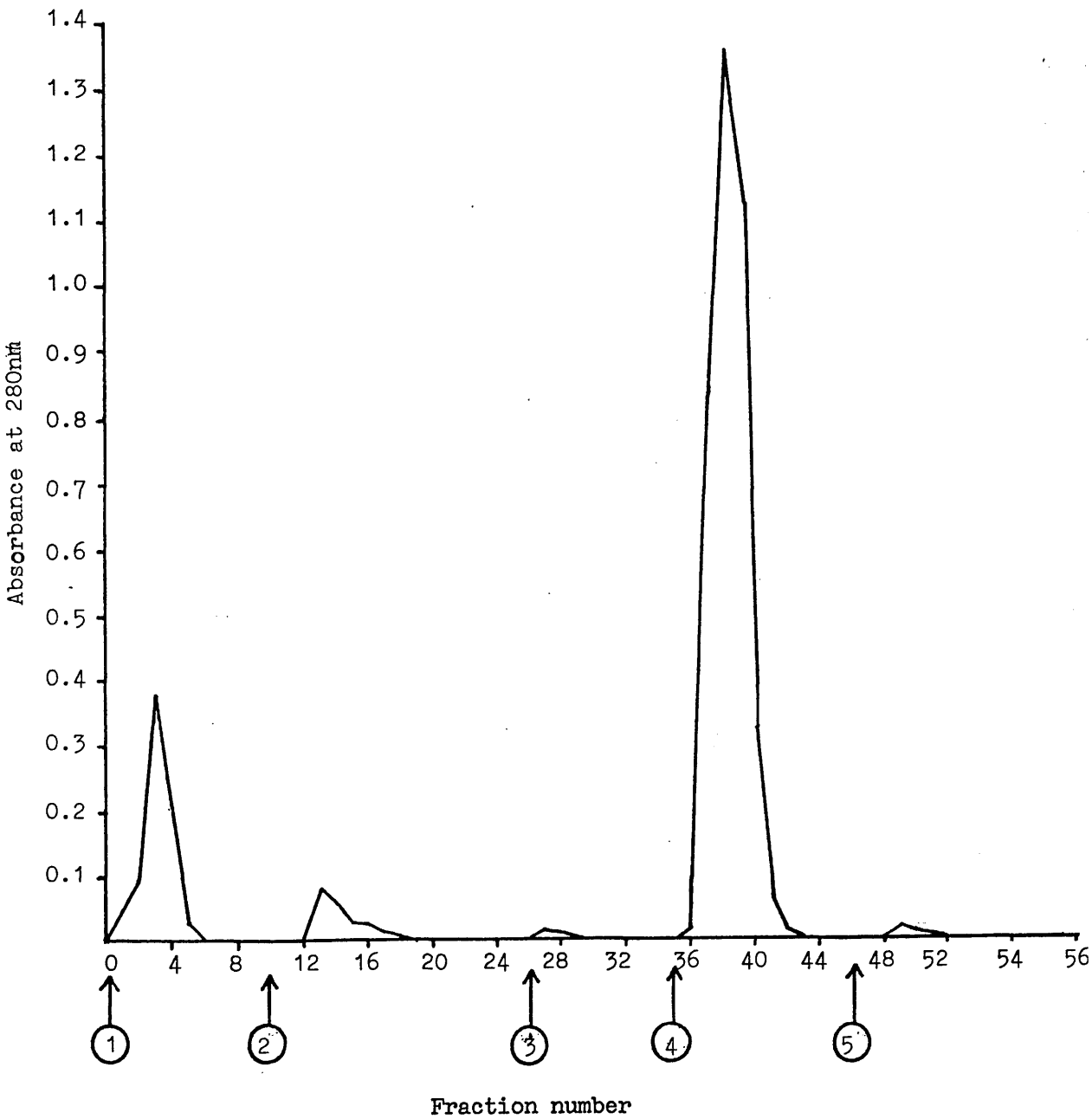
The immunofluorescence technique was performed using rabbit polyclonal $F(ab')_2$ anti-CR1 or a commercially available monoclonal antibody to CR1 as the first layer antiserum. In total 3 layers of antiserum were used to increase the sensitivity of the assay. Both the second and third layer antisera were commercially supplied, and were pre-absorbed with human erythrocytes prior to use, to prevent any non-specific interaction with the erythrocytes. The third layer antiserum was conjugated to fluorescein isothiocyanate (FITC).

The monoclonal anti-CR1 antibody was purified using affinity chromatography (Fig. 17). A total of 0.711mg of the normal mouse IgG applied did not bind to the column and was washed through with PBS, 0.226mg was eluted with 0.1M glycine-HCl (pH 2.5). Therefore 2mg of the 3mg of mouse IgG applied remained irreversibly bound to the column, blocking all the high affinity binding sites on the Sepharose 4B conjugated sheep anti-mouse IgG. The monoclonal antibody was then applied, the culture fluid in which it was supplied passed straight through the column and had a total protein content of 3.728mg. The peak eluted with 0.1M glycine-HCl (pH 2.5, fractions 48 to 52) was pooled, dialysed into PBS and concentrated, the recovery was 26µg/ml. The company who supplied the monoclonal antibody estimated the presence of 28ug/ml of specific antibody therefore 26µg/ml represented a 93% recovery.

Indirect immunofluorescence on human erythrocytes using the monoclonal antibody to CR1 as the first layer was unsuccessful since it was below the level of detection. This was probably a consequence of using a monoclonal antibody, as one would expect only one antibody molecule to bind per receptor and therefore the amount

FIG. 17. Elution profile for purification of monoclonal anti-CR1 antibody using a sheep anti-mouse IgG affinity chromotography column.

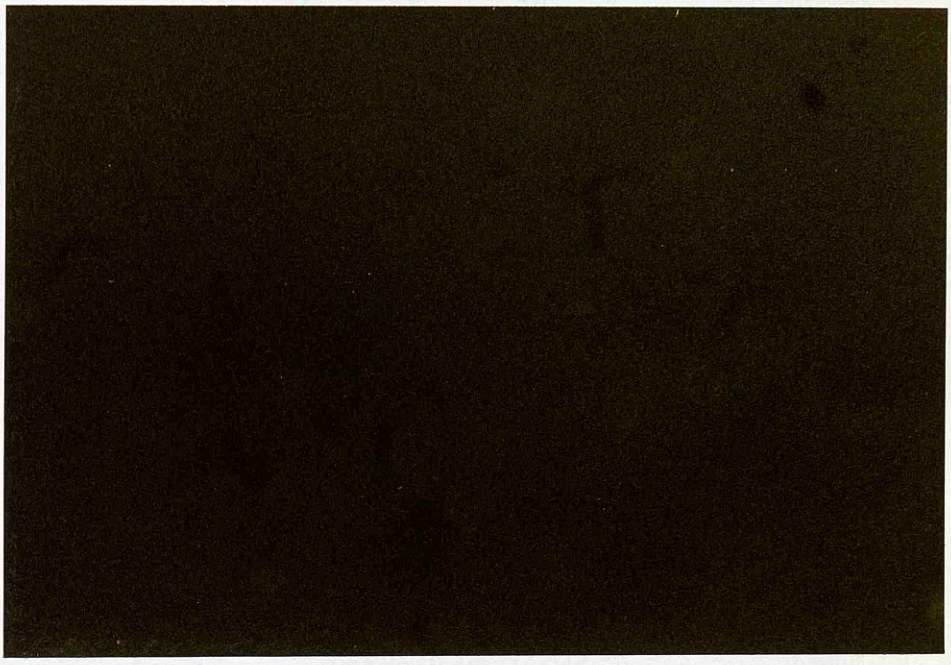
1) Application of normal mouse IgG and elution with PBS,
2) Application of 0.1M Glycine-HCl pH 2.5,
3) Application of PBS,
4) Application of monoclonal antibody and elution with PBS,
5) Application of 0.1M glycine-HCl pH 2.5


Absorbance at 280nm
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4
8
12
16
20
24
28
32
36
40
44
48
52
54
56
1
2
3
4
5
Fraction number

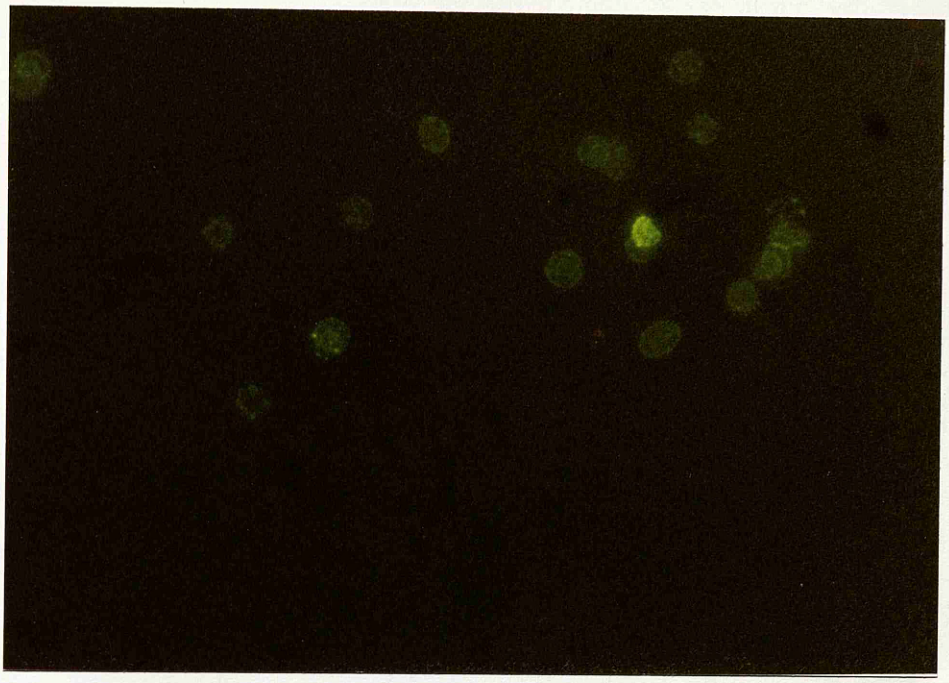
of FITC-conjugated antibody binding would be reduced. This is in comparison to using a polyclonal antiserum as the first layer, this would have multiple binding sites on a single receptor and consequently the amount of FITC-conjugated antiserum binding would be greatly enhanced and detectable. Results were obtained using the $F(ab')_2$ fragment of the polyclonal rabbit anti-CR1 as the first layer. However, the success of this technique was spasmodic with no consistency in the assay repeatability. For example, a blood sample from a given normal individual might have given positive fluorescence whereas a second sample from the same individual might not have done so. The most probable reason for this is that the number of CR1 on the erythrocytes is so low that often they are below the limit of sensitivity of the indirect immunofluorescence technique. The ability to detect CR1 in a few cases suggested that these individuals had high CR1 numbers. The failure to detect CR1 by immunofluorescence on the erythrocytes of some of these individuals, on repeated testing, could suggest that CR1 numbers in normal people vary from time to time. However, the lack of consistency could simply indicate the lack of reproducibility of the immunofluorescence technique when it is used in situations where the antigen density is very low.

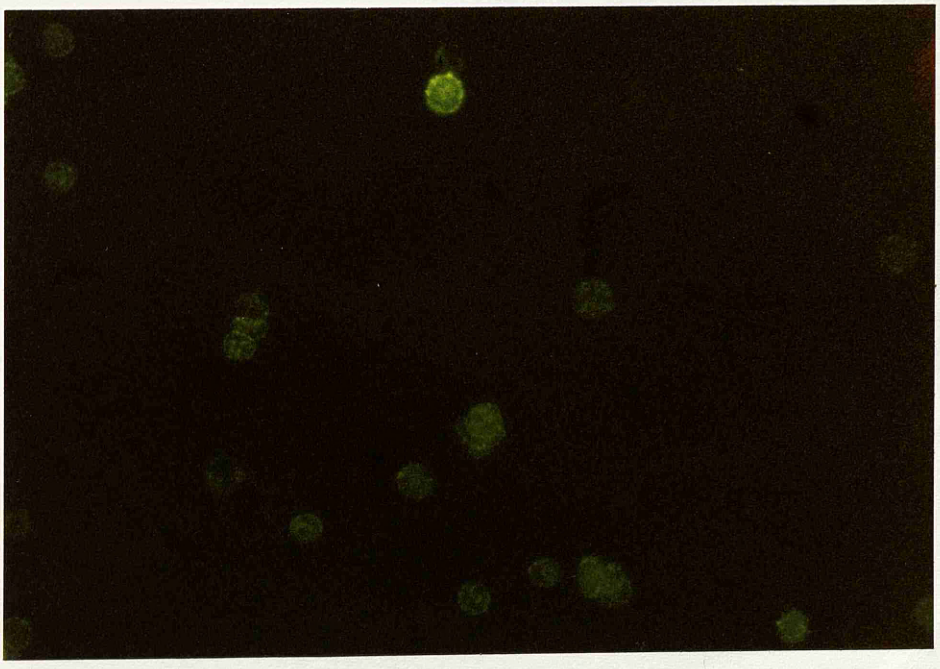
The results of an indirect immunofluorescence assay performed on the erythrocytes of a normal individual are shown in Plates 5, 6 and 7. Control erythrocytes (Plate 5) were incubated with NRS $F(ab')_2$ as the first layer antibody rather than $F(ab')_2$ anti-CR1 (5.4). There was no non-specific binding of this fragment to the erythrocytes as indicated by the lack of fluorescence. In contrast cells incubated with $F(ab')_2$ anti-CR1 showed positive fluorescence (Plates 6 and 7), these cells did exhibit a fluo-

PLATE 5 Indirect immunofluorescence on human erythrocytes using NRS $F(ab')_2$ as the first layer antiserum (control).



PLATES 6 and 7 Indirect immunofluorescence on human erythrocytes using $F(ab')_2$ anti-CR1 as the first layer antiserum. There are foci of fluorescence on the cell surface



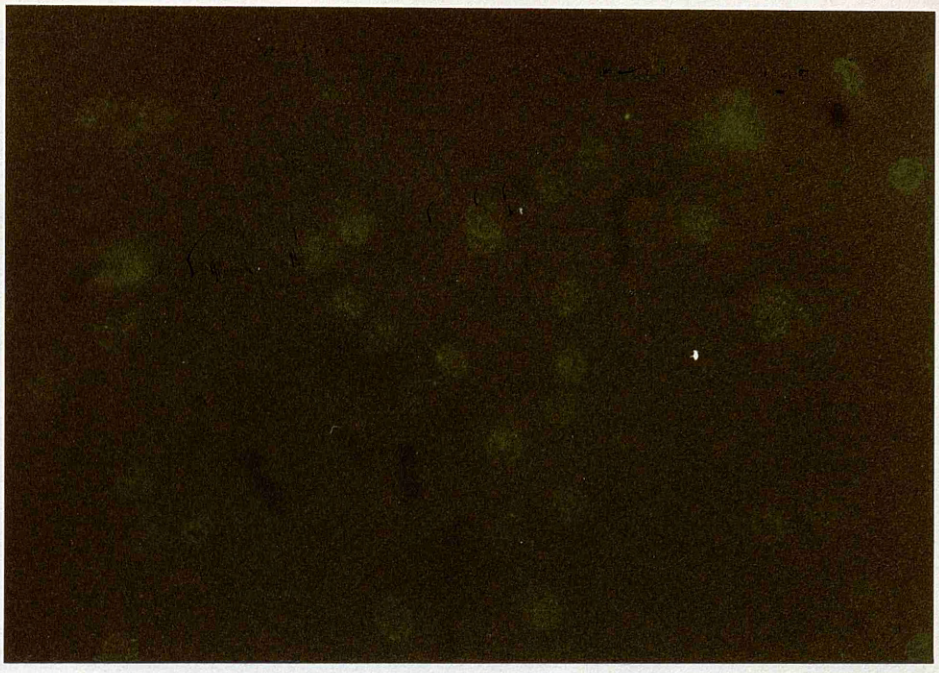


rescent haze over the cell surface. However there were discrete fluorescent bright spots and by adjusting the focus of the microscope and therefore the plane of vision, these were seen on all the cells.

Trypsin treatment of the cells resulted in a faint background staining in the negative controls when high doses were used (Plate 8). The trypsin has probably altered the cell surface in such a manner as to increase the non-specific binding of the first or second layer antiserum or the FITC-conjugated antiserum. Erythrocytes which had been incubated with 1mg/ml trypsin (5.7) no longer had any fluorescent bright spots on their surface but rather a faint halo (Plate 9). With 0.01mg/ml trypsin bright fluorescent specks were present on some of the cells (Plate 10). Plate 11 is the control. With the lowest dose of trypsin (0.0001mg/ml) there was a normal pattern of fluorescence (Plate 12). Plate 13 is the control. Therefore, trypsin removes CR1 from the erythrocyte surface in a dose-dependent fashion. This dose-dependent removal of CR1 was also visualized and semi-quantitated using haemagglutination (5.9). Untreated cells agglutinated to a titre of 1/64, those treated with 1mg/ml trypsin demonstrated no agglutination, with 0.01mg/ml trypsin there was partial agglutination and with 0.0001mg/ml trypsin their agglutination titre was 1/32 (Plate 14), a dilution one-fold less than that for untreated erythrocytes. Therefore, although the immunofluorescence technique failed to show reduced CR1 levels when the lowest concentration of trypsin was used, the haemagglutination assay results indicated that even with this low dose 0.0001mg/ml trypsin, some reduction in CR1 had been achieved. A difference in titre of one dilution (1/64 to 1/32) may represent a genuine difference in CR1 levels between the trypsinized erythrocytes and the untreated erythrocytes. However,

PLATE 8 Indirect fluorescence on human erythrocytes treated with 1mg/ml trypsin and using NRS $F(ab')_2$ as the first layer antiserum (control)

PLATE 9 Indirect immunofluorescence on human erythrocytes treated with 1mg/ml trypsin and using $F'ab')_2$ anti-CR1 as the first layer antiserum



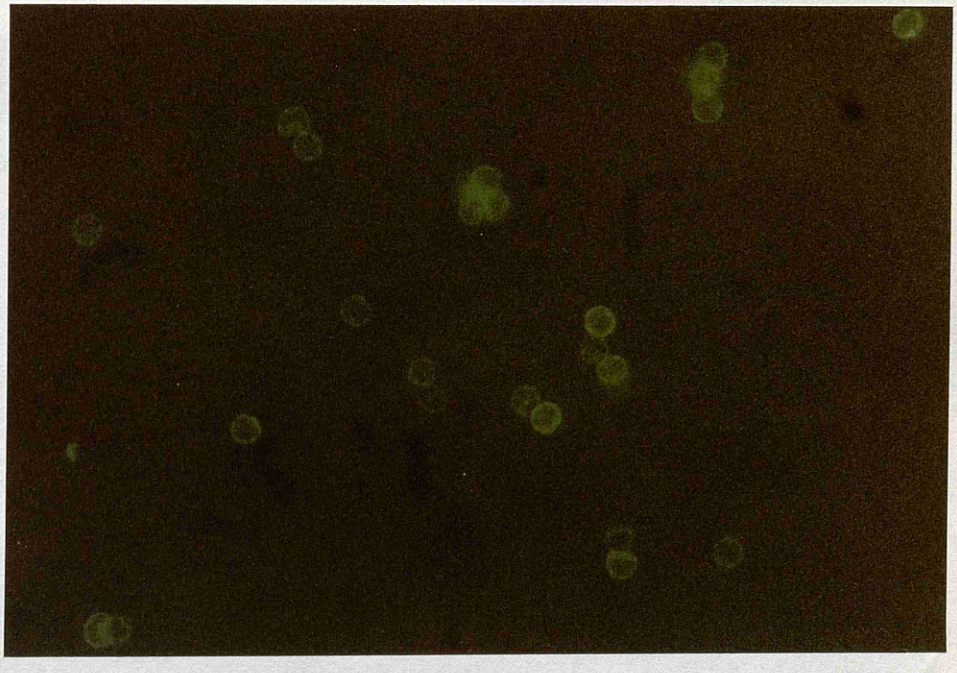


PLATE 10 Indirect immunofluorescence on human erythrocytes treated with 0.01mg/ml trypsin and using $F(ab')_2$ anti-CR1 as the first layer antiserum

PLATE 11 Indirect immunofluorescence on human erythrocytes treated with 0.01mg/ml trypsin and using NRS $F(ab')_2$ as the first layer antiserum (control)

PLATE 12 Indirect immunofluorescence on human erythrocytes treated with 0.0001mg/ml of trypsin and using $F(ab')_2$ anti-CR1 as the first layer antiserum

PLATE 13 Indirect immunofluorescence on human erythrocytes treated with 0.0001mg/ml of trypsin and using NRS $F(ab')_2$ as the first layer antiserum (control)

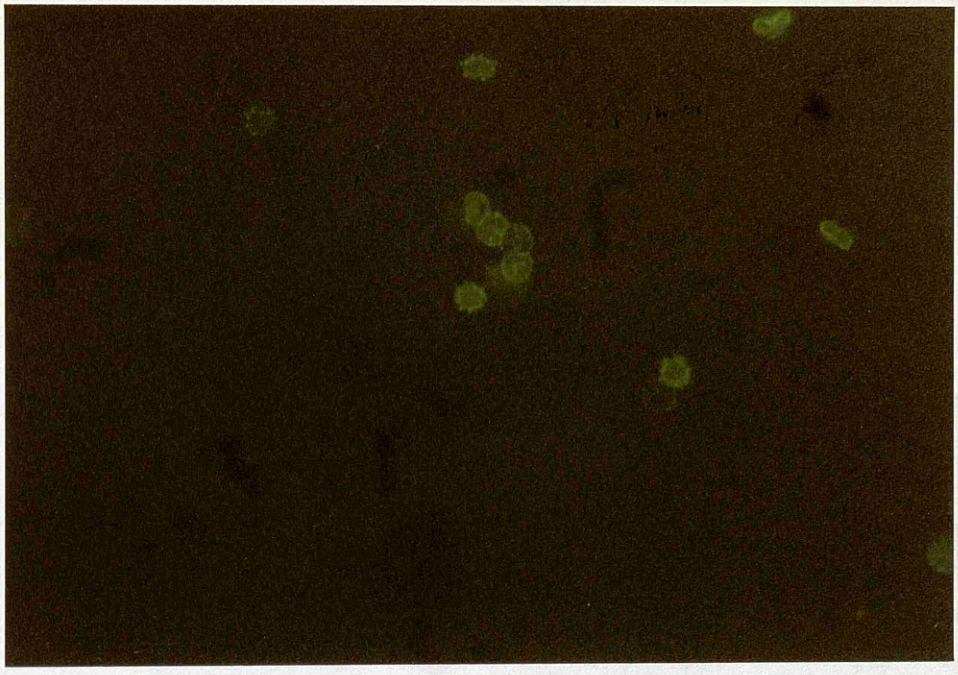
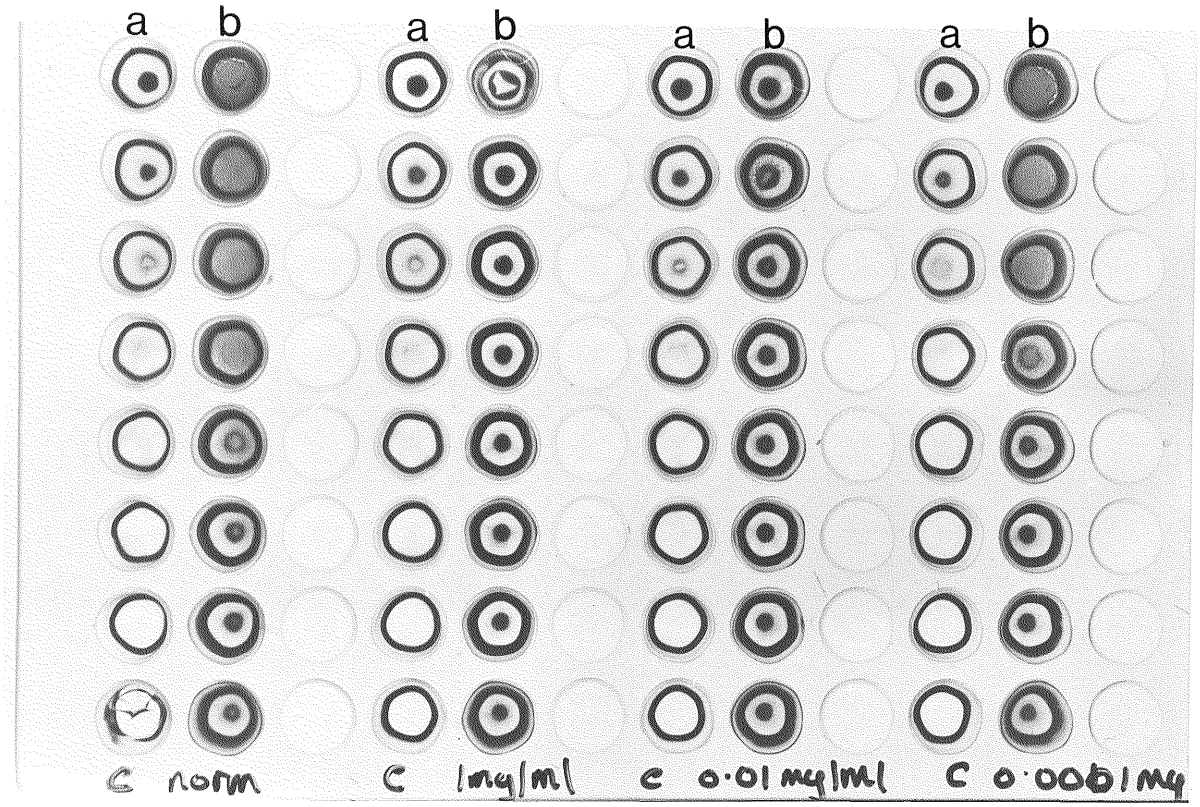


PLATE 14 Haemagglutination of EAC43b by human erythrocytes and erythrocytes treated with 1mg/ml, 0.01mg/ml and 0.0001mg/ml trypsin (lane b, lane a = control)


a b
a b
a b
a b
C norm
C 1mg/ml
C 0·01 mg/ml
C 0·0001 mg

the error limit for a haemagglutination assay is considered to be ±1 dilution, which could account for the discrepancy seen between the haemagglutination and immunofluorescence results.

In addition, when trypsinized erythrocytes were stored overnight at 37°C their haemagglutination titre remained unchanged.

The number of CR1 sites/erythrocyte as determined using the RIA (4.7) was not measured on the cells used in the experiments described. However, the number of CR1 sites/erythrocyte was determined on the same individual at a later date. The untreated cells had 1488 CR1 sites/erythrocyte, cells treated with 1mg/ml trypsin had no receptors (with the total and non-specific binding curves overlapping as described in Chapter 4) and those treated with 0.01mg/ml trypsin had 482 CR1 sites/erythrocyte. The lowest dilution was not tested.

DISCUSSION

CR1 within the membrane of human erythrocytes has been shown to be distributed in punctuate discrete clusters, as demonstrated by indirect immunofluorescence. This distribution pattern has been reported for CR1 on human leucocytes (Petty et al 1981, Fearon et al 1981, Abrahamson and Fearon 1983, Jack and Fearon, 1984). CR1 on leucocytes has been extensively investigated using immunofluorescence techniques; however to date no such work has been reported for erythrocytes.

Indirect immunofluorescence was performed on normal human erythrocytes using either polyclonal rabbit $F(ab')_2$ anti-CR1 or a commercially available monoclonal antibody to the receptor

as the first layer antiserum. The assay system using the monoclonal antibody was not sufficiently sensitive to detect the receptor by immunofluorescence. Using the polyclonal antibody to the receptor, positive immunofluorescence was obtained on several occasions. However, this assay system had obvious limitations. Firstly, a certain density of receptors seemed to be required to give positive immunofluorescence despite using 3 layers of antiserum which should have increased the assay sensitivity (compared to direct immunofluorescence). This necessity for a certain receptor density probably accounts for the failure to visualize CR1 on some of the erythrocyte samples tested. Due to this limitation further experiments using the immunofluorescence technique became impossible. For example, it would have been interesting to examine the distribution pattern of CR1 on erythrocytes of different ages, the effects of storage at $4^{o}C$ and $37^{o}C$ on the CR1 distribution pattern, and, whether CR1 was re-expressed on trypsinized erythrocytes.

Stored (overnight at $37^{o}C$) trypsinized erythrocytes did not recover their receptors as assessed by haemagglutination. This result can be interpreted in 3 ways: a) CR1 had been re-expressed but the assay system was not sufficiently sensitive to detect them, b) there is no internal pool of CR1 in erythrocytes or c) there is an internal pool but it is not being expressed.

Thus immunofluorescence, haemagglutination and RIA showed the dose-dependent removal of CR1 from the erythrocyte cell surface by trypsin. The immunofluorescence technique was used to show foci of fluorescence suggesting that CR1 was present as clusters within the cell membrane and not diffusely distributed.

Several groups have examined CR1 on leucocytes using immunofluorescence. Petty et al (1981) using immunofluorescence

and photobleaching recovery experiments showed the lack of lateral diffusion of the CR1 clusters in the cell membrane, whilst HLA antigens and lipid probes were randomly distributed and moved freely within the membrane plane. Thus they interpreted this to mean that the CR1 clusters represented a specialization of the cell surface and that the distribution was physiologically relevant, based on the observation that C3b deposited on particulate surfaces tended to be as clusters and hence would easily interact with the pre-clustered CR1.

Fearon et al (1981) investigated this further and looked at the ability of CR1 to mediate adsorptive pinocytosis, based on the knowledge that other membrane receptors distributed in such a manner, e.g. low density lipoprotein receptors on fibroblasts, could mediate this function. Like Petty et al (1981), they demonstrated the punctuate distribution of CR1 on leucocytes and excluded the possibility that this distribution was a manifestation of the sampling procedure or due to prior binding of C3b to the receptor. They demonstrated the ability of these receptors to become internalized at 37°C if cross-linked with $F(ab')_2$ anti-CR1. This process was not dependent upon microfilaments as cytochalasin B did not inhibit the internalization and this distinguishes adsorptive endocytosis from phagocytosis which is dependent upon the cell's cytoskeleton. As a result of their experiments, they suggested that the receptors may be associated with another cytoplasmic structural protein called clathrin and that CR1 were clustered within clathrin coated pits and it was by this mechanism that soluble immune complex endocytosis was mediated.

This was confirmed by Abrahamson and Fearon (1983) who looked at the binding and the fate of internalized material. This was

studied using fluorescent and electron microscopic examination of cells which had been labelled with monospecific $F(ab')_2$ anti-CR1 and anti-$F(ab')_2$ conjugated with rhodamine or ferritin. They examined PMNs and mixed leucocyte populations from various normal donors and found that at 37°C the antibody cross-linked receptors became internalized, the half time being 5 mins and there after the ferritin conjugated antibody was delivered to the lysosomes within 20 mins. Using electron microscopy the exact fate of the ferritin granules could be followed. On the cell surface most of the ferritin was within endocytic invaginations having cytoplasmic bristle coats (coated pits), they were not found associated with uncoated invaginations or large phagocytic processes. Intracellularly, ferritin was primarily present within 100nm coated vesicles which were 0.5μm beneath the cell surface with eventual delivery to the lysosomes.

Thus, there seems to be agreement, that CR1 is clustered within clathrin-coated pits on the membranes of human leucocytes, and that the cross-linking of these receptors by the appropriate ligand results in their internalization. These are by no means the only receptors to be clustered within clathrin-coated pits, and this clustering probably represents an important process for communicating between the intracellular and extracellular compartments. For example, cultured human fibroblasts have been shown to take up low density lipoprotein, epidermal growth factor, α_2-macroglobulin and insulin by this mechanism (Pearse and Bretscher, 1981). The function of the coated vesicle seems to vary with cell type, with the most frequent role being absorption and secretion (Pearse 1976). The low density lipoprotein receptor on human fibroblasts has become the prototype model for studying receptor mediated endocytosis via coated pits. From

this model several properties of coated pits have been established:

1) the structures are transient, continually being formed and pinched off,

2) life span is less than 5 mins ,

3) coated pits may contain several receptor types and

4) the major component of the pits is clathrin (Brown and Goldstein 1979).

However, certain pertinent points remain unanswered, for example, how does the receptor escape degradation in the lysosomes and how does the clathrin coated pit become coupled to the receptor, is it constitutive or is it a result of the ligand binding? (Pearse and Bretscher 1981). Abrahamson and Fearon (1983) demonstrated the lack of clathrin coated pits beneath areas where ferritin granules were bound, at temperatures lower than 37^{o}C, nor was monovalent Fab' anti-CR1 sufficient to induce endocytosis (Fearon et al, 1981), indicating that for coupling of CR1 with clathrin coated pits receptor perturbation is required (i.e. cross-linking) and perhaps there is a critical cytomechanical event required for endocytosis.

Therefore the clustered CR1 receptors on leucocytes may provide a mechanism for the elimination of soluble C3b-bearing complexes from the extracellular milieu. This however does not answer why CR1 on erythrocytes appeared clustered, as visualized using indirect immunofluorescence. This possibly arose as a result of the assay procedure or is this a natural state, having some physiological relevance. This would require further investigation.

C H A P T E R 6

PURIFICATION OF HUMAN C3, AND DEVELOPMENT OF AN RIA TO QUANTITATE THE NUMBER OF FUNCTIONAL CR1 SITES PER CELL.

INTRODUCTION

Human C3 was purified from fresh human plasma to enable the development of an RIA which would measure the number of functional binding sites on CR1 bearing cells for the natural ligand C3b.

C3 was purified using a modification of the method described by Tack and Prahl (1976) and consisted of fractionating human plasma with polyethylene glycol (PEG) followed by chromatographic separation using an anion exchange column and a hydroxylapatite column. This procedure yields in addition to C3, P, B, I, H, C2, C4 and C5.

Prior to the commencement of this project, the CR1 levels on human erythrocytes had been measured semi-quantitatively using IAHA (Miyakawa et al, 1981, Inada et al 1982) or quantitatively using radiolabelled CR1 specific antiserum (Wilson et al 1982, Iida et al 1982). These RIAs measured the number of antigenic sites per cell for the CR1 specific antibody (CR1 sites/cell) and the development of such an RIA was discussed in Chapter 4. However, in addition to this type of RIA, Wilson et al (1982) developed an RIA to quantitate the number of functional sites per cell using radiolabelled dimeric C3b. This functional RIA described by Wilson et al (1982) was used as the basis for the development of an RIA to measure the number of functional CR1 sites per cell. In addition this assay would enable the distinction between receptors which may be antigenically detectable but perhaps blocked and therefore functionally inactive.

For the development of this assay dimeric human C3b was chosen to be the radiolabelled ligand, based on the observations of Arnaout et al (1981) that dimeric C3b binds to CR1 with a greater affinity than monomeric C3b. Two methods were used to chemically

cross-link C3b, firstly using dithiobissuccinyl propionate (DTBSP) which yields polymers which can be reduced into their natural monomeric state or secondly dimethyl suberimidate which produces polymers which are stable and non-reducible.

The C3b dimers were then separated from the remaining monomeric C3b and polymeric C3b using sucrose gradient fractionation or gel filtration, for use in the RIA.

MATERIALS AND METHODS

6.1 Preparation and fractionation of plasma with PEG

Unless otherwise stated all purification steps were performed at 4°C. Two litres of fresh human plasma was collected from the Blood Transfusion Service, this was immediately put on ice and the following protease inhibitors were added: 0.02% sodium azide (w/v), 10mM benzamidine, 0.5mM PMSF (in dimethyl formamide) and 1mM EDTA (from a 0.086M isotonic EDTA stock solution). With continuous stirring 15% (w/v) PEG (mol.wt. 4000) in 100mM sodium phosphate buffer pH 7.4. containing 150mM NaCl, 15mM EDTA and 0.5mM PMSF was added, to give a final concentration of 5% PEG, and stirred for a further 30 mins on ice. This was then spun at 5000g for 15 mins, the supernatant was retained and the pellet discarded. To 3 litres of the supernatant 1 litre of 28% (w/v) PEG (in the aforementioned buffer for 15% PEG) was added to give a final PEG concentration of 12% (w/v). This was allowed to stand for 30 mins on ice and then was centrifuged at 7000g for 20 mins. The supernatant was retained and frozen at -70°C; from this C2 and B can be further purified. The pellet was then resuspended in a

minimum volume of starting buffer for the first column (6.2).

6.2 Diethylaminoethyl (DEAE) Sephacel anion exchange column

Column: 5 x 90cm column containing 1766cm^3 of degassed DEAE Sephacel equilibrated with starting buffer.

Starting buffer: 10mM Tris/HCl pH 7.8, conductivity 2mS at 4°C plus 50mM ε-amino-n-caproic acid (EACA), 5mM EDTA and 10mM benzamidine.

The protein sample in starting buffer was applied to the column and washed through with 2 column volumes of starting buffer, until the elution fractions had a protein content of less than 0.1mg/ml. A 5 litre 0.3M NaCl linear salt gradient in starting buffer was then used to elute P, I, H, C5, C3 and C4. The flow rate of the column was between 100 and 150ml per hour and 23ml fractions were collected. The pH and conductivity of every 5th. fraction was measured as was the protein concentration using the absorbancy at 280nm. The column was screened for P, I, H, C5, C3 and C4 using radial immunodiffusion plates containing the appropriate antiserum to detect these components (6.4). The tubes containing P, I, H, C5 and C4 were pooled and stored at -70°C with 10mM benzamidine and 0.5mM PMSF. The partially purified C3 was further cleaned up by passage over a hydroxylapatite column.

6.3 Hydroxylapatite anion exchange column

Column: 5 x 60cm column containing 1177cm^3 of hydroxylapatite equilibrated in starting buffer.

Starting buffer: 25mM potassium phosphate buffer pH 7.4, 100mM KCl and 50mM EACA

The partially purified C3 pool from the DEAE Sephacel column was concentrated to 300ml by ultrafiltration using a YM 10 membrane (2.5) and applied to the column and the following stepwise elution scheme was used: 2 litres starting buffer, 2 litres 25mM potassium phosphate buffer, pH 7.4 plus 2M KCl and 50mM EACA, 2 litres starting buffer, 2 litres 0.125 potassium phosphate buffer pH 7.4 plus 100mM KCl and 50mM EACA, 2 litres 0.4M potassium phosphate buffer pH 7.4, plus 100mM KCl and 50mM EACA. A flow rate of 120ml per hour was used and 15ml fractions were collected into tubes containing 1ml of 100mM EDTA pH 7.4. The pH, conductivity and protein content of every 5th. tube was monitored. The presence of C3 and C5 was established using radial immunodiffusion with anti-C3 and anti-C5 antiserum (6.4). Tubes containing C3 were pooled and concentrated by ultrafiltration using a YM 10 membrane (2.5). The C5 pool was stored at -70^{o}C with 10mM benzamidine and 0.5mM PMSF.

The C3 purity was tested reduced and non-reduced on 4% SDS-PAGE phosphate tube gels (2.10). The protein concentration was determined using Folin assay (2.4), radial immunodiffusion (6.4) and nephelometry (nephelometry was kindly performed by Mrs. J. Veitch). The haemolytic activity of C3 was determined using a C3 haemolytic titration (6.5).

6.4. Detection and quantitation of complement components using radial immunodiffusion

The DEAE Sephacel column was screened for P, I, H, C5, C3 and C4 using radial immunodiffusion. As this purification procedure is well documented the whole column was not screened for all 6 components. The areas screened for each component were determined using the conductivity at which they were expected to be eluted from the column after application of the salt gradient.

Complement component	Area screened
P	Exclusion peak
I	1.5 to 3.5mS
H	4 to 7mS
C5	5 to 8mS
C3	6 to 10mS
C4	8 to 12mS

Radial immunodiffusion plates were set up as described in (2.8), using the following dilutions of monospecific antisera in agarose type (b).

Anti-P antiserum	1/1000 dilution
Anti-I "	1/50 "
Anti-H "	1/25 "
Anti-C5 "	1/30 "
Anti-C3 "	1/25 "
Anti-C4 "	1/35 "

A standard curve for C3 was obtained by using doubling dilutions of normal human serum (NHS) containing a known concentration of C3.

6.5. Haemolytic titration of human C3

This assay was used to determine the haemolytic activity of the purified C3. The method used EAs (antibody coated SRBCs) sensitised with C1, C4 and oxidised C2 (^{oxy}C2). Oxidised C2 was used to give a more stable and haemolytically active classical pathway C3 convertase. Cell lysis was brought about by adding the terminal components in the form of C5 and guinea-pig serum depleted in components 3, 4 and 5 (GPR345, guinea-pig serum treated with potassium thiocyanate and hydrazine). The test sample of C3 was titrated into the system and the number of haemolytic units of C3 per ml could be calculated from the degree of lysis it caused.

Reconstitution of EAC4s: Frozen EAC4s (antibody coated SRBCs sensitized with human C4) were kindly donated by Mrs. L. Jones and were reconstituted according to the following protocol. The cells were quickly thawed in a 37°C water bath and the following were added:

0.23ml	of	D50S	incubated	for 5 mins	at 37°C
1.00ml	of	Mannitol GVB^{++}	incubated	for 2 mins	at 37°C
2.00ml	of	"	"	"	"
8.00ml	of	"	"	" 5 mins	"
5.00ml	of	DGVB^{++}	"	" 2 mins	"
10.00ml	of	"	"	" 2 mins	"
10.00ml	of	"	"	" 2 mins	"
10.00ml	of	"	"	" 10 mins	"

The EAC4 suspension was then spun for 4 mins at 1000g at R.T. The supernatant was discarded and the cell pellet was washed thrice in DGVB^{++} or until the cells stopped lysing. The cells were then standardized to 1x10^{8}/ml (2.14) in DGVB^{++}.

Preparation of EAC$\bar{1}$4s: All the following steps except where otherwise stated were performed on ice with ice cold reagents

and buffers. Purified human C1 was diluted to 1/1000 in $DGVB^{++}$, heated to 37°C and added to an equal volume of EAC4s at 37°C. These were mixed and incubated for 15 mins at 37°C in a shaking water bath. The cells were then spun at 1000g at R.T. for 5 mins. The supernatant was removed and the cells were resuspended to their original volume in $DGVB^{++}$.

Preparation of $EAC\overline{14^{oxy}2}$: Purified human C2 was oxidised by adding 100µl of C2 to 100µl of oxidising mixture (10µl KI/I_2 plus 2ml 0.1M KPO_4 pH 6.0) and incubated at R.T. for 5 mins. The volume was made up to 5ml and added to an equal volume of $EA\overline{C14}s$. The cells were incubated for a further 10 mins at 30°C then spun at 1000g for 5 mins at R.T, the supernatant was discarded and the cell pellet resuspended to the original volume in $DGVB^{++}$. To test the activity of the $EAC\overline{14^{oxy}2}$, 300µl of 1/15 dilution of normal rat serum (C rat) in 0.04M EDTA/GVB^{++} was added to 100µl of $EAC\overline{14^{oxy}2}$ and incubated at 37°C. If the cells are correctly sensitised this should result in lysis within 5 mins. These cells can now be used in the C3 haemolytic assay.

Assay for haemolytic C3: A 1/100 dilution of GPR345 was made in $DGVB^{++}$ and to this human C5 was added to give a final dilution of 1/200. This was used as the buffer in which C3 was doubly diluted starting at 1/1000. To 100µl of the diluted C3 100µl of $EAC\overline{14^{oxy}2}$ was added and the tubes were incubated in a shaking water bath at 37°C for 1 hr. Two ml of saline was added to the tubes and these were then spun at 1000g for 5 mins. The OD of the supernatants at 414 nm were measured. The controls were as follows:

Reagent blank (RB) = $EAC\overline{14^{oxy}2}$ + GPR345 + buffer + saline

100% lysis (100%) = " + " + " + water

Complement colour (CC)= GPR345 + " + saline

Cell blank (CB) = $EAC\overline{14^{oxy}2}$ + buffer + buffer + saline

The percentage lysis of the $EAC14^{oxy}2$ in the presence of C3 was determined using the following formula:

$$\% \text{ lysis} = \frac{OD_{414} \text{ sample} - OD_{414} \text{ RB}}{OD_{414} \text{ 100\%} - OD_{414} \text{ RB}} \times 100 = Y$$

$$Z \text{ value} = -\ln (1 - Y)$$

By calculating the Z value for each dilution of C3 and plotting the Z value against the C3 dilution a straight line graph is obtained from which the number of haemolytic units of C3 per ml can be calculated.

6.6 Formation of C3b

Purified human C3 was converted to C3b by trypsinization. The method was based upon those used by Arnaout et al (1981) and Nilsson, Mandle & McConnell-Mapes (1975)

0.6% w/w trypsin to C3 was incubated at 37°C for 3 mins. Then a 2-fold weight excess of soya bean trypsin inhibitor (SBTI) to trypsin was added and incubated for a further 2 mins at 37°C to stop further cleavage. The conversion of C3 to C3b and the presence of any further breakdown fragments was analysed using IEP (2.9) with a 1/10 dilution of rabbit anti-human C3c antiserum in the troughs to precipitate the C3 fragments. C3b is more negatively charged than C3 so it migrates further towards the anode; the presence of C3b was also confirmed using 4% SDS-PAGE tube gels (2.10).

6.7 C3b polymerization using DTBSP

One ml of C3b at 5mg/ml in 0.05M sodium phosphate buffer pH 7.5 was added to 25µl of DTBSP at 3.7mg/ml. After thorough mixing the solution was incubated at R.T. for 2 hrs. Then 5µl of 1M Tris/HCl pH 7.5 was added to prevent polymerization and then the polymerized C3b was dialysed against 0.05M sodium phosphate buffer pH 7.5 to remove any remaining DTBSP. A sample was then run on 4% SDS-PAGE phosphate tube gels (2.10) to check whether the polymerization had been successful.

The method was repeated using 5µl, 10µl, 15µl or 20µl of DTBSP at 3.7mg/ml.

6.8 C3b polymerization using dimethyl suberimidate

A 50mM stock solution of dimethyl suberimidate was made up in a buffer composed of equal volumes of 200mM NaOH and 500mM triethanolamine pH to 8 with concentrated HCl.

The C3b and dimethyl suberimidate stock solution were mixed to give a final protein concentration of 1mg/ml and 10mM dimethyl suberimidate. After mixing the solution was left at R.T. for 1 hr. A sample was then run on 4% SDS-PAGE phosphate tube gels (2.10) to check whether the polymerization had been successful.

The method was repeated using dimethyl suberimidate at a final concentration of 10mM, 20mM or 40mM or using a 0.2M Tris/HCl buffer pH 8 (Wilson et al, 1982) as opposed to the NaOH-triethanolamine buffer.

6.9 Sucrose gradients

Sucrose solutions were made up in PBS. Gradients composed of 1ml of 10%, 20%, 30% and 40% or 1ml of 7.5%, 15%, 22.5% and 30% were formed on ice in 13 x 51mm polyallomer centrifuge tubes. 500µl of the sample to be fractionated was carefully layered onto the top of the gradient and spun at 4°C for 16 hrs at 105,000g on a Beckman model L2-65B ultracentrifuge. 200µl fractions were then collected from the tube base using a sucrose gradient fractionator. The protein content of each fraction was analysed using Folin assay (2.4) and the polymer content analysed on 4% SDS-PAGE phosphate tube gels (2.10).

6.10 Separation of C3b polymers by gel filtration

Two types of gel filtration columns were used, both were equilibrated in 0.05M sodium acetate pH 5.6 plus 1M NaCl and 1ml fractions were collected.

a) 1.5 x 86cm Sephadex G150 superfine column

b) 2.6 x 90cm Sephadex S200 column

The protein and polymer content of each fraction was analysed as described for sucrose gradients (6.9)

RESULTS

The method used to separate C3 from human plasma employs the linear polymer PEG as an agent for fractionating the plasma proteins followed by chromotography. The 5% (w/v) PEG removes the bulk of the plasma fibrinogen, and the 12% (w/v) PEG

precipitates C4, C3 and C5 leaving in solution albumin and transferrin. A brief resumé of the protocol used to purify C3 is given in Fig. 18. The elution profiles for the DEAE Sephacel and hydroxylapatite columns are shown in Fig. 19 and Fig. 20 respectively.

The DEAE Sephacel column yielded: P, I, H, C5, C3 and C4. The column was screened using radial immunodiffusion with monospecific antiserum against the aforementioned complement components. The results of these plates are expressed as D^2(mm) in Fig. 19. P was eluted between fractions 35 and 70; however this is not marked in Fig. 19 as the rings were very faint and despite counterstaining for 24 hrs with a second layer antibody (1/10 dilution of sheep anti-rabbit antiserum) the rings could not be accurately measured. Pools were made of the fractions containing P, I, H and C4 and these were stored at -70°C. Tubes 340 to 360 were pooled, to give a C3 fraction slightly contaminated with C5, after concentration this was applied to the hydroxylapatite column.

C3 and C5 were resolved on the hydroxylapatite column by using a stepwise elution scheme (Fig. 20). Only the high molarity phosphate buffer (0.4M potassium phosphate buffer) applied at fraction 360 eluted any protein from the column. This contained C3 as determined by radial immunodiffusion with no contaminating C5. The C3 was eluted between tubes 375 and 450 in two peaks, three pools were made, pools 1 and 2 consisting of peaks 1 and 2 respectively and pool 3 the residual tubes.

	Column fractions	Volume
Pool 1	385 - 420	600ml
2	421 - 430	200ml
3	375-384, 430-450	500ml

FIG. 18 Protocol used for human C3 purification

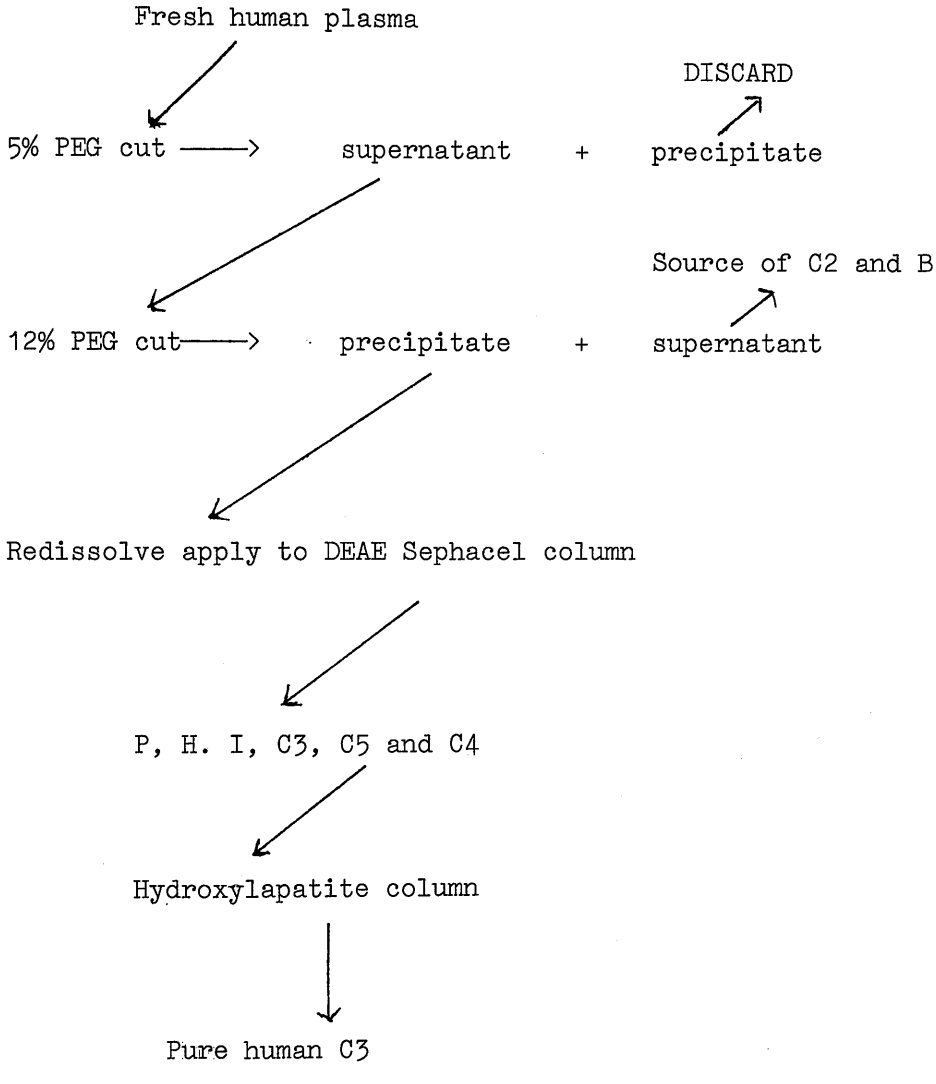

Fresh human plasma
DISCARD
5% PEG cut
supernatant
+
precipitate
Source of C2 and B
12% PEG cut
precipitate
+
supernatant
Redissolve apply to DEAE Sephacel column
P, H. I, C3, C5 and C4
Hydroxylapatite column
Pure human C3

FIG. 19 Elution profile from DEAE Sephacel column showing conductivity (mS at $0^{o}C$ ---), protein (OD_{280}nm .-.-.) and complement component levels as measured by radial immunodiffusion (D^{2}mm ——)

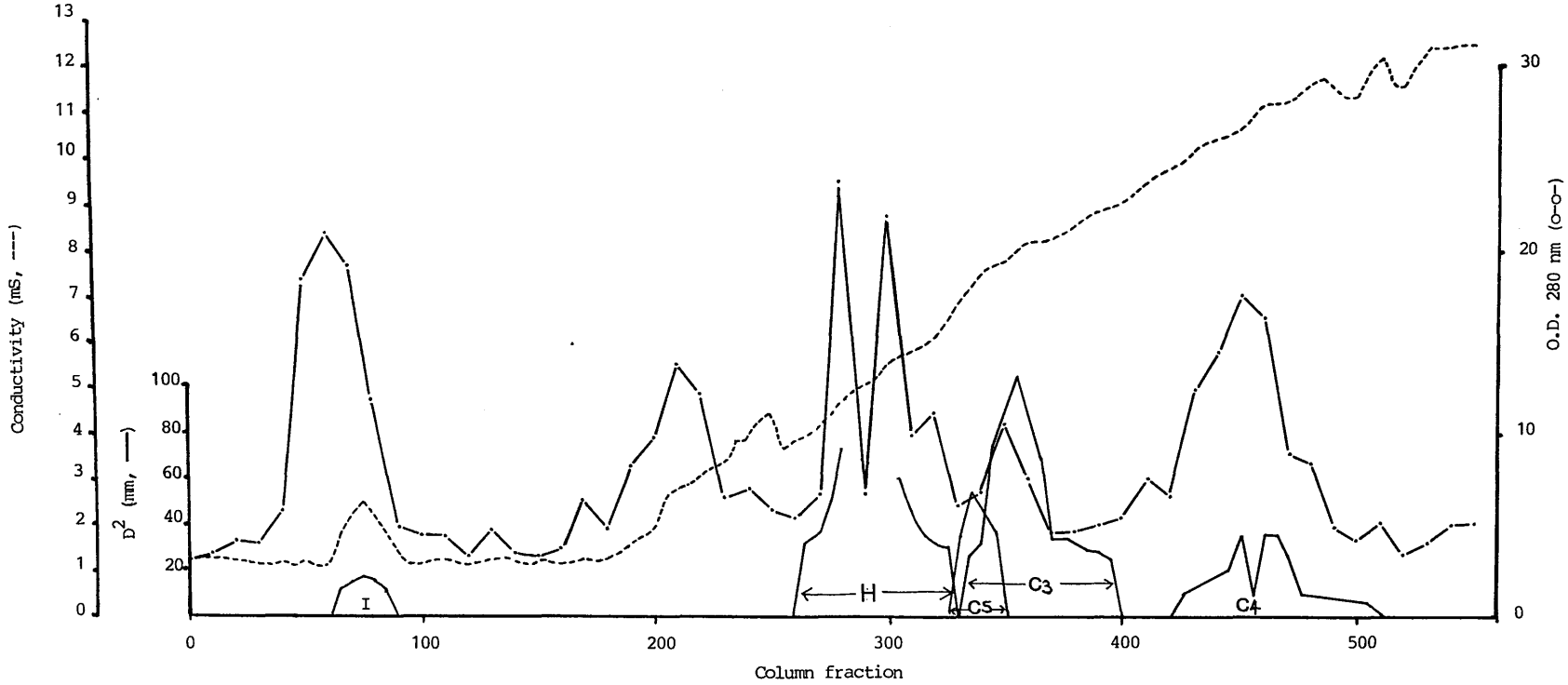

Conductivity (mS, ---)
D^2 (mm, —)
O.D. 280 nm (o–o–)
Column fraction
I
H
C3
C5
C4

FIG 20. Elution profile from hydroxylapatite column showing conductivity (mS at $0^{\circ}C$ ---), protein (OD_{280nm} .-.-) and C3 level as measured by radial immunodiffusion (D^2_{mm} ——)

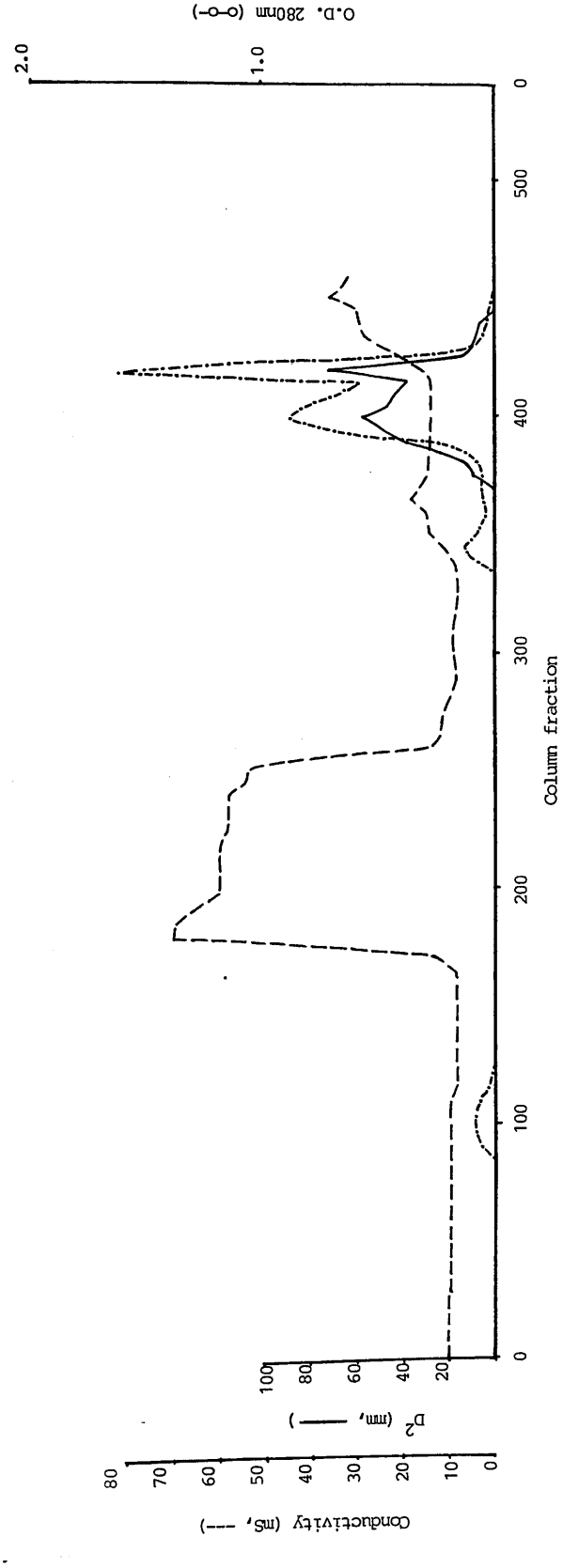
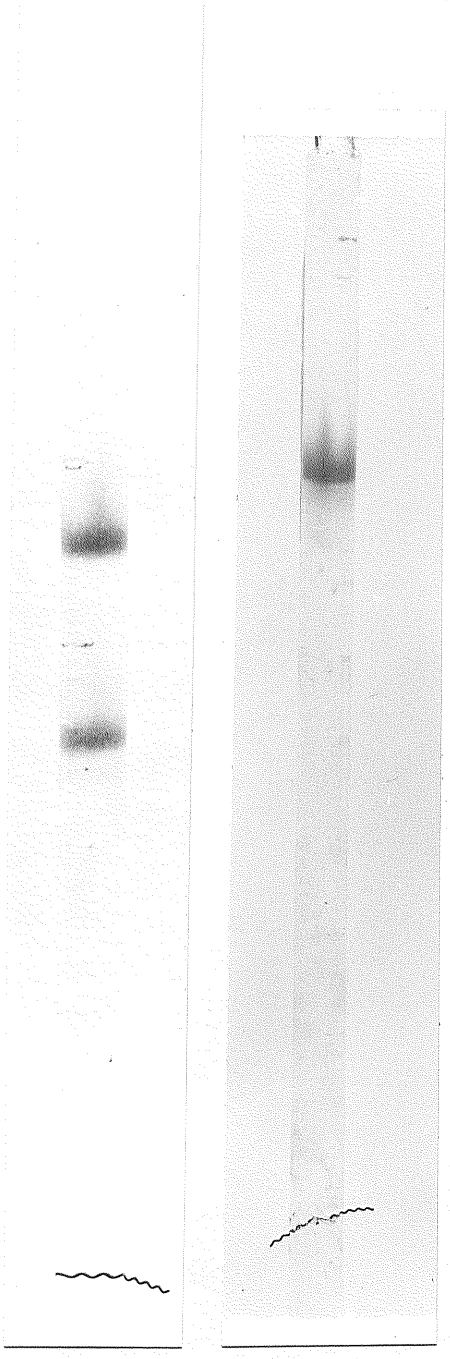

O.D. 280nm (o—o—)
2.0
1.0
0
500
400
300
200
100
0
Column fraction
D² (mm, ——)
100
80
60
40
20
Conductivity (mS, ---)
80
70
60
50
40
30
20
10
0

PLATE 15 Purified C3 (reduced and non-reduced) run on 4% SDS-PAGE phosphate gels.



These pools were concentrated by ultrafiltration, then the protein content was measured using Folin assay, nephelometry and radial immunodiffusion. The results of these assays are summarized in Table 16 together with the profile of protein recovery for the whole purification procedure. It is clear that the protein concentrations as quantified by these three methods in some instances give conflicting results. For example, the protein content of pools 2 and 3 as determined by Folin assay do not correspond with those determined by radial immunodiffusion. This is probably due to the high concentrations of PMSF and benzamidine in these pools interfering with the Folin assay and it is for this reason that protein measurements could not be made using OD_{280}nm. There was also some difference in the level of C3 in pools 1 and 2 as determined by nephelometry and radial immunodiffusion. The reason for the lower values of C3 as determined using nephelometry is that this technique requires the presence of carrier proteins for an accurate determination of the C3 concentration.

The purity of C3 was tested using 4% SDS-PAGE phosphate gels (Plate 15). Pools 1 and 2 were tested; non-reduced the molecular weight was 170,000 daltons and reduced the α chain was 120,000 and the β chain 80,000 daltons.

The recovery of C3 in mg was 30% (using C3 protein concentration determined by radial immunodiffusion) based on an estimated value of 1mg/ml of C3 in the starting material. The haemolytic titration of pools 1 and 2 (Fig. 21a and 21b) showed pool 1 (Fig.21a) to have 310,000 haemolytic units/ml and pool 2 (Fig. 21b) to have 8,333 haemolytic units/ml.

TABLE 16. Profile of protein recovery during C3 purification

Stage of purification	Volume	Protein	Total protein	C3 concentration determined by:	
				Nephelometry	Radial immuno-diffusion
Plasma	2000 ml	60 mg/ml	135000 mg	NT	NT
5% PEG cut (Total	3000 ml	16 mg/ml	48000 mg	NT	0.9 mg/ml
5% PEG cut (Precipitate	-				
5% PEG cut (Supernatant	3000 ml	15.3 mg/ml	46000 mg	NT	0.83 mg/ml
12% PEG cut (Total	4000 ml	11.5 mg/ml	46000 mg	NT	ND
12% PEG cut (Supernatant	4000 ml	3.1 mg/ml	12400 mg	NT	ND
12% PEG cut (Precipitate (redissolved)	280 ml	120 mg/ml	33600 mg	NT	NT
Sephacel column Pool 1	300 ml	5.6 mg/ml	1680 mg		
Hydroxylapatite column					
Pool 1	100 ml	4.375 mg/ml		4 mg/ml	4.4 mg/ml
Pool 2	22 ml	3.125 mg/ml		4.75 mg/ml	5.53 mg/ml
Pool 3	8 ml	2.125 mg/ml		NT	5.7 mg/ml

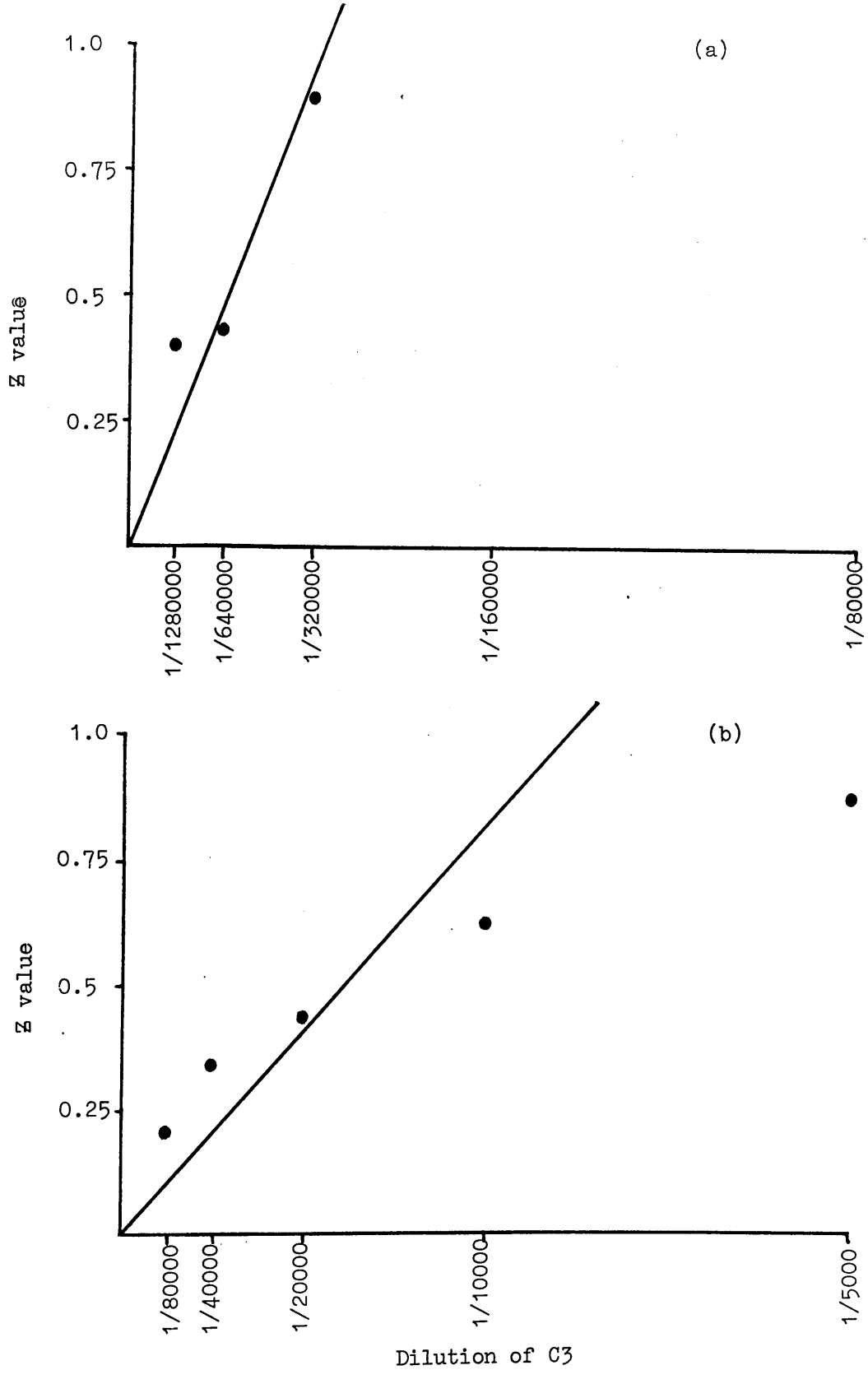
NT = not tested

ND = not determinable

FIG 21 Haemolytic titration curves for purified human C3

(a) Pool 1

(b) Pool 2


(a)
1.0
0.75
0.5
0.25
Z value
1/1280000
1/640000
1/320000
1/160000
1/80000
(b)
1.0
0.75
0.5
0.25
Z value
1/80000
1/40000
1/20000
1/10000
1/5000
Dilution of C3

C3 was successfully cleaved to C3b using trypsin (6.6) and this was confirmed by IEP, with C3b being more electronegative than C3 thus travelling further towards the anode. This, together with the use of 4% SDS-PAGE tube gels, showed that there were no further C3 breakdown products. The C3b produced by this method was used for polymerization. DTBSP and dimethyl suberimidate were used to chemically cross-link C3b. The results of these two methods will be discussed separately.

DTBSP was initially used to polymerize C3b and the polymerized molecules were then separated according to size using sucrose gradients (20% / 30% / 40% / 50%, 6.9). By using a sucrose gradient fractionator, fractions could be collected from the base of the tube; 200µl fractions were collected and this gave 11 fractions per gradient, with fraction 1 containing the highest molecular weight oligomers. The fractions were then screened for protein by Folin assay (2.4) and the polymer content was analysed on 4% SDS-PAGE phosphate tube gels (2.10). This method of polymerization produced very little dimeric C3b. In an attempt to optimize the dimeric C3b yield the amount of DTBSP in the reaction mixture was titrated. Five µl, 10µl and 15µl of DTBSP were used instead of 25µl and this did reduce the extent of C3b polymerization. After fractionation on 20% / 30% / 40% / 50% sucrose gradients the protein profile for the gradients (Fig 22) showed a peak in fractions 7 and 8 for all three concentrations of DTBSP used. However, the gels did not show an increase in dimer formation, but of trimer and monomer. As the yield of dimer was poor despite adapting the method it was decided to try another means of cross-linking C3 (dimethyl suberimidate) and use 7.5% / 15% / 22.5% / 30% sucrose gradients to try and give a better

separation of monomeric, dimeric and trimeric C3b.

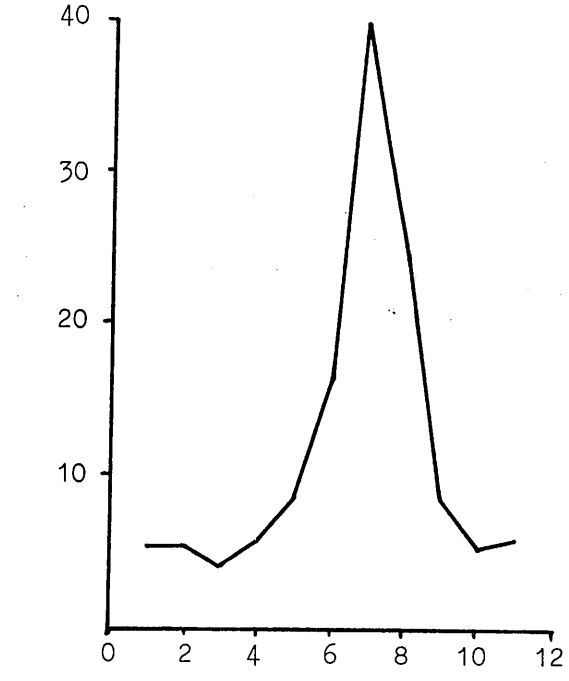
The use of dimethyl suberimidate to polymerize C3b did yield fewer high molecular weight oligomers, as demonstrated by the small amount of protein in the lowest fractions of the gradient (Fig. 23), however it also produced a lot of monomeric C3b in which the α and β chains had become cross-linked. Gradient fractions 2, 3 and 4 did contain dimer contaminated with monomer. It was decided to adapt the method by increasing the amount of dimethyl suberimidate used, to try and increase the degree of polymerization. In addition, the polymerization was performed in a 0.2M Tris/HCl pH 8.0 buffer according to the method described by Wilson et al (1982).

Increasing the concentration of dimethyl suberimidate from 10mM to 20 or 40 mM did not alter the degree of C3b polymerization. The method described by Wilson et al (1982) gave a low yield of dimer and a high percentage of monomeric C3b.

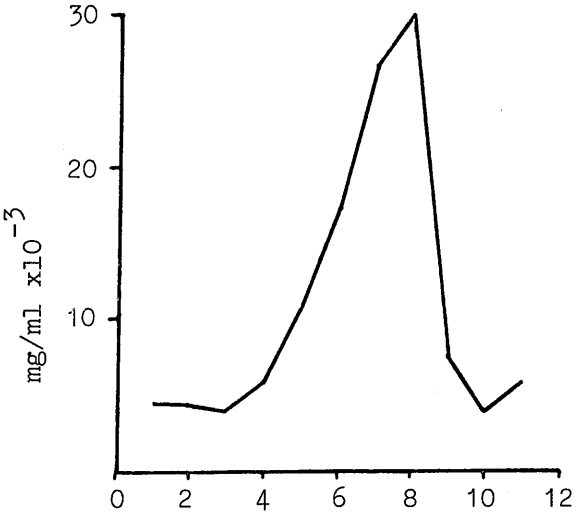
By using 10mM dimethyl suberimidate it was possible to get a pool containing dimer and monomer C3b (50:50 as determined by the visualization of band densities on the 4% SDS-PAGE phosphate tube gels). These could not be resolved further despite using gel filtration (6.10).

It was evident that dimer contaminated with monomer would have to be used in the RIA and that using 10mM dimethyl suberimidate was the best means for producing dimer. However, on a second attempt of this method C3b did not become polymerized and we were informed by the company supplying this chemical that it had a very short shelf life and probably had decomposed rendering it non-functional.

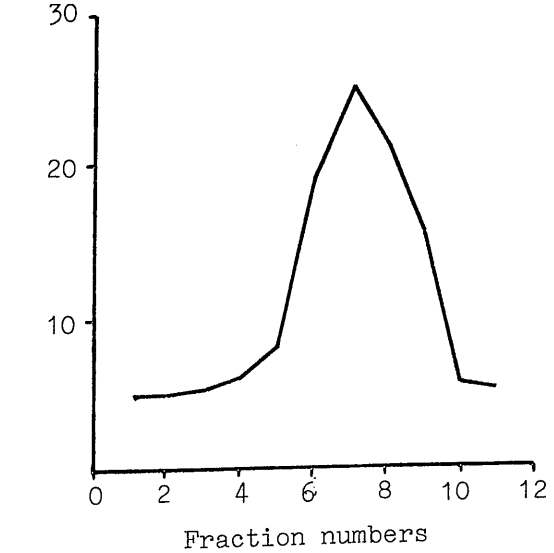
FIG. 22 Effects of a) 5µl, b) 10µl and c) 15µl of DTBSP on the profile of C3b from 20% / 30% / 40% / 50% sucrose gradients.



a) 5ul DTSBP

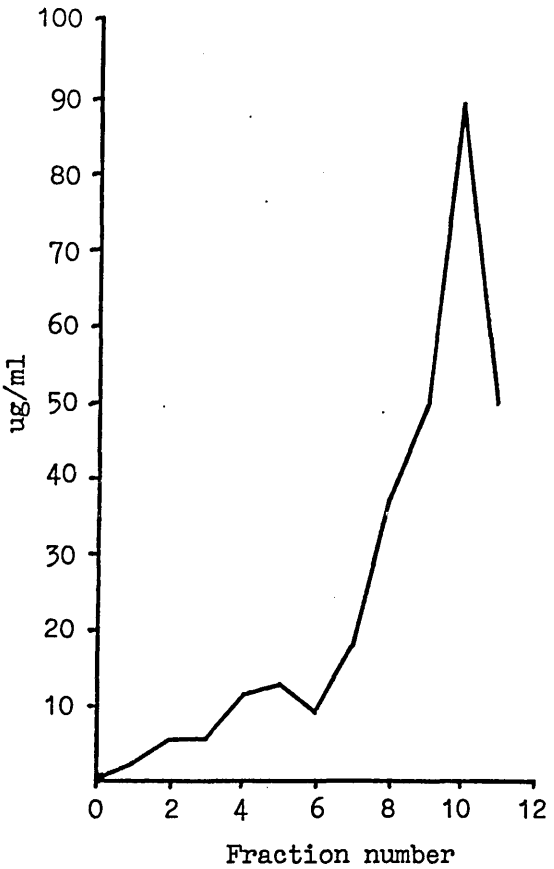


b) 10ul DTSBP



c) 15ul DTSBP

FIG. 23 Profile of protein recovery of dimethyl suberimidate treated C3b from 7.5% / 15% / 22.5% / 30% sucrose gradient.


100
90
80
70
60
50
40
30
20
10
ug/ml
0
2
4
6
8
10
12
Fraction number

The method using DTBSP and 7.5% / 15% / 22.5% / 30% sucrose gradients was then repeated. This cross-linked C3b up to a pentameric form (Plate 16), with fractions 1 and 2 containing high molecular weight oligomers (Plate 17), fractions 3 and 4 containing dimeric, trimeric, tetrameric and pentameric C3b (Plate 18), fractions 5 and 6 monomeric, dimeric and trimeric C3b (Plate 19), fractions 7 and 8 monomeric and dimeric C3b (Plate 20) and finally, fractions 9, 10, 11 and 12 having monomeric C3b with breakdown fragments in fractions 11 and 12. The fractions (7 and 8) containing dimer contaminated with monomer were pooled and concentrated, and from a starting concentration of 3mg/ml, 0.8mg/ml was recovered in the form of dimer contaminated with monomer.

DISCUSSION

C3 was purified successfully from fresh human plasma and converted to C3b using trypsin; however as is clear from the results section pure C3b dimer was not attained despite trying two methods of chemical cross-linking (DTBSP and dimethyl suberimidate) and fractionation (sucrose gradients and gel filtration). The use of dimethyl suberimidate as a cross-linking agent was abandoned because of the spontaneous decomposition of this linking agent. DTBSP gave dimeric C3b; however the protein recovery was low; another problem with this method of cross-linking was that the links spontaneously broke down after long periods of storage, as the links formed by DTBSP are reducible unlike those formed by dimethyl suberimidate. Due to these difficulties and the low yield of dimeric C3b it was decided to abandon the development of the functional RIA.

PLATE 16 DTBSP polymerized C3b (a=reduced, b= non-reduced) on 4% SDS-PAGE phosphate gels


a
b

PLATE 17. High molecular weight oligomers of DTBSP polymerized C3b,
(a) fraction 1 and
(b) fraction 2
from the sucrose gradient (run non-reduced on 4% SDS-PAGE phosphate gels)

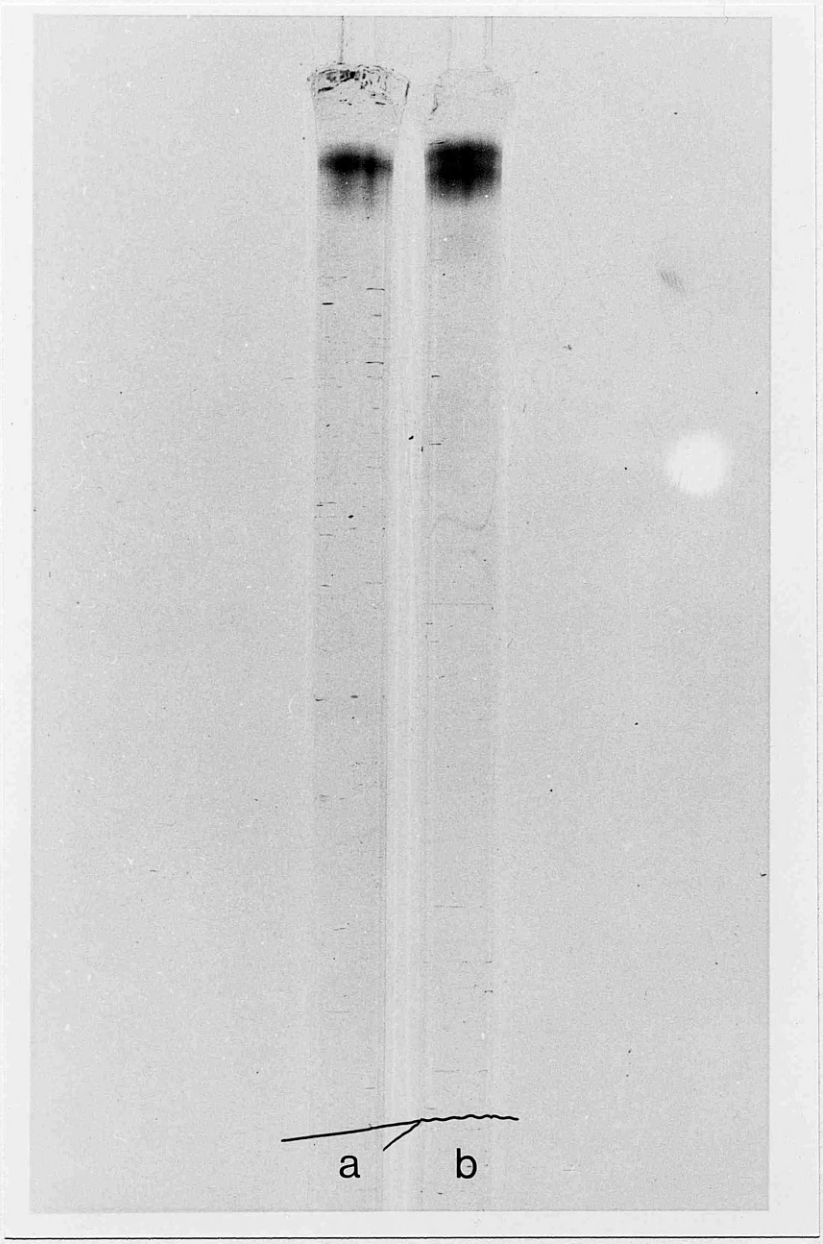

a
b

PLATE 18 Dimeric, trimeric, tetrameric and pentameric DTBSP polymerized C3b,

(a) fraction 3 and

(b) fraction 4

from the sucrose gradient (run non-reduced on 4% SDS-PAGE phosphate gels)

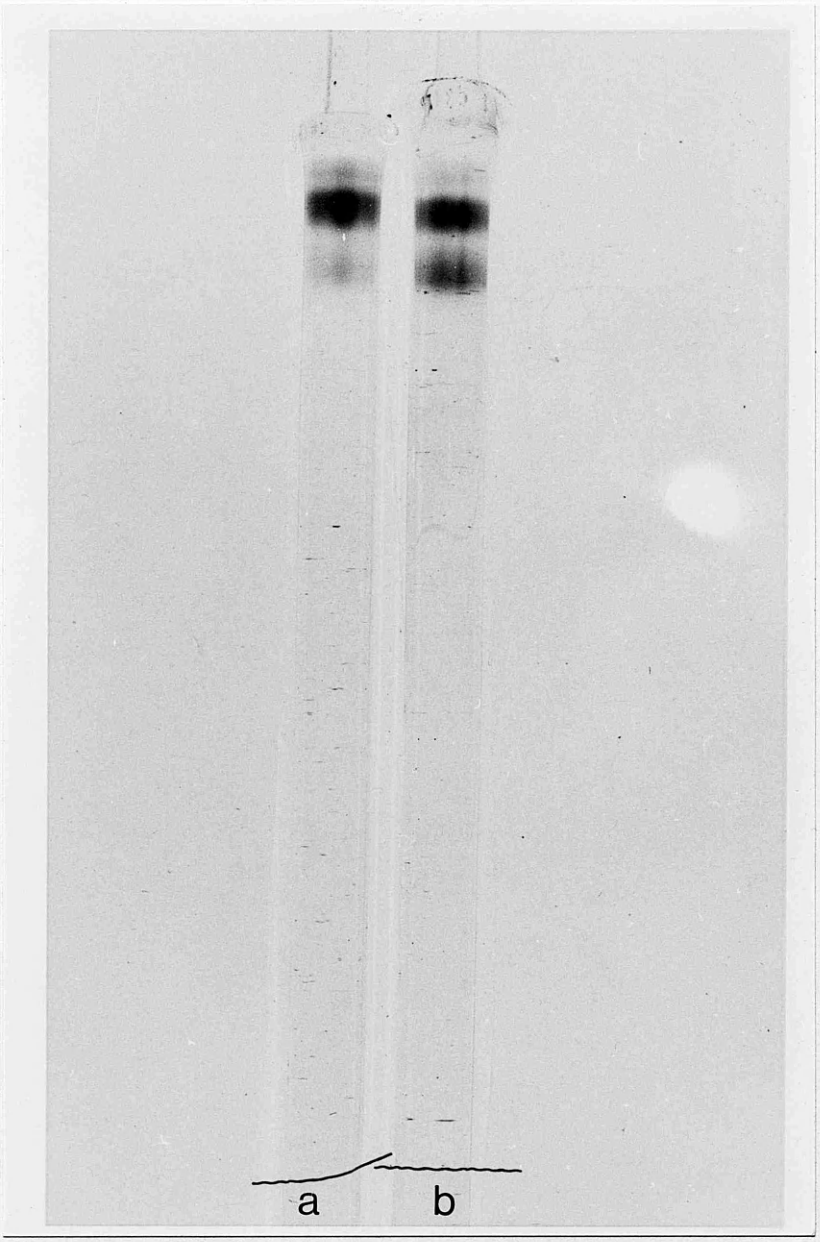

a
b

PLATE 19 Monomeric, dimeric and trimeric DTBSP polymerized C3b,
(a) fraction 5 and
(b) fraction 6
from the sucrose gradient (run non-reduced on 4% SDS-PAGE phosphate gels)

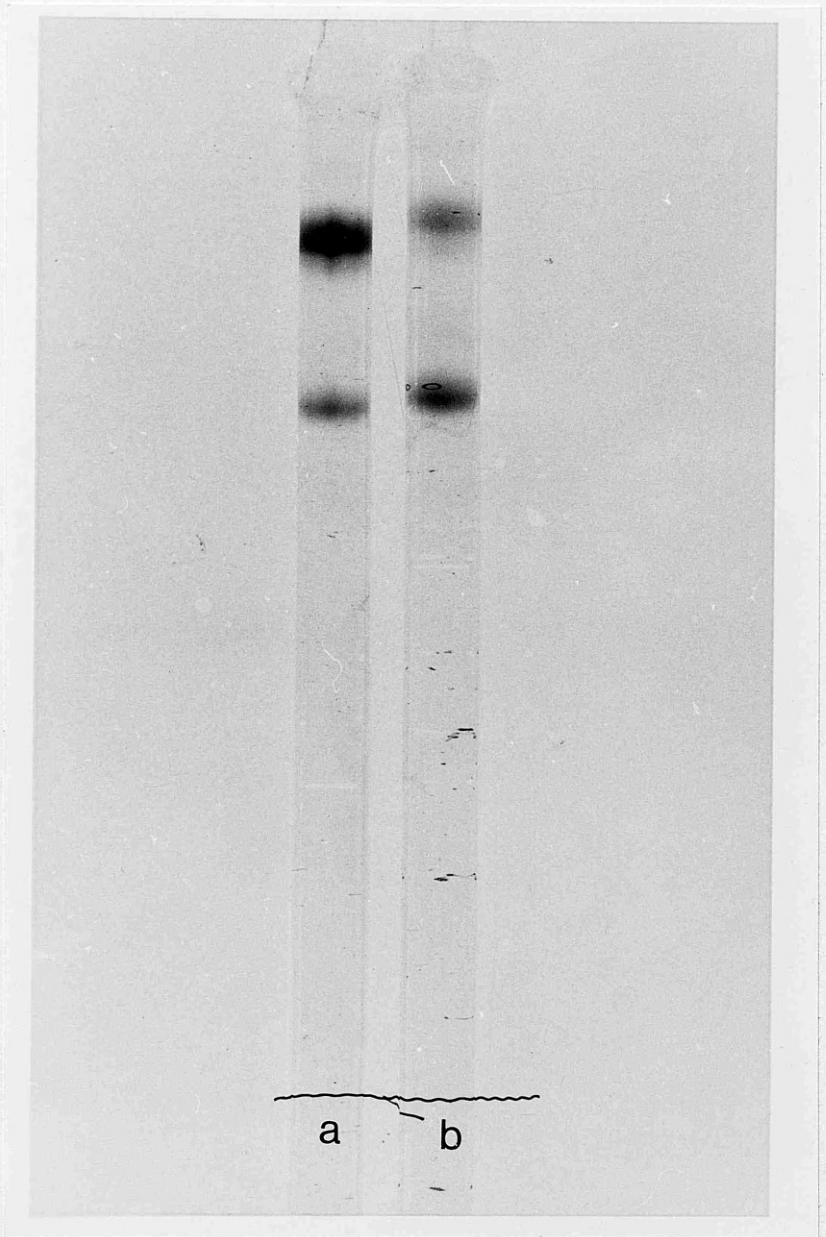

a
b

PLATE 20 Monomeric and dimeric DTBSP polymerized C3b,

(a) fraction 7 and

(b) fraction 8

from the sucrose gradient (run non-reduced on 4% SDS-PAGE phosphate gels)


a
b

CHAPTER 7

CR1 LEVELS ON THE ERYTHROCYTES OF NORMAL INDIVIDUALS, SLE AND RA PATIENTS.

INTRODUCTION

Before this project was started four studies had been done on the erythrocyte CR1 levels in normals, SLE and RA patients. The results of these studies are discussed extensively in chapter 1. However to briefly summarize; all four groups found the SLE patients to have lower CR1 levels on their erythrocytes compared to the normal controls. The causative factor, however was an area of dispute. Wilson et al (1982) and Miyakawa et al (1981) present data to support the view that CR1 numbers are inherited, whilst Iida et al (1982) and Inada et al (1982) believe that low CR1 levels could be a secondary manifestation of the disease process. Thus the aim was firstly, to compare CR1 levels on the erythrocytes from normals, SLE and RA patients. Secondly, to see whether CR1 levels in these groups varied with time and thirdly to examine CR1 levels in normal and SLE families. Thus it was hoped to determine whether CR1 levels are inherited or not. In addition extensive serological studies were performed on the RA and SLE patients to see if any of the results of routine serological tests correlated with erythrocyte CR1 levels.

MATERIALS AND METHODS

7.1 Normal controls

A total of 70 blood samples from 44 normal individuals were studied. The age range was from 18 to 65 and consisted mainly of laboratory personnel and members of the normal families.

7.2 SLE patients

A total of 41 blood samples from 33 SLE patients were studied (Tan et al,1982). Immediately prior to venous blood sampling the SLE patients were examined for evidence of disease activity. The criteria used for the assessment of the SLE patients fulfilled the ARA classification scheme which is as follows: Active disease, continuing clinical activity of involved organ system(s) e.g. polyarthritis, vasculitis, lupus pernio rash, proven presence of pleurisy or pericarditis, continuing active urinary sediment with cells and casts alone or in addition to proteinuria when present. Inactive disease was diagnosed when none of the above were present. Serology was not used to define disease activity. The blood samples were kindly supplied by Dr. Asad Zoma and Prof. K. Whaley. The disease activity of the patients was not disclosed until after the CR1 determinations had been completed.

7.3 RA patients: A total of 25 blood samples from 20 RA patients were studied (Ropes et al 1959). Immediately prior to venous blood sampling the RA patients were examined for evidence of disease activity.

7.4 Quantitation of CR1 sites/erythrocyte

The RIA to quantitate the number of CR1 sites/erythrocyte was performed as described in 4.7.

7.5 Serological studies: Ten ml of blood was collected and allowed to clot in a plain glass tube, the serum was removed and stored at -70°C until required. Serum samples from SLE and RA patients were examined for total haemolytic complement (CH50), C4, C3, B and $C\bar{1}$-INH levels (Whaley 1985). In addition samples from RA patients were examined for rheumatoid factor levels using latex and RIA, and SLE patients were examined for antinuclear antibody and DNA binding capacity (Webb and Whaley 1974). Some SLE samples were also examined using the Crithidia test for double stranded DNA and for immune complex levels. All the serological tests were performed very kindly by the staff of the immuno-pathology laboratory, except the determination of immune complex levels by PEG precipitation and conglutinin binding; these were kindly performed by Dr. S. Cobb. In the latter assay fixed bovine serum conglutinin binds immune complexes containing iC3b. In the former assay circulating immune complexes are precipitated with PEG and their IgG content measured by radial immunodiffusion.

7.6 Serial studies: These were performed on 9 normal individuals, 8 SLE patients and 4 RA patients. The time periods between individual determinations were variable.

7.7 Family studies: In total six families were studied, two normal families and 4 families where 1 or 2 members had SLE. The families were chosen on the basis that a) all immediate family members were alive, b) the family was willing to take part in the study and c) was geographically accessible. The CR1 sites/erythrocyte in an individual family were all performed at the same time.

7.8 Statistical analysis: Where applicable the data were analysed using the Mann-Whitney U-test for non-parametric data.

RESULTS

Erythrocyte CR1 levels in patients and normal individuals

The CR1 sites/erythrocyte were studied in three groups; a group of normal controls randomly picked and the SLE and RA patient groups, comprising of patients picked at random by the clinicians supplying the blood samples. The distribution of CR1 sites/erythrocyte in these three groups is given in Fig. 24.

The distribution of CR1 numbers in the controls (n = 70) was unimodal, the mean level was 3320 CR1 sites/erythrocyte with a range from 0 to 21692 CR1 sites/erythrocyte. The mean level of CR1 sites/erythrocyte in the SLE group (n = 41) was 1541 and like the normal group the range was wide, from 0 to 15766 CR1 sites/erythrocyte. However this can be attributed

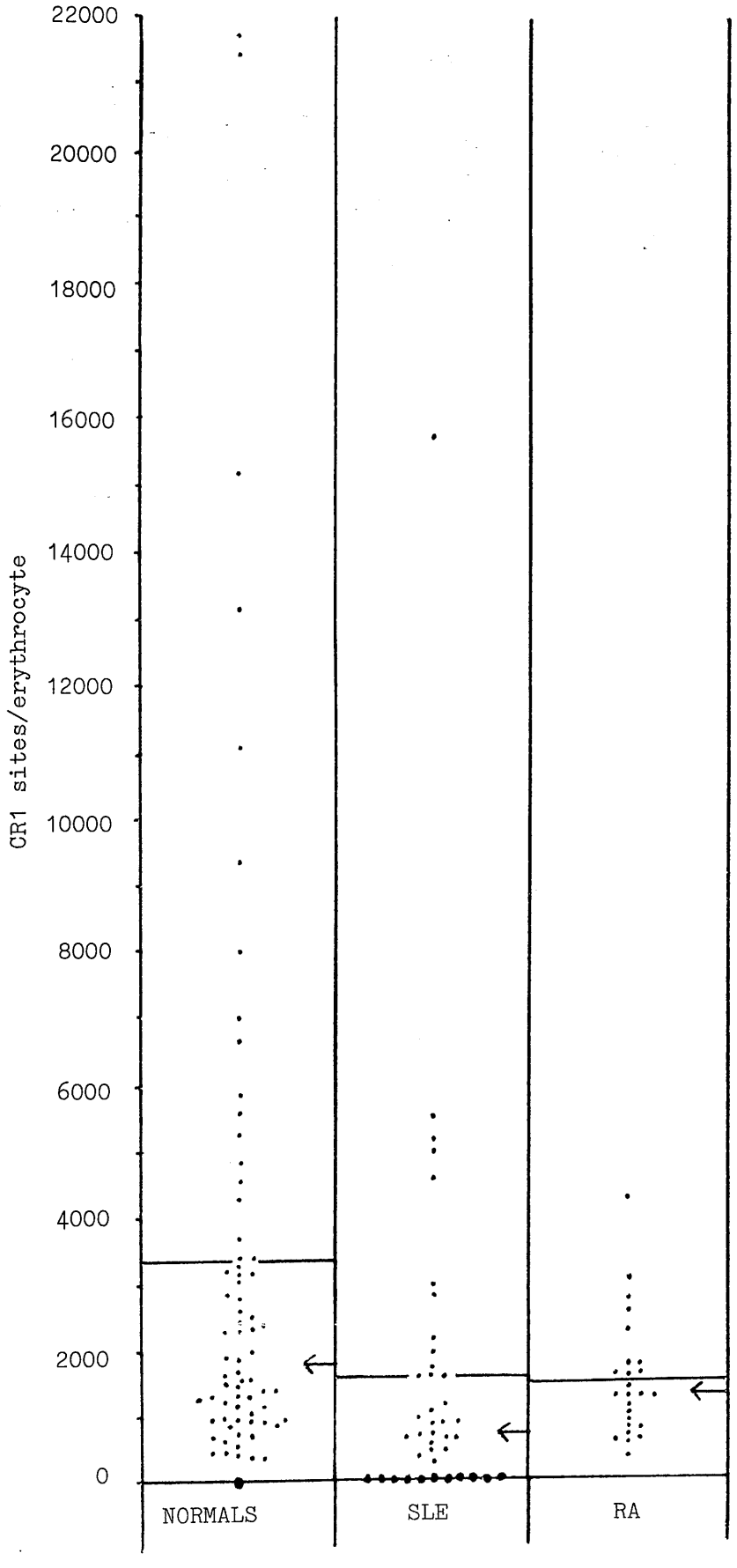
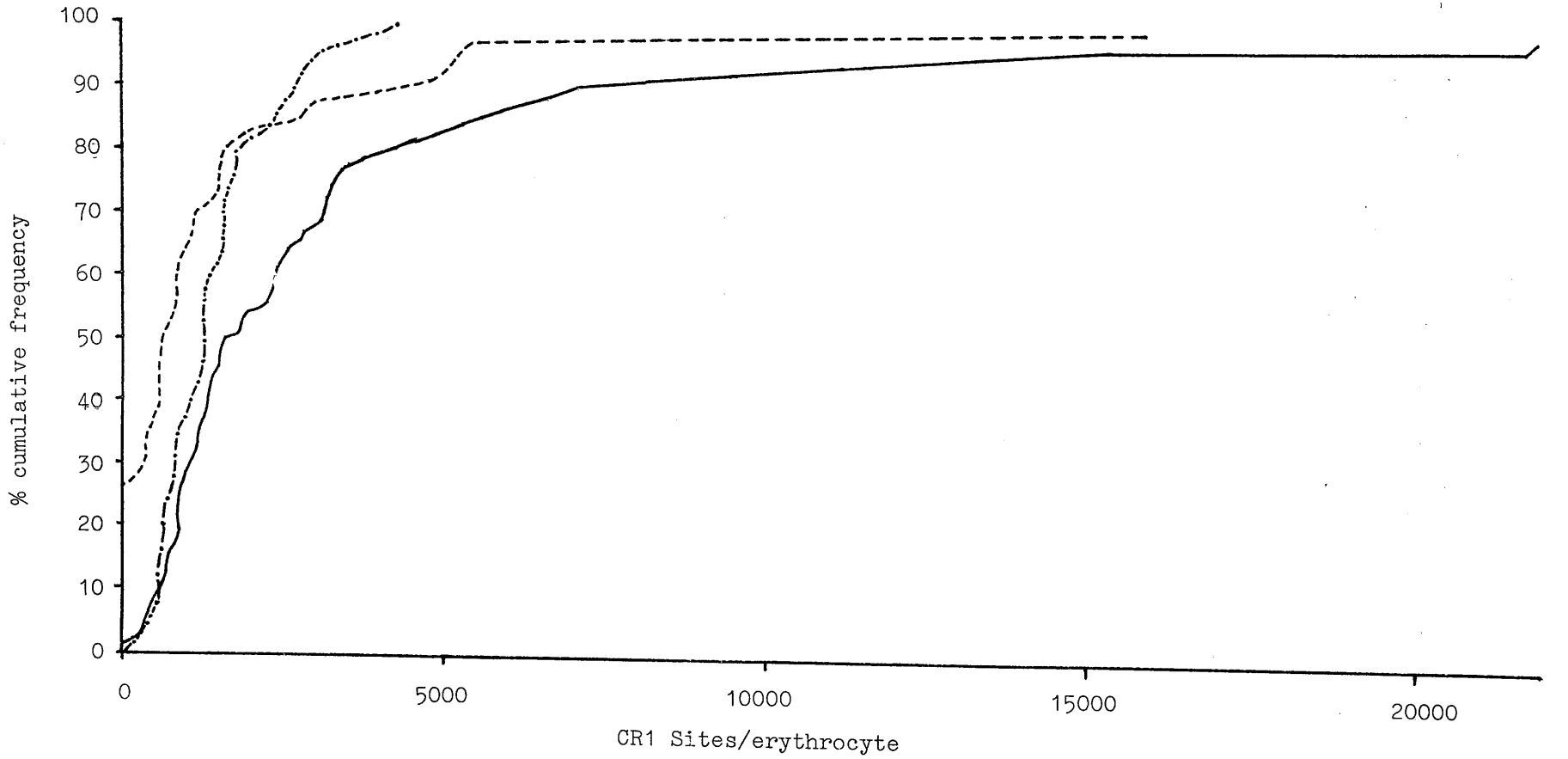

22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
CR1 sites/erythrocyte
NORMALS
SLE
RA

FIG. 24 Distribution of CR1 sites/erythrocyte in normal individuals and patients with SLE and RA. The horizontal lines represent the mean values and the arrows the median values.

to one outlying point (15766 CR1 sites/erythrocyte) with the remaining being clustered between 0 and 5500 CR1 sites/erythrocyte. In the SLE patient group 11 of the blood samples studied had no receptors, in comparison to one in the normal group. The mean for the SLE patients was significantly lower than for the normal controls ($p < 0.001$). The mean for the RA group ($n = 25$) studied was 1410 CR1 sites/erythrocyte, which is significantly less than the normal mean ($p < 0.05$). The range of CR1 sites/erythrocyte (350 to 4259) was narrower than the normal controls and the SLE patients. However, unlike the SLE patients there were no RA patients who had zero receptors on their erythrocytes. The mean CR1 sites/erythrocyte for the SLE and RA patients differed significantly ($p < 0.05$).

These results were replotted as cumulative frequency curves which gave a clearer indication of the differences and similarities between the normal, SLE and RA groups studied (Fig.25). The cumulative frequency curve for the normals is sigmoidal with only minor irregularities in this shape between 50 and 77%. Eighty per cent of the normal population studied had 4000 CR1 sites/erythrocyte or less. The remaining 20% constituted the large range from 4000 to 21692 CR1 sites/erythrocyte. The curve for the RA patients was also sigmoidal paralleling the normal curve to the 50% point after which it deviated towards the SLE curve. This observation indicated that the RA group studied did not have many patients with very high or very low CR1 numbers. Although the cumulative frequency curve for the SLE group was also sigmoidal, 27% of the patients had no receptors at the time of study. The curve is displaced towards the origin in comparison to the curve for the normals,

FIG. 25 Cumulative frequency curves of CR1 numbers in normal individuals (——), patients with SLE (----) and RA (·-·-·-)


100
90
80
70
60
50
40
30
20
10
0
% cumulative frequency
0
5000
10000
15000
20000
CR1 Sites/erythrocyte

clearly indicating that this group have fewer CR1 sites/ erythrocyte than normals but that they also exhibit a wide receptor range.

Relationship between CR1 levels and other laboratory tests

The relationship between CR1 numbers and the results of the other laboratory tests were investigated in the SLE and RA patients (Tables 17 and 18). Complement levels and rheumatoid factor levels (7.5) were assessed in the RA patients. Complement levels, the levels of antinuclear antibodies (ANF) and DNA binding antibodies (7.5) were assessed in the SLE patients. Unfortunately these were not performed on every blood sample tested for CR1 levels. Most of the complement levels in the SLE patients (Table 17) and RA patients (Table 18) were normal, with no correlation between CR1 sites/erythrocyte and the parameters measured (see table below, the normal ranges for these assays are given in Table 19).

	C3	C4	B	$C\bar{1}$-INH	CH50	RF RIA
RA patients	0.4	0.11	0.37	0.23	0.21	-0.2
SLE patients	-0.08	0.06	-0.22	-0.20	-0.23	-

Clearly CR1 levels do not correlate with the serum levels of complement components in the SLE or RA patients, nor do they correlate with circulating rheumatoid factor (RF) levels in the RA patients. The ANF and DNA binding values were assessed in the SLE patients.

TABLE 17 Results of CR1 sites/erythrocyte, C3 (μg/ml), C4 (μg/ml) B (μg/ml) $C\bar{1}$-INH (μg/ml) and CH50 (units/ml) in 34 samples from SLE patients

Patient	Disease activity	CR1	C3	C4	B	$C\bar{1}$-INH	CH50
S.C.	Active	0	694	174	146	306	83
D.D.	Inactive	0	943	306	158	317	191
H.McG	Active	0	903	230	186	410	273
C.McG.	Active	0	1243	199	223	224	308
J.M.	Active	0	1020	299	230	270	201
E.McH.	Active	0	1700	302	240	368	234
A.C.	Active	0	1476	486	258	275	205
J.M.	Active	0	1134	620	258	582	199
E.McH.	Active	0	1587	294	270	474	269
J.McD.	Active	0	1515	414	204	417	-
R.H.	Active	240	767	156	208	234	-
C.F.	Inactive	377	938	154	178	198	179
C.F.	Inactive	465	1050	347	202	203	160
M.S.	Inactive	476	1337	426	173	332	244
J.G.	Inactive	576	1579	339	207	310	226
S.S.	Inactive	637	1370	377	281	287	159
M.G.	Active	645	1411	337	242	350	180
M.G.	Active	654	1296	216	286	290	-
B.D.	Active	680	890	213	198	478	184
M.McD.	Inactive	772	1129	243	187	322	267
K.E.	Inactive	886	963	238	143	252	276
M.B.	Inactive	898	952	270	137	268	218
I.T.	Inactive	936	904	404	212	390	242
J.M.	Inactive	1062	1351	333	175	327	249
M.G.	Active	1126	1334	222	289	220	155
B.D.	Active	1160	839	179	389	374	176
I.M.	Inactive	1561	874	189	227	268	203
C.B.	Inactive	1611	968	164	196	251	148
J.G.	Inactive	1977	1427	326	283	254	259
L.B.	Inactive	4553	931	336	127	241	159
S.S.	Inactive	4954	1253	284	241	291	229
M.G.	Inactive	5197	1530	400	292	350	128
B.D.	Inactive	5506	1152	190	160	196	176
M.G.	Inactive	15766	976	359	134	275	175

TABLE 18 Results of CR1 sites/erythrocyte, C3 (μg/ml), C4 (μg/ml), B (μg/ml) $C\bar{1}$-INH (μg/ml), CH50 (units/ml) determinations and rheumatoid factor (RF) levels (RF latex and RF RIA) in 23 RA patients.

Patient	CR1	C3	C4	B	$C\bar{1}$-INH	CH50	RF latex	RF RIA
A.J.	334	1887	894	378	414	173	(+)ve	30400
K.C.	530	1164	472	243	370	198	(+)ve	9000
M.M.	532	1242	515	185	360	259	(+)ve	1067
J.D.	606	1314	542	231	528	357	(-)ve	-
R.H.	629	860	179	226	296	131	(+)ve	19652
M.H.	846	1583	457	301	402	343	(-)ve	<800
E.F.	925	1328	300	257	300	238	(+)ve	5550
A.R.	1056	1553	286	287	374	235	(+)ve	7000
C.D.	1221	959	288	186	244	210	(+)ve	9000
M.R.	1231	1720	451	358	484	198	(+)ve	11750
M.B.	1238	1709	212	304	234	184	(+)ve	10000
M.C.	1250	1850	509	394	354	266	(+)ve	10500
J.S.	1350	1831	368	242	238	305	(+)ve	10568
R.H.	1556	931	235	182	272	140	(+)ve	5533
B.F.	1582	2528	629	364	485	250	(+)ve	2850
K.C.	1598	2046	652	372	506	324	(+)ve	40000
M.C.	1712	1887	454	325	312	313	(+)ve	15363
M.C.	1742	2208	628	346	234	270	(+)ve	12121
CMcN.	2275	1830	361	274	412	262	-	-
P.B.	2525	1882	382	328	442	316	(+)ve	7350
M.B.	2731	2336	956	412	432	246	(+)ve	13200
M.S.	3038	-	-	-	-	-	-	<800
H.M.	4259	1762	488	372	463	278	(+)ve	4700

TABLE 19 Ranges for laboratory serological tests

	Normal range
C3	720-1800 µg/ml
C4	199-574 µg/ml
B	149-421 µg/ml
$C\bar{1}$-INH	160-370 µg/ml
CH50	150-250 units/ml
RF latex	(-)
RF RIA	Less than 800 units/ml
DNA binding assay	0 —> 27% Normal
	27 —> 33% Borderline (B)
	33% —> Raised (R)
Antinuclear factor assay (ANF)	Homogeneous staining pattern (H)
	Speckled " " (S)
	Nucleolar " " (N)
Crithidia	Negative
	Weakly positive
	Strongly positive

In the 31 blood samples studied there was no correlation between CR1 sites/erythrocyte and antinuclear factor or anti-DNA antibody levels; however all the SLE patients did have antinuclear factor antibodies and 44% had anti-DNA antibodies (Table 20).

Relationship between CR1 levels and disease activity

The SLE patient group was further sub-divided into those who had active disease and those in whom the disease was in remission at the time of study. This division was based on the clinical assessment as described in 7.2 and the information was only made available following the completion of all the CR1 assays.

The distribution of CR1 sites/erythrocytes in SLE patients with active and inactive disease is shown in Fig. 26. The mean for the patients with active disease ($n = 17$) was 356 CR1 sites/erythrocyte, whilst for inactive disease ($n = 24$) the mean was 2428, these two groups differ significantly ($p<0.001$). Other distinguishing features illustrated by this graph are: a) the very high percentage (53%) of patients with no detectable receptors on their erythrocytes when studied during an active disease phase and b) the difference in the range of CR1 numbers between active and inactive disease states, with the range for the active groups being much narrower (0 to 1160 CR1 sites/erythrocyte) than the inactive group (0 to 15977 CR1 sites/erythrocyte). The range of the latter

TABLE 20 Results of CR1 sites/erythrocyte, ANF (titre and staining pattern) and DNA binding (%) in 31 SLE patients with varying disease activity (ACTIVE, INACTIVE).

Patient	Disease activity	CR1	ANF	DNA
E.McH	Active	0	1/1000 H + S	98.1% R
A.C.	Active	0	1/256 H	Normal
S.C.	Active	0	-	96% R
C.McG	Active	0	1/64 H	-
H.McG	Active	0	1/64 H + S	-
J.M.	Active	0	1/1000 S + H	Normal
E.McH	Active	0	1/1000 H	93.5% R
J.McD	Active	0	1/1000 H	-
D.D.	Inactive	0	-	Normal
C.F.	Inactive	377	1/1000 H	Normal
C.F.	Inactive	465	1/256 H	Normal
M.S.	Inactive	476	1/1000 S	Normal
J.G.	Inactive	576	1/64 H	Normal
S.S.	Inactive	637	1/64 H + N	69.4% R
M.G.	Active	645	-	36.7% R
M.G.	Active	654	1/1000 H + S	-
B.D.	Active	680	1/256 H	54.7% R
M.McD	Inactive	772	1/1000 H	Normal
K.E.	Inactive	886	-	Normal
I.T.	Inactive	936	1/1000 H + S	Normal
J.M.	Inactive	1062	1/1000 S	Normal
M.G.	Inactive	1126	1/1000 S + H	43.9% B
B.D.	Active	1160	1/256 H	30.7% B
I.M.	Inactive	1561	1/1000 H	Normal
C.B.	Inactive	1611	1/256 H	30.8% B
J.G.	Inactive	1977	1/256 H	Normal
L.B.	Inactive	4553	W (+)ve	Normal
S.S.	Inactive	4954	1/256 H	68.7% R
M.G.	Inactive	5197	1/1000 S	61.2% R
B.D.	Inactive	5506	1/256 H + S	Normal
M.G.	Inactive	15766	1/256 H	55.4% R

FIG. 26 Distribution of CR1 sites/erythrocyte in SLE patients with active and inactive disease. The horizontal lines represent the means and the arrows the median values.

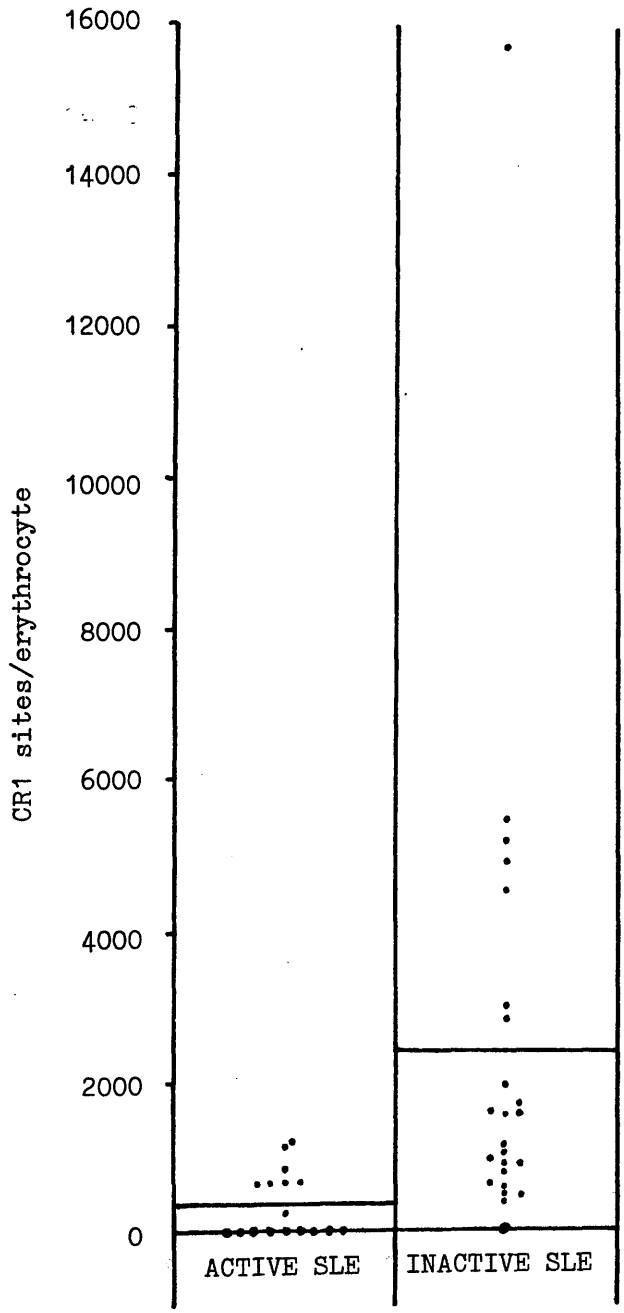

16000
14000
12000
10000
8000
6000
4000
2000
0
CR1 sites/erythrocyte
ACTIVE SLE
INACTIVE SLE

TABLE 21 Results of CR1 sites/erythrocyte, ANF (titre and staining pattern), Crithidia test for dsDNA and DNA binding (%) in 8 active SLE patients and 16 inactive SLE patients.

Patient	Disease activity	CR1	ANF	Crithidia	DNA
E.McH	Active	0	1/1000 H+S	St(+)ve	98.1% R
A.C.	Active	0	1/256 H	(-)ve	Normal
S.C.	Active	0	-	St(+)ve	96% R
C.McG	Active	0	1/64 H	(+)ve	44.3% R
R.H.	Active	240	-	St(+)ve	95.1% R
M.G.	Active	645	-	(+)ve	36.7% R
B.D +	Active	680	1/256 H	(-)ve	54.7% R
B.D.	Active	1160	1/256 H	(+)ve	30.7% R
D.D.	Inactive	0	-	(-)ve	Normal
C.F.	Inactive	377	1/1000 H	(-)ve	Normal
C.F.+	Inactive	465	1/256 H	(-)ve	Normal
J.G.	Inactive	576	1/64 H	W(+)ve	Normal
S.S.	Inactive	637	1/64 H+N	(+)ve	69.4% R
M.McD	Inactive	772	1/1000 N	(-)→(+)ve	Normal
K.E.	Inactive	886	-	(-)ve	Normal
M.B.	Inactive	898	-	(-)ve	-
I.T.	Inactive	936	1/1000 H+S	(-)ve	Normal
J.M.	Inactive	1062	1/1000 S	(-)ve	Normal
M.G.	Inactive	1126	1/1000 S+ H	W(+)ve	43.9% B
I.M.	Inactive	1561	1/1000 H	W(+)ve	Normal
C.B.	Inactive	1611	1/256 H	(-)ve	30.8% B
J.G.	Inactive	1977	1/256 H	W(+)ve	Normal
L.B.	Inactive	4553	W(+)ve	(-)ve	Normal
M.G.	Inactive	5197	1/1000 S	St(+)ve	61.2% R

group resembles more the range of CR1 sites/erythrocyte found in the normal group (0 to 21692), however, both groups of SLE patients still differ significantly ($p<0.001$) from the normal group. Clearly SLE patients have reduced CR1 numbers and this reduction is more marked when the disease is undergoing an exacerbation.

The possible relationship between CR1 levels and serological parameters was further studied in the active and inactive SLE patients in the hope that the division of the SLE group may highlight any correlations present which may have been masked when studying the SLE group as a whole. However, there was no correlation between CR1 numbers, complement levels and disease activity. All the patients studied, both active and inactive had antibodies to nuclear factors (Table 21), however in the active group 88% had a positive DNA binding test compared to 27% in the inactive group, 75% of the active group had a positive Crithidia test compared to 38% in the inactive group. In neither the inactive or active SLE patient groups did CR1 levels correlate with ANF titre or DNA binding levels. Therefore although there was clearly an increase in autoantibodies to dsDNA in active disease states (as determined by DNA binding assay and Crithidia test) this does not have any direct correlation with the CR1 levels.

As SLE is considered to be the prototype human immune complex disease sera from 4 patients with active SLE and 8 with inactive disease were examined for immune complexes (Table 22). There was no correlation between CR1 and immune complex levels detected by either the conglutinin binding assay or PEG IgG in patients with inactive disease. In patients with active

TABLE 22 Results of CR1 sites/erythrocyte, conglutinin binding assay (μg equivalent/ml) and PEG IgG (mg/%) in 12 SLE patients, 4 in an active disease state and 8 in an inactive state.

Patient	Disease activity	CR1	Conglutinin binding assay	PEG IgG
S.C.	Active	0	54	5.6
C.McG	Active	0	22.2	5.85
M.G.	Active	645	61.5	18.2
B.D	Active	680	78	4.1
D.D.	Inactive	0	58.5	7.1
M.S.	Inactive	476	34.5	4.45
M.M.	Inactive	772	201	4.35
K.E.	Inactive	886	18	2.7
M.B.	Inactive	898	45	2.35
J.M.	Inactive	1062	17.4	4.9
C.B.	Inactive	1611	3.75	10.9
L.B.	Inactive	4553	34.5	2.22

disease there was a correlation (r=0.79) between immune complex levels (as detected by the conglutinin binding assay) and CR1 levels, but this was not found using the PEG IgG method of immune complex quantitation.

Serial measurements of CR1

Serial studies were performed on 9 normal individuals, 4 RA patients and 8 SLE patients. These were performed to determine whether CR1 levels varied with time. The time periods between individual determinations were variable. The 9 normal individuals studied were all laboratory personnel who were healthy when the blood samples were taken. The results (Table 23) were variable and therefore each individual will be discussed separately.

E.H. Over 52 weeks this individual shows very little change in receptor numbers, the receptors being present at low levels.

I.N. This individual exhibits widely fluctuating receptor levels over a 71 week time period, with the receptor levels changing even in a one week period, i.e. week 30, 3118 CR1 sites/erythrocyte, week 31, 4557 CR1 sites/erythrocyte and again that week, a blood sample taken 2 days later showed the erythrocytes to have 2827 CR1 sites/erythrocyte.

A.F. Within an 8 week period this individual has constant CR1 levels, however 40 weeks later the CR1 numbers had increased nearly six fold.

TABLE 23 Serial studies of CR1 sites/erythrocyte in 9 normal individuals, examined at variable time intervals, and expressed as weeks (wk) from the first determination.

E.H.	♀	0wk	29wk	51wk	52wk						
		0	353	335	449						
I.N.	♀	0wk	22wk	30wk	31wk	31wk	41wk	57wk	60wk	68wk	71wk
		1320	463	3118	4557	2827	7019	2572	9389	21692	15180
A.F.	♀	0wk	8wk	48wk							
		942	838	4840							
R.S.	♀	0wk	7wk	8wk	23wk						
		1856	2307	2730	1084						
L.J.	♀	0wk	16wk	37wk	42wk						
		1161	3713	988	13116						
K.W.	♀	0wk	52wk	56wk	93wk						
		3130	1260	1488	3082						
A.M.	♀	0wk	39wk								
		1435	5227								
L.M.	♀	0wk	58wk								
		2403	2340								
K.G.	♀	0wk	26wk								
		8009	11072								

R.S. This individual has relatively constant CR1 levels over a 23 week period.

L.J. This individual exhibits widely fluctuating CR1 levels.

K.W. The 0 week and 93 week CR1 levels and the 52 and 56 week CR1 levels are very similar in this individual.

A.M. The two CR1 determinations made on the erythrocytes of this individual are vastly different.

L.M. Despite the long interval between these two blood samples the CR1 levels are remarkably close.

K.G. These two determinations of CR1 levels in this individual with a 26 week gap do not differ to the extent seen in, for example, L.J. but are not as close as seen in, for example, L.M.

Clearly there is a considerable degree of change in CR1 levels in some normal individuals, with no pattern to the variations.

The serial studies on the RA patients are shown in Table 24 and the complement and RF factors in Table 25. Individual M.C. has fairly constant CR1 levels, however R.H., M.B. and K.C. do show some degree of change in their receptor levels. Unfortunately the serial studies on the RA patients was limited, making it difficult to make any associations between CR1 changes, complement and RF levels.

Serial studies of CR1 numbers were performed on 8 SLE patients (Table 26) together with their complement levels, ANFs and DNAs (Table 27). Three patients serially studied remained inactive throughout, one of these patients (C.F) had stable low receptor

levels, the remaining two (S.S. and J.G.) showed an increase in their receptor levels within 3 weeks. Two patients remained active whilst being serially studied, patient E.McH had no receptors on 3 successive determinations of receptor levels and the second patient's (C.McG) receptor levels increased despite remaining active. Three patients had changes in their disease status whilst being serially studied, and the relationship between disease activity and CR1 levels in these three patients is illustrated in Fig. 27. In all three patients (M.G., J.M. and B.D.) receptor levels increased during periods of disease remission and decreased during phases of exacerbation. Patient M.G. (Fig. 27a) shows an increase in receptor levels despite remaining active and likewise patient J.M. (Fig. 27b) shows an increase in receptor levels despite remaining inactive. However in patient M.G. the next serial study (Fig 27(a)3) showed another increase in the receptor levels and also that the patient had entered into a phase of disease remission. There was no obvious correlation between CR1 levels and complement, ANF and DNA binding levels in the 3 patients extensively serially studied, however in 2 out of these three patients there were incomplete serological data.

Family studies

CR1 sites/erythrocyte were measured in the families of two normal individuals and five patients with SLE, the criteria used for the choice of these families is described in 7.7.

TABLE 24 Serial studies of CR1 sites/ erythrocyte in 4 RA patients, examined at variable time intervals, expressed as weeks from the first determination.

R.H.	♀	0wk	3wk	
		629	1556	
M.C.	♀	0wk	2wk	4wk
		1712	1742	1250
M.B.	♀	0wk	11wk	
		1238	2731	
K.C.	♀	0wk	10wk	
		530	1598	

TABLE 25 Complement and RF levels in the 4 RA patients studied serially

Patient	Week	CR1	C3	C4	B	C1-INH	CH50	RF latex	RF RIA
R.H.	0wk	629	860	179	226	296	131	(+)ve	19652
	3wk	1556	931	235	182	272	140	(+)ve	5533
M.C.	0wk	1712	1887	454	325	312	313	(+)ve	15363
	2wk	1742	2208	628	346	234	270	(+)ve	12121
	4wk	1250	1850	509	394	354	266	(+)ve	10500
M.B.	0wk	1238	1709	212	304	234	184	(+)ve	10000
	11wk	2731	2336	956	412	432	246	(+)ve	13200
K.C.	0wk	530	1164	472	243	370	198	(+)ve	9000
	10wk	1598	2046	652	372	506	324	(+)ve	40000

TABLE 26 Serial studies of CR1 sites/ erythrocyte in 8 SLE patients, examined at variable time intervals, expressed as weeks from the first determination. Disease activity is stated as (A) for active and (IA) for inactive.

M.G.	O+	0wk	40wk	59wk	74wk
		645(A)	1126(A)	5197(IA)	654(A)
E.McH.	O+	0wk	53wk	55wk	
		0(A)	0(A)	0(A)	
C.McG.	O+	0wk	36wk		
		0(A)	860(A)		
J.M.	O↓	0wk	8wk	25wk	54wk
		0(A)	1062(IA)	1536(IA)	0(A)
B.D.	O+	0wk	16wk	32wk	49wk
		680(A)	2817(IA)	1160(A)	5506(IA)
C.F.	O+	0wk	2wk		
		465(IA)	377(IA)		
S.S.	O+	0wk	3wk		
		637(IA)	4954(IA)		
J.G.	O+	0wk	3wk		
		576(IA)	1977(IA)		

TABLE 27 Complement levels, ANF (titre and staining pattern) and DNA binding (%) in the 7 SLE patients study (A=active disease, IA=inactive disease)

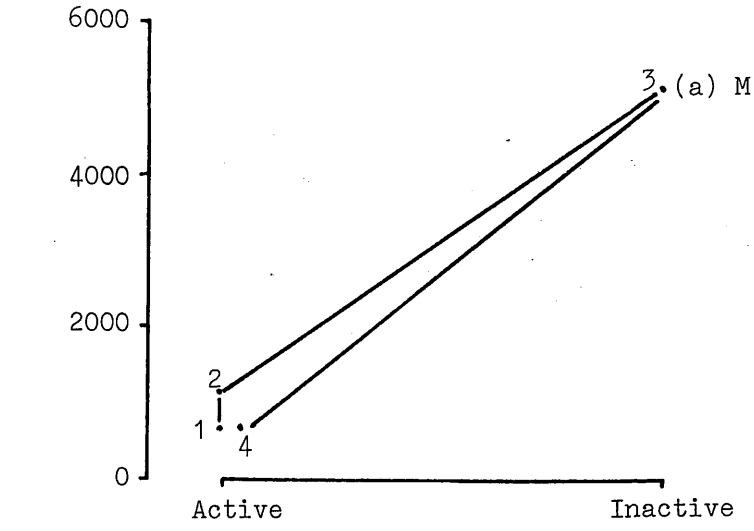
Patient	Week	CR2	C3	C4	B	$C\bar{1}$-INH	CH50	ANF	DNA
M.G (A)	0wk	645	1411	337	242	350	138	-	36.7%R
(A)	40wk	1126	1334	222	289	220	155	1/1000 S+H	43.9%R
(IA)	59wk	5197	1530	400	292	350	128	1/1000 H+S	61.2%R
(A)	74wk	654	1152	190	160	196	176	1/256 H+S	-
E.McH(A)	0wk	0	-	-	-	-	-	-	-
(A)	53wk	0	1700	302	240	368	234	1/1000 H+S	98.1%R
(A)	55wk	0	1587	294	270	474	269	1/1000 H	93.5%R
J.M (A)	0wk	0	-	-	-	-	-	-	-
(IA)	8wk	1062	1351	333	.175	327	249	1/1000 S	Normal
(IA)	25wk	1536	-	-	-	-	-	-	-
(A)	54wk	0	1134	620	258	582	199	1/1000 S+H	Normal

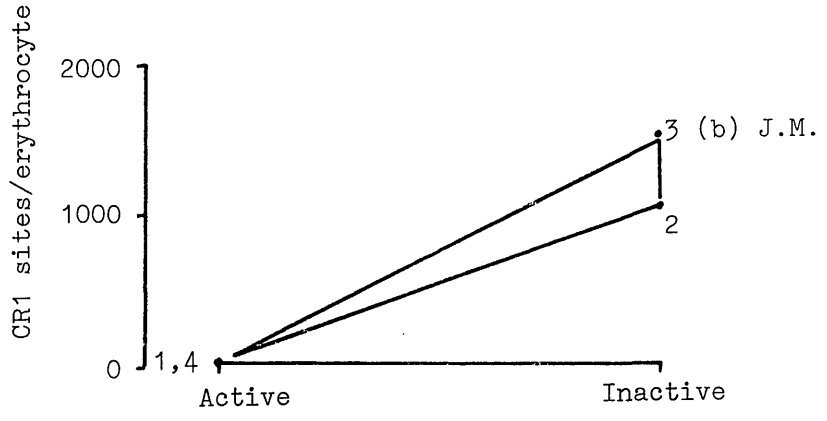
contd..

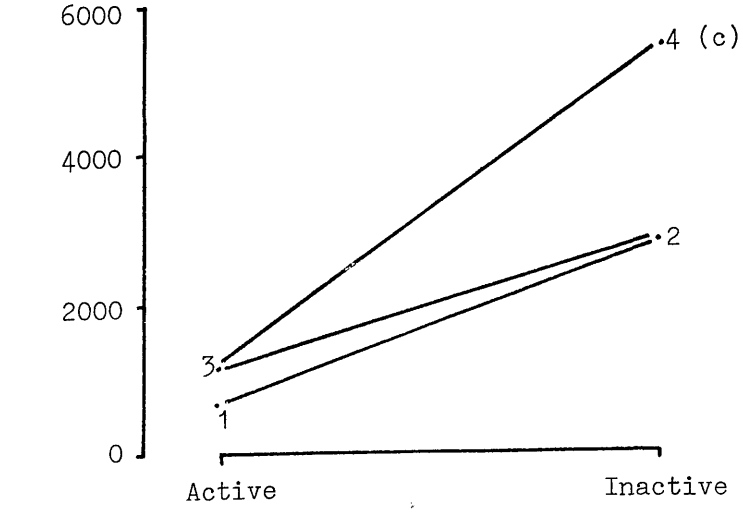
Patient	Week	CR2	CR3	C4	B	$\bar{C1}$-INH	CH50	ANF	DNA
B.D(A)	0wk	680	890	213	198	478	184	1/256 H	-
(IA)	16wk	2817	-	-	-	-	-	-	-
(A)	32wk	1160	839	179	389	374	176	1/256 H	30.7%B
(IA)	49wk	5506	1152	190	160	196	176	1/256 H+S	Normal
C.F(IA)	0wk	465	1050	347	202	203	160	1/64 H+N	69.4%R
(IA)	2wk	377	938	154	178	198	179	1/1000 H	Normal
S.S(IA)	0wk	637	1370	377	281	287	159	1/64 N+H	69.4%R
(IA)	3wk	4954	1253	284	241	291	229	1/256 H	68.7%R
J.G(IA)	0wk	576	1579	339	207	310	226	1/64 H	Normal
(IA)	3wk	1977	1427	326	283	254	259	1/256 H	Normal

FIG. 27 Graphic representation of serial studies on 3 SLE patients (a) M.G, (b) J.M. and (c) B.D, showing changes in the number of CR1 sites/erythrocyte with disease activity.

•1, •2 etc. represent serial samples


6000
4000
2000
0
3
(a) M.G.
2
1
4
Active
Inactive


CR1 sites/erythrocyte
2000
1000
0
3
(b) J.M.
2
1,4
Active
Inactive


6000
4000
2000
0
4
(c) B.D.
2
3
1
Active
Inactive

For ease of presentation of these data, individuals have been divided into one of three groups, based on their CR1 numbers. The 3 groups were made arbitarily, low ($\leq$ 1000 CR1 sites/erythrocyte), medium (1001 to 3999 CR1 sites/erythrocyte) and high ($\geq$ 4000 CR1 sites/erythrocyte). In the normal control group studied 20% had low receptors, 60% medium and 20% high (taken from cumulative frequency curve Fig. 25).

Normal family (a) (Fig. 28) had four members, the father had medium receptor levels, whilst the mother and two daughters had low receptor levels (there were no serological data on this family). The second normal family (Fig. 28 (b)) consisted of 5 members. The father had medium receptor levels and the mother low receptor levels, likewise one daughter, however her two sisters had medium receptor levels. None of the members of these families had antinuclear or anti-DNA antibodies in their sera and CH50, C4, C3, B and $C\bar{1}$-INH levels were normal.

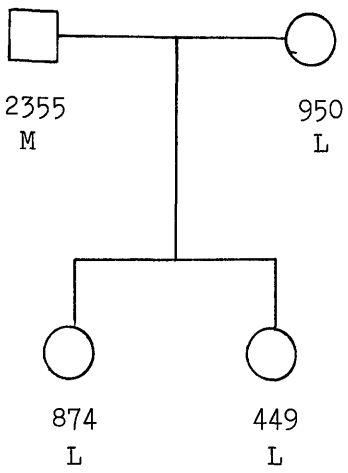
At the time family (a) (Fig. 29) was studied the family member with SLE had medium receptor levels (1062 CR1 sites/erythrocyte), as did his mother, father and brother. His paternal aunt had high receptor levels. However, this patient had been studied 2 months previously when his disease status was active and he had undetectable receptors.

The patient in the second family (Fig. 29 (b)) had low receptor levels, her mother, father and sister had medium receptor levels whilst her brother had high receptor levels.

The remaining 2 families studied each contained a set of identical twins. Both twins of family (a) (Fig. 30) had clinical SLE. However, only one twin of family (b) (Fig. 30)

FIG. 28 CR1 sites/erythrocyte in two normal families

Normal family (a)



L = low

M = Medium

H = High

Normal family (b)

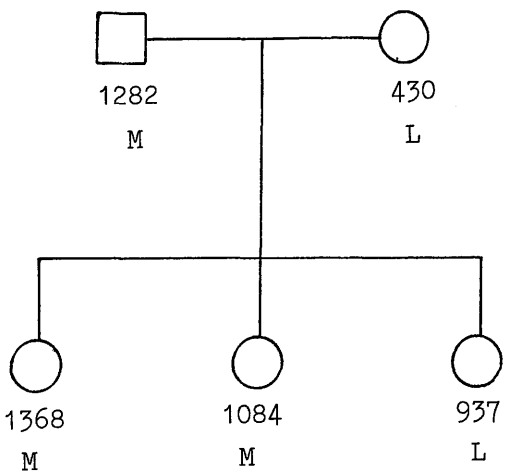
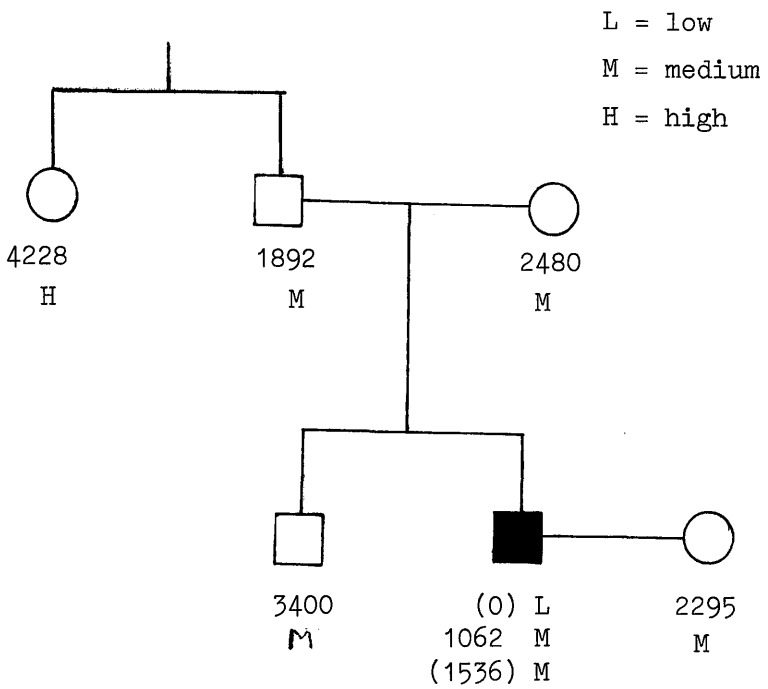


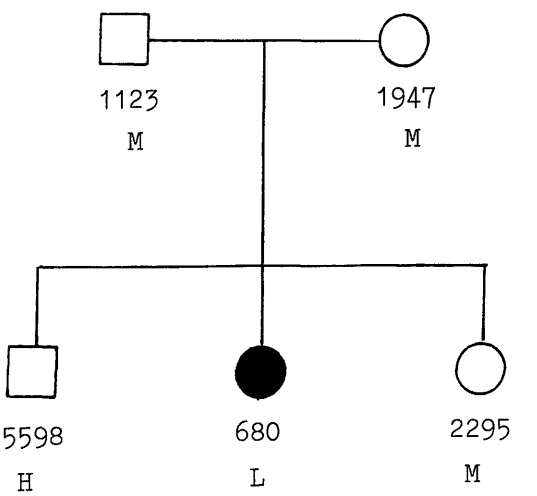
FIG. 29 CR1 sites/erythrocyte in two families where one member is an SLE patient

(● or ■ family member with SLE)

SLE family (a)



SLE Family (b)



had clinical SLE; her twin sister only had serological evidence of SLE. Monozygosity was established on the basis of physical appearance, HLA tissue typing and analysis of the blood groups ABO, Rh, Kell, Lewis, Duff and Kidd.

In the first family (Fig 30 (a)) one twin had medium (1977) and the other had high (4954) receptor numbers (both were inactive at the time of study). The brother had medium receptor levels whilst the mother had high receptor levels. Unfortunately the father refused to give a blood sample. In the second family (Fig 30 (b)), the twin with serological evidence of SLE (*●) had medium receptor levels (1047) whilst her twin (inactive at the time of study) had low levels (377). Both parents had medium receptor levels and their sister had low levels.

Except for the patients with SLE none of the SLE family members had clinical or serological evidence of SLE.

DISCUSSION

Many studies have now been performed in which the levels of CR1 on the erythrocytes from normal individuals and patients with immune complex diseases have been investigated. In particular patients with SLE have been studied with a view to a possible understanding of the role of CR1 in determining the possible fate of circulating immune complexes which are prevalent and possibly phlogistic in SLE. In occasional studies RA patients have also been studied as they also have phlogistic circulating immune complexes.

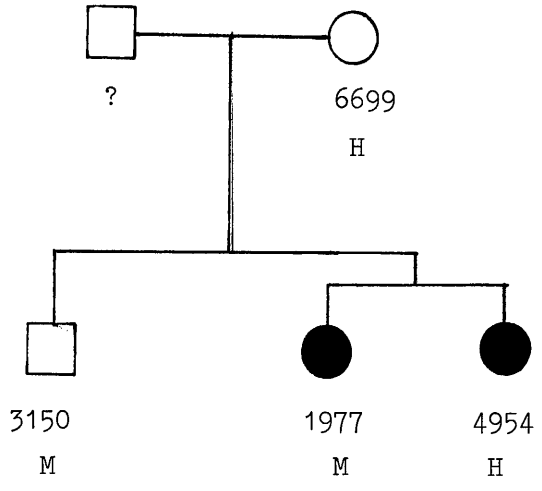
FIG. 30 CR1 sites/erythrocyte in two families where there are twins with SLE
(● individual with SLE,
*● individual with serological SLE only).

L = low

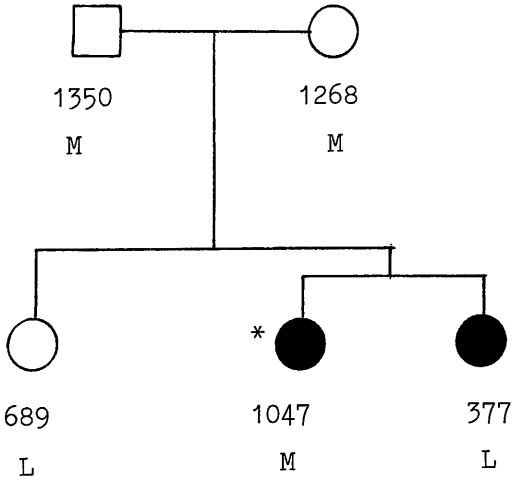
M = medium

H = high

SLE family (a)



SLE family (b)



CR1 levels have been determined by two methods, firstly by IAHA or modifications of this method or using an RIA with either monoclonal or polyclonal CR1 specific antiserum. In this project erythrocyte CR1 levels were studied in a group of normal individuals, SLE and RA patients and the receptor levels were quantitated using an RIA utilizing radiolabelled polyclonal rabbit $F(ab')_2$ anti-CR1.

Due to different methods used to quantitate receptor levels, it is difficult to make direct comparisons between the data obtained by different laboratories. Therefore the results obtained in this project will be discussed together with the general trends found by other workers.

Erythrocyte CR1 levels in patients and normal individuals

The range and mean erythrocyte CR1 levels in normal individuals obtained by other workers quantitating receptor levels using RIA are given below.

Mean CR1 levels	CR1 range	No. of samples tested	
5014±155	100 to 8500	113	Wilson et al 1982
1410±625	-	52	Iida et al 1982
-	145 to 1214	86	Walport et al 1985
-	168 to 1877	124	Minota et al 1984
707±202	300 to 1400	77	Ross et al 1985

The mean found in this study was 3320 with a range of 0 to 21692 CR1 sites/erythrocytes. This range is much wider than those found by other workers, and the mean value is the second highest. However, the use of a monoclonal antibody to determine the number of receptors by Iida et al 1982, Minota et al 1984, Walport et al 1985 and Ross et al 1985 would account

for their lower ranges and mean values. In fact the aforementioned workers have found very similar CR1 levels on the erythrocytes of their normal individuals. This is probably a result of using a monoclonal anti-CR1 antibody to determine the number of receptors. Wilson et al (1982) used a polyclonal anti-CR1 antiserum, their determined mean value of 5014±155 CR1 sites/erythrocyte is closer to the values attained in this project, however their range (100 to 8500 CR1 sites/erythrocyte) is much narrower.

Another problem concerning the interpretation of published data by other groups is the manner in which the RIA was executed. The assay technique used throughout this thesis for the determination of CR1 sites/erythrocytes was based on the method described by Wilson et al (1982) and involves incubating incremental amounts of ^{125}I-F(ab')$_2$ anti-CR1 with standardized quantities of erythrocytes. With the quantitation of specifically bound cpms at a range of input cpm values a Scatchard plot can be constructed and therefore the number of CR1 receptor sites determined. However the groups analysing receptor levels with a monoclonal antibody only incubate erythrocytes with a single saturating dose of radiolabelled monoclonal anti-CR1 antibody.

Iida et al (1982) however created a standard curve using purified CR1 from which they could calculate the number of receptors for the level of monoclonal binding observed. Yet, for Walport et al (1985) the saturating dose was determined using approximately 10 erythrocyte specimens. If this sample of the normal population did not contain individuals with very high receptor levels they may not pick up such people in future assays,

and this may lead to artificially narrow ranges of CR1 numbers.

Wilson et al (1982), Minota et al (1984), Walport et al (1985) and Ross et al (1985) further analysed their normal groups by constructing histograms of the number of individuals against CR1 levels. Walport et al (1985) make no comment about their histogram although it does show some degree of clustering of receptor sites. Ross et al (1985) believed their histogram showed a bimodal distribution of receptor numbers; however extensive statistical analysis confirmed that it was unimodal. Wilson et al (1982) visualized a bimodal distribution (Fig. 5) and further subdivided one group to give a trimodal distribution of CR1 sites/erythrocyte in his normal control group. They used this as the basis for the proposal that CR1 levels were inherited by two autosomal codominant alleles H and L, HH coding for high, HL for medium and LL for low receptor levels. The fact that the expected phenotype frequencies predicted by the Hardy-Weinberg equilibrium did not differ significantly from the observed frequencies further supported their argument. However their histogram distribution pattern was not analysed statistically and although there is clearly a bimodal distribution pattern it is difficult to support their decision to arbitrarily subdivide one cluster to give 3 groups where the phenotype frequency coincides with the expected Hardy-Weinberg equilibrium for 2 codominant alleles.

The histogram drawn from the data of Minota et al (1985) shows 3 distinct clusters of CR1 sites in the normal controls, and the observed phenotype frequencies do not differ significantly from those found by Wilson et al (1982). However, this group

stated that if the alleles were codominant, the expected frequencies would be LL 25%, HL 50%, HH 25% and not, as observed by themselves (and Wilson et al 1982) LL 11% (12%), HL 53% (54%) and HH 36% (34%), with their results showing a strong deviation in favour of the HH phenotype. They therefore state that their data do not support a codominant allele control of receptor levels but favour a multigene control of CR1 expression.

The CR1 levels on normal erythrocytes found in this project (Fig. 24) appeared unimodal in their distribution. On the advice of the statistician, Dr. P. Boyle, these results were replotted as a cumulative frequency curve (Fig. 25), as if any genetic subgroups of receptor numbers were present in the normal population studied there would be indentations in the cumulative frequency curve. As can be seen in Fig. 25 the curve is smooth and sigmoidal apart from a few minor irregularities between 50 and 77% and this may be due to sampling error or may reflect on the size of the population used. However, the data does not support the presence of 3 groups within our normal population bearing low, medium and high CR1 receptor phenotypes on their erythrocytes.

The results attained by IAHA or modifications of this method are difficult to compare with those determined using the RIA. Several studies have been performed using IAHA, however there are considerable problems with this method which is only semi-quantitative. These have been highlighted by Minota et al (1984) and Uko et al (1985). Both these groups studied the ability of serial dilutions of aggregated human IgG (AHG) coated with complement (guinea pig) to agglutinate human erythrocytes, The results being expressed as the highest dilution of AHG to show positive agglutination.

Both Uko et al (1985) and Minota et al (1984) found that patients and normal erythrocytes demonstrated extreme bimodality in their haemagglutination patterns, with a group expressing no receptors, as indicated by no haemagglutination and a group with very high receptor levels with haemagglutination titres of approximately 2^8. Therefore using this technique there was a clear cut discrimination between individuals with no receptors and those with very high receptor levels, with no intermediate values. Minota et al (1984) further showed that IAHA negative erythrocytes very often had intermediate levels of receptors, determined using the RIA, thus suggesting that a critical number of CR1 sites/erythrocyte are required for positive IAHA in the presence of AHG bearing limited amounts of C3b. This would account for the bimodality seen in IAHA titres and consequently makes IAHA data difficult to interpret, and this must always be borne in mind.

There seems to be no correlation between CR1 levels with either the sex, blood group (ABO) or age of an individual (Iida et al 1982).

To date all workers studying erythrocyte CR1 levels in SLE patients have found them to be significantly reduced compared to normals; this is in concordance with the results found in this project. There have been only two studies which have determined CR1 levels in RA patients. In this study RA patients had significantly fewer receptors ($p<0.05$) than normals; this confirms the result of Iida et al who also found their RA patients to have significantly fewer receptors ($p<0.02$) than the normals. Ross et al (1985) however found their RA patients to have only slightly but significantly lower CR1 levels ($p<0.01$)

than the normals. In both of these studies the SLE patients had much reduced CR1 levels in comparison to the RA patients.

The cumulative frequency curves for the SLE and RA patients (Fig. 25) like the normal curve are sigmoidal with few irregularities in shape indicating no presence of genetic sub-groups within these two patient groups. It also clearly shows that SLE patients have fewer receptors than normals with a large percentage (27%) having no receptors and that the RA patients had no patients with 0 receptors and none with very high receptor levels.

Relationship between CR1 levels and other laboratory tests

The possibility that there was a link between low CR1 levels in SLE and RA with clinical serological parameters was investigated. In the RA patients there was no correlation between CR1 levels and C3, C4, B, $C\bar{1}$-INH and CH50 levels, nor with RF levels as determined by RIA. In the SLE patients, likewise, there was no correlation between CR1 levels and C3, C4, B, $C\bar{1}$-INH and CH50 levels. In the SLE patients the levels of antinuclear factor antibodies (ANF) and autoantibodies specific for double stranded (ds) DNA were assessed. There was no correlation between CR1 levels in the SLE patients and the levels of ANF or anti-DNA antibodies.

No other workers in this field have examined the correlation between CR1 levels, RF, ANF and anti-dsDNA antibody levels.

Iida et al (1982) correlated haemolytic C4 levels with CR1 levels; the correlation coefficient was low. Wilson et al (1982) examined CH50, C4 and C3 levels in 22 SLE patients and found no correlation with CR1 levels. This is in agreement with the results found in this thesis.

Relationship between CR1 levels and disease activity

The division of the SLE patients into those who were active at the time of study and those who were inactive (Fig.26) shows clearly that receptor numbers are related to disease activity. The mean for the active SLE patients was 356 CR1 sites/erythrocyte as opposed to 2428 CR1 sites/erythrocyte for the inactive SLE patients. These two patient groups differed significantly ($p<0.001$). It is also clear that the high percentage of individuals with zero receptors were found amongst those patients with active disease.

The relationship between CR1 levels and serological parameters was studied in the active and inactive SLE patients. There was no correlation found between CR1 levels, complement levels, ANF titres and autoantibodies to dsDNA (as detected by the DNA binding assay or Crithidia test). However, there was a much higher percentage of positive DNA binding assays (88%) in active disease as opposed to inactive disease (27%). This was further supported by the Crithidia test (75% positive in active disease, 38% in inactive disease). This confirms the relationship between the presence of anti-dsDNA antibodies and active SLE.

A limited number of sera from SLE patients (4 active and 8 inactive) were examined for circulating immune complexes (conglutinin binding assay and PEG precipitation of IgG containing complexes, Table 22). A positive correlation was only found between CR1 levels and conglutinin binding complexes ($r=0.79$) in the serum from the active SLE patients.

Several groups have also examined the relationship between circulating immune complexes and CR1 levels. However, the variety of techniques used to detect and quantitate circulating immune complexes make it difficult to compare data, as certain immune

complexes may be detected by one method and not another or vice versa.

Iida et al (1982) studied the presence of circulating immune complexes in 20 serum samples from SLE patients using the Raji cell and solid-phase C1q-binding assays. Although they found CR1 levels to correlate negatively with the results of the C1q-binding assay ($r=0.49$, $p<0.05$) and not the Raji cell assay ($r=0.32$, $p>0.2$), the former correlation coefficient is of only borderline significance while the latter is not statistically significant. Wilson et al (1982) found only 2 out of 22 SLE patients to have raised levels of circulating immune complexes. Inada et al (1982) examined 103 serum samples from 82 SLE patients for circulating immune complexes by measuring the ability of the patient's sera (heat-inactivated) to agglutinate standardized human erythrocytes in the presence of a complement source (guinea-pig serum); the control was the same assay performed with a known quantity of AHG. They observed that 55 serum specimens had circulating immune complexes ranging from 4μg/ml to 512μg/ml, of these patients 95% had defective CR1 levels as measured by a modified IAHA. In a later paper Inada et al (1983) showed that 110 out of 184 serum samples from SLE patients had circulating immune complexes. Of these 106 (96%) had reduced CR1 levels, however, 54 out of 74 samples (72%) had low CR1 but no circulating immune complexes.

All these results tend to support the view that CR1 levels do not correlate exactly with the levels of circulating immune complexes. However, an association cannot be ruled out as the assay systems used to detect circulating immune complexes may not pick up those which may be related to CR1 levels.

Serial measurements of CR1

Serial studies were performed on erythrocyte specimens from 9 normal individuals, 4 RA and 8 SLE patients to examine whether there were temporal changes in CR1 receptor levels. Unfortunately, the time intervals between the CR1 determinations on a single individual were variable. However, the message is clear that CR1 levels do vary with time, both in normal individuals and patients. Limited serial studies on RA patients show that some exhibit larger variations than others (Table 24); because of the numbers involved in this study no correlations could be made between changes in CR1 levels and changes in complement or RF levels. The most significant observation was the changes in CR1 levels with disease activity in the SLE patients. Three patients had changes in their disease activity whilst being serially studied (Fig. 27); clearly receptor levels decrease during periods of disease exacerbation and increase during phases of remission. Despite incomplete serological data on these patients there were no apparent correlations between changes in CR1 levels with complement, ANF or DNA binding levels. A large, carefully designed prospective study would be required to examine this problem.

To date 6 groups have studied CR1 levels serially in SLE patients. There have only been 3 studies on the longitudinal variation in CR1 numbers in normal individuals. In all cases the studies have been small in numbers, the erythrocyte specimens have been examined irregularly and not all studies have examined serological parameters. The results of these studies are conflicting. Miyakawa et al (1981) examined CR1 levels using IAHA. Serial erythrocyte samples from 50 SLE patients showed no variation in their IAHA activity despite being tested

two or more times. However, the majority of these patients were in remission at the time of study and consequently may not have expressed great variations in their CR1 levels. Three of their patients were followed from active disease into remission and their IAHA activity still remained low. These results must be interpreted with caution as the IAHA technique used is relatively insensitive and unsuitable for the detection of intermediate receptor levels (as discussed earlier). Therefore, Miyakawa et al (1981) stated that there were no changes in receptor levels with time and this led them to the conclusion that the defect was persistent and therefore inherited. It is possible however, that any change in receptor levels would not have been detected by this screening method.

Wilson et al (1982) performed serial studies on 24 normal individuals and 10 SLE patients over a one to four month period. However they only present the mean variation in receptor levels (436±71 CR1 sites/erythrocyte for normals, 361±87 CR1 sites/erythrocyte for SLE patients) and not the variation in receptor levels of given individuals. Furthermore the SLE patients were not studied during different disease status; however they do state that most of their patients were in remission at the time of study. The results of Wilson et al (1982) could indicate that individuals do exhibit fluctuations in their receptor numbers, however they do not make any comments about the results from the serial studies.

Iida et al (1982) followed four SLE patients through inactive and active disease phases, and showed that 2 of these patients retained low receptor levels (as determined by RIA) when their disease status became inactive, whilst two patients showed large increases (80% and 176%). Similar studies in normal volunteers

showed that their CR1 levels were remarkably stable.

Like Iida et al (1982), Inada et al (1982) performed serial studies on patients during different disease activities (using IAHA) and likewise they found an increase in receptor levels during disease remission. This was coupled with a drop in circulating immune complex levels but no significant change in CH50 or C3 levels.

A single serial study by Nojima et al (1985) using radiolabelled AHG as the ligand to detect CR1, showed no change in receptor levels despite changes in clinical activity, but once again this assay technique may pose the same problems as IAHA.

Longitudinal studies by Walport et al (1985) were performed extensively on two individuals; they showed little change; however it is possible that like 3 of the normals studies in this thesis (E.H., R.S. and L.M.) these two people do not exhibit large variations in their CR1 levels, or possibly as their assay system only uses one point determinations of receptor levels, wide fluctuations may be outwith the limits of the assay and therefore not detected. They also looked at CR1 levels on the erythrocytes of 15 SLE patients and 13 normals on two successive occasions and showed that there was no change. However there was no mention of the time interval between the two samples.

Finally, Uko et al (1985) followed serially 7 normals and there was minimal variation in their CR1 levels as determined by IAHA, but SLE patients showed considerable changes and in 3 cases there were extreme changes. Some individuals showed persistently low levels. In six patients extensively serially studied there was generally an inverse relationship between CR1 levels and C3 activation or autoantibodies to dsDNA and therefore clinical activity.

The conclusions drawn from the serial studies performed in this project are a) given normal individuals can have relatively stable receptor levels, moderately or highly variable receptor levels, b) a similar situation is found in the RA patients and c) a similar situation is found in SLE patients but in addition there is an association between disease activity and receptor status. This last statement strongly supports the view that reduced CR1 levels in SLE are acquired. Similar serial studies on SLE patients by Iida et al (1982), Inada et al (1982) and Uko et al (1985) support this view. However, Miyakawa et al (1981) and Nojima et al (1985) find the defect to be persistent and postulate that low levels are inherited. The serial studies performed on normals by other workers (Iida et al, 1982, Walport et al 1985, Uko et al 1985) partially support the findings of this thesis; in all the aforementioned studies normal individuals exhibit minimal variation in their CR1 levels. Such a situation was found in 3 individuals studied in this project, however 6 individuals demonstrated changeable receptor levels. The possible causative factors will be discussed in the next chapter.

Family studies

Throughout this chapter data from other laboratories and results found during this project have been discussed and clearly there are two not necessarily incompatible views on the causation of low erythrocyte CR1 levels in SLE. Firstly, that they are inherited or secondly, that they are acquired as a secondary manifestation of the disease process. The data presented so far from this thesis supports the view that low CR1 levels in SLE are acquired. However, establishing the

validity of either of these hypotheses is crucial because if the abnormality is inherited then clearly it is a factor which predisposes to SLE. Alternatively if low CR1 numbers are acquired then an understanding of how the abnormality occurs will shed light on the pathogenesis of the condition and might even suggest new therapeutic approaches.

To determine whether receptor levels are inherited or not, extensive family studies have been performed by all workers in this field. In this project, two normal families and four SLE families were studied, with two of the SLE families luckily containing one set of identical twins. For ease of presentation 3 groups of receptors were assigned, those with low ($\leqslant$ 1000 CR1 sites/erythrocyte), medium (1001 to 3999 CR1 sites/erythrocyte) and high ($\geqslant$ 4000 CR1 sites/erythrocyte) receptor levels. It must be stressed that this division is arbitrary. uch arbitrary divisions have also been made by Walport et al (1985) for the examination of CR1 levels in their families; however Wilson et al (1982) and Nojima et al (1985) based their divisions on their observed trimodal distribution of CR1 levels seen in their normal population. The division by the latter two groups resulted in the percentage frequency of low, medium and high CR1 phenotypes not differing significantly from the Hardy-Weinberg equilibrium for two autosomal codominant alleles (discussed previously). The conclusions which can be drawn from the family studies performed in this project are:

a) There is clustering of a given CR1 phenotype within an individual family, for example, in normal family (a) (Fig. 28) there is a clustering of low receptors and in normal family (b) (Fig. 28) there is a clustering of low phenotypes around the low to medium borderline. Likewise excluding the SLE patient, a

similar situation is seen in the SLE families. Both SLE families (a) and (b) (Fig. 29) and twins SLE family (a) (Fig.30) have medium to high receptor levels, with the remaining twins SLE family (b) (Fig. 30) having medium to low receptor levels.

b) The phenotype of the patient's family could not account for the CR1 levels found in the patients. This is particularly so in SLE families (a) and (b) (Fig. 29). In family (a) the affected male started with 0 receptors (during active disease state) however as he went into remission his receptor levels resemble those of his family. The patient in family (b) was in an active disease state and unlike the rest of her family had low receptor levels.

c) Identical twins do not have the same receptor levels.

The results of these family studies do tend to contradict each other. On the one hand there seems to be clustering of CR1 phenotypes in an individual family tending to favour the inheritance of receptor; however the active SLE patients (SLE families (a) and (b) (Fig. 29)) do not have CR1 phenotypes akin to their immediate families and identical twins with SLE do not have the same CR1 levels (Fig. 30). These latter two findings favour the acquisition of low CR1 levels in SLE.

The findings of Walport et al (1985) support the above findings. As previously mentioned they could not assign HH and LL phenotypes to CR1 levels based on their population data. There 3 normal families illustrated clustering of receptors; however in the four SLE families the patients have much lower CR1 values than the remaining members of the families.

Nojima et al (1985) studied CR1 levels in 3 SLE families (by measuring the binding of complement coated ^{125}I-AHG). All 3 SLE patients were inactive and had low receptors, 10 out of 11

family members had intermediate receptor levels and 1 low. They also state that the mean levels for the patient's relatives were lower than the normals but not significantly. Based on these findings and the persistent defect in one patient despite changes in disease activity they considered the defect to be genetic. Obviously one serial study is not sufficient to make any claims about CR1 persistence with disease activity. It must be pointed out that the SLE families studied by Nojima et al (1985) may just be members of the population who have low CR1 levels, like the two normal families studies in this project; they also make no mention about the disease activities of these three patients.

Miyakawa et al (1981) made the original claim that low CR1 levels are inherited (as determined by IAHA), based on the persistence of low CR1 levels with disease activity and this was further supported by the high frequency (6 out of 24, 25%) of the defect found in the relatives of patients with erythrocyte CR1 levels. However, closer inspection of the data presented by this group indicates that only 24 family members of 15 SLE patients were tested, therefore it would seem impossible to draw any conclusive statement about CR1 inheritance when they could have only been testing approximately 2 or less family members per SLE patient. The results of their serial studies have been discussed earlier.

Minota et al (1985) performed serial studies on 3 normal families and 5 SLE families, however they make no comment on their results.

Wilson et al (1982) present the strongest data from family studies supporting the inheritance of CR1 levels, by two codominant alleles. In seven normal families there was appropriate segregation

of the LL, LH and HH phenotypes and in nine SLE families the healthy relatives had fewer receptors than normal subjects. In addition the pedigree analysis of these 9 families supported the diallelic model.

The results of this study supports the view that defective CR1 levels in SLE patients may be acquired. This is based on the findings that; a) active SLE patients have fewer receptors than inactive SLE patients, b) in individual patients CR1 levels change with disease activity, c) identical twins with SLE do not have the same CR1 numbers, and d) SLE patients with low CR1 phenotypes can belong to genotypically high CR1 families. The data from the normal population studies does not support the inheritance of CR1 levels; however, the numbers in this study (n = 70) are not large enough to make a firm conclusion. The family studies data does tend to support the inheritance of CR1 levels within an individual family or at least a heritable component in the expression of erythrocyte CR1 numbers. These views are compatible with those cited by Walport et al (1985) and Uko et al (1985).

In conclusion, CR1 levels can be genetically determined (clustering of CR1 phenotypes in families) and a recent abstract by Wilson et al (1985) presents data demonstrating an element linked to the CR1 gene which controlled the quantitative expression of CR1 (but was not related to the structural polymorphism). Equally though CR1 phenotypes can be acquired particularly in active SLE. Therefore low CR1 levels in SLE may be genetically determined or a secondary manifestation of the disease process per se.

The possible causal factors for acquired low CR1 levels will be discussed in the next chapter.

CHAPTER 8

INVESTIGATION INTO THE MEANS OF ACQUIRED CR1 REDUCTIONS IN SLE PATIENTS

INTRODUCTION

As a result of the work described in Chapter 7, the following conclusions were reached regarding erythrocyte CR1 levels in normal individuals, SLE and RA patients:
a) Genetic factors probably play a role in controlling the erythrocyte CR1 phenotypic expression, the supportive evidence being the clustering of either high, medium or low CR1 levels in the six families studied. b) In a number of SLE patients reduced erythrocyte CR1 levels seem to be acquired. Several pieces of evidence support this view; the variability of given SLE patients' CR1 levels with disease activity, the predominance of low CR1 levels during active disease phases, the finding that identical twins with SLE do not have the same CR1 levels and SLE patients with low CR1 phenotypes can belong to families with medium to high CR1 phenotypic expression. These latter findings suggest that CR1 levels in some SLE patients may be affected by environmental factors.

An explanation for the acquisition of low CR1 levels during periods of disease activity in SLE is as yet unknown. In this chapter preliminary investigations were undertaken to try and elucidate this area. A number of distinct possibilities exist which may explain the reduction and these fall broadly into two categories: a) removal of the receptors and b) blockade of the receptors. The former possibility was not studied but could be envisaged to occur during the circulation of the erythrocyte where the receptors may be removed by plasma or cellular enzymes. Alternatively the binding of immune complexes to the erythrocyte CR1 could be followed by release of the complexes together with the receptor. In this chapter I have investigated the possibility that reduced CR1 levels could be due to receptor

blockade. This could arise as a consequence of immune complexes binding to the erythrocytes, or the binding of anti-erythrocyte antibodies, or anti-CR1 antibodies all leading to the blockade of the receptor making it inaccessible to the radiolabelled $F(ab')_2$ anti-CR1 probe.

In this chapter the in vitro effect of opsonized immune complexes on erythrocyte CR1 levels was examined. In addition, erythrocytes from patients were examined for membrane-bound immune complexes.

MATERIALS AND METHODS

8.1 Immune complexes

a) BSA anti-BSA immune complexes; the quantitative precipitin curve and equivalence point for these complexes were determined by Dr. J. K. Naama who kindly donated the antiserum. The complexes formed at equivalence, consisted of 50µl of BSA (0.35mg/ml) and 100µl of a 1 in 2 dilution of rabbit anti-BSA antiserum.

b) Thyroglobulin anti-thyroglobulin immune complexes; anti-thyroglobulin antiserum was prepared by immunizing two rabbits with 2ml of bovine thyroglobulin (0.5mg/ml) emulsified with an equal volume of Freund's complete adjuvant. The rabbits were given two booster injections at 21 day intervals, using the previous protocol, but replacing Freund's incomplete for complete adjuvant. Seven days after the last injection the rabbits were exsanguinated and the serum was recovered from the blood using the method described in 2.13. The IgG fraction of the antiserum was purified as described in 4.3, and standardized to a concentration of 1.2 mg/ml. Bovine thyroglobulin was used at 1mg/ml in PBS.

The following concentrations of antigen were added to nine tubes: 0, 10, 20, 50, 100, 150, 200, 250, 350 and 450µg. The volume in each tube was adjusted to 550µl by the addition of PBS and 100µl of IgG anti-bovine thyroglobulin. The tubes were incubated for 1 hour at 37°C and overnight at 4°C and then centrifuged at 1500g for 5 mins at 4°C. The supernatants were removed and the pellets consisting of insoluble antigen-antibody complexes were washed twice in ice cold PBS. Finally the pellets were dissolved in 1ml of 0.1M NaOH. The protein content of the tubes was determined by measuring the absorbancy at 280nm (2.4), the OD_{280} was plotted against the antigen input in µg (Fig. 31), and the equivalence point determined. The equivalence complexes consisted of 50µl of thyroglobulin (1mg/ml) and 100µl of rabbit IgG anti-thyroglobulin (1.2mg/ml).

c) DNA anti-DNA immune complexes: The serum from an SLE patient with a high DNA binding capacity (96%) was used as the anti-DNA antibody source. The serum was heat-inactivated for 1 hour at 56°C and then diluted 1 in 10 before use. A quantitative precipitin curve was performed as described for the thyroglobulin anti-thyroglobulin immune complexes. However, despite repeated testing an equivalence point was not convincingly demonstrated, so immune complexes consisting of 50µg or 200µg of DNA (calf thymus) and 100µl of a 1/10 dilution of the SLE serum were used for the experiments.

8.2 Opsonization of immune complexes and incubation with normal erythrocytes.

Blood group O Rhesus positive erythrocytes were used for these experiments and autologous serum was used for opsonization

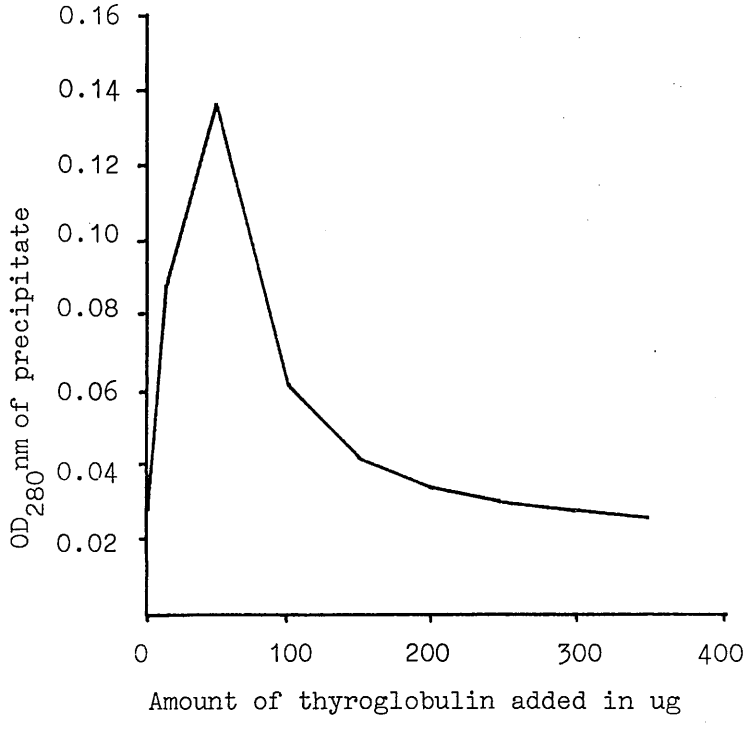

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
OD_{280}^{nm} of precipitate
0
100
200
300
400
Amount of thyroglobulin added in ug

FIG. 31 Quantitative precipitin curve for thyroglobulin anti-thyroglobulin immune complexes.

of the immune complexes. The erythrocytes and serum were prepared as described in 4.5 and 7.5. For experiments employing BSA anti-BSA immune complexes the erythrocytes were suspended in RPMI plus 0.1% (w/v) gelatin as opposed to 1% BSA.

The respective antigen and antibody were warmed for 5 mins at 37°C and the desired quantities were mixed to form immune complexes at equivalence (8.1). To this 500µl of warmed (37°C) fresh autologous normal serum was added and the antigen, antibody, serum mixture was incubated for a further 7 mins at 37°C with occasional shaking. The opsonized immune complexes were then added to pre-warmed O Rh positive erythrocytes at 2.5×10^8/ml. Quantities of 5 to 100µl of immune complexes were added per 200µl of cells at 2.5×10^8/ml and after a 7 mins incubation at 37°C the cells were either put directly onto ice or washed thrice in ice cold buffer and re-standardized to 2.5×10^8/ml.

8.3. Determination of erythrocyte CR1 levels using polyclonal anti-CR1 pre- and post-incubation with immune complexes

The number of CR1 sites/erythrocyte pre- and post-incubation with 50µl of immune complexes per 200µl of erythrocytes (2.5×10^8/ml) was performed as described in Section 4.7.

8.4. Determination of erythrocyte CR1 levels using monoclonal anti-CR1 pre- and post-incubation with immune complexes.

Mouse ascites fluid containing the monoclonal antibody to CR1 E11 was kindly given by Dr. N. Hogg. The antibody was puri-

fied from the fluid using the following method; to 1ml of the ascites fluid enough 0.1M acetic acid was added to drop the pH to 4.5. This was monitored using pH paper, then caprylic acid was added to give a final suspension of 5%. The tube was vigorously shaken by hand then centrifuged at 10000g for 7 mins. The supernatant was removed and retained, the pellet was discarded. To the supernatant an equal volume of saturated ammonium sulphate (SAS) was added dropwise, the mixture was left to stir at R.T. for 2 hours, after which the suspension was centrifuged at 10000g for 7 mins. The supernatant was discarded, the pellet was resuspended in PBS and dialysed overnight at $4^{o}C$ into PBS to remove any traces of SAS. This pool contained the mouse monoclonal antibody E11. Its presence was confirmed using double immunodiffusion in agarose (2.7), where it gave a positive reaction against sheep anti-mouse IgG antiserum.

The RIA using E11 to determine the number of CR1 sites/ erythrocyte was performed as follows: E11 was labelled as described in 2.15, erythrocytes were washed and standardized to $2.5x10^{8}$/ml (4.5) and 200ul aliquots were put into a series of microcap centrifuge tubes. Ten micrograms (10µg) of unlabelled E11 was added to the first 3 tubes which were then incubated for 30 mins at $4^{o}C$ with continuous mixing, after which 1µg of ^{125}I-E11 was added to all 6 tubes. The tubes were incubated for a further hour at $4^{o}C$ with continuous mixing and then the cells were separated from the supernatant by centrifugation over 300µl of separating fluid (4.7) at 8500g for 5 mins. The supernatants were discarded and the cpm in the pellets measured in a γ-counter. The number of CR1 sites was obtained by subtracting the mean cpm value for the tubes pre-incubated with cold E11 from the mean cpm

value for the tubes incubated with labelled E11 alone. This value was converted into the number of molecules of IgG anti-CR1 bound per erythrocyte by multiplying by the correction factor (cf).

$$Cf = \frac{4.03 \times 10^{12}}{^{125}\text{I-E11 input in cpm}} \Big/ 5\times10^{7}$$

Although this means of CR1 determination was extensively criticised in Chapters 4 and 7, this one point determination assay was employed to give a quick overall picture of receptor levels, as the number of tubes in each experiment was too great to perform the full titration procedure from which Scatchard plots and therefore exact CR1 levels could be derived.

8.5 Kinetics of binding of immune complexes to erythrocytes.

These experiments were performed using thyroglobulin ^{125}I-anti-thyroglobulin immune complexes at equivalence (8.1), the immune complexes were opsonized as described in 8.2. To a given volume of pre-warmed erythrocytes at 37°C the opsonized immune complexes were added (50µl of immune complexes per 200µl of erythrocytes at 2.5×10^{8}/ml). The suspension was kept at 37°C and at given time intervals duplicate 200µl samples were removed and placed on ice. At the end of the experiment, the 200µl samples were centrifuged over 300µl of separating fluid at 8500g for 5 mins, to separate the cell pellets from the supernatants. The supernatants were removed and the cpm in the pellets measured in a γ-counter. The control for this experiment was the use of radiolabelled immune complexes which had not been opsonized.

8.6 Dose response binding of immune complexes to erythrocytes.

These experiments were performed using thyroglobulin anti-thyroglobulin immune complexes (8.1); depending upon the experiment these were used labelled or unlabelled. The immune complexes were opsonized as described in 8.2. To given volumes of pre-warmed erythrocytes at 37°C increasing amounts of immune complexes were added, from 5µl to 100µl of opsonized immune complexes per 200µl of erythrocytes at 2.5×10^8/ml. The cells and immune complexes were incubated at 37°C for 7 mins, followed by immersion in an icebath. Duplicate samples (containing 5×10^7 cells) were removed from the tubes and centrifuged over separating fluid as described in 8.5; the supernatant was removed and the cpm in the pellets counted in a γ-counter. Variations on this experiment were: a) incubating erythrocytes and immune complexes at 4°C as opposed to 37°C, b) treating the serum with 10mM isotonic EDTA or 20mM MgEGTA and c) using unopsonized immune complexes.

8.7 Probing of erythrocytes for surface bound components.

Normal erythrocytes and patients's erythrocytes were probed for surface bound IgG or C3 fragments, and normal erythrocytes treated *in vitro* with immune complexes were examined to confirm the binding of the immune complexes to the cell surface. The following antisera were used for these experiments; rabbit anti-human C3c, donkey IgG anti-rabbit IgG, sheep anti-human IgG, rabbit anti-BSA, rabbit IgG anti-thyroglobulin and human IgG anti-DNA. The antisera were labelled using the procedure described in 2.15, and were then tested by double immunodiffusion in

agarose (2.7) against the antigen specific for the antiserum to ensure that the antiserum had not been denatured during the radiolabelling. For all the assays the same protocol was followed. Duplicate 200µl samples of the erythrocytes ($2.5x10^8$/ml) to be tested were placed in microcapped centrifuged tubes and to this 10µl of the radiolabelled antiserum was added. The tubes were incubated for 1 hour at 4°C with continuous mixing, after which the cells were separated from the supernatant by centrifugation over 300µl of separating fluid (4.7) at 8500g for 2 mins. The supernatant was removed and discarded and the cpm in the pellet was measured in a γ-counter. The cpm input into the system was determined by counting alone 10µl of the radiolabelled antiserum. The binding of the antiserum to the cells was expressed as counts per minute, or the number of antibody molecules bound per cell.

RESULTS

The in vitro effect of opsonized immune complexes on erythrocyte CR1 levels.

Artificial immune complexes (BSA anti-BSA, thyroglobulin anti-thyroglobulin and DNA anti-DNA) were opsonized by incubating with fresh human serum for 7 mins at 37°C (Horgan and Taylor 1984). The serum used was derived from the same individual as the erythrocytes used for the experiment. The opsonized immune complexes were then incubated with the standardized normal erythrocytes (50µl of immune complexes per 200µl of erythrocytes at $2.5x10^8$/ml) for 7 mins at 37°C. After washing with ice-cold RPMI-BSA (or in the case of the BSA anti-BSA immune complexes RPMI plus 0.1% gelatin)

to remove excess immune complexes, the number of CR1 sites/ erythrocyte were determined (8.3). The number of CR1 sites/ erythrocyte prior to the incubation with the immune complexes was ascertained by handling a second batch of cells in a similar manner omitting the addition of the immune complexes.

In the two assays performed with opsonized BSA anti-BSA immune complexes at equivalence the number of erythrocyte CR1 remained unchanged (Table 28). Three assays were performed using opsonized thyroglobulin anti-thyroglobulin immune complexes at equivalence. These immune complexes brought about partial or total blockade of the erythrocyte CR1 (Table 28). An equivalence point could not be obtained for the DNA anti-DNA immune complexes; two concentrations of calf thymus DNA were used to form the immune complexes. In an initial test when 50μg of DNA was used to form the immune complex, this reduced the receptor levels by 64%, whilst the use of 200μg of DNA reduced receptor levels by 80% (Table 28). On the basis of these observations in all future experiments, opsonized DNA anti-DNA immune complexes were formed using 200μg of DNA. In two further experiments the opsonized DNA anti-DNA immune complex completely blocked the accessibility of the receptor to the ^{125}I-F(ab')$_2$ anti-CR1 probe.

To confirm that the immune complexes had bound to the cell surface, the cell surfaces were probed for bound C3 fragments and/or the antigen used to form the immune complex. Bound C3 fragments (C3b, iC3b and C3c) were probed for using ^{125}I-rabbit anti-human C3c and ^{125}I-rabbit anti-BSA, ^{125}I-rabbit IgG anti-thyroglobulin and ^{125}I-human IgG anti-DNA were used to probe for BSA, thyroglobulin and DNA respectively (8.7). Unfortunately, both cell bound C3 fragments and antigen were not examined in all

TABLE 28. CR1 levels on normal erythrocytes pre- and post-incubation with opsonized immune complexes (BSA anti-BSA, thyroglobulin anti-thyroglobulin and DNA anti-DNA).

Opsonized immune complexes	CR1 sites/erythrocyte	
	Pre-incubation	Post-incubation
BSA anti-BSA	3713	3678
	7019	7572
Thyroglobulin anti-thyroglobulin	3265	0
	13116	7844
	26135	0
DNA anti-DNA	20921	7575 (50μg DNA) 4197 (200μg DNA)
	18800	0
	2550	0

the experiments, nor in these experiments was any attempt made to correlate the level of probe binding with the degree of receptor blockade. The results are shown in Tables 29, 30 and 31; in all cases there was an increase in the level of probe binding after the cells had been incubated with the immune complexes indicating that the immune complexes had bound to the erythrocyte cell surface.

Kinetics of binding of immune complexes to erythrocytes

The binding of thyroglobulin immune complexes at equivalence opsonized with fresh human serum was studied in four individuals (8.5). It was found that the immune complexes in the absence of serum had the ability to bind to the cell surface; these non-specifically bound cpm were subtracted from the total bound cpm (immune complexes bound in the presence of serum) to give the specifically bound cpm. Samples were taken at various time intervals during a 60 mins period and a representative example of a kinetic binding curve is given in Fig. 32. Similar shaped binding curves were found in two individuals; in the fourth individual there was no binding above the non-specific cpm value; however this individual was known to have consistently low CR1 levels and at the time of this study only had 140 CR1 sites/erythrocyte as determined using the monoclonal antibody E11 (8.4). The 3 individuals who did bind the immune complexes were known to have high CR1 levels and the individual's erythrocytes used for the experiment shown in Fig 32, had 630 CR1 sites/erythrocyte at the time of study (8.4). In these 3 individuals peak binding was always at 15 mins.

TABLE 29 Levels of radiolabelled anti-BSA (results expressed as cpm, mean of two determinations) bound to normal erythrocytes pre- and post-incubation with opsonized BSA anti-BSA immune complexes, together with CR1 levels

Pre-incubation	Post-Incubation	Change in CR1 sites
800	11392	3713 ⟶ 3678
599	12085	7019 ⟶ 7572

TABLE 30. Levels of radiolabelled anti-human C3c and anti-thyroglobulin (results expressed as cpm, mean of two determinations) bound to normal erythrocytes pre- and post-incubation with opsonized thyroglobulin anti-thyroglobulin immune complexes together with CR1 levels.

^{125}I-anti-C3c binding		^{125}I-anti-thyroglobulin binding		Change in CR1 sites
Pre	Post	Pre	Post	
NT	NT	877	3030	13116→7844
552	2941	NT	NT	3265→ 0
419	866	3364	20854	26135→ 0

NT = not tested

TABLE 31 Levels of radiolabelled anti-human C3c and anti-DNA (results expressed as cpm, mean of two determinations) bound to normal erythrocytes pre- and post-incubation with opsonized DNA anti-DNA immune complexes together with CR1 levels.

^{125}I-anti-C3c		^{125}I-anti-DNA		Change in CR1 sites
Pre	Post	Pre	Post	
565	777	NT	NT	20921—> 7575
565	936	NT	NT	20921—>4197
382	2700	280	2220	18848—>0
1482	1598	12512	17821	2550—>0

NT = not tested

FIG 32. Kinetics of binding of thyroglobulin anti-thyroglobulin immune complexes to normal erythrocytes.

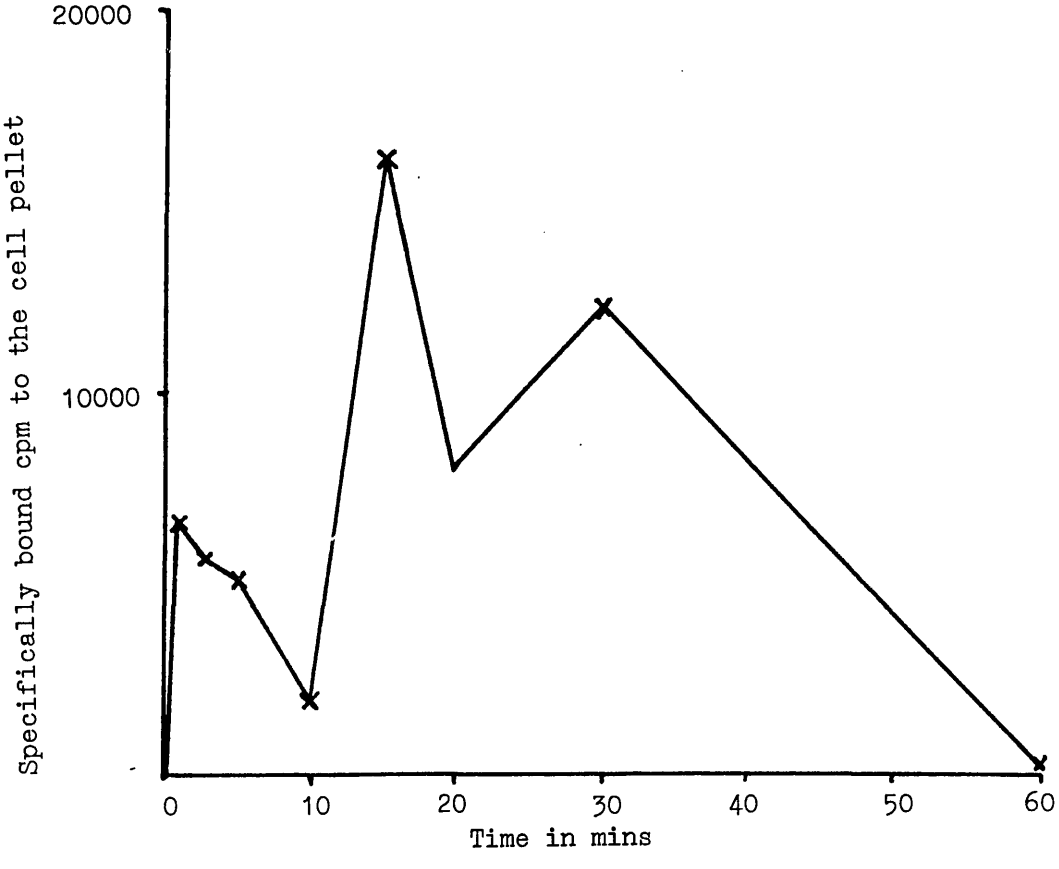
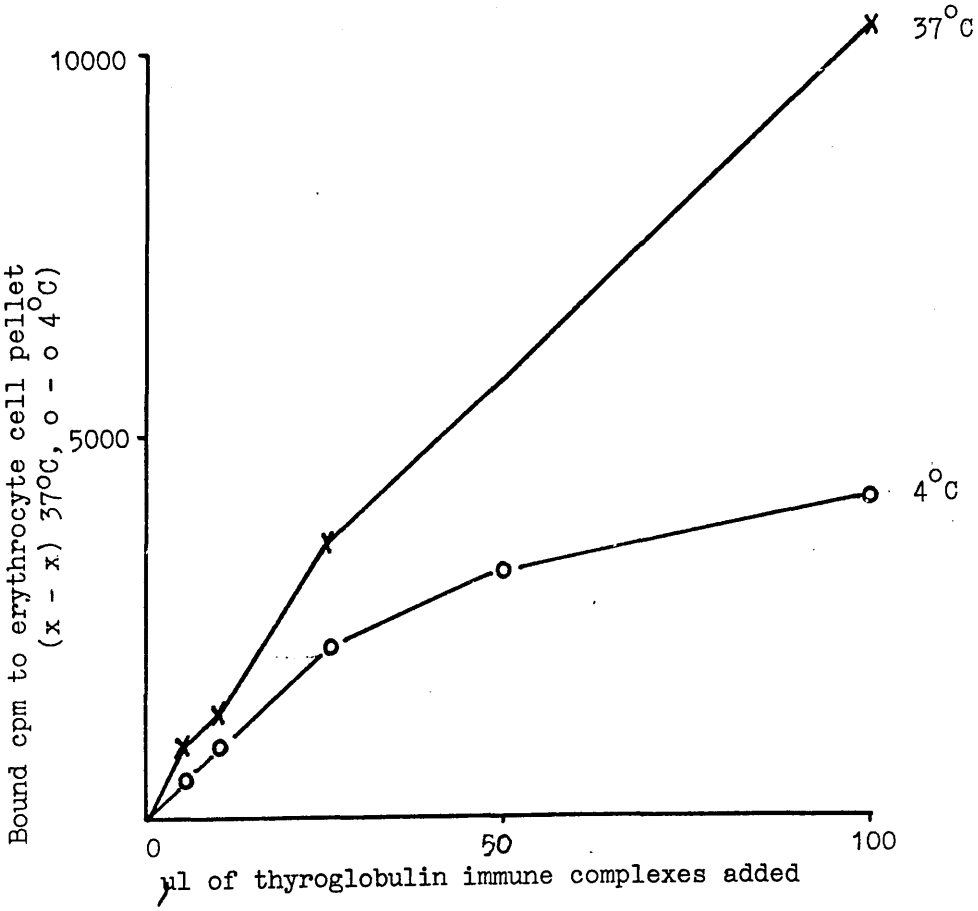

20000
10000
Specifically bound cpm to the cell pellet
0
10
20
30
40
50
60
Time in mins

FIG 33. Dose-dependent binding of opsonized thyroglobulin anti-thyroglobulin immune complexes at $37^{o}C$ (x-x) and $4^{o}C$ (o-o) to normal erythrocytes

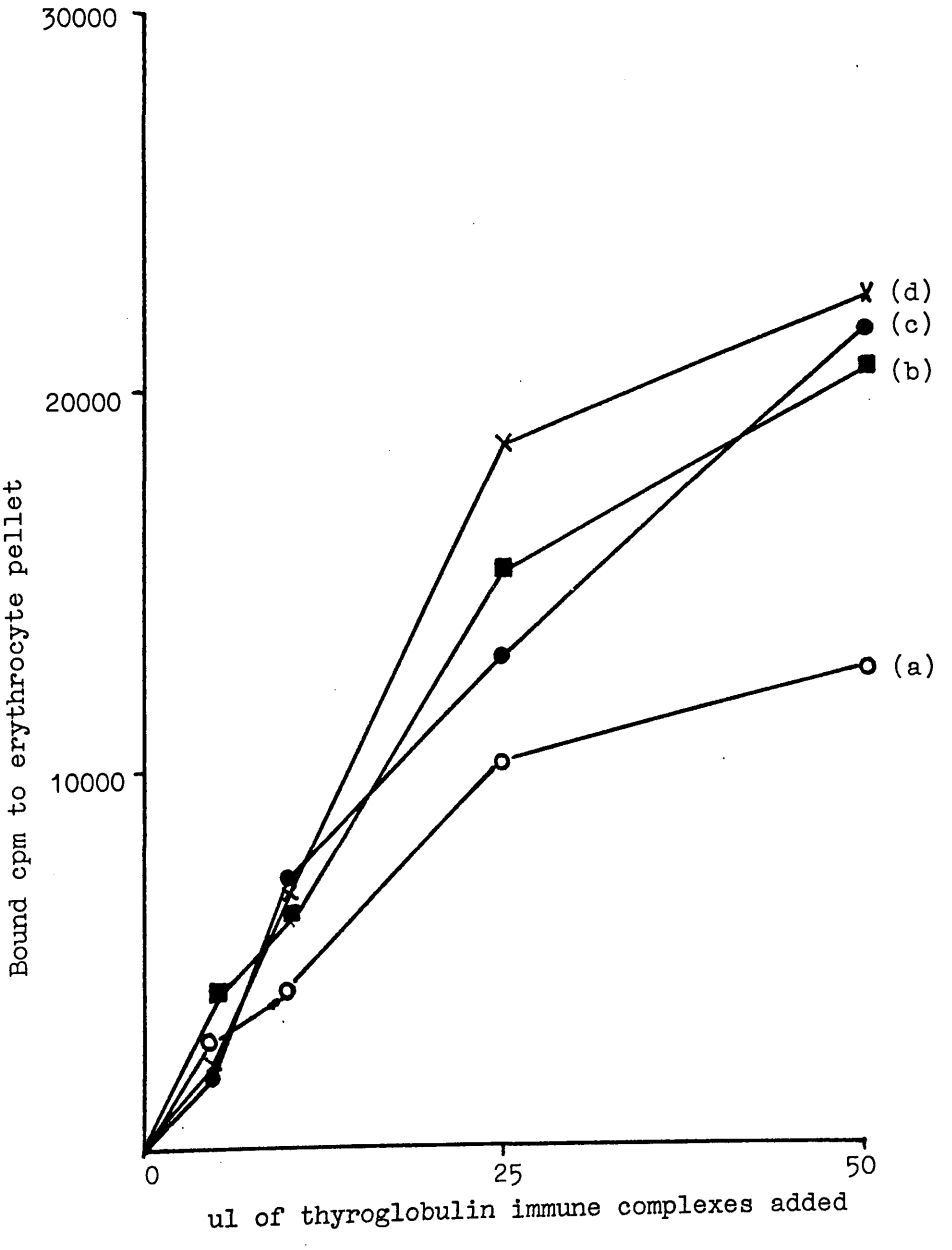

37°C
4°C
10000
5000
0
50
100
Bound cpm to erythrocyte cell pellet
(x - x) 37°C, o - o 4°C)
µl of thyroglobulin immune complexes added

Dose response binding of immune complexes to erythrocytes.

Using radiolabelled thyroglobulin immune complexes at equivalence 5 to 100µl of immune complexes were incubated with 200µl of erythrocytes ($2.5x10^8$/ml) for 7 mins and then the amount of immune complex bound to the cell surface was evaluated (8.6). This experiment was performed on erythrocytes from 3 people and a typical result is shown in Fig. 33. At 37°C there was a dose-dependent binding of the immune complexes to the erythrocytes; the binding at 4°C was reduced in comparison to the binding at 37°C. Higher doses of immune complexes were not tested. However, with the doses used there was no plateau in the immune complex binding. Unfortunately, CR1 levels were not quantitated in these experiments.

Further experiments of this type were performed to evaluate the complement requirements of this binding reaction. The experiments were performed as described in 8.6 and the dose-dependent binding of the immune complexes to the erythrocytes was analysed in; a) buffer alone, b) serum, c) serum treated with 20mM Mg EGTA and d) serum treated with 10mM EDTA. A representative result is shown in Fig. 34. Clearly the radiolabelled thyroglobulin anti-thyroglobulin immune complexes are binding in the absence of serum and this was consistently found. In Fig. 34 the binding of the immune complexes in the presence of serum was enhanced; however neither 20mM Mg EGTA or 10mM EDTA had any effect on the binding of the immune complexes to the cell. To evaluate whether the immune complexes affected the receptor levels a preliminary experiment was performed where 50µl of cold immune complexes were incubated with 200µl of erythrocytes ($2.5x10^8$/ml), in the presence of buffer alone, serum or serum treated with 10mM EDTA; the receptor levels were then

FIG. 34 Dose-dependent binding of thyroglobulin anti-thyroglobulin immune complexes to normal erythrocytes in the presence of a) buffer alone (o), b) serum (■), c) 10mM EDTA treated serum (Δ) and d) 20mM Mg EGTA treated serum (x)


30000
20000
10000
Bound cpm to erythrocyte pellet
(d)
(c)
(b)
(a)
0
25
50
ul of thyroglobulin immune complexes added

quantitated using the monoclonal antibody E11 (8.4). The results are tabled below.

Assay conditions	CR1 sites/erythrocyte
Buffer alone	1221
Immune complexes + buffer	1082
Immune complexes + serum	266
Immune complexes + serum + 10mM EDTA	902

Although the immune complexes had bound to the erythrocytes only immune complexes incubated with normal serum reduced the receptor levels significantly.

A further study investigated whether increasing doses of cold opsonized thyroglobulin immune complexes brought about a reduction in receptor levels as measured by the monoclonal E11 (8.4). The presence of immune complexes bound to the erythrocyte cell surface was confirmed using ^{125}I-rabbit IgG anti-C3c, ^{125}I-donkey IgG anti-rabbit IgG and ^{125}I-rabbit IgG anti-thyroglobulin (8.7). The level of binding of the probes being expressed as the number of IgG molecules bound per erythrocyte. The results are shown in Figs. 35, 36 and 37. Clearly as the input of immune complexes increased, the binding of the radiolabelled probes increased (Fig 35, Fig 36 and Fig 37) and there was a concominant decline in the receptor levels (Fig. 35).

Examination of levels of erythrocyte bound IgG and C3 fragments.

The presence of erythrocyte bound IgG and/or C3 fragments were probed for using radiolabelled rabbit anti-human C3c and

FIG. 35 Changes in levels of CR1 (measured by E11) and binding of ^{125}I-IgG anti-rabbit IgG to normal erythrocytes incubated with increasing amounts of opsonized thyroglobulin immune complexes.

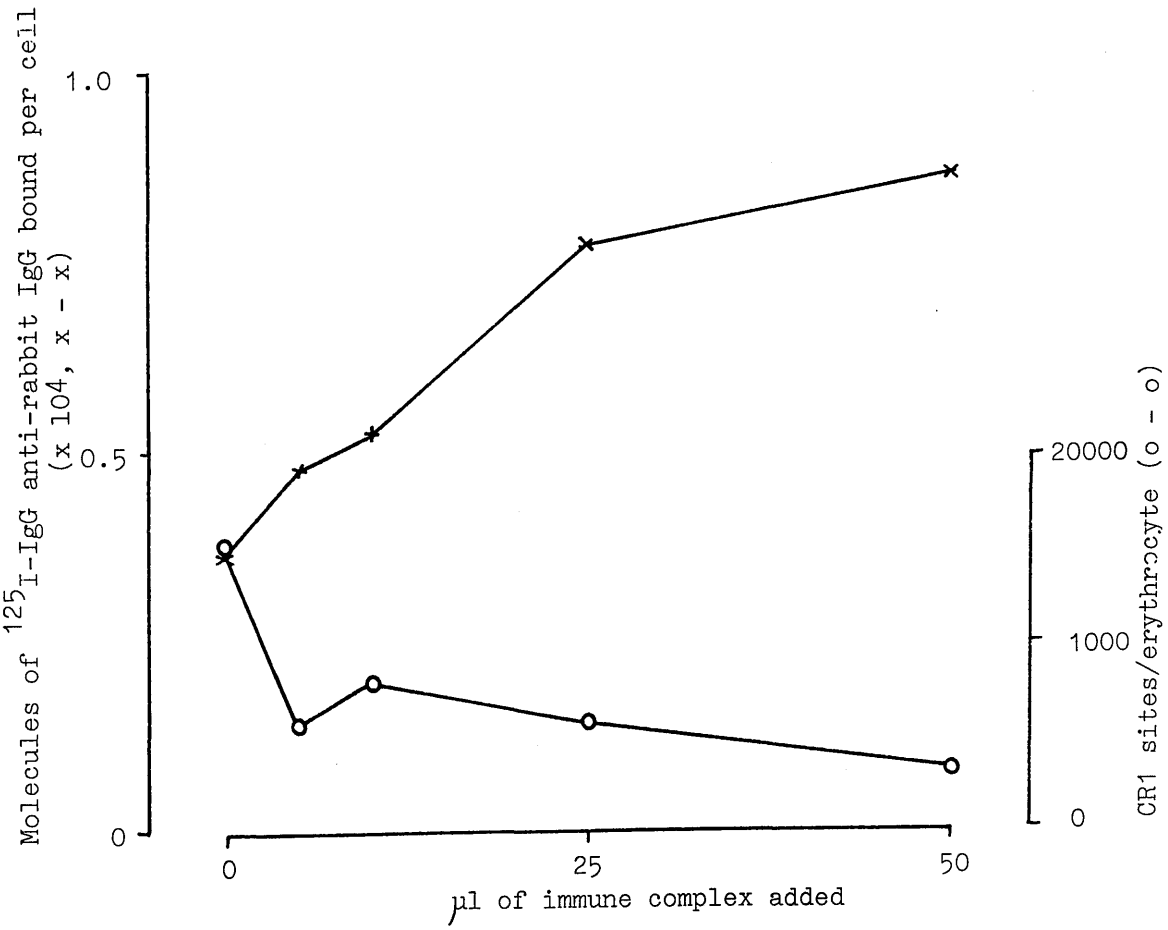

Molecules of ^{125}I-IgG anti-rabbit IgG bound per cell (x 104, x - x)
1.0
0.5
0
0
25
50
µl of immune complex added
20000
1000
0
CR1 sites/erythrocyte (o - o)

FIG. 36 Changes in the levels of CR1 (measured by E11) and binding of ^{125}I-IgG anti-human C3c to normal erythrocytes incubated with increasing amounts of opsonized thyroglobulin immune complexes.

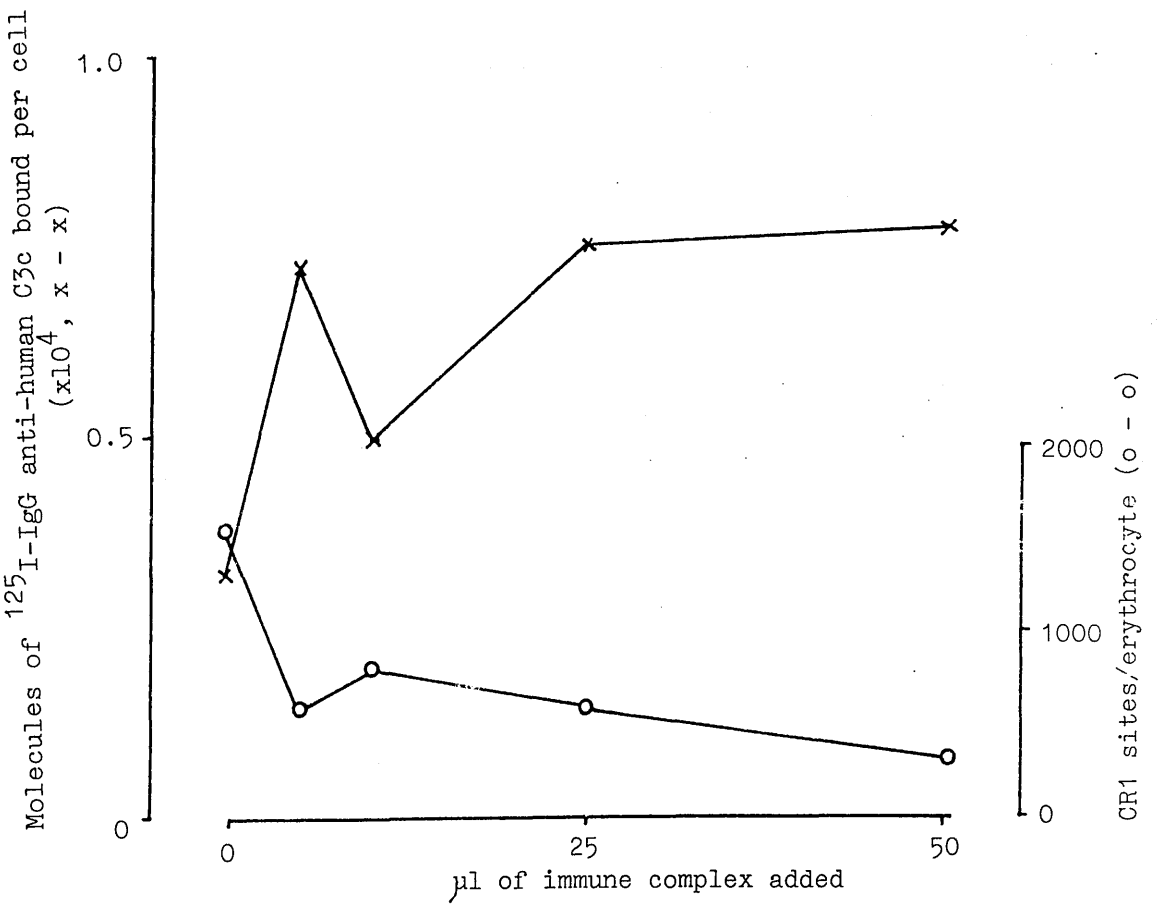

Molecules of ^{125}I-IgG anti-human C3c bound per cell (x10^4, x - x)
1.0
0.5
0
0
25
50
µl of immune complex added
2000
1000
0
CR1 sites/erythrocyte (o - o)

FIG. 37 Changes in levels of CR1 (measured by E11) and binding of ^{125}I-IgG anti-thyroglobulin to normal erythrocytes incubated with increasing amounts of opsonized thyroglobulin immune complexes.

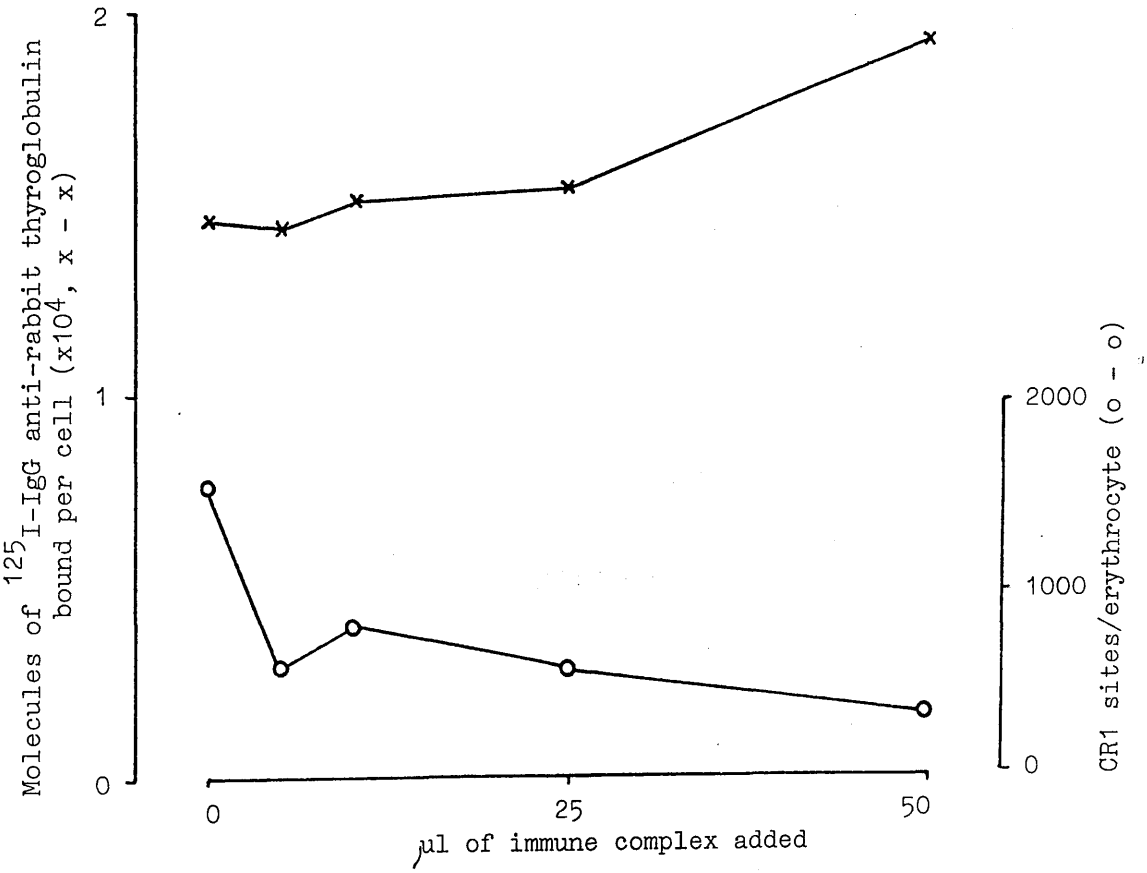

Molecules of ^{125}I-IgG anti-rabbit thyroglobulin bound per cell (x10^4, x - x)
2
1
0
0
25
50
µl of immune complex added
2000
1000
0
CR1 sites/erythrocyte (o - o)

TABLE 32. Binding of ^{125}I-rabbit anti-human C3c and ^{125}I-sheep anti-human IgG to the erythrocytes from 2 normal individuals and 4 SLE patients.

	cpm of ^{125}I-anti-C3c bound	cpm of ^{125}-anti-IgG bound
Normal (1)	1098	NT
Normal (2)	779	6014
SLE (1)	1702	6260
SLE (2)	868	6347
SLE (3)	838	5535
SLE (4)	1648	6351

sheep anti-human IgG (8.7). The erythrocytes from two normal individuals and 4 SLE patients were examined to try and establish whether these molecules bound to the erythrocyte cell surface could directly or indirectly block CR1, rendering it inaccessible to ^{125}I-F(ab')$_2$ anti-CR1. The results are shown in Table 32, and are expressed as the mean cpm bound to the erythrocyte pellet (mean of 2 determinations). There is very little variation in the level of ^{125}I-sheep anti-human IgG bound to the 5 erythrocyte samples tested. There was slightly more variation however, in the levels of ^{125}I-rabbit anti-human C3c bound.

DISCUSSION

Nelson's paper of 1953 has the title "The immune-adherence phenomenon: An immunologically specific reaction between micro-organisms and erythrocytes leading to enhanced phagocytosis", and in this paper Nelson demonstrated the binding of antibody sensitised Treponema pallidum in the presence of complement to normal human erythrocytes. The time reaction for maximal binding was approximately 5 minutes, and similar results were found using the bacterium Diplococcus pneumoniae. From his results Nelson proposed that red blood cells preferentially bound sensitised opsonized micro-organisms and presented them to the leucocytes therefore enhancing phagocytosis.

In 1981 Siegel et al (1981) put forward the view that erythrocytes are an essential component of the immune system, and they were the first to propose that erythrocytes have a primary role in the removal of circulating opsonized immune

complexes from the fluid phase of the circulation by virtue of the erythrocyte's CR1, to bind C3 containing complexes. They stressed that the erythrocytes would be the main carrier system as they possess 95% of vascular CR1 on their surfaces (based on the numerical data of Fearon 1980) and because of the red blood cell to white blood cell ratio a circulating immune complex would be 500 to 1000 times more likely to interact with an erythrocyte as opposed to a white blood cell. They emphasised that this system would be physiologically advantageous, preventing the deposition of harmful immune complexes and allowing the leucocytes to function normally without being overwhelmed by circulating immune complexes.

This view is supported by Cornacoff, Zeger and Herbert (1981), who demonstrated the avid binding of soluble ^{125}I-BSA anti-BSA immune complexes to human erythrocytes; the reaction was complement dependent and larger immune complexes bound more effectively. Again this may be of physiological advantage selectively removing larger circulating immune complexes. Aikawa et al (1979) also demonstrated the IC size dependent binding effect using immune complexes derived from the serum of SLE patients.

Medof and Oger (1982) investigated the in vitro binding of immune complexes to erythrocytes in detail. Using radiolabelled BSA anti-BSA immune complexes they first examined the effects of the antibody : antigen ratio and the ratio of serum to immune complexes on their binding to the erythrocytes. Using conditions where the immune complexes were opsonized for 30 mins and then incubated with the erythrocytes for a further 45 minutes, they demonstrated optimal binding of immune complexes (65%) formed in 4 times antibody excess with a serum:immune complex ratio of 1:16. The high antibody ratio may be necessary for

effective complement fixation. The critical serum dilution probably represents a compromise, i.e. at lower dilutions although C3b may be produced the levels of I may be sufficiently high to break it down into non-CR1 binding forms and at higher serum dilutions insufficient C3 may be turned over to allow immune complex binding to the erythrocyte. However, using these conditions, Medof and Oger (1982) showed that the immune complexes bound preferentially to the erythrocytes, although less bound per cell compared to the binding to the leucocytes, thus confirming the hypothesis of Siegel et al (1981) that immune complexes would bind preferentially to erythrocytes by virtue of their total higher CR1 concentration.

Kinetic studies by Medof and Oger (1982, using undiluted serum) showed a bell shaped binding curve, with maximum binding of the immune complexes occurring in 4 mins and dramatically dropping off by 8 to 10 mins. The kinetics of binding was tested in four people and although the bell shaped curve was the same for all four individuals, the levels of peak immune complex binding varied from 30 to 65%; this probably reflects the different erythrocyte CR1 densities. They found that binding release could be delayed using diluted or hypocomplementemic serum and Medof and Oger (1982) suggest that sustained immune complex binding may possibly have clinical implications in diseases with prevalent hypocomplementemia and high immune complex load. For example a) the equilibrium of free to bound immune complexes could be dramatically altered by the availability of complement components and regulators, and b) sustained binding of the immune complexes to the erythrocytes could paralyse the system's ability to bind large immune complex loads; this may allow the immune

complexes to interact with leucocyte CR1 bearing populations and may cause aberrent immune responses.

Similar work, investigating the immune complex binding properties of erythrocytes have been performed by Cornacoff et al (1983) using an *in vivo* model. They studied the fate of large preformed ^{125}I-BSA anti-BSA immune complexes (10,000 to 288000 S) infused into primates, as a means of testing whether erythrocyte CR1 acted as a removal mechanism for circulating immune complexes *in vitro*. After infusion immune complexes were shown to have bound to the erythrocytes within 30 seconds, the great majority bound to the erythrocytes and were cleared after passage through the liver, spleen and gut. Lower levels bound to the leucocytes, but were cleared more slowly by the liver, spleen and gut. The immune complexes which bound to the erythrocytes induced no haemolysis and they speculated this was due to CR1's inhibitory effects on the complement system.

The size of the immune complexes used in these experiments was large, although optimal for binding in their system, This however raises questions about the biological significance of these results particularly as the infusion of antigen and antibody alone did not result in the immune complexes binding to the erythrocytes. This may be a reflection on the immune complex size perhaps being of insufficient size for binding, or that the complexes were cleared too quickly to be detected by the experimental system.

The experimental data discussed do provide strong experimental evidence to support the hypothesis presented by Siegel et al (1981) that erythrocyte CR1 acts as an immune complex carrier system. Thus defects in this system could reduce the ability of affected individuals to handle an immune complex load.

Reduced erythrocyte CR1 levels do exist, particularly in SLE patients, and the aim of the work performed in this thesis was to examine this defect and to try to establish whether it was acquired or inherited; the results are extensively discussed in Chapter 7, as are those of other workers in this field. As a result of the studies it became clear that low CR1 levels in some SLE patients were acquired, with the CR1 levels varying with disease activity, strikingly lower in active disease as opposed to inactive disease, and the finding that identical twins with SLE do not have the same CR1 levels supports this view.

There are two possible explanations for low erythrocyte CR1 levels; a) receptor blockade and b) receptor removal. The latter was not investigated in this thesis; however the following means of receptor removal can be considered; a) cleavage of the receptor by plasma enzymes or b) removal of the receptor by the immune complex during passage through the circulation. The first possibility seems an unlikely explanation as normal erythrocytes incubated with physiological levels of the following enzymes; thrombin, cathepsin C and D, kallikrein and plasmin showed no change in their CR1 levels. Elastase was used at two concentrations 0.1% and 1%; only the higher dose reduced the receptor levels. This enzyme is an intracellular constituent of phagocytic cells and it seems unlikely that concentrations of elastase required to reduce receptor levels would be found in the circulation (personal communication Miss A. W. Fyfe). The second candidate for receptor removal "stripping" as a result of the interaction of the immune complex bearing erythrocyte with the cells of the fixed mononuclear phagocytic system during circulation through the liver and spleen is supported by Ross et al (1985). However the <u>in vivo</u> work performed by Cornacoff et al (1983), where they infused radiolabelled BSA

immune complexes into primates showed that erythrocytes cleared of immune complexes still possessed the ability to bind more immune complexes. Thus the erythrocytes had not been damaged after passage through the liver or spleen, similar work performed in vitro by Medof, Prince and Oger (1982) produced similar results. However, it must be stressed that the fixed mononuclear phagocytic systems of patients with immune complex diseases may not function normally and may indeed bring about receptor removal. In support of this argument recent work by Walport et al (1985b) demonstrated the loss of CR1 from normal erythrocytes after transfusion into SLE patients (e.g. 493 to 202 CR1 per erythrocyte in 112 hours). The finding of a soluble form of CR1 by Yoon and Fearon (1985) which they believed to be derived from the erythrocytes (the plasma CR1 being identical in molecular structure to the erythrocyte CR1), raises the possibility that CR1 may be lost from erythrocytes. They investigated this by incubating normal erythrocytes for up to 4 hours at $4^{o}C$ to see whether receptor loss occurred. No reduction was seen. Similarly, this was found in the storage experiments performed in Chapter 4. Yet at $4^{o}C$ all cell processes would be minimal and if energy was required for receptor loss the process may not have occurred. More relevant information would have been obtained by performing the experiments at a higher temperature. It is however possible that receptor loss does occur, despite the mechanism being unknown.

The second means of reducing receptor levels, receptor blockade, was the subject of the investigations performed in this chapter. As discussed at the beginning of this section evidence has accumulated to support a role for erythrocyte CR1 as an immune complex carrier. With this view in mind the possibility

that immune complexes bound to the erythrocyte cell surface could block CR1 was investigated. Three types of immune complexes were opsonized and incubated in vitro with normal erythrocytes and their effects on CR1 levels determined.

The three types of antigen used for these studies were BSA (60000 daltons), thyroglobulin (660000 daltons) and calf thymus DNA (molecular weight not determined); these antigens vary markedly in size and consequently would yield immune complexes of vastly different sizes. The BSA and thyroglobulin containing immune complexes were used at equivalence. Despite using serum from an SLE patient with a high anti-DNA antibody titre (96% DNA binding capacity) a quantitative precipitin curve with DNA failed to yield an equivalence point. This could be due to the nature of the DNA antigen which is highly soluble in aqueous solutions. In addition, anti-DNA antibodies tend to bind to the DNA monogamously, rather than forming cross links between individual DNA molecules, consequently the DNA immune complex would be more soluble (Lennek et al 1981). The doses of DNA used were therefore chosen arbitrarily.

Investigations on the binding of three types of opsonized immune complexes to normal erythrocytes showed that although the smallest of the three types of complexes (BSA anti-BSA) became bound, CR1 levels were unaffected (Table 29). In contrast the larger complexes formed with thyroglobulin or DNA brought about partial or complete receptor blockade (Tables 30 and 31). It is thus evident that the degree of CR1 blockade may be related to the size of the antigen. It is probable that BSA anti-BSA complexes which have reacted with complement are considerably smaller than similar complexes formed with thyroglobulin or DNA. It is also probable that the number of C3b molecules bound per

complex is also greater in these two latter types of complex. Thus the greater quantities of C3b would increase the binding affinity of the complex to CR1. (This has been demonstrated *in vitro* by Arnaout et al (1983b), they showed that polymerized C3b bound with greater affinity than monomeric C3b to erythrocyte CR1) and they may therefore not be displaced by $F(ab')_2$ anti-CR1. The larger size of the complexes would also mean that many CR1 sites were inaccessible to the anti-CR1 probes. A point to be noted from these experiments is that the degree of receptor blockade is not related to the number of CR1 sites, i.e. an individual with high CR1 levels may be blocked totally by immune complexes, whilst erythrocytes with a low CR1 level may only be partially blocked.

In these experiments the presence of the immune complexes on the erythrocyte cell surface was confirmed by the binding of radiolabelled probes for C3 fragments and the appropriate antigen under study (8.7). The levels of probe binding are expressed as cpm (Tables 29, 30 and 31) and in all cases there was an increase in probe binding to cells after they had been incubated with the immune complexes. The variable levels of binding could reflect the amount or accessibility of the antigens within the complex.

With the demonstration that *in vitro* immune complexes could bind and block erythrocyte CR1 it was decided to investigate the binding reactions further. Thyroglobulin anti-thyroglobulin immune complexes were chosen for these preliminary experiments as; a) they were known to bind and block receptors and b) large supplies of thyroglobulin and antiserum were available.

The first study examined the kinetics of immune complex binding to normal erythrocytes from four individuals; in one

individual (with known low receptor levels) there was no binding above background. In the remaining 3 individuals (representative result from one individual is shown in Fig. 32), there were different phases of immune complex binding and release, with maximum binding occurring at 15 mins and with complete release by 60 mins.

Kinetic binding curves for BSA immune complexes performed by Medof and Oger (1982) were bell shaped with the complex dissociation being mediated by I (Medof and Prince 1983a) but further more in depth studies showed sequential rebinding and immune complex release. They proposed that the first phase of dissociation mediated by I, left sufficient bound C3b to form an alternate pathway C3 convertase and this brought about rebinding (Medof and Prince 1983a). Together with this, the longer the immune complexes had been incubated with the erythrocytes, the less well they bound to fresh erythrocytes, PMNs or monocytes; and by analysing the immune complexes on sucrose gradients, they showed demonstrable reductions in the complex size (Medof and Prince 1983b), Thus indicating the immune complex handling by the erythrocytes had altered their physical and binding properties.

Horgan et al (1984) studied the kinetics of binding of ^{3}H-DNA anti-DNA immune complexes to erythrocytes and their subsequent release. The binding was rapid (less than 5 mins) with a slow release; however the rate of release was affected by both the antibody:antigen ratio and the molecular weight of the DNA used. For example, using high molecular weight DNA (6×10^{6} daltons) the t $\frac{1}{2}$ for release was 60 mins and with lower molecular weight DNA (2 to 6×10^{5} daltons) the t $\frac{1}{2}$ was 20 mins, and rebinding did not occur. Using small complexes (less than 2.5×10^{5} daltons)

there was minimal binding. Analysis of the released immune complexes showed no size change but an increase in C3 breakdown products. A lack of size change may not be unexpected due to the nature of the DNA molecule but clearly there had been modifications of the C3b.

The effects of size on binding and release may reflect the amount of antibody bound and therefore complement fixation, with large complexes fixing more complement therefore binding with higher affinity and for longer to erythrocytes and vice versa.

Clearly the kinetics of binding and release are influenced by antigen and antigen size, resulting in different binding and processing by the erythrocytes. The different phases of binding and release from the erythrocytes seen with the thyroglobulin immune complexes (Fig. 32) was a constant finding and could represent handling of the immune complexes by the erythrocyte CR1 and the complement system, as described by Medof and Prince (1983 a and b); however this was not investigated further.

The next line of investigation was to see whether the immune complexes bound to erythrocytes in a dose-dependent fashion, and to establish the complement dependency and temperature sensitivity of this reaction.

At 37°C there was a dose-dependent binding reaction of thyroglobulin immune complexes to erythrocytes. Using 5µl to 100µl of complexes per 200µl of erythrocytes (2.5×10^8/ml), 3 individuals were tested and saturation of the receptors was never achieved with the doses of immune complex used. The binding reaction was greatly diminished when the experiments were performed at 4°C (Fig. 33). Similar results were found by Pedersen et al (1980) using DNA immune complexes, and studies on the effects of temperature by Horgan and Taylor (1984) showed lower binding

rates of DNA immune complexes to erythrocytes at low temperatures (less than 22°C) but above 22°C they assumed binding levels found at 37°C. The results of my work indicate that at 4°C the receptors are not in a state which allows receptor binding, therefore increasing the temperature either allows the production of energy for membrane reorganisation or allows an increase in membrane fluidity. Horgan and Taylor (1984) favour the latter (especially as erythrocytes produce low levels of energy anaerobically), with the low temperature inducing membrane rigidity which would inhibit any "long range" receptor organization.

The complement dependency was next investigated. EDTA (10mM) prevents both the turnover of the classical and the alternative pathways and Mg EGTA (20mM) prevents classical pathway turnover. Both these chemicals prevented the lysis of EAs by normal human serum and therefore were working effectively; however as can be seen in Fig. 34 they did not diminish the binding of the immune complexes to the erythrocytes. This raises the question of how the immune complexes were binding to the cell surface. The manner in which the immune complexes bound to the cell surface was presumed to be via C3b interacting with CR1 and evidence from other laboratories supported this assumption (Taylor et al, 1985; Medof and Oger 1982). However the experimental results attained using EDTA and Mg EGTA to specifically block complement turnover seem to suggest another binding mechanism. A recent paper by Virella, Shuler and Sherwood (1983) demonstrated the non-complement-dependent adsorption of soluble immune complexes to human erythrocytes. Such results were found in these experiments (Fig. 34(a) immune complex plus buffer); however, the binding in the presence of serum was greatly enhanced (Fig. 34(b).

An experiment quantitating the receptors using the monoclonal

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E11 pre- and post-incubation with complexes alone, immune complexes plus serum and immune complexes plus EDTA (10mM) treated serum, showed that although the immune complexes had bound to the erythrocytes only immune complexes incubated with serum bound to and blocked CR1 (results page 259). This restored faith in the view that the immune complexes were binding via C3b to CR1 and blocking the receptor, but this does not explain the results found using 10mM EDTA and 20mM Mg EGTA. A possible explanation could be that although the C1 macromolecule would be dissociated due to the chelation of calcium ions (by EDTA and Mg EGTA), the C1q moiety may still bind to the immune complexes, and it is possible that erythrocytes like lymphocytes, monocytes and neutrophils (Ross 1982) have C1q receptors allowing the binding of the complexes to the cell surface. The binding of the complexes in the absence of serum could be a non-specific "sticking" effect, or may be due to interactions with Fcγ receptors, although their presence on erythrocytes has not been described.

Based on the findings that opsonized immune complexes bound dose-dependently to erythrocytes and blocked receptor, it was decided to investigate whether this dose-dependent binding coincided with dose-dependent blockade. The levels of cold immune complexes on the erythrocytes' cell surface were monitored by the number of molecules of ^{125}I-rabbit IgG anti-C3c, ^{125}I-donkey IgG anti-rabbit IgG and ^{125}I-rabbit anti-thyroglobulin bound. As is shown in Figures 35, 36 and 37, as the amount of immune complexes added to the system increased the receptor levels decreased and there was a concominant increase in the number of IgG molecules of the radiolabelled probes bound to the erythrocytes.

The results of the studies performed in this chapter do clearly show that <u>in vitro</u> opsonized immune complexes can reduce erythrocyte CR1 levels and perhaps this process could account for

diminished receptor levels particularly in diseases where there is a high immune complex load such as SLE. If this was the case, one would perhaps expect to be able to detect immune complex associated IgG and/or C3 fragments bound to the erythrocyte surface of SLE patients.

The erythrocytes from six individuals (2 normals, 4 SLE patients) were examined for surface bound IgG and/or C3b/iC3b/C3c (as detected by anti-C3c antiserum, Table 32). Normal (1) had constantly low CR1 levels, normal (2) had constantly high receptor levels, there is a small difference in their levels of ^{125}I-anti-C3c bound (1098 cpm compared with 779 cpm respectively). Of the four SLE patients only (4) had active disease at the time of study, the remaining 3 having inactive disease. They showed little variation in the levels of ^{125}I-anti-IgG bound and this is not significantly different from the normal value. The levels of ^{125}I-anti-C3c bound showed greater variation. The largest binding values were attained using the erythrocytes from patients (1) (937 CR1 sites/erythrocyte) and (4) (1126 CR1 sites/erythrocyte) and indeed patient (1) with the lower receptor value does have a higher level of ^{125}I-anti-C3c binding (CR1 levels in patients (2) and (3) were not evaluated). These results may indicate that there is an association between CR1 levels and amount of surface bound C3 fragments. However, the sample size was very small, more meaningful information would be gained by performing a prospective study, examining the erythrocyte cell surface of normals and patients for different immunoglobulin isotypes and for C3 and C4 which as well as being a CR1 ligand is adsorbed onto the erythrocyte cell surface to give the Chido and Rodgers blood groups.

Various other workers have performed such studies to try and

establish whether receptor blockade by immune complexes, immunoglobulin or complement components could contribute to receptor reduction on erythrocytes. The results are conflicting. In a study by Wilson et al (1982) they found no correlation between CR1 levels, bound C3 or auto-antibodies directed against CR1. In a later paper Wilson et al (1985) describe the case of an individual woman with SLE who had auto-antibodies to CR1; 34 additional SLE patients and 28 healthy donors also tested had no such auto-antibodies. In two studies by Inada et al (1982, 1983), they found some of their patients with unspecified immune complex diseases to have erythrocyte bound IgG and/or C3 or C4 as detected by a direct Coombes' test; however the significance of their results is questionable, as they point out in their own discussion (Inada et al 1982) the results could not be reproduced using more conventional serological techniques. Ross et al (1985) found no correlation between the presence of immune complexes or fixed C3b/iC3b with CR1 levels on the erythrocytes from active SLE patients. However, there was a correlation between CR1 levels and the levels of erythrocyte fixed C3d-g. In 12 patients studied through periods of disease exacerbation there was an inverse relationship between the levels of fixed C3d-g and CR1. However, they do not believe that reduced receptor levels are caused by immune complexes blocking CR1 because they were unable to detect surface immunoglobulins or C3b/iC3b, and the in vitro incubation of DNA immune complexes with erythrocytes did not cause a receptor reduction. These results must be interpreted with care. Firstly, the amount of immune complex required to bring about receptor blockade in vivo is unknown, this may be of particular importance in patients with phenotypically low CR1 levels where very small quantities of immune complexes may cause

blockade, and these levels may be undetectable by radiolabelled antisera to C3 and/or IgG. Secondly, C3d-g only binds very weakly to CR1 and therefore may not be bound to CR1 but another C3 receptor and only coincidentally related, or maybe bound covalently to the cell membrane. Thirdly, it is difficult to imagine by what means C3d-g alone becomes deposited on the erythrocyte cell surface; perhaps the C3d-g is masking immune complexes bound to the cell surface. Fourthly, in the in vitro immune complex experiments performed, it is possible that the DNA complex used did not have CR1 blocking characteristics. For example, the BSA complexes used for the blockade experiments described in this chapter bound to the erythrocytes and did not block the receptor; this may be the case in the experiments performed by Ross et al (1985). Walport et al (1985) also state that reduced receptor levels are not caused by receptor blockade as their monoclonal antibody to CR1 (E11) can detect occupied receptors (i.e. binding of E11 is not affected by immune adherence). However, the results of the experiments reported in this chapter using E11 clearly show its inability to detect receptors to which immune complexes are bound (Fig. 35). Therefore, in the papers where the CR1 levels have been quantitated using E11 (Walport et al 1985; Ross et al 1985) low patient erythrocyte CR1 levels could be attributed to blockade by immune complexes.

This chapter has dealt with possible mechanisms leading to the acquisition of low CR1 levels on erythrocytes. There are two mechanisms which could be used to explain the reduction of receptor levels. Firstly, receptor removal, (this was not investigated but has been discussed in this chapter) and secondly, receptor blockade. Preliminary studies were performed investigating this area. These encompassed an examination into whether circulating

immune complexes could bind to erythrocytes and temporarily (or otherwise) block the receptors. Using 3 types of antigens (BSA, thyroglobulin and DNA) to form the immune complexes, in vitro studies showed that although all these complexes when opsonized bound to the erythrocytes only the larger thyroglobulin and DNA immune complexes caused receptor blockade (as detected by ^{125}I-F(ab')$_2$ anti-CR1). Studies in vitro using thyroglobulin immune complexes demonstrated the dose-dependent binding of the complexes, and their subsequent dose-dependent blockade of the receptor (as detected by ^{125}I-E11) and the temperature sensitivity of the binding reaction. Binding kinetics showed multi-phasic binding and release of the immune complex from the erythrocyte cell surface. This is unlike the binding curves of Medof and Oger (1982) using BSA anti-BSA complexes, or Horgan and Taylor (1984) using DNA anti-DNA immune complexes; the differences observed may only reflect the different natures of the immune complexes studied. As pointed out by Taylor et al (1983) the fate of a circulating immune complex is largely governed by how they are recognised and processed by cells and tissues, consequently their physical properties play a major role in this recognition process. Influencing factors would be antibody isotype, antibody avidity, mode of antibody binding, antigen size, solubility, stability and composition of the immune complex and the degree of complement fixation (Pedersen et al 1980; Taylor et al 1985).

Initial in vitro studies have shown that immune complexes do bind to and block erythrocyte CR1 and this could be implicated as a possible means of low CR1 acquisition in diseases such as SLE where immune complexes are believed to manifest the disease process.

The possibility of receptor removal by proteolysis is another means of acquiring low receptor levels. Evidence from our laboratory suggests that proteolysis by plasma enzymes does not bring about a reduction in erythrocyte CR1 levels. However, in vivo studies performed by Brown and Nelson (1973) demonstrated the sequestration of EC43 in the liver of rabbits after their intravenous injection. By dissecting out and fixing the liver at various time periods post-administration of the EC43, the fate of the EC43 could be followed. Within 2 mins of injection, the EC43 had adhered to the cell membrane of the Kupffer cells, from which protrusions were beginning to force their way into the red cell membrane. Seven to 25 mins later small red blood cell fragments were detected in the Kupffer cell cytoplasm. Thus cells bearing C3b or iC3b in the circulation had bound to the Kupffer cells, and as the C3 ligands were removed from the cell surface small fragments of membrane were removed also. This process leads to immune spherocytosis. Clearly when erythrocytes bearing C3b-coated complexes bind to the Kupffer cells, parts of the membrane could be removed including CR1.

C H A P T E R 9

FINAL DISCUSSION

The complement system consists of at least 20 chemically and immunologically distinct serum proteins, these are capable of interacting with one another, with antibody and with cell membranes and such interactions lead to biological activity.

The complement system has been shown to have a role in inflammation, opsonization of immune complexes, modulation of immune complexes, cell lysis and the inhibition of the activities of viruses and bacteria.

Together with the serum proteins of the complement system, there are also a group of membrane receptors for complement components found on the cell surfaces of both immune and non-immune cells. The subject of this thesis was CR1, the receptor for the major C3 cleavage fragment C3b. The first description of a C3 receptor was by Duke and Wallace in 1930; they described the adherence of human erythrocytes to trypanosomes in the presence of antibody and complement. Similarly, Nelson (1953) observed the preferential binding of antibody and complement coated Treponema pallidum to erythrocytes when added to whole blood, and termed this reaction immune adherence. It is now known that this reaction is mediated by the interaction of CR1 on the erythrocytes with the C3 breakdown fragments on the immune complex.

This receptor is not confined to human erythrocytes, but is also found on granulocytes, monocytes, macrophages, B lymphocytes, some T lymphocytes, glomerular podocytes and mast cells (Arnaout and Colten, 1984; Fearon, 1984). The ligand specificity of the receptor was originally thought to be for C3b,

iC3b and C4b (as described in Chapter 1) but it is now known to bind C3b, C4b, iC3b, iC4b, C3i and C3c (personal communication Dr. G. Ross, 1986).

To date studies on CR1 have shown it to have 3 major functions; a) enhancing the phagocytosis of opsonized immune complexes by monocytes, macrophages and neutrophils, b) acting as an inhibitor of complement turnover and c) functioning as a transport mechanism carrying circulating immune complexes to the fixed mononuclear phagocytic system. The latter role was proposed by Siegel et al (1981) who believed that the role of the erythrocyte in the circulation was not purely respiratory, but that it also formed an integral and important part of the immune system. Experimental evidence both in vitro and in vivo support this hypothesis (Medof and Oger, 1982; Cornacoff et al, 1983; Horgan and Taylor, 1984; Taylor et al, 1985; Jepsen et al, 1986). Bearing in mind the three aforementioned roles of CR1, it is clear that a defect in CR1 would have major implications upon the functioning of the immune system.

CR1 levels have been evaluated on the erythrocytes of patients with immune complex diseases where there are often associated complement deficiencies, with particular attention being paid to the disease SLE. The first defect in the erythrocyte CR1 levels of SLE patients was described in 1981 by Miyakawa et al. They studied the erythrocytes from 56 SLE patients using IAHA and showed that 66% of their patients had no CR1 (IAHA negative) in comparison to 4% of the controls. Studies subsequent to this investigation from geographically widespread localities, have confirmed these findings (assays performed using IAHA and RIA, as discussed in Chapter 7). In addition the

the defect was found not to be confined solely to the disease SLE; patients with other types of rheumatic disorders (RA and Sjögren's syndrome) and probable immune mediated disorders (cold agglutinating disease and paroxysmal nocturnal haemoglobinuria) also had reduced erythrocyte CR1 levels (Iida et al, 1982; Ross et al, 1985).

Studies investigating erythrocyte CR1 levels in normals and SLE patients have striven to determine whether the low CR1 levels seen in SLE patients are an acquired secondary manifestation of a complex immunologically mediated disease process or a predisposing inherited defect. This distinction is clearly important as an acquired defect may be open for clinical treatment. The data found by other workers in this field are extensively discussed in Chapter 7. Clearly, there are scientists who believe that CR1 levels are inherited (Miyakawa et al, 1981; Wilson et al, 1985; Minota et al, 1985) and those who believe that they can be acquired (Inada et al, 1982; Iida et al, 1982; Walport et al, 1985). The results of the studies performed in this thesis suggest that genetic factors do play some role in the determination of erythrocyte CR1 levels, this is indicated by the clustering of CR1 phenotypes (i.e. high, medium or low) within individual families. However, some of the patients studied may have acquired low erythrocyte CR1 levels. The supportive evidence for this statement is ; a) acquisition of low CR1 levels during periods of disease exacerbation as shown by serial studies (Fig. 27), b) lower mean CR1 levels in the SLE patients in an active disease phase (356 CR1 sites/erythrocyte) as opposed to an inactive disease phase (2428 CR1 sites/erythrocyte, $p < 0.001$), c) SLE

patients with low CR1 levels belonging to families with medium to high CR1 levels (Figs. 29 and 30), d) identical twins with SLE had different CR1 levels. Therefore, some individuals with SLE seem to acquire low erythrocyte CR1 levels and this seems to be associated with disease activity.

It is clearly well established that low erythrocyte CR1 levels are associated with SLE and other immunologically mediated diseases and one can consider how this would affect the pathogenesis of the disease, for example in SLE. The potential consequences of low receptor levels could be conceived to be the following: 1) Decreased carriage of circulating immune complexes by the erythrocytes to the fixed mononuclear phagocytic system of the liver and spleen; this could consequently affect the rate at which immune complexes were cleared and the amount left circulating. Therefore, immune complexes left in the circulation would have an enhanced chance of being deposited. A contributory factor would be the finding by Horgan and Taylor (1984) that DNA complexes bind less rapidly to erythrocytes from SLE patients than normals (30 mins as opposed to 4 mins), so there would be reduced binding which would occur more slowly. 2) Reduced cleavage of C3b on the immune complexes to the smaller breakdown fragments iC3b and finally C3d-g by the action of I and CR1 (Medof and Prince, 1983a; Horgan et al, 1984). This reduction in C3d-g bearing complexes would inhibit the interaction of immune complexes with phagocytic cells bearing CR3 and CR4 (C3d-g receptor), and hence phagocytosis and immune complex clearance by this mechanism would be minimised. 3) As there would be reduced immune complex interaction with CR1, there would be less modulation of the circulating immune complexes size brought about by the interaction of CR1 and the complement system (Medof and Prince, 1983b).

In addition, if one considers that the SLE patients may have already elevated circulating immune complexes and/or hypocomplementaemia, the situation may become aggravated and a vicious circle could become established. For example, low CR1 levels would be unable to minimise the circulating immune complex load, this in turn would result in continuous complement activation and perhaps finally there would be deposition of the immune complexes and the subsequent initiation of an inflammatory response, all leading to tissue injury.

CR1 is not found solely on erythrocytes and the defect in erythrocyte CR1 levels seen in SLE patients may not be confined to the erythrocyte alone. In this thesis the technique used to quantitate the erythrocyte CR1 levels was modified and performed without success on the human leucocyte populations (Chapter 4). Pursuing this work further Miss A. W. Fyfe successfully adapted the RIA method and has shown that the PMNs from SLE patients have lower CR1 levels than normal PMNs. She also observed a variation in the CR1 levels of PMNs with disease activity corresponding with changes in the erythrocyte CR1 levels. Similar studies have not yet been performed on monocytes, macrophages, lymphocytes or mast cells. A study by Kazatchkine et al (1982) however did show the lack of CR1 on the kidney podocytes of SLE patients with diffuse proliferative glomerulonephritis but not the non-proliferative form. It would therefore be of importance to establish whether this defect was restricted to certain cell types or a widespread defect, and what effects this would have on the immune system's ability to cope with circulating immune complexes.

The evidence supporting an acquired defect in CR1 levels has been presented as has the possible implications this may

have on the functioning of the immune system and its ability to handle circulating immune complexes. However, the question which still remains to be answered is how low CR1 levels on erythrocytes are acquired? Three possibilities can be considered. Firstly receptors may be lost during erythropoiesis, however in-depth studies examining CR1 levels at the different stages of erythrocyte differentiation would be required to answer this possibility. Secondly, receptor removal. This could possibly occur due to the action of plasma proteases on the erythrocyte CR1, or they may be removed together with the attached immune complexes by the fixed mononuclear phagocytic system during their passage through the liver and spleen. The finding by Yoon and Fearon (1985) of a soluble plasma form of CR1 proposed to be derived from the erythrocytes, supports the possibility that by some manner CR1 can be released and/or removed from the erythrocyte's cell membrane. In addition, Wilson et al (1982) found that older normal erythrocytes possessed fewer CR1 than younger ones and this data may also favour receptor removal. This area obviously requires further investigation; for example, does serum or serum factors (from normals and patients) affect receptor levels, do circulating soluble CR1 levels correspond with erythrocyte CR1 levels and can assay conditions be established which examine receptor loss? This area of receptor removal was not investigated in this thesis. Thirdly, receptor blockade. In this thesis preliminary studies were undertaken to establish whether receptor blockade by immune complexes could contribute to receptor reduction. Opsonized immune complexes (BSA anti-BSA, thyroglobulin anti-thyroglobulin, DNA anti-DNA) were incubated in vitro with normal erythrocytes. All three complex types were shown to have bound to the erythrocyte cell surface, but only

those formed with thyroglobulin or DNA brought about partial or total receptor blockade (as determined using both polyclonal and monoclonal anti-CR1). Thus stressing the importance of the immune complex size on its efficacy to block receptors, with the larger antigens forming complexes which sterically block the receptors more effectively than those formed with smaller antigens. An extended study on the effect of immune complexes on receptor levels would be required to see whether parameters such as antibody isotype, avidity, type of antibody-antigen interaction, immune complex stability and solubility affect to different extents receptor blockade. For example, Pedersen et al (1980) believe that in vitro studies performed with small globular antigens such as BSA are irrelevant when studying diseases such as SLE where the antigen (DNA) is large and of variable size. Studies would also be required to confirm that the in vitro findings are not a test tube artifact. In vivo studies have been performed by Taylor et al (1985) using DNA complexes and they examined their binding to guinea pig platelets (which bear CR1, whilst their erythrocytes do not). They demonstrated binding and clearance of 75% of the complexes within 5 mins, the reaction's complement dependency and that the binding and release of DNA and BSA immune complexes were not the same. So more in-depth in vivo studies using different types of immune complexes may help elucidate whether immune complex blockade in vivo could contribute to the acquisition of low CR1 levels.

As mentioned earlier, if the defect of CR1 levels on erythrocytes in some SLE patients is acquired, it opens up the possibility of developing means of increasing the circulating erythrocyte CR1 levels. The most obvious remedy would be the

transfusion of normal erythrocytes with a high CR1 level into an affected patient. This has been performed by 2 groups, yielding conflicting results. Inada et al (1983) transfused a patient with normal erythrocytes with a high CR1 level, this brought about a reduction in the patient's circulating immune complex levels and an improvement of their condition. A similar study performed by Walport et al (1985b) caused a reduction in the CR1 levels on the transfused erythrocytes. Despite the conflicting results, this manner of treatment cannot be dismissed and further investigation into this area seems warranted.

A very evident problem with this means of treatment or any other means which may be used to try and rectify the CR1 levels is in determining whether the patient has genetically or acquired low CR1 levels. To define this, serial studies and family studies would be required to assess whether the observed patient CR1 phenotype was inherited or acquired, as treatment of an inherited defect may have no potential benefit. It is for the same reason that an individual's CR1 levels would be of no value as a diagnostic criteria for disease activity, unless the patient's normal inherited CR1 level was known, and continued serial studies performed to evaluate whether an acquired CR1 deficiency concorded with disease activity.

The aim of this thesis was to examine CR1 levels on the peripheral blood cells of normal individuals, SLE and RA patients. This was successfully performed on the erythrocytes but not the leucocytes. More in-depth studies performed on the SLE patients showed that some of these patients had acquired low erythrocyte CR1 levels. Preliminary investigations into the means of low

CR1 acquisition concentrated on the possibility that opsonized circulating immune complexes could bind to erythrocytes and block the receptors. Personally I favour the view that acquired low CR1 levels are caused by receptor blockade. Although, CR1 is known to be very sensitive to proteolysis (personal communication Dr. R. Sim), it is hence easy to believe that they could be removed from the erythrocyte, however, it is more difficult to explain why CR1 levels increase so rapidly in some individuals. For example, normal individual L.J. (Table 23) had a 1227% increase in her receptor levels in 5 weeks and SLE patient S.S. (Table 26) increased her receptors by 677% in 3 weeks. It is difficult to explain these increases on the production of new erythrocytes possessing a higher CR1 level, as the half-life of these cells is 120 days. However, blockade of receptors could explain such changes, with a removal of the blocking factor exposing more CR1 and vice versa. Daily serial studies would clarify how quickly and how often CR1 levels change. It may also show whether there is any diurnal variation or any relationship with, for example, food intake, exercise or stress.

In conclusion, while the results of my study indicate that low CR1 levels could be due to receptor blockade, it has to be realised that, as with other biological systems, the exact mechanism whereby low CR1 numbers arise probably involves many other additional factors, the nature of which remains to be elucidated.

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