A THESIS ENTITLED

COMPLEMENT ACTIVATION BY IMMUNE COMPLEXES

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GHAZI RAJAB AUDA

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

This thesis is dedicated to my devoted wife Zahra, my father and mother, and to my children Thuha, Hosam and Ali.

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SUMMARY

The binding of bacterial, viral or parasitic antigens with specific antibody and the formation of immune complexes (IC) in the circulation is a natural event in infectious diseases. In autoimmune immune complex diseases, such as RA and SLE, the development of circulating IC depends on the binding of autoantibodies to autoantigens. In either case complement is activated with the generation of activation products. As the extent of complement activation is likely to be proportional with the disease severity, the measurement of complement activation products in biological fluids could be used as a measure of disease activity.

In order to be able to measure complement activation in biological fluids, three avidin-biotin sandwich ELISA procedures were developed for the quantification of the C1s:C1r:C1-inhibitor (C1s:C1-INH), C3bBbP (C3:P) and SC5b-9 (C5b-9) complexes. The three assays were optimized and had sensitivity limits of 0.75ng C1-INH/ml for C1s:C1-INH, 0.92ng C3/ml for C3:P and 0.9ng C5/ml for C5b-9. All three complement activation complexes were found to be stable during 4 hours incubation periods at 20^{o}C and 37^{o}C.

These ELISA procedures were used for the measurement of the complement activation complexes in the serum and plasma of patients with rheumatoid arthritis and systemic lupus erythematosus. Significantly elevated levels in all three complexes were found in both groups of patients ($p<0.0001$) in comparison with normals. There were significant correlations between the three complement activation complexes and serum levels of rheumatoid factors (IgM-RF, IgG-RF and IgA-RF) in the sero-positive rheumatoid arthritis patients. However, as the same results were obtained when $F(ab')_2$ fragments of ELISA-coating antibodies were used to measure complement activation products, and as the addition of purified IgM-RF to sera which were sero-negative for IgM-RF, did not influence the ELISA results, it was concluded that these correlations were not the result of RF interference with the assays.

Experiments were undertaken to determine the properties of IC which resulted in complement activation. Complement activation was stimulated *in vitro* with nascent and preformed BSA anti-BSA and thyroglobulin anti-thyroglobulin IC, at antigen antibody ratios ranging from 16-times antibody-excess to 16-times antigen-excess. Activation was quantified by measuring levels of C1s:C1-INH, C3:P and C5b-9. The interpretation of the data was assessed by measuring levels of C4a, C3a and C5a on the same samples, as indices of activation of C1, C3 and C5 respectively.

The result of the investigation showed:

1) Nascent and preformed IC produced dose-dependent increase in complement activation as assessed by C1s:C1-INH, C5b-9, C4a, C3a and C5a formation, whereas a clear dose-response effect was not seen with C3:P

2) The antigen antibody ratio influenced complement activation. When thyroglobulin anti-thyroglobulin IC were studied, more complement activation occurred (as assessed by C1s:C1-INH, C4a, C3a and C5a) when IC were formed at 4-times antibody-excess. When C5b-9 generation was measured, activation was greatest when IC were formed at 16-times antibody-excess. When BSA anti-BSA IC were used, C1 activation was greatest at equivalence, whereas C5b-9 activation was greatest at 16-times antibody-excess.

3) The antigen in the IC influenced complement activation, as BSA containing IC activated complement more efficiently at equivalence, while IC containing thyroglobulin activated complement more efficiently in antibody-excess. In addition, C5 activation was greater with thyroglobulin anti-thyroglobulin IC than with BSA anti-BSA IC.

4) Preformed IC activated C3 and C5 better than nascent IC, whereas nascent IC produced more C1 activation than did preformed IC.

5) The role of the alternative pathway was established by repeating the experiments in serum containing EGTA ($10mM.L^{-1}$) and $MgCl_2$ ($2.5mM.L^{-1}$). C1 activation did not occur, while C3a and C5a production were reduced by approximately 50%. Thus, half of the C3 and C5 convertase activity generated was dependent upon the alternative pathway.

6) Differences between the rate and extent of formation of C1s:C1-INH and C4a and C5b-9 and C5a showed that although each pair is considered to be an index of C1 activation and C5 activation respectively, the results show that C1s:C1-INH and C5b-9 are not absolute measures of C1 and C5 activation respectively.

7) Levels of C3:P showed no relationship to those of C3a and thus it is unlikely that C3:P determinations can be used as an index of C3 turnover.

The explanation for reduced levels of C3:P with high concentrations of IC at certain antigen-antibody ratios was investigated. Although the explanation was not forthcoming, it was shown that it was not due to insolubility of the IC.

As complement activation in the blood occurs in the presence of blood cells (mainly erythrocytes), the effect of erythrocytes on IC-mediated complement activation was investigated. Levels of C4a, C3a and C5a were unaltered when activation occurred in the presence of erythrocytes, which demonstrated that they do not affect complement activation. However, in the presence of erythrocytes the levels of the C1s:C1-INH were increased, levels of C3:P were reduced and those of C5b-9 were probably elevated slightly. The explanation for increased C1s:C1-INH was not clear, but could have been related to the binding of IC to erythrocytes which render IC-bound C1 more susceptible to the action of C1-INH. Reduced levels of C3:P were shown to depend on the presence of the C3b receptor (CR1) on erythrocytes. The precise mechanism by which CR1 affects C3:P levels requires investigation, but the two main possibilities are binding of C3:P to CR1 or degradation of C3b in the C3:P complex by the action of factor I with CR1 acting as a cofactor.

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Abbreviations

4xAb	IC at 4-times antibody-excess
4xAg	IC at 4-times antigen-excess
16xAb	IC at 16-times antibody-excess
16xAg	IC at 16-times antigen-excess
Ab(μg/ml)	Concentration of IgG antibody component of IC in the reaction mixture
B	Factor B
BSA	Bovine serum albumin
C1	First component of complement
C1-INH	C1 inhibitor
C1q	Subcomponent of the first component of complement
C1r	Subcomponent of the first component of complement
C1s	Subcomponent of the first component of complement
C2	Second component of complement
C3	Third component of complement
C4	Fourth component of complement
C4bp	C4 binding protein
C5	Fifth component of complement
C6	Sixth component of complement
C7	Seventh component of complement
C8	Eighth component of complement
C9	Ninth component of complement
CH50	Total haemolytic activity of complement classical pathway
CNBr	Cyanogen bromide
CoVF	Cobra venom factor
CR1	Complement receptor type one

D	Factor D
DAF	Decay accelerating factor
$DGVB^{++}$	Dextrose gelatin veronal-buffered saline with cations
$DGVB^{=}$	Dextrose gelatin veronal-buffered saline without cations
$D5W^{++}$	Isotonic dextrose containing cations
$D5W^{=}$	Isotonic dextrose without cations
DE52	Diethyl aminoethyle cellulose
E	Sheep erythrocytes
EA	Antibody-sensitized sheep erythrocytes
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycoltetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
Eq	IC at equivalence
GVB^{++}	Isotonic veronal-buffered saline containing gelatin and cations
$GVB^{=}$	Isotonic veronal-buffered saline containing gelatin but without cations
H	Factor H
HRF	Homologus restriction factor
I	C3b/C4b inactivator (Factor I)
IAB	Immune adherence buffer
IC	Immune complexes
IgG	Immunoglobulin G
IEP	Immunoelectrophoresis
kD	kilodaltons
MCP	Membrane cofactor protein
MIP	Membrane inhibitory protein
MW	Molecular weight
NHS	Normal human serum

OD	Optical density
OPD	Orthophenylenediamine
P	Properdin
PBS	Phosphate-buffered saline
PIP	Prevention of immune precipitation
PMSF	Phenylmethyl sulphonyl fluoride
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RIA	Radioimmunoassay
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide-gel electrophoresis
SLE	Systemic lupus erythematosus
SOL	Solubilisation
TEMED	N,N,N',N'-Tetramethylethylendiamine
VBS	Veronal-buffered saline

CHAPTER

ONE

INTRODUCTION

1.1. HISTORY OF THE COMPLEMENT SYSTEM

Complement activity was probably first described by John Hunter, who noted that putrefaction proceeded more slowly in blood than in other tissues (Osler, 1976). In 1889 Buchner described a heat labile protective activity which he termed "alexin", and this was thought to be enzymatic in nature. In 1894 Pfeiffer observed that the intraperitoneal inoculation of *Vibrio cholera* bacilli into guinea pigs which had recovered from an earlier infection with this organism, resulted in their rapid destruction.

In 1896 and 1898 Bordet confirmed Pfeiffer's findings by showing that fresh serum from immune animals contained a heat-sensitive bacteriolytic substance which was active in the presence of specific antibodies. He also demonstrated that in the presence of specific antibody fresh serum was able to lyse erythrocytes and suggested that haemolytic assays could be used as an easy way to demonstrate the presence of alexin in sera.

In 1900 Ehrlich and Morganroth introduced the term "complement" (something which completes or makes perfect), as a more appropriate term than alexin. In 1901 Bordet and Gengou formulated the complement fixation test, which proved helpful in identifying antibodies to different antigens. Friedberger (1910) showed that fresh serum lost its haemolytic activity after incubation with varying substances including agar, and this treatment produced anaphylactic shock when the serum was injected into guinea pigs.

In 1911 Sachs and Omorokow described a heat-stable functional activity of complement which was destroyed when the serum was treated with cobra venom. In 1913 Weil showed that this component acts after the first and second components of complement, and called it C3, as C4 was unknown at that time. In 1914, Coca reported that C3 activity was abolished by incubating serum with yeast.

During the 1940's Ecker and Pillemer explored the chemical constituents of the complement system for the first time, and attempted to determine their properties.

These studies were based on euglobulin precipitation, heat-sensitivity, treatment with zymosan or cobra venom and ammonia-sensitivity. The conclusions obtained from these studies were as follows:

a) Complement component (1) is a heat-labile globulin which is precipitated by dialysis against distilled water (0^{o} C for 18-36 hours).

b) Complement component (2) is heat-labile and not precipitated by dialysis.

c) Complement component (3) is heat-stable protein which adsorbed strongly to polysaccharides (for example zymosan).

d) Complement component (4) is heat-stable protein but it is sensitive to dilute alkali (for example ammonia).

The subsequent use of more advanced protein fractionation techniques has permitted the purification and characterization of the proteins which constitute the complement system.

1.2. NOMENCLATURE OF COMPLEMENT COMPONENTS

It is now known that the plasma complement system consists of at least twenty proteins (Table 1.1). For the sake of simplicity the plasma complement system is divided into 4 group of proteins; those which comprise the classical pathway, the alternative pathway, the terminal complex and finally a group of control proteins.

The components of the classical pathway and the terminal complex are denoted by the letter C followed by a number and are listed in the order of their interaction as follows: C1, C4, C2, C3, C5, C6, C7, C8 and C9. C1 is a trimolecular complex containing C1q, C1r and C1s (Lepow et al, 1963 ; Ziccardi and Cooper, 1977). The alternative pathway components are termed "factors" and each component is represented by a letter: factor B, factor D and factor P. Sometimes the prefix factor is omitted and they are described as B, D and P. Control proteins are referred to by their trivial names, eg C1-inhibitor (C1-INH), C3b inactivator (factor I), C4 binding protein (C4bp) and factor H.

When a complement component is fragmented by proteolysis, the cleavage products

are denoted by lower case letters; eg. C3a, C3b, C3c and C3d. When a complement component is composed of more than one chain, the different polypeptide chains are designated by Greek letters, the largest being α then β and so on; eg C4α, C4β and C4γ. In the past when a complement protein acquires an enzymatic activity, a horizontal bar was placed over the symbols, eg. $C\bar{1}$, $C\bar{1}s$, $\bar{D}$, and $C4\overline{b2}a$. With the exception of C1 and its subcomponents, the use of bars over letters has been dropped because of difficulties in using them on word processors.

Table 1.1 Characteristics of complement proteins.

Component	Molecular Weight	Electrophoretic Mobility	Serum Conc.(μg/ml)	Polypeptide Chain Structure	Genetic Polymorphism	Cleavage Products
C1q	400 000	α	75	18(6x3)	+	---
C1r	90 000	β	100	1	?	A&B
C1s	90 000	β	80	1	?	A&B
C4	204 000	β	430	3	+	C4a, C4b C4c, C4d
C2	100 000	β	20	1	+	C2a, C2b
B	93 000	β	150	1	+	Ba, Bb
D	25 000	α	2	1	?	---
P	220 000	γ	30	4	?	---
C3	190 000	β	1300	2	+	C3a, C3b C3c, C3dg
C5	180 000	β	75	2	-	C5a, C5b
C6	128 000	β	60	1	+	---
C7	121 000	β	60	1	+	---
C8	153 000	γ	80	3	+	---
C9	79 000	β	50	1	-	---
C1-INH	105 000	α	180	1	+	---
C4bp	540 000	β	?	8	?	---
Factor I	90 000	β	50	2	-	---
Factor H	150 000	β	300	1	?	---
S-protein	88 000	α	505*	1	?	---
Anaphylatoxin Inactivator	300 000	α	?	8	?	---

(Table cited from McPhaden et al, 1982)
(*) Cited from Müller-Eberhard, 1986.

1.3. THE CLASSICAL PATHWAY

The classical pathway C3 convertase, C4b2a, is generated by the interaction of five proteins, C1q, C1r, C1s, C4 and C2 (Fig 1.1). This process is regulated by three plasma proteins, C1-inhibitor (C1-INH, Pensky et al, 1961; Ratnoff et al, 1969), C4 binding protein (C4bp) and factor I (C3b/C4b inactivator, Gigli et al, 1985).

1.3.1. C1 Macromolecule

C1 is a macromolecular complex composed of five molecules, one C1q and two of each of C1r and C1s (Naff et al, 1964; Dodds et al, 1978). Approximately 70% of C1 is present in serum in the complex macromolecular form (Ziccardi and Cooper, 1977). The C1 macromolecule is held together by calcium ions, (Ca^{++}) and after treatment with the chelating agent EDTA, C1 dissociates into its three subcomponents (Colomb et al, 1984). Activation of C1, initiates activation of the classical pathway (Lepow et al, 1963).

C1q

The recognition subcomponent of C1, is a large complex molecule having a molecular weight of about 410kD, which, because of its extreme cationic nature, expresses slow $\gamma 2$ mobility on immunoelectrophoresis (Cooper, 1985). Electron-microscopic examination of C1q showed that the molecule is composed of three distinct parts: a central subunit, six peripheral globular heads and six fine strands connecting the central core to the heads (Shelton et al, 1972).

Biochemically, C1q is composed of 18 polypeptide chains, 6A, 6B and 6C, which are assembled to form six triple helices where each triple helix is composed of 1A, 1B and 1C chain. Disulphide bridges are present between each A and B chains (A-B) and 2C chains (C-C). The interchain disulphide bonds between the C chains together with hydrophobic and electrostatic interactions join the six triple-helices together to form the central core. The C-terminal end of each triple helix terminates in a globular head which contains the immuonoglobulin binding site of C1q (Reid and Porter, 1976).

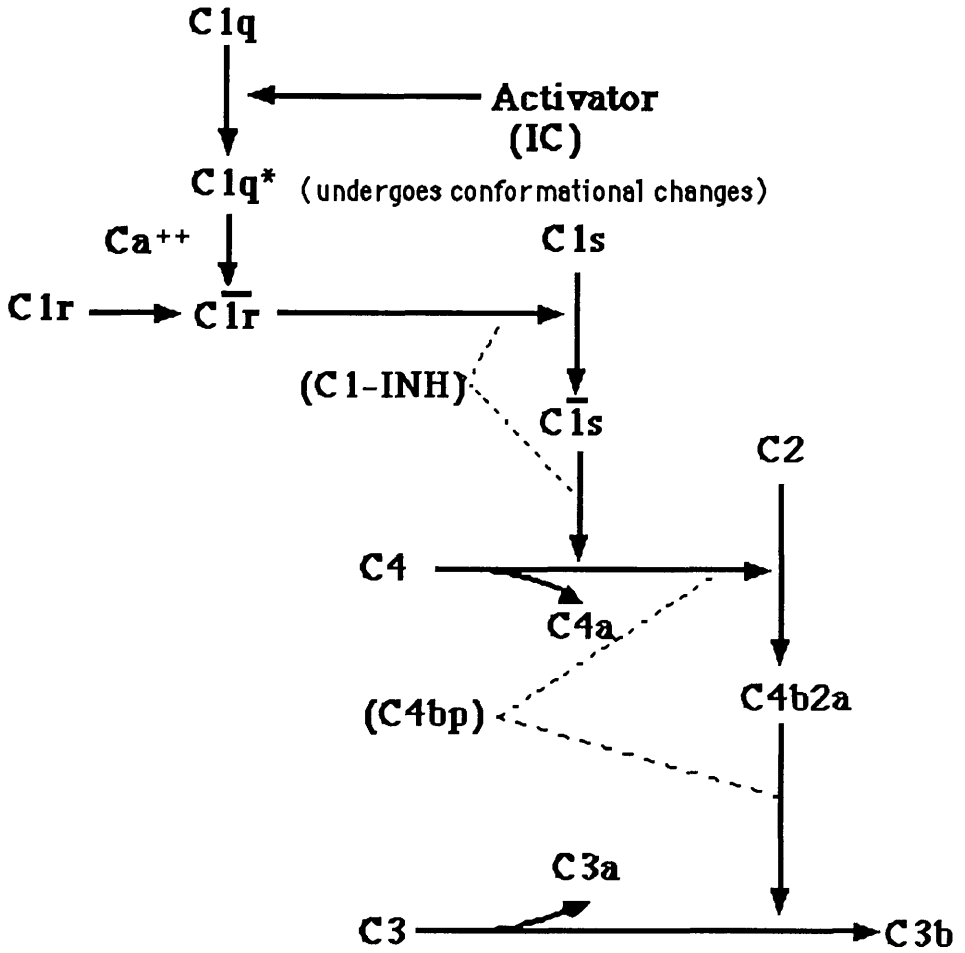


Figure 1.1: Diagrammatic representation of the classical pathway of complement activation.

Amino acid analysis of C1q revealed that it contains a high proportion of glycine, hydroxylysine and hydroxyproline, which are the major constituents of collagen and the proteins which compose the cellular basement membranes. Moreover, the amino acid sequence of the A, B, and C chains revealed the repeating triplet Gly-X-Y which is peculiar to collagen and is responsible for the triple helical structure (Reid, 1976). It has been found that the point of divergence of the collagen like strands corresponds with an interruption in the Gly-X-Y triplet (Reid, 1977; Porter and Reid, 1978). The average divergence angle of each of the stalks with the axis of the central stalk is approximately 60^{o} (Gilmour et al, 1980).

Apart from its function as the recognition subcomponent of C1, C1q offers the assembly site for the zymogens C1r and C1s, which exist as a C1r2:C1s2 tetramer. The association constant as well as the attachment site of this tetramer with C1q will be discussed later.

The C-terminal globular heads of C1q bind to the CH2 domain of IgG (Kehoe and Fougereau, 1969) and the CH3 domain of IgM (Hurst et al, 1975). IgA, IgD and IgE do not bind C1q whether in the monomeric or aggregated form (Ishizaka and Ishizaka, 1969; Augener et al, 1971; Garred et al, 1989).

Human IgG subclasses exhibit different binding affinities to C1q; IgG3 binds most efficiently, then IgG1 followed by IgG2. IgG4 does not have any demonstrable C1q binding activity (Schumaker et al, 1976). This has been attributed to the steric hindrance created by the proximity of the $F(ab')_2$ to the Fc portion of the immunoglobulin (Isenman et al, 1975). Recently it has been demonstrated, using human monoclonal antibodies, that although IgG1 is less efficient in binding C1q than IgG3, the number of C4 molecules activated by the C1 macromolecule is 10 fold higher for IgG1 than for IgG3. This behaviour was not due to favoured binding of C4 to IgG1, but appeared to depend upon increased activation of C4 by C1s when C1 is bound to IgG1 (Bindon et al, 1988).

The affinity of C1q to monomeric immunoglobulin is weak, with the association

constant ranging from $4x10^3$ to $5x10^4$ M^{-1} (Sledge and Bing, 1973; Schumaker et al, 1976; Hughes-Jones and Gardner, 1978). Thus, plasma IgG is incapable of binding and/or activating C1 (Borsos and Rapp, 1965). The affinity of C1q for immunoglobulin increases with aggregation by chemical (Tschopp et al, 1980) or physical (Dodds and Porter, 1979) procedures, or after the formation of immune complexes (IC). The association constant of C1q binding to complexed IgG was determined as 10^7-10^8 M^{-1} (Hughes-Johnes and Gardner, 1978; Lin and Fletcher, 1978). This increase in affinity may be attributed to the polyvalent binding of C1q to the complexed IgG. The binding of C1q to IC is temperature independent (Borsos et al, 1964) and after binding it undergoes a reversible conformational change (Dodds et al, 1978), which has been confirmed by the detection of neo-antigenic determinants within C1q (Golan et al, 1982).

The number of IgG molecules which can be bound by a single C1q molecule remains controversial. Schumaker et al (1976) reported that a single C1q molecule can bind from 12-18 IgG molecules, while Tschopp et al, 1980, who used chemically cross-linked IgG dimers and electron-microscopy, showed that the C1q molecule accepts only 3 IgG dimers. There is also disagreement over the number of complexed IgG molecules which are required for activation. Goers et al (1977) reported that a single IgG molecule complexed with monovalent hapten is capable of activating the classical pathway; others (Hyslop et al, 1970) suggested that complexes with less than 4 IgG molecules are incapable of activating complement; while other workers have reported that two or three complexed IgG molecules are sufficient (Cohen, 1968; Mannik et al, 1971). Kijlstra et al (1981) found that four or more IgG molecules per complex are required to activate complement. The differences in the conclusions of these groups is probably due to variations in the experimental procedures.

C1r

The purified proenzyme form of C1r is present in serum as a Ca^{++}-independent

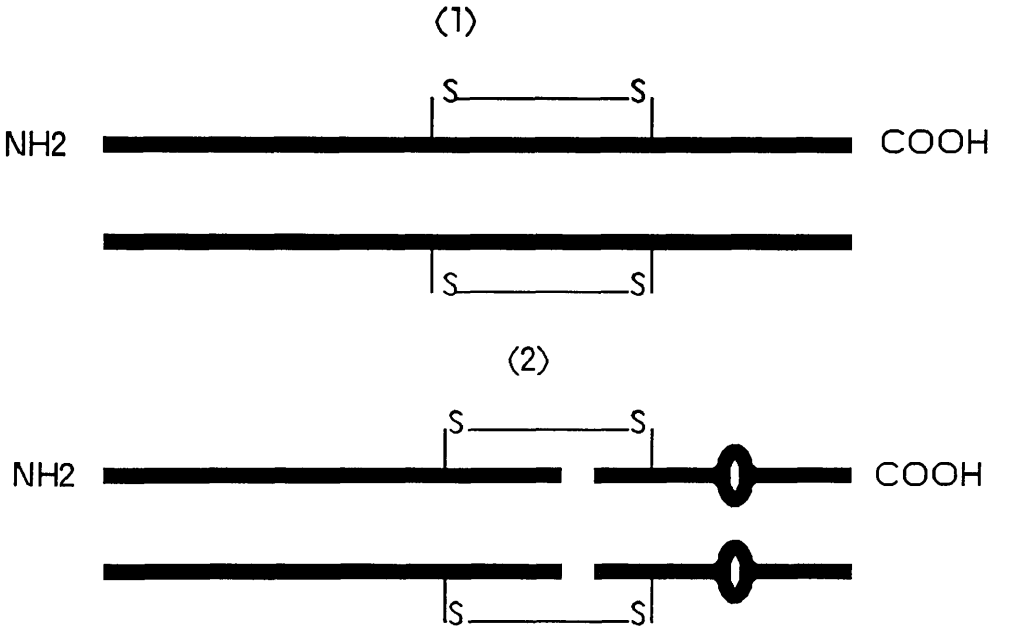


Figure 1.2: Schematic representation of the subcomponent C1r. The proenzyme form (1) is composed of two non-covalently linked monomers. During activation (2) each monomer is cleaved near its C-terminus, giving rise to the formation of two disulphide linked chains. The active enzymatic site is located on the C-terminal light chain (Assimeh et al, 1978).

dimer, which is composed of two single glycosylated polypeptide chains (Fig 1.2) having a molecular weight of 83-91kD (Sim et al, 1977; Arlaud et al, 1980). The electrophoretic mobility of C1r varies according to the availability of Ca^{++}. In the presence of Ca^{++} it exhibits γ mobility whereas in the presence of EDTA it has β mobility (Ziccardi and Cooper, 1976a). Electron-microscopy revealed that the C1r dimer consists of two dumb-bell-shaped molecules which are bound together near the junction between the rod and one of the globular domains to form an assymetrical X (Fig 1.3) with two closely located inner globules and two outer globules (Weiss et al, 1986).

Upon activation each subunit of the proenzyme form of C1r is cleaved into two disulphide-linked polypeptide chains, designated as A and B. The A chain, 51-56kD, is derived from the N-terminus, while the B chain, 27-36kD, which contains the enzymatically active site of the protein (Fig 1.2) is derived from the C-terminus (Sim et al, 1977; Arlaud and Gagnon, 1983; Arlaud et al, 1987).

The A chain is composed of 446 amino acid residues (MW 51200), with two carbohydrate moieties attached to the polypeptide chain via asparagine residues at positions 108 and 204 (Arlaud et al, 1987). Amino acid sequencing of the B chain showed that it contains 242 amino acid residues (MW 27096), and exhibits strong homology with other mammalian serine proteases. The active site serine residue is located at position 191 and carbohydrate moieties are attached via asparagine residues at positions 51 and 118 (Arlaud and Gagnon, 1983).

C1s

C1s consists of a single polypeptide chain with molecular weight of about 86kD. It exists in two forms; the proenzyme or precursor form and the activated form (Valet and Cooper, 1974). On immunoelectrophoresis the proenzyme form migrates as a β-globulin, while the activated form behaves as an α-globulin (Sakai and Stroud, 1973; Laurell et al, 1976).

Electron-microscopic investigations reveal that monomeric C1s is an asymmetrical

dumb-bell (Fig 1.3), where the large spherical globular domain contains the catalytic site of the activated molecule. The smaller domain is located on the N-terminus of the polypeptide chain and contains the Ca^{++}-binding interaction domain upon which dimerization and interaction with C1r2 depend (Tschopp et al, 1980). Thus, under physiological conditions, C1r and C1s exist in plasma as a tetramer consisting of two molecules of C1r and two molecules of C1s (C1r2:C1s2) (Laurell and Martensson, 1974).

The proenzyme C1s is the natural substrate of C1r, and upon activation it is cleaved into two chains of unequal size which are connected by disulphide bonds (Naff and Ratnoff, 1968). The heavy chain is designated as the A chain with molecular weight of 59kD while the light chain or B chain has molecular weight of 28kD and bears the enzymatically active site (Arlaud et al, 1977).

C1s consists of 673 amino acid residues (Mackinnon et al, 1987). The heavy chain derived from the N-terminus (422 amino acid residues) is similar to that of C1r and contains 5 domains, while the C-terminal derived light chain (251 residues) contains the serine protease domain of C1s (Mackinnon et al, 1987). An overall amino acid sequence homology of 40.5% exists between C1s and C1r, and the location of cysteine residues for disulphide bond formation is identical (Kusumoto et al, 1988).

Modelling of the light chains of C1r and C1s proteolytic (A) chains reveal that the greatest difference between these two subcomponents lies in the external loops of their three-dimensional structure (Carter et al, 1984). This possibly explains the differences in their substrate specificities.

C1r2:C1s2 tetramer

Electron-microscopic visualization of the C1r2:C1s2 tetramer which had been reconstituted from equimolar amounts of C1r2 and C1s in the presence of Ca^{++}, showed it to be an elongated S-shaped structure comprising domains of unequal sizes (Tschopp et al, 1980). The arrangement of these domains has been investigated, using

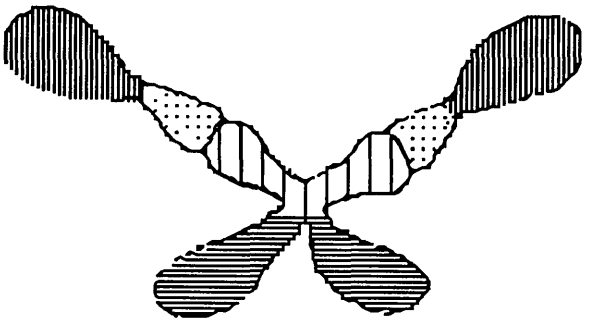


Figure 1.3: Schematic representation of the C1r2:C1s2 complex. The X shaped C1r2 molecule is centrally located with the binding domains (| | |) attached with those of the terminally located C1s molecules (::::::). The catalytic domains of C1r (≡≡≡) and those of C1s (||||||||) are oriented towards the exterior of the tetramer (Weiss et al, 1986).

a ferritin labelled avidin-biotin system, and revealed that the tetramer is joined through the interaction of the N-terminal domains (smaller domains) of C1r and C1s, while the C-terminal domains of C1r are joined in the centre of the tetramer and those of C1s are located at the distal ends of the tetramer (Fig 1.3) (Weiss et al, 1986). The C1r2:C1s2 tetramer interacts with the collagen-like part of C1q, as pepsin-digested C1q, which lacks the globular heads of the molecule, was found to compete with intact C1q in the C1r2:C1s2 tetramer interaction (Reid et al, 1977). Further, the association constant of the tetramer with pepsin-digested C1q was determined to be $2x10^7$ M^{-1} which is similar to that with intact C1q (Siegel and Schumaker, 1983). Electron-microscopy of reconstituted C1 macromolecules, from purified C1q, C1r and C1s, revealed that the C1r2:C1s2 complex appeared as a folded structure around the point at which the C1q stalks diverge from the central core (Strange et al, 1982). Despite the low association constant of the C1r2:C1s2 tetramer for C1q, approximately 70% of the C1 subcomponents in plasma circulate within the C1 macromolecule (Ziccardi and Cooper, 1977). The association constant of the tetramer for C1q increases approximately 10-fold when C1 binds to IC (Hughes-Jones and Gorick, 1982).

1.3.2. Activation of C1

The precise mechanism of C1 activation remains an enigma. It is generally accepted that a conformational change occurs in the C1q molecule after the binding of C1 to IC. This acts as the initiation signal for C1r activation which can only occur after some rearrangement within the C1r molecule which results in the exposure of a susceptible peptide bond to an enzymatic site on the same molecule and thus to the cleavage of C1r (Dodds et al, 1978).

Ziccardi and Cooper (1976a, b) reported that after incubation for 10 minutes at 37°C, purified C1r acquires the ability to activate C1s. SDS-PAGE analysis revealed that C1r underwent auto-activation with molecular cleavage. Many workers have confirmed this phenomenon (Assimeh et al, 1978; Lin and Fletcher, 1980; Kasahara et

al, 1985). However, Dodds et al (1978) suggested that the activation of purified C1r in solution was most probably due to traces of contaminating proteinases. They reported that the rate of activation of purified C1r showed no correlation with its concentration, indicating that the activation noticed was not due to an intramolecular auto-catalytic process. Thus, present evidence suggests that activation of C1r will only occur within the intact C1 macromolecule.

The molecular arrangement of the three C1 subcomponents that permit this auto-catalytic process is unclear. All the present models of C1 activation assumes that a conformational change occurs in C1q. Hanson et el (1985) speculated that upon binding of C1q heads to an activator, the stalks may be held in a slightly more open or more closed conformation which in turn initiates activation of the C1 proenzyme. Alternatively, the globular heads of C1q, which are bound to antibody, rotate and produce rotation of the collagen-like stalks around their axis. Such a change would initiate a conformational change in C1r2:C1s2 complex with activation of C1 (Kilchherr et al, 1985). The flexible structure of the C1q molecule would allow either process to occur.

The arrangement of C1r2:C1s2 tetramer around the C1q molecule must permit C1r activation. A number of models have been proposed. Colomb et el (1984) suggested that the flexible rod-like structure of the C1r2:C1s2 tetramer is folded around two arms of C1q in a figure eight shape, with the catalytic domains of C1r and C1s inserted inside the cone defined by C1q stalks. Cooper (1986) assumed that the C1r2:C1s2 tetramer forms two identical C-shaped C1r:C1s which stacked together vertically and wrapped around the outside of the C1q stalks near their attachment with the central core. This model was presented as allowing free access of C1r2:C1s2 to C1-INH, C4 and C2. Another model has been presented, in which the catalytic domains of C1r are located inside the cone formed from the C1q stalks, and the rest of the tetrameric complex is wound outside the rest of the stalks where during activation the catalytic domain of C1s folds back to the inside of the cone, allowing contact with the catalytic domains of C1r and activation of C1s by activated C1r take place (Weiss et al, 1986).

Each of these models has its supporters and opponents and further structural studies will be required to resolve the outstanding issues.

1.3.3. Activators of C1

Immune complexes are the main class of C1 activators (already discussed), but it has also been shown that many "non-immune activators" are capable of binding and activating C1. Gram-negative bacterial lipopolysaccharides, polyanions, certain viruses and some gram-positive bacteria can also bind C1q and activate the classical pathway of complement. The lipid-A component of gram-negative bacterial lipopolysaccharides is responsible for the binding and activation of C1 (Vukajlovich et al, 1987). Studies on intact bacterial cells and mycoplasma showed that *Escherichia coli, Klebsiella pneumoniae* and *Mycoplasma pneumoniae* bind directly to C1 via C1q. Experiments on a mutant strain of *Salmonella minnesota* showed that bactericidal action of serum is dependent on C1, C4, C2 and calcium ions. Peitch et al (1987), described two classes of non-antibody activators of the classical pathway of complement. These activators were categorized according to their activation potency into "strong" activators such as cardiolipin vesicles, which are mitoplasts derived from human heart mitochondria, and semi-rough strain of *Escherichia coli* designated as J5. The weak activators include smooth strains of *E. coli*, DNA and human heart mitochondria. Strong activators activate C1 in the presence of C1-INH, while the weak activators do not.

Another activator of the classical pathway is a polysaccharide derived from the venom of the tropical ant species *Pseudomyrmyx*. This polysaccharide was found to cause depletion of the fourth and the second complement components, through its activation of C1 (Schultz et al, 1980).

1.3.4. C1-inhibitor and regulation of C1

C1-inhibitor (C1-INH) is a heat labile α-glycoprotein (100kD), consisting of a single chain polypeptide chain (478 amino acid residues) (Davis et al, 1986; Bock et al, 1986), which can regulate many serum proteases such as plasmin (Harpel and Cooper,

1975), kallikrein (Schapira et al, 1982), C1r and C1s. Its serum concentration is about 137μg/ml so that the C1-INH:C1 molar ratio is about 7:1 (Schumaker et al, 1987). C1-INH exerts two regulatory activities on C1; 1) It prevents spontaneous activation of C1 under physiological conditions or during the presence of weak non-immune activators like heparin or DNA or weak immune activators like IC formed at antigen-antibody ratios far from equivalence. 2) It is a stoichiometric inhibitor of C1. Upon activation of complement by IC, C1-INH does not prevent C1 activation, since this type of activation is beyond its positive regulatory control. The reason for that can be attributed to the extremely rapid rate of C1 activation induced by IC (Ziccardi, 1982a,b; Ziccardi, 1983, 1984). However, C1-INH interacts with C1 by binding to the active sites of C1r and C1s leading to irreversible dissociation of the C1r2:C1s2 tetramer into two (C1-INH)2-C1s-C1r complexes (Sim et al, 1979). The released complex, (C1-INH)2-C1r-C1s, is stable under conditions that dissociate non-covalently linked complexes (Harpel and Cooper, 1975). During binding to C1 a small peptide is released from C1-INH (Salvesen et al, 1985), with the formation of a covalent bond between C1-INH and C1r2:C1s2 (Chesne et al, 1982). Investigation of the interaction of C1-INH and activated C1 on IC showed that C1-INH first binds rapidly to C1s and then more slowly to C1r. When binding to C1r is completed the C1 macromolecule is dissociated and (C1-INH)2C1r-C1s complex is liberated to the fluid-phase (Sim et al, 1979; Ziccardi and Cooper, 1979), leaving C1q bound to the activator. The activator (IC) bound C1q may be controlled by C1q-inhibitor that prevents the binding to fresh C1r2:C1s2 (Conradie et al, 1975).

1.3.5. Component C4

The fourth component of complement, C4 (Fig 1.4), consists of three polypeptide chains α, β, and γ, with molecular weights of 93, 78 and 30kD respectively. These chains are bound by disulphide bridges and non-covalent forces. On electrophoresis, C4, migrates as a β-globulin (Schreiber and Müller-Eberhard, 1974). Staining of

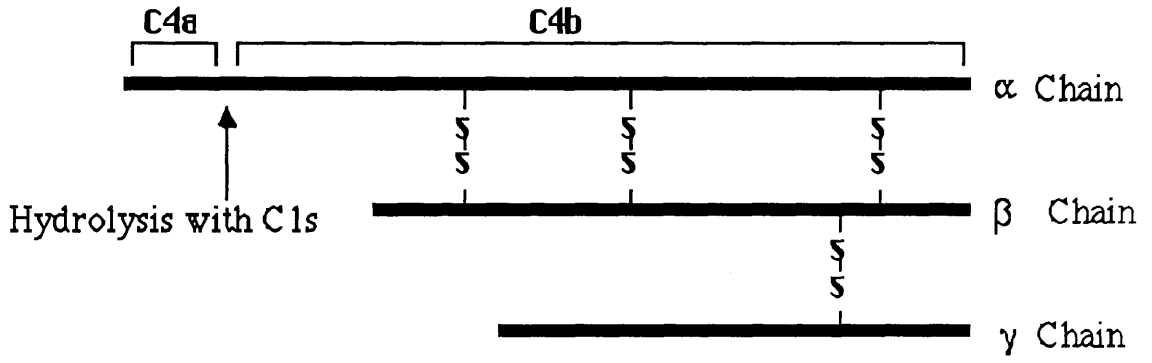


Figure 1.4: Schematic representation of C4 molecule which is composed of 3 polypeptide chains; α chain (93kD), β chain (78kD) and γ chain (30kD). These are linked by disulphide bridges. During activation the α chain is cleaved with C1s with the liberation of a 6kD fragment (C4a).

reduced C4 on SDS-PAGE with periodic acid Schiff (PAS) reagent revealed that carbohydrate is attached to all three chains (Gigli et al, 1977).

C4 is synthesised as a single chain precursor, pro-C4, which is then cleaved twice during secretion to become the disulphide-bonded three-chain plasma molecule (Hall and Colten, 1977). The complete amino acid sequence of the pro-C4 molecule has been determined and shown to be composed of 1722 amino acids (Belt et al, 1985). The arrangement of the polypeptide chains in pro-C4 are in the order β-α-γ (Goldberger and Colten, 1980). C4 is a highly polymorphic protein and its synthesis is controlled by two genes, located in the HLA class III region of the major histocompatability complex (MHC) on chromosome 6 of man (O'Neill et al, 1978; Raum et al, 1981). These two genes (C4A and C4B) which are closely linked and thought to have occurred as a result of gene duplication (Belt et al, 1985), encode two structurally and functionally distinct C4 isotypes (C4A and C4B) and are encoded by these two genes (O'Neill et al, 1978). The structural differences between C4A and C4B are confined to their C4d fragments, in which five primary amino acid sequence variations have been detected at residues 94, 141, 142, 145 and 146 out of the total 346 amino acid residues which compose C4d (Hellman et al, 1984). These variations are responsible for the functional differences between C4A and C4B.

The C4 molecule has an internal thiol-ester bond located in the α-chain. Upon activation with C1 or C1s, the α-chain is cleaved at the N-terminus with the liberation of a short polypeptide fragment (C4a, 6kD) into the fluid-phase (Budzko and Müller-Eberhard, 1970). Simultaneously with the cleavage of the α-chain, the thiol-ester is exposed to allow the larger fragment C4b (135kD) to bind covalently to the activating surface (Janatova and Tack, 1981). This binding of C4b occurs after the generation of a metastable reactive acyl group which is transferred from the thiol to a hydroxyl or amino group on the acceptor molecule (Campbell et al, 1981). The isotype C4B reacts preferentially with hydroxyl groups to form ester bonds, while C4A tends to react with amino groups on the target surface to form amide bonds (Law et al, 1984).

1.3.6. Component C2

C2 is a β-globulin composed of a single polypeptide chain with a molecular weight of 102kD and a serum concentration of 15μg/ml (Kerr and Porter, 1978). The carbohydrate content of C2 is about 15.9% (Tomana et al, 1985). C2 and the alternative pathway component factor B are the products of closely linked genes within the MHC class III region, which in the human is located on chromosome 6 (Robson and Lamm, 1984). Electron-microscopic examination of C2 and B revealed that both proteins have three globular domains of similar size (Smith et al, 1984). Analysis of the deglycosylated forms of C2 and B showed that they had similar molecular weights (Smith et al, 1984). Thus, the higher carbohydrate content of C2 in comparison with factor B (MW 93000) which contains only 8.6% (Tomana et al, 1985) may account for the molecular weight difference between the two native components. The structural and functional similarities of C2 and factor B support the hypothesis that the two loci encoding them arose by gene duplication.

C2 is activated by C1s in the absence of C4b but its binding to target bound C4b makes it more susceptible to cleavage by C1 or C1s (Gigli and Austen, 1969). C1s cleaves C2 at an x-lys bond with the production of two fragments of unequal size, C2a (73kD) and C2b (34kD) (Polley and Müller-Eberhard, 1968; Kerr and Porter, 1978; Kerr, 1979). The C-terminal fragment C2a carries the enzymatic site of the classical pathway C3 and C5 convertases (Kerr, 1979).

1.3.7. Assembly of the classical C3 convertase (C4b2a)

During the activation of C4 by C1 in the presence of IC, a very small proportion (7-10%) of activated C4 (C4b) becomes covalently bound to the IC. The thiolester of the remainder reacts with water to become fluid-phase C4b (Goers and Porter, 1978). The IgG binding site for C4b is located on the N-terminal half of the IgG heavy chain (Fd region) or on the antigen (Campbell et al, 1980; Ejzemberg et al, 1983). The molar ratio of bound C4b to IgG in the C4b-IgG complex is 1:1 (Campbell et al, 1980). Surface bound C4b has an Mg^{++}-dependent binding site for C2, which is also a natural

substrate for C1s (Kerr, 1980). The convertase (C4b2a) may be formed in the fluid-phase by adding C1s to a mixture of C4 (or C4b) and the C2 (but not pre-activated C2) (Müller-Eberhard et al, 1967). The mechanism of binding of C2 to surface bound C4b and the generation of the classical C3 convertase, C4b2a, has been investigated by a number of workers (Müller-Eberhard et al, 1967; Nagasawa and Stroud, 1977; Kerr, 1980). It has been shown that the C2 molecule binds to surface bound C4b through its C2b domain by Mg^{++}-dependent binding site on C4b (Oglesby et al, 1988). Once bound, C2 is cleaved by C1 into C2b and C2a, the smaller fragment, C2b, is liberated to the fluid-phase, and the larger fragment, C2a, which carries the enzymatic site of the classical pathway C3 convertase, C4b2a, remains attached to C4b. The convertase is composed of an equimolar complex of C4b and C2a. Once this bimolecular complex has been formed, Mg^{++} is no longer required (Kerr, 1980). C4b2a is an extremely labile enzyme, having a half life of 10 seconds at $37^{o}C$. Oxidation of C2 with a weak solution of iodine produces a stable C3 convertase in comparison to that produced with native C2 (Kerr, 1980). The classical C3 convertase exhibits similarities with the alternative C3 convertase (Table 1.2).

1.3.8. Assembly of the classical C5 convertase (C4b2a3b)

The classical C5 convertase is generated after the binding of one or more activated C3 molecules to the C3 convertase, C4b2a (Cooper and Müller-Eberhard, 1970). The role of C3b in this enzyme is to bind to and modulate the configuration of C5 to render it susceptible to cleavage by C4b2a (Vogt et al, 1978). It has been shown that a nascent C3b molecule generated by the C3 convertase, binds covalently to the α-chain of C4b through an ester bond with the generated C4b-C3b dimer exhibiting high-affinity ($Ka=2.1x10^{8}M^{-1}$) for the C5 molecule (Kinoshita et el, 1987). Blocking the surface-bound C4b-C3b dimer with anti-C4 produced an 80% reduction in C5 binding to EAC4b3b cells. Thus, it was concluded that the C4b-C3b dimer serves as the binding site for C5 (Kinoshita et el, 1987). C4b-C4b dimers have been reported to be produced

during C4 activation (Campbell et al, 1980), and because of the structural similarity between C4 and C3 (Sottrup-Jensen et al, 1985), the possibility exists that C4b-C4b dimers behave like C4b-C3b dimers in binding and presenting C5 for cleavage by C4b2a. The observation that C5 activation occurred when C4b2a prepared with oxidized C2 was added to C3-deficient serum, supports this notion (Kitamura et al, 1984).

1.3.9. Regulation of C4b2a/C4b2a3b assembly

C4b2a is an unstable enzyme with a half-life of 1.5 minutes at 30°C and 10 seconds at 37°C. The catalytic subcomponent C2a dissociates rapidly from C4b to enter the fluid-phase in the inactive form (Kerr, 1980). However, if C1 is still present, fresh convertase can be formed by the binding and activation of fresh C2 (Borsos et al, 1961). Apart from its intrinsic lability, the formation of C4b2a is regulated by specific plasma proteins at different stages of its generation. These proteins are C4 binding protein (C4bp), C3b/C4b inactivator (I), decay accelerating factor (DAF), membrane cofactor protein (MCP), complement receptor type 1 (CR1) and complement receptor type 2 (CR2). Recently it has also been demonstrated that nascent C3b and C4b fragments produce an inhibitory feedback on IC mediated complement activation by binding to the catalytic domains of C1r and/or C1s (Ziccardi, 1986).

C4 Binding protein (C4bp)

C4bp, is a glycoprotein (570kD) that regulates the classical pathway C3 convertase as a result of its binding to C4b (Scharfstein et al, 1978). It is composed of seven 70kD, identical subunits which are linked at the C-terminus by disulphide bridges, giving the molecule a "spider"-like structure (Dahlback et al, 1983). Under physiological conditions, C4bp can bind 4 C4b molecules (Ziccardi et al, 1984). C4 bp regulates the classical pathway C3 convertase by; 1) binding C4b to prevent uptake of C2, 2) by acting as a cofactor for I mediated degradation of C4b and 3) by accelerating the decay of the pre-existing convertase by dissociating C2a from C4b2a (Fujita et al,

1978; Gigli et al, 1979; Nagasawa et al, 1980).

C1 which has escaped the regulatory action of C1-INH will catalyse the formation of C4b2a, which can be regulated by C4bp. It has been noted that certain IC, such as some mixed cryoglobulins, are capable of binding and activating C1, but incapable of generating the classical C3 convertase (Hayday et al, 1980). It has been proposed that C4 bp and I inactivate C4b and inhibit C3 convertase formation (Brown et al, 1983).

C3b/C4b Inactivator (I)

Factor I (88kD) is a serine protease composed of two polypeptide chains (38kD and 50kD) present in plasma in the active form (Fearon, 1977). The smaller chain contains the active site of the enzyme. In order to exert its proteolytic activity on C3b, I requires H (Whaley and Ruddy, 1976), or CR1 (Medof et al, 1982) as cofactors, and for inactivation of C4b it requires C4 bp (Fujita et al, 1978; Scharfstein et al, 1978). I produces two cleavage sites on the α-chain of C4b yielding three polypeptides of 47kD, 25kD, and 17kD. The 47kD peptide (C4d) is released from the N-terminus and remains covalently attached to the target surface, while the remaining part of C4b (C4c) which consists of the intact β and γ chains and the two α chain fragments are released into the fluid-phase (Fujita and Nussenzweig, 1979).

Membrane-associated regulatory proteins

These including decay accelerating factor (DAF), membrane cofactor protein (MCP), complement receptor type 1 (CR1).

DAF: This membrane protein consists of a single polypeptide chain (70kD) which is attached to the membrane through a C-terminal phosphotidyl inositol glycolipid anchor (Medof et al, 1986). It is widely distributed in tissues, including erythrocytes, leucocytes, platelets, epithelial cells and connective tissues (Kinoshita et al, 1985; Medof et al, 1985). DAF accelerates the decay of the C3 and C5 convertases of the classical and the alternative pathways by binding to the C2a and Bb components respectively (Fujita et al, 1987). It has also been suggested that DAF functions by

competitively inhibiting the uptake of C2 or factor B, and preventing the assembly of the C3 convertases (Kinoshita et al, 1986).

MCP: Membrane cofactor protein is a 45-75kD glycoprotein occurring in all human leucocytes and platelets, but does not appear to be present on erythrocytes (Cole et al, 1985; Seya et al, 1986). It binds to C3b and C4b and acts as a cofactor for their cleavage by factor I. In this respect it is approximately 50 times more effective than CR1 or H (Seya et al, 1986). Cleavage of C3b by factor I in the presence of MCP only progresses to the iC3b stage (see below).

CR1: This component will be discussed later.

1.3.10. Component C3

C3 is the most abundant complement component in serum (serum concentration 1.3mg/ml) and plays a central role in complement activation, where it is involved in the generation of the classical pathway C5 convertase and in the the formation of the alternative pathway C3 and C5 convertases. It is synthesised as a single chain precursor, pro-C3, which is then cleaved once during secretion to become the disulphide-bonded two-chain plasma molecule (Brade et al, 1977).

C3 is a β globulin, which contains 2.7% carbohydrate and has a molecular weight of 190kD. The molecule is composed of two polypeptide chains (Fig 1.5), α (120kD) and β (70kD) which are held together by disulphide bridges and non-covalent bonds (Tack and Prahl, 1976; Law and Levine, 1977). The α chain of C3 contains an internal thiolester bond formed between a γ–carbonyl group of a glutamic acid residue and a thiol group on an adjacent cysteine residue. This bond is separated from the N-terminus by a mass of about 46kD (Pangburn and Müller-Eberhard, 1980; Sim et al, 1981; Tack et al, 1980). Upon activation, the α-chain of C3 is cleaved by C3 convertase, with the liberation of a 9kD peptide, C3a from the N-terminus. The remaining part of the molecule C3b (181kD) consisting of the cleaved α–chain and the intact β-chain (Sim et al, 1981; Tack et al, 1979), simultaneously with the conversion

of C3 to C3b the internal thiolester undergoes hydrolysis to provide nascent C3b with the capacity to covalently bind to the activating surfaces through the transfer of an acyl group from the thiol on C3b to a hydroxyl group on the acceptor surface (Law et al, 1980). This binding capability is metastable and exists for approximately 60 microseconds (Janatova et al, 1980, Law et al, 1980; Kulics et al, 1983). Nascent C3b binding to activating surfaces is mediated through covalent and hydrophobic forces (Law and Levine, 1977). Binding of C3b to IgG is probably dependent on the distribution of carbohydrate in the antibody molecule (Capel et al, 1978). It has been shown that C3b binds covalently to IgG through certain binding sites on the Fc and Fd portion of the gamma chain (Gadd and Reid, 1981; Kulics et al, 1983). This is determined by the distribution of available hydroxyl groups that can react with the labile binding site on the thiol group of nascent C3b. C3b molecule provides binding sites for C4b, C5, factor B, properdin, factor H and factor I, thus C3b can be described as a crowded molecule.

1.3.11. Breakdown fragments of C3

Conversion of C3b into iC3b involves factor I mediated cleavage of the α–polypeptide chain of C3b (Fig 1.5), at two closely adjacent sites with the release of a 3kD fragment, C3f. The resulting molecule, iC3b, remains covalently bound to the surface. The iC3b molecule (Fig 1.5) is composed of two α-chain fragments (αcdg, 68kD and αb, 43kD) which remain bound to the intact β-chain by disulfide bridges (Harrison and Lachmann, 1980; Sim et al, 1981). Conversion of C3b to iC3b requires factor H (Whaley and Ruddy, 1976), CR1 (Fearon, 1979) or MCP (Seya et al, 1986) which act as cofactors for factor I. iC3b does not bind to B, properdin, C5 but does bind to CR2 and CR3 (Ross, 1980) and weakly recognizes CR1 (Micklem et al, 1984).

Further degradation of the iC3b α-chain produced two fragments, C3dg (α2D) (40kD), which remain covalently attached to the activator surface, as it contains the thiolester bonds, and C3c (140kD) which is liberated into the fluid phase. The enzyme that is responsible for this cleavage remains unidentified. However, it has been

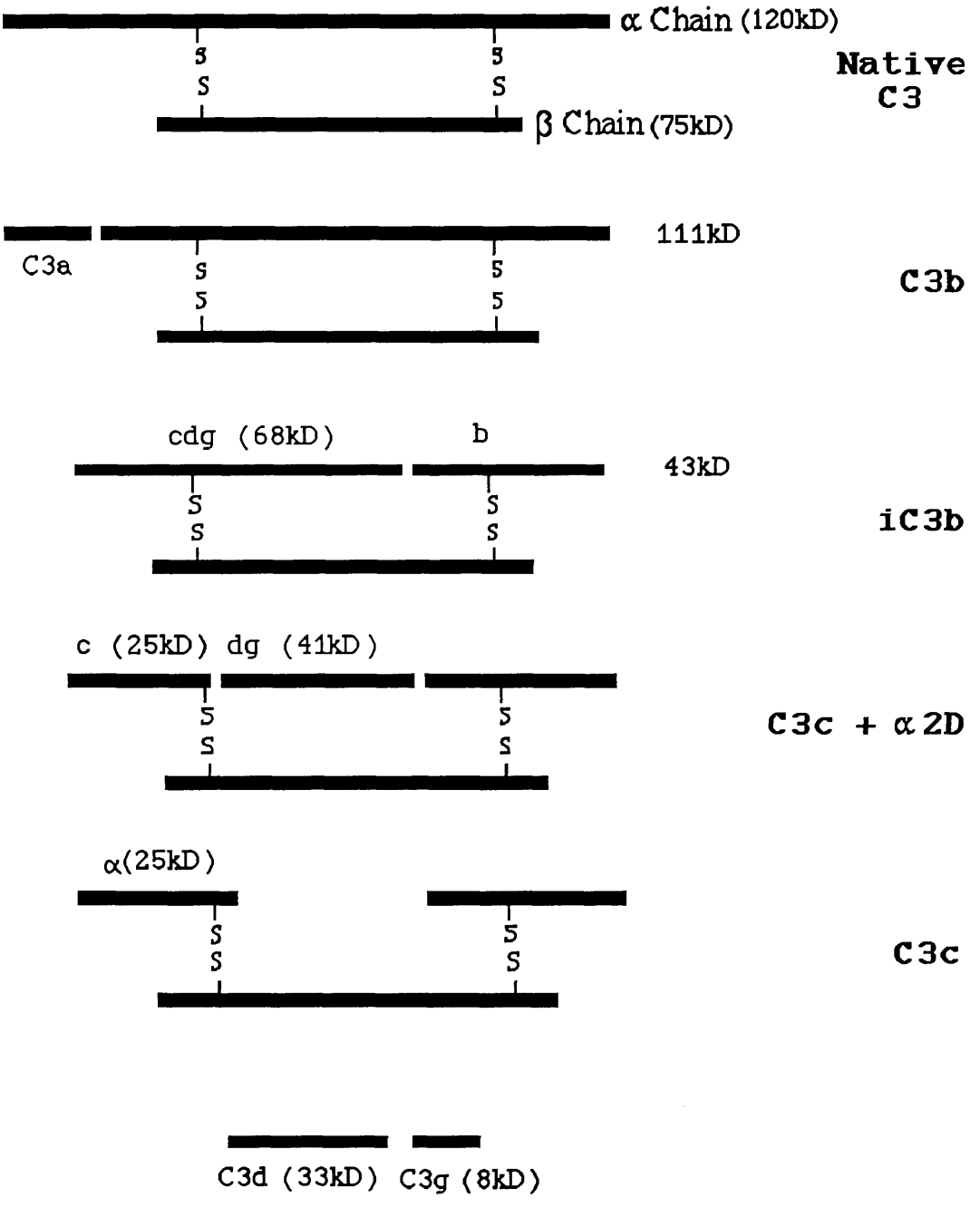


Figure 1.5: Diagrammatic representation of C3 molecule and its degradation fragments.

suggested that factor I may be responsible for this cleavage in the presence of CR1 (Medicus et al, 1983). Cleavage of C3dg by leucocyte tryptic enzymes produces two fragments, C3d and C3g (Lachmann et al, 1982). The cleavage fragments of C3 have the ability to bind cell membrane receptors, such as CR1 to C3b, CR2 to C3d and iC3b and CR3 to iC3b.

1.4. THE ALTERNATIVE PATHWAY OF COMPLEMENT

In 1925 Whitehead and colleagues described the action of the yeast cell powder (zymin) on the third component of complement. Pillemer and associates (1941) purified the insoluble polysaccharide fraction of yeast following tryptic digestion, and this fraction was called "zymosan" by San Clemente and Ecker in 1943. Pillemer et al (1954) described first the existence of an alternative pathway of complement activation, and reported that C3 could be inactivated by a complex that was generated from the interaction between serum properdin and zymosan. They called it the C3 shunt, or the properdin pathway, and postulated that the sole events in this pathway are dependent on a serum protein which they called "properdin" which is derived from the Latin word "*perdere*" which means to eradicate or destroy. Considerable doubt arose regarding the concept of the properdin or alternative pathway for complement activation, and it was not until the 1960s when further research proved its existence.

Schur and Becker (1963) showed that IC formed with the $F(ab')_2$ fragment of antibody were able to deplete the terminal complement components, while sparing the classical pathway components. The discovery that endotoxin and zymosan produced activation of C3 in guinea pigs with an inherited deficiency of C4 proved conclusively the existence of the alternative pathway (Ellman et al, 1971; Frank et al, 1971; Root et al, 1972).

The alternative pathway of complement activation is now known to be composed of three proteins C3, B and D which interact to generate the amplification C3 (C3bBb) and C5, ($(C3b)_nBb$) convertases. These enzymes are regulated by properdin, H, I,

complement receptor type 1 (CR1), decay accelerating factor (DAF) and membrane cofactor protein (MCP).

1.4.1. Proteins and their activation

Component C3

C3 (section 1.3.10) is the basic component for the initiation of the alternative pathway of complement, where the binding of C3b to B and the cleavage of B by D leads to the generation of the alternative C3 convertase, C3bBb, and the binding of one or more extra C3b molecules converts the enzyme to a C5 convertase. The extra C3b molecule in the C3 convertase binds C5 and renders it susceptible to cleavage by Bb (Vogt et al, 1978; Isenman et al, 1980). It has recently been shown that the extra C3b molecule binds to the original C3b in the C3 convertase to form the C5 convertase (Kinoshita et al, 1987).

Factor D

Factor D is a single chain plasma protein having a molecular weight between 23-25kD with a plasma concentration of 2μg/ml (Lesavre and Müller-Eberhard, 1978). In the presence of Ca^{++} it has γ electrophoretic mobility which changes to β in the presence of EDTA (Davis et al, 1979). The complete amino acid sequence of factor D revealed that the protein is composed of 222 amino acid residues, and exhibits structural homology with plasmin (40%), trypsin (35%) and thrombin (30%) (Niemann et al, 1984). It is now known that factor D and the adipocyte enzyme adipsin are identical (White et al, 1989). Factor D is present in human plasma in the active form only and is not inhibited by serum protease inhibitors or by spontaneous decay. It is a substrate specific enzyme, cleaving factor B only when it is in the Mg^{++} dependent C3b bound form (Lesavre and Müller-Eberhard, 1978).

Factor B

This protein was recognized by Pillemer et al (1953) who described it as a heat labile factor that forms a complex with zymosan and has the ability to inactivate the late

components of complement without consumption of C1, C4 and C2. Factor B was reported to bind to cobra venom factor, which is cobra C3, to form a stable enzyme which was capable of cleaving C3 (Müller-Eberhard et al, 1966). Factor B (MW 93000) is a β-globulin consisting of a single polypeptide chain and containing 7.3% carbohydrate (Curman et al, 1977). Synthesis of factor B is encoded on a single locus, Bf, which contains about 10 alleles of which BfS and BfF are the most common (Mauff et al, 1978). The structural similarities between factor B gene (Bf) and that of C2 as well as their close association, within class III of the MHC, has led to the assumption that these two loci have resulted from gene duplication (Perlmutter et al, 1984). Complete amino acid sequencing of B revealed that the molecule is composed of 739 amino acid residues, and cleavage by D produces two fragments, Ba and Bb with 234 and 505 amino acid residues respectively (Gagnon, 1984).

Cleavage of the C3b-bound Factor B by D at an arginyl-lysine bond near the N-terminus, resulted in the liberation of a 33kD peptide, Ba, to the fluid-phase (Lesavre et al, 1979). The Bb fragment, 60kD, which contains the enzymatic site, remains attached to C3b, probably through its N-terminus (Medicus et al, 1976; Lesavre and Müller-Eberhard, 1978) giving rise to the alternative C3/C5 convertase, C3bBb and $(C3b)_nBb$ (Medicus et al, 1976; Müller-Eberhard and Schreiber, 1980).

Properdin

Properdin is composed of identical 53kD glycoprotein subunits (Minta and Lepow, 1974). Electron-microscopy of purified protein revealed a heterogeneous mixture of cyclic dimers, trimers, tetramers and high molecular weight oligomers. Monomers are undetectable, and the functional activity of properdin was found to be directly proportional with the increase of the oligomer size (Smith et al, 1984). During activation of the alternative pathway, the central event is the interaction between C3b, B and D and the generation of the alternative C3/C5 convertase. The role of properdin is to amplify activation by binding to the convertase and to render it more stable by slowing the intrinsic decay of Bb from the enzyme (Fearon and Austen, 1975). Studies on the interaction of properdin with C3, its split products and factor B, failed to detect

any interaction with C3 or iC3b, while there was a weak interaction with cell bound C3b. This interaction was enhanced in the presence of Bb (Farries et al, 1988a). Binding of properdin to C3bBb is mediated through direct interaction between Bb and P, which results in increasing the affinity of both of these components to C3b (Farries et al, 1988b).

1.4.2. Activation of the alternative pathway of complement and activators

Activation of the alternative pathway (Fig 1.6) is comprised of three phases: 1) The initiation of the convertase formation, 2) Discrimination between self and non-self and 3) The phase of the positive feed-back amplification.

Assembly of the initial C3 convertase

The mechanism which results in the generation of the first C3b molecule, which is essential for the initiation of the alternative pathway, is still uncertain. However it is proposed that a native C3 molecule undergoes a conformational change, which permits it to interact with factor B, in the fluid-phase. Cleavage of B by D results in the formation of the complex, C3Bb, which possesses a C3 convertase activity (Fearon and Austen, 1975; Schreiber et al, 1978). Accordingly, this mechanism permits a continuous low grade formation of a fluid-phase C3 convertase. It has been suggested that the initial conformational change in C3 occurs because the internal thiolester bond undergoes hydrolysis at a slow rate giving rise to a functionally active C3 molecule C3(H_2O). This molecule has similar properties to C3b as it is able to bind B to generate C3 convertase [C3(H_2O)Bb] before it becomes susceptible to inactivation by H and I (Isenman et al, 1981; Pangburn et al, 1981). This assumption is supported by the fact that C3 haemolytic activity decays spontaneously in aqueous solution (Müller-Eberhard and Nilsson, 1960). This unstable,low-efficiency, fluid-phase C3 convertase cleaves C3 and a proportion of the resulting C3b molecules binds to cell membranes and proteins in the vicinity of the convertase. The deposition of C3b molecules on

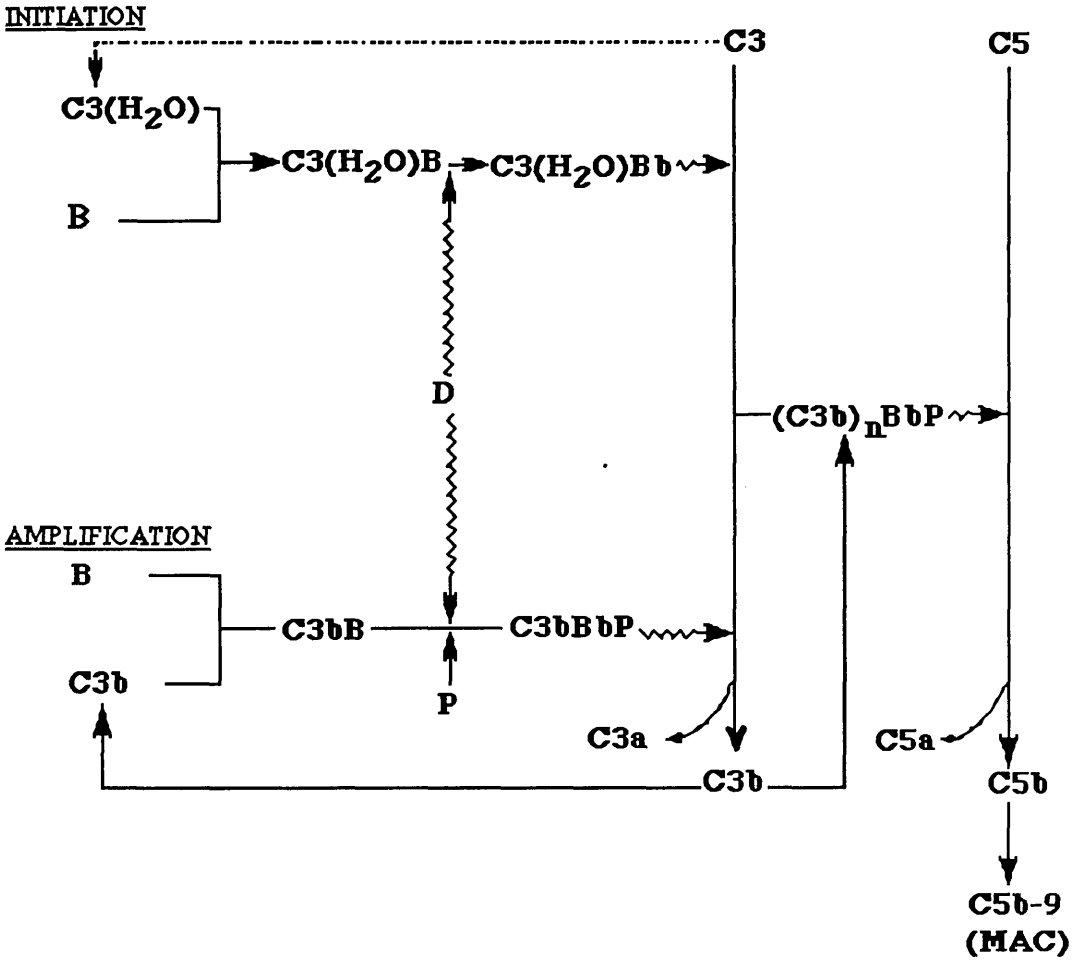


Figure 1.6: Diagrammatic representation of the alternative pathway of complement activation.

surfaces results in the formation of more C3 convertase, C3bBb, and each newly formed C3b molecule has the potential to form alternative pathway convertase together with B and D with the result of more rapid production of C3b on surface and in solution (Fearon and Austen, 1977; Schreiber et al, 1978). The incorporation of additional C3b molecules to the C3bBb results in the formation of $(C3b)_nBb$ which expresses a C5 convertase activity. The enzyme, C3bBb, is unstable due to the decay of Bb from the complex, and can be reconstituted after the addition of more B in the presence of D (Fearon et al, 1973). The half-life of the enzyme is 90 seconds at 37°C (Fearon and Austen, 1975) and is shortened by the regulatory protein H which binds to C3b and displaces Bb from the complex (Whaley and Ruddy, 1976; Weiler et al, 1976). Properdin increases the half-life to 20 minutes at 37°C (Fearon and Austen, 1975; Ross and Medof, 1985).

Discrimination between self and non-self

As C3b binds to all surfaces, yet only a few are activators of the alternative pathway, it follows that the composition of the surface to which the C3b has bound must be able to be recognized as an activator or a non-activator. This ability to discriminate between activators and non-activators is the basis for alternative pathway activation.

Activators of the alternative pathway include a wide variety of immune and non-immune activators. The immune activators include complexed IgA and IgG, including its sub-classes which are unable to activate the classical pathway, such as IgG4. The essential site on the immunoglobulin molecule for the assembly of the alternative pathway C3/C5 convertase was found to be located on the $F(ab')_2$ region of the molecule (Reid, 1971; Fujita et al, 1977).

The non-immune activators of the alternative pathway include surfaces of bacteria and fungi (Pangburn and Müller-Eberhard, 1978).

Amplification phase of alternative pathway activation

Once C3b has bound to the surface of an alternative pathway activator, uncontrolled

assembly of the C3 convertase occurs. As a result of further C3 cleavage more C3b binds to the surface to form more C3 convertase. Some of the C3b binds covalently to C3b which is already bound, and that then forms the alternative C5 convertase $(C3b)_nBbP$ (Kinoshita et al, 1987). Thus, on the surface of an alternative pathway activator the uncontrolled assembly of C3/C5 convertases prepares microorganisms for phagocytosis by coating them with C3b and for lysis by assembly of the membrane attack complex (see below).

1.4.3. Regulation of the alternative pathway of complement

On non-activating surfaces factor H controls the expression of C3bBb by binding to the C3b subunit and mediating three inhibitory effects; 1) preventing uptake of B by C3b, 2) accelerating the decay dissociation of C3bBb by displacing Bb from the complex and 3) acting as a cofactor for the inactivation of C3b by I (Whaley and Ruddy, 1976; Conrad et al, 1978). Thus, under normal circumstances alternative pathway complement activation cannot occur on the surfaces of non-activators. The affinity of surface bound C3b to H is inversely related to the activation potency of that surface. Activating surface bound C3b and C3/C5 convertase are relatively protected from the regulatory effect of H and I (Fearon and Austen, 1977a). It has been proposed that the nature of the constituents of the activating surface modulate the binding of H to bound C3b. For instance, the absence of sialic acid on certain activating surfaces is associated with increased binding of B to C3b and reduced binding of H to C3b (Kazatchkine et al, 1979). C3b bound to IgG is also protected against inactivation by H and I in comparison with free C3b or C3b bound to other serum proteins (Fries et al, 1984).

Regulation of the alternative pathway by CR1, DAF and MCP has been discussed elsewhere (sections 1.3.9 and 1.8).

Table 1.2: Similarities between the classical and alternative pathways.

Classical Pathway		Alternative Pathway	
C4b	1. Binds C2 (Mg^{++}-Dependent) 2. Degraded by factor I + C4bp	**C3b**	1. Binds C2 (Mg^{++}-Dependent) 2. Degraded by factor I + H
C2	1. Single polypeptide chain 2. Cleaved by C1s into C2a + C2b 3. Enzymatic site on larger fragment (C2a) 4. Decays from C4b2a 5. Structural genes on chromosome 6	**B**	1. Single polypeptide chain 2. Cleaved by D into Ba + Bb 3. Enzymatic site on larger fragment (Bb) 4. Decays from C3bBb 5. Structural genes on chromosome 6
C1s	Serine protease cleaves C4 and C2	**D**	Serine protease cleaves B
I	Enzymatically inactivates C4b in the presence of C4bp	**I**	Enzymatically inactivates C3b in the presence of H
C4bp	1. Binds to C4b 2. Cofactor for I in C4b cleavage 3.Accelerates decay of C4b2a	**H**	1. Binds to C3b 2. Cofactor for I in C3b cleavage 3. Accelerates decay of C3bBb

(Cited from Whaley, 1987).

1.5. THE MEMBRANE ATTACK COMPLEX (MAC)

Although Buchner noticed that cell-free human serum was able to lyse bacteria in 1889, it was not until 1959 that Green et al, reported that complement produces holes in cell membranes. The "One-hit" theory which was postulated by Mayer in 1961, attributed each single membrane lesion to a single "effective" complement molecule, and that a single lesion was sufficient to induce lysis of an erythrocyte. It is known that the membrane attack complex consists of five proteins, C5, C6, C7, C8 and C9 (Nelson et al, 1966; Lachmann and Thompson, 1970; Götze and Müller-Eberhard, 1970). In 1972 Mayer suggested that the late five complement proteins assemble to form a large hydrophobic protein channel within the lipid bilayer of the target cell membrane. It was later suggested that polymerized tubular C9 in the C5b-9 complex comprises the structural basis for the MAC channel (Podack and Tschopp, 1982a). This hypothesis was confirmed by Ramm et al (1985) using resealed erythrocyte ghosts and finding that each complex is comprised of one molecule of C5b, C6, C7, C8 and varying numbers of C9 molecules in the polymerised form.

1.5.1. The MAC proteins and their organization

C5

C5 is a 190kD glycoprotein composed of two chains, α (115kD) and β (75kD) which are linked by disulphide bridges. The general structure of C5 is similar to that of C3 and C4, and there is 27% and 25% homology respectively with their amino-acid sequence (Wetsel et al, 1988). C5 lacks a thiolester bond (Law et al, 1980; DiScipio et al, 1983) which is present on C3 (Tack et al, 1980) and C4 (Campbell et al, 1981, Harrison et al, 1981). This occurs as a result of the replacement of the cysteine and glutamine residues which are required for the thiolester formation, with serine and alanine respectively (Lundwall et al, 1985).

C5 is synthesized as an intracellular single molecule precursor, pro-C5, in hepatocytes (Patel and Minta, 1979) and macrophages (Ooi and Colten, 1979). Pro-C5 which is synthesized in the β–α chain orientation (Lundwall et al, 1985), is processed

and secreted as a two-chain glycoprotein molecule (Tack et al, 1979). The gene encoding C5 in humans is located on chromosome 9 (Wetsel et al, 1988).

C6 and C7

These are similar single chain β–glycoproteins with molecular weights of 128 and 121kD respectively, each having a serum concentration of 50-70μg/ml (Arroyave and Müller-Eberhard, 1971; Podack et al, 1979). The complete amino acid sequence of C6 revealed that the molecule is composed of 913 amino acids (DiScipio and Hugli, 1989), while that of C7 is 821 amino acid residues with the N-terminal two thirds of the C7 molecule exhibiting homology with the C8α, C8β and C9 (DiScipio et al, 1988). The genetically determined polymorphisms of both C6 and C7 are controlled by closely linked loci (Hobart et al, 1978) and their structural similarities support the notion that both molecules exist as a result of gene duplication.

C8

C8 is a γ-glycoprotein (150kD) (Manni and Müller-Eberhard, 1969) which is composed of 3 non-identical polypeptide chains. The α (64kD) and the γ (22kD) are linked by disulphide bridges, while the β chain (64kD) is non-covalently bound to the α and γ chain complex (Kolb and Müller-Eberhard, 1975). Synthesis of each of the three C8 subunits is controlled by separate genes. The genes encoding the α and β chains in the human are located on chromosome 1, whereas the gene encoding the γ chain is located on chromosome 6 (Kaufman et al, 1989).

Both α and β chains of C8 are essential for its functional activity, as a C8 derivative composed of only α and β-chains was found to be as functionally active as the normal C8 protein (Brickner and Sodetz, 1984). The β–chain carries the recognition domain through which C8 molecule binds the C5b-7 trimolecular complex (Monahan and Sodetz, 1981). The α-chain is also responsible for the cytolysis generated by C5b-8, through its integration into the target cell membrane (Stackel et al, 1983; Amiguet et al,

1985). C8α acts as a receptor for C9 and promotes its polymerization. Distinct domains on C8α are responsible for each of these effects.

C9

C9 is a single chain α–globulin with a molecular weight of 79kD (Hadding and Müller-Eberhard, 1969), which consists of at least five independently folding domains (Stanley and Hertz, 1987). One of these domains probably corresponds to the membrane insertion region of C9, as it is resistant to proteolysis after the membrane insertion of C9 (Hammer et al, 1977). The complete amino acid sequence of C9 shows that the molecule consists of 537 residues, and the C-terminal half is characteristically hydrophobic in nature which is important in the insertion of C9 into cell membranes (DiScipio et al, 1984).

Recently it has been demonstrated that the gene encoding C9 is located on chromosome 5 (Abbott et al, 1989).

1.5.2. Activation and assembly of MAC

Assembly of the MAC (Fig 1.7) is initiated after the cleavage of the N-terminus of the α chain of C5 at the peptide bond 74/75 (Fernandez and Hugli, 1978; Tack et al, 1979) by the classical pathway C5 convertase, C4b2a3b (Goldlust et al, 1974), or the alternative pathway C3/C5 convertase, $(C3b)_nBbP$ (Schreiber et al, 1978) with the liberation of a short polypeptide, C5a, (MW 11200) into the fluid-phase. During C5 cleavage a labile binding site, which is available for about 2-3 minutes at 37°C, is generated on C5b and this fragment is capable of forming a stable complex with C6 (Cooper and Müller-Eberhard, 1970). The presence of C3b on the target membrane potentiates the hydrophobic interaction of C5b with the membrane (Hammer et al, 1976). Electron microscopic examination reveals that the C5b-6 complex (MW 325000) is an elongated and slightly curved structure (Podack et al, 1980). This complex may be released from the target surface and the binding of C7 generates a

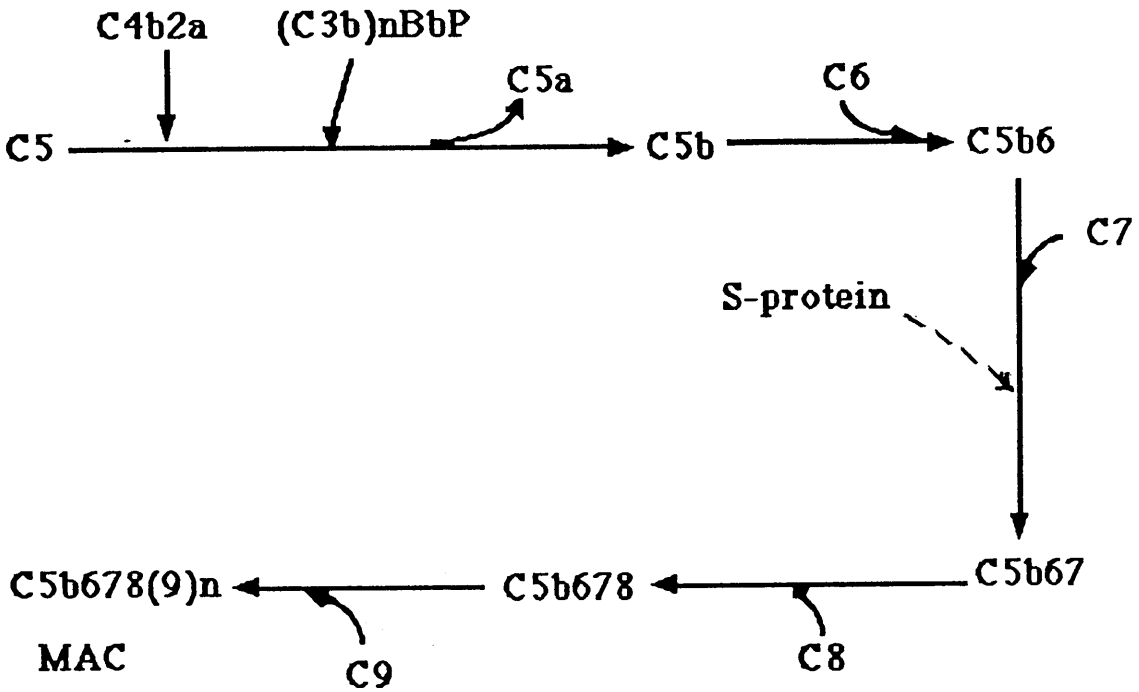


Figure 1.7: Diagrammatic representation of the assembly of the membrane attack complex.

stable binding site which enables the trimolecular complex (C5b-7) to anchor itself firmly on the lipid surface of the target membrane through a hydrophobic interaction. Assembly of C5b-7 in the fluid-phase may result in its binding to any membrane lipid surface in its immediate vicinity (Lachmann and Thompson, 1970). The C5b-7 complex inflicts no harm on the membrane until the binding of the C8 and C9. C8 binds the membrane bound C5b-7 through its β chain and this binding results in the insertion of C8α into the membrane lipid bilayer (Monahan and Sodetz, 1981). The C5b-8 complex has the ability to lyse erythrocytes slowly providing a large number of complexes are present (Gee et al, 1980). Morgan et al (1987) demonstrated the lytic effect of C5b-8 on nucleated mammalian cells and also showed it to be dependent on the presence of a large number of C5b-8 molecules. Assembly of C5b-9 occurs with the binding of C9 to the α-chain of C8. C9 undergoes a slow conformational change with the exposure of hydrophobic regions which are capable of inserting themselves into the target membrane (Whitlow et al, 1985). The next C9 molecule then binds to the already inserted C9 molecule and undergoes the same configurational changes and inserts itself into the cell membrane. Thus, polymerization and insertion of C9 occurs by the sequential binding of individual C9 molecules in the same manner (Podack and Tschopp, 1982b). After the activation of the first C9 molecule, which is a C5b-8 dependent process, the polymerization of C9 becomes independent of C5b-8 (Silversmith and Nelsestuen, 1986). The completed form of MAC is composed of one molecule of each of C5b, C6, C7, C8, and 12-16 molecules of C9 (Tschopp et al, 1982; Bhakdi and Tranum-Jensen, 1984), with the final number of C9 molecules being dependent on the concentration of C9 in the medium (DeLisi et al, 1980). The MAC is a hollow structure with a characteristically hydrophobic exterior, which facilitates penetration through the membrane lipid bilayer, and a hydrophilic interior, which allows the influx of water and ions freely into the cell with the end result of its destruction (Mayer, 1972). It has been shown, using phospholipid vesicles containing trapped macromolecules of different sizes, that the extent of the release of each type of these molecules is proportional to the C9 content of MAC, where smaller molecules can

be released with lower C9/C5b-8 ratio while higher ratios are required for the release of larger molecules (Malinski and Nelsestuen, 1989).

Different cells, like erythrocytes and nucleated cells, exhibit different sensitivity to the lytic action of complement. This was demonstrated by the experiments of Mayer and colleagues (Koski et al, 1983) who showed that unlike erythrocytes which require a single hit for their lysis, nucleated mammalian cells require multiple hits for their lysis. This behavior of nucleated cells was found to be associated with a shorter life-span of C5b-9 on the surface of these cells (1-3 minutes at 37^{o}C) (Morgan et al, 1984; Ramm et al, 1983a) in comparison with erythrocytes where the MAC remains for several days (Ramm et al, 1983b). On nucleated cells the MAC is either internalized and degraded or capped and shed into the surrounding medium (Carney et al, 1985).

1.5.3. Regulation of MAC

MAC assembly and insertion are regulated at a number of stages in the generation sequence. Apart from C4bp, H and I which regulate C5 convertase expression, two plasma proteins, S protein (Podack et al, 1977), and the newly described Sp 40:40 (Murphy et al, 1988) regulate MAC expression. S protein binds to the hydrophobic binding site on C5b67 so that this complex, which is assembled on or is closely adjacent to a cell membrane is unable to be inserted (Podack et al, 1977). The remaining components (C8 and C9) are still able to be assembled on the complex but C9 polymerization is inhibited. The resulting complex is hydrophilic SC5b-9 (MW 668000), and is composed of one molecule each of C5b, C6, C7, C8 and three molecules of C9 and three molecules of S protein can be detected in the fluid-phase (Podack et al, 1977). The SC5b-9 complex has no haemolytic activity (Kinoshita et al, 1979). The Sp 40:40 protein appears to act at the C5b67 stage, although its mode of action has not yet been clarified. In addition to these plasma proteins, there are at least three membrane proteins which protect cells against attack by homologous complement and are therefore called homologous restriction factors. DAF which regulates the

expression of the C3/C5 convertase and exhibits homologous restriction, has been discussed above.

Homologous restriction factor (HRF) is a protein of 70 kD which is identical with C8 binding protein (C8bp) (Zalman et al, 1986) and probably identical with the membrane inhibitory protein (MIP) (Watts et al, 1988; Yamamoto et al, 1990). HRF/C8bp/MIP recognizes homologous C8 and prevents its insertion into the cell membrane. It also inhibits the binding of C9 to C8 and thus prevents C9 polymerization.

A third homologous restriction factor has been described recently. It is a 18.5-20 kD protein which has been called HRF 20 and has been identified as the protein recognized by monoclonal antibodies to CD59 (Davies et al, 1989; Okada et al, 1989). The mode of action of HRF20/CD59 has not yet been elucidated. The presence of three distinct types of homologous restriction factors on cell membranes indicates that strict regulation of MAC assembly and insertion is maintained in order to protect cells from autologous complement attack. The distribution of these homologous restriction factors on different cells and in different tissues has not been ascertained.

1.6 BIOLOGICAL ACTIVITIES OF THE COMPLEMENT SYSTEM

The role of the complement system in maintaining human health can be illustrated by the development of diseases particularly immune complex diseases or severe recurrent bacterial infections in individuals who are congenitally deficient of an individual complement component or a regulatory protein. These disorders include SLE, glomerulonephritis, recurrent bacterial infections and angioedema. The biological activities of complement can be classified as cytolytic, pro-inflammatory and opsonic, although other miscellaneous activities have been identified. The biological activities of complement comprise the following:

1.6.1. Cytolytic (Discussed earlier, see section 1.5)

1.6.2. Opsonic

The opsonic effects of complement are mediated by the interaction of complement-coated targets with membrane complement receptors. The major ligands are C3b, its cleavage products iC3b and C3dg, and C4b, although receptors for other complement components such as C1q have been defined. Although stimulation of phagocytosis and bacterial killing are the events which come naturally to mind when one considers opsonization, the covalent binding of C3b to IC plays a significant role in protection against immune complex diseases. The interaction of complement with IC is considered in details later (see section 1.9).

After their reaction with specific antibodies and in the presence of serum complement, micro-organisms were observed to adhere to human erythrocytes and phagocytes (Nelson, 1953). The nature of this behavior was attributed to the deposition of C3b molecules (Gigli and Nelson, 1968) and/ or C4b (Cooper, 1969) which act as ligands for the C3b/C4b receptor (CR1) which is present on the surfaces of different cells, including erythrocytes, polymorphnuclear leucocytes, macrophages and monocytes (Fearon, 1980). The adherence between complement coated micro-organism or immune complexes and human erythrocytes is called immune adherence. This process is important for the removal of these opsonized particles from the blood (Medof et al, 1982a).

Macrophages possess receptors for C4b/C3b (CR1) and iC3b (CR3) (Lay and Nussenzweig, 1968, Fearon, 1980). The latter receptor is a member of the LFA-1 family of leucocyte adhesion molecules (Sanchez-Madrid et al, 1983) and is probably the most important receptor in the adhesion between C3b/iC3b coated targets and macrophages.

On resident macrophages and monocytes, C3b/iC3b will permit binding of the target to the phagocytes but will not promote phagocytosis unless IgG antibody is also bound (Mantovani, 1975). Although IgG antibody alone will trigger phagocytosis, the

presence of C3b/iC3b potentiates this effect (Mantovani, 1975). In contrast, on activated macrophages C3b/iC3b-coated target will trigger phagocytosis in the absence of antibody (Wright and Silverstein, 1983). Resident macrophages and monocytes will phagocytose C3b/iC3b coated targets in the absence of IgG antibody only if the target is a particulate alternative pathway activator. This occurs because alternative pathway activating particles are able to bind to specific receptors on phagocytes (Czop et al, 1978). This receptor-ligand interaction appears to substitute for the Fcγ receptor-ligand interaction.

Another form of synergism between Fc receptors and CR1 has recently been documented, which involves the clearance of IC from the circulation. Simultaneous infusion of IC with a murine monoclonal antibody (FcγIII) to FcγRIII resulted in the prevention of the liver and spleen macrophages to clear the infused IC which remain bound to the monkey erythrocytes through the CR1 (Kimberly et al, 1989).

1.6.3. Pro-inflammatory

The anaphylatoxins C3a, C4a and C5a have direct effects on the microvasculature which is characterized by vasoconstriction, platelet aggregation and increased plasma leakage to the extravascular environment. The three anaphylatoxins act on mast cells to release histamine which also increase vascular permeability (Johnson et al, 1975). The active site of C3a was reported to be the octapeptide C-terminal portion and removal of of the C-terminal arginine residue, by carboxypeptidase N, abolished the biological activity of C3a. The product of this reaction, $C3a_{des\text{-}Arg}$, can not bind to C3a receptors (Hugli and Erickson, 1977). Removal of C-terminal arginine from C5a and C4a likewise removes their anaphylatoxin activity. C5a is the most potent anphylatoxin, C3a the next while C4a is the least potent.

C5a and $C5a_{des\text{-}Arg}$ are potent chemotactic agents which lead to the accumulation of neutrophils, eosinophils and monocytes at the site of inflammation (Damerau et al, 1978; Fernandez et al, 1978). C5a and $C5a_{des\text{-}Arg}$ also produce neutrophil aggregation and activation. Thus, they stimulate release of prostaglandins (Goldstein et al, 1978) which

produce smooth muscle relaxation and vasodilatation and stimulate release of toxic oxygen radicals (Maly et al, 1983) and proteolytic enzymes (Harlan et al, 1981; McCarthy and Henson, 1979) both of which may produce endothelial damage with vascular leakage.

1.6.4 Miscellaneous

Perfusion of isolated rat femora with IC-activated serum caused a massive increase in the release of leucocytes from the bone marrow. This is due to the presence of a C3-derived leucocyte migration factor (Rother, 1972). The active fragment has been identified as an acidic peptide with molecular weight of 10-12kD that arises from cleavage of one of the α-chain fragments of the C3c, and has been designated as C3e (Ghebrehiwet and Müller-Eberhard, 1979). The C3e fragment was also found to stimulate neutrophils to secrete lysosomal enzymes (Ghebrehiwet, 1984). Another fragment (C3d-K) which is produced by the digestion of iC3b with kallikrein was found to possess a C3e-like activity (Meuth et al, 1983). Leucocytosis has also been produced with a synthetic nonapeptide that corresponds to the N-terminal part of C3d-K (Hoeprich et al, 1985). It is probable that C3e and C3d-K are overlapping peptides.

1.6.5. Regulation of immune response

Although C3-deficient individuals appear to be otherwise immunologically normal, there is a body of experimental evidence which shows that cleavage products of C3 and other complement components may influence immune responses.

In vitro studies have shown that target cell-bound C3b plays an important role in potentiating antibody-dependent cell mediated cytotoxicity (Ghebrehiwet et al, 1979). The C3d-K fragment of C3 inhibits proliferation of human T-lymphocytes (Meuth et al, 1983). Depletion of C3 with cobra venom factor was shown to prevent the development of B-cell memory cells in thymectomised mice (Klaus and Humphrey, 1977). The interaction of complement with lymphocyte function may be attributed to

the presence of receptors for C3 fragments on the surfaces of these cells. It has been reported that the cytotoxicity of killer (K) and natural killer (NK) cells was doubled in the presence of complement (Yefenof et al, 1984). This finding of the effect of C3 on NK cells is not in agreement with earlier report which stated the inhibitory effect of this component on these cells (Charriaut et al, 1982). Bird and Lachmann (1988) reported that an intact classical pathway was necessary for IgG4 synthesis by B-cells. The Bb fragment of B has been shown to share antigenic determinants with the B-cell growth factor which is secreted by activated T-cells, and has also been shown to act as a growth factor for B-Cells (Peters et al, 1988).

1.7. COMPLEMENT AND IMMUNE COMPLEXES

1.7.1. Solubilisation of immune complexes

During the interaction of complement with preformed insoluble IC, it has been observed that the covalent binding of C3 to these complexes resulted in the disruption of their lattice structure rendering them soluble (Miller and Nussenzweig, 1975). The process of solubilisation of IC was shown to be dependent on an intact alternative pathway, as sera which had been depleted of the alternative pathway components D, B or P failed to solubilise the immune precipitates unless the missing component had been replaced (Takahashi et al, 1978). The results of these experiments were supported by the observation that solubilisation could be produced by incubating immune precipitates with the purified components of the alternative pathway (C3, B, D, properdin, H and I) (Fujita et al, 1981). However, although C2 or C4 deficient sera were shown to be able to solubilise immune precipitates, the onset was delayed and the final extent of solubilisation was reduced in comparison with solubilisation in normal serum. Furthermore, complexes formed with $F(ab')_2$ fragments were shown to be solubilised more slowly than complexes formed from whole IgG molecules (Miller and Nussenzweig, 1975). It has therefore been concluded that although the classical pathway is not essential for solubilisation to occur, it is required for maximum speed

and efficiency (Czop and Nussenzweig, 1976).

Solubilisation of IC proceeds in three phases. The first phase is the deposition of C3b on the activating complexes, which occurs rapidly when the classical pathway is activated (Takahashi et al, 1978). However, when the alternative pathway alone is in operation, the deposition of C3b occurs slowly as it takes time for the fluid-phase turn-over to deposit sufficient C3b on the IC to generate an efficient amplification convertase (Czop and Nussenzweig 1976). As a result of the initial deposition of C3b on the IC, the second phase of solubilisation occurs, in which bound C3b molecules generate the positive feed-back loop through the assembly of the alternative C3 convertase, C3bBbP, with the result of more C3b deposition on the IC lattice. The inability of the classical pathway alone to induce solubilisation may be due to the lower level of C4 and C2 compared with C3 and B or to the short half life of the classical pathway C3 convertase, C4b2a, in comparison with C3bBbP. The other possibility is that C3b generated from the alternative C3 convertase tends to propagate in many sites beyond the original convertase site due to the generation of more molecules of the alternative pathway C3 convertases (Ross and Medof, 1985). Prior to the onset of solubilisation large number of C3b molecules are incorporated into the precipitate. This step has been investigated by incubating IC with serum for a few minutes, and the presolubilised complexes were washed and then incubated with purified C3. This resulted in rapid incorporation of C3b into the complexes prior to solubilisation taking place (Takahashi et al, 1977). The final phase of disruption of the IC lattice probably depends upon the intercalation of C3b (Takahashi et al, 1978). This intercalation possibly reduces the antibody affinity for the antigen (Miller, 1977), as IC formed from high affinity antibody are solubilised more slowly than complexes made from low affinity antibody (Czop and Nussenzweig, 1976; Johnson et al, 1987). Alternatively binding of C3b on the hinge region of IgG reduces the antibody flexibility and interferes with the cross linking of antigen. Deposition of C3b on the Fc portion of IgG may interfere with the Fc-Fc interactions which are known to promote lattice growth (Nisonoff and Pressman, 1958; Moller, 1979; Rodwell et al, 1980). The solubilised IC lose their ability to

activate the classical or the alternative pathways of complement system and accordingly called the "end-stage" IC (Takahashi et al, 1977).

Solubilisation of IC may play an important role in preventing tissue damage by already deposited IC. This suggestion has been supported by the finding that there is a delay in IC removal from the glomeruli of de-complemented rabbits with experimental serum sickness (Bartolotti and Peters, 1978).

1.7.2. Prevention of immune precipitation (PIP)

It has been suggested (Schifferli et al, 1980) that IC are unlikely ever to be formed in the absence of complement. Thus, solubilisation may be a laboratory artifact and one should study the effects of complement on the solubility of IC as they are formed. Indeed, when IC were formed in serum at equivalence or antibody-excess they remained soluble, whereas when formed in buffer they precipitated (Schifferli et al, 1980). This complement-mediated process is called prevention of immune precipitation (PIP) or inhibition of immune precipitation (IIP).

Sera which had been inactivated by heat (56^{o}C for 30 minutes) or EDTA were unable to sustain PIP, while sera which had been depleted of B, D or properdin retained PIP activity (Schifferli et al, 1980; Naama et al, 1983). Thus, the alternative pathway does not appear to play a significant role in PIP and by implication PIP is dependent upon an intact classical pathway. This conclusion was supported by the finding that IC formed in sera which were deficient of one of the C1 subcomponents, C4, C2 or C3 precipitated, whereas they remained soluble in C5-deficient or C7-deficient serum (Schifferli et al, 1980, 1982; Naama et al, 1983). Thus, the classical pathway is essential for PIP, but the terminal components beyond C3 are not required. This conclusion was confirmed by showing that IC remained soluble when formed in the presence of purified C1, C4, C2 and C3 and the addition of C5b-C9 or the alternative pathway components did not improve the efficiency of the process (Naama et al, 1984, 1985). The incorporation of C3 into the IC lattice is essential for PIP with molar ratio

of C3b to IgG being 2:5 (Naama et al, 1985). In the case of solubilisation, the molar ratio of C3:IgG is 1:1 (Fujita et al, 1981), which indicates that less C3 is required to prevent the precipitation of IC than that required for the solubilisation of IC precipitates.

PIP is probably mediated through the binding of C3b to Fd region of IgG to prevents the formation of Fc-Fc interactions which are important for the lattice formation (Moller, 1979; Rodwell et al, 1980) but it is also probable that C3b binding to the antigen may prevent the formation of some antigen-antibody bonds. The properties of antigen and antibody are thought to play an important role in the ability of IC to precipitate when formed in serum and their ultimate precipitation in tissues. *In vitro* studies have shown that IC formed from a small antigen, such as bovine serum albumin (BSA), can be prevented from precipitation easily whereas IC formed from a larger antigen, such as bovine thyroglobulin, are relatively difficult to keep in solution (Whaley, 1987). The nature of antibody was also found to affect the IC precipitation, where IC formed from IgA monoclonal anti-DNP antibodies precipitate rapidly, whereas IgG1 and IgM antibodies produced IC which remained soluble (Johnson et al, 1987).

Since the classical pathway is essential for PIP but not for solubilisation and as patients with deficiencies of the classical pathway components are predisposed to develop IC diseases, it can be concluded that prevention of immune precipitation is more important than solubilisation.

1.8. INTERACTION OF COMPLEMENT-PROCESSED IC WITH HUMAN ERYTHROCYTES (THE ROLE OF COMPLEMENT RECEPTOR TYPE 1, CR1)

Complement receptor type 1, CR1, is a large single chain membrane glycoprotein. It mediates the binding, processing and transport of C3b or C4b bearing IC, or particles and plays a role in the regulation of complement activation (Hourcade et al, 1988). Four polymorphic variants of human CR1 have been identified, CR1-A (220kD); CR1-

B (250kD); CR1-C (190kD) and CR1-D (280kD) (Dykman et al, 1985a, b). The cells which express CR1 include polymorphnuclear leucocytes, monocytes, macrophages, B lymphocytes, some T lymphocytes, dendritic reticular cells in germinal centers and glomerular podocytes. The number of CR1 antigenic sites per cell is different among blood cell types, with 950, 2100, 4800 and 5700 on erythrocytes, B-lymphocytes, monocytes and polymorphonuclear leucocytes respectively (Fearon, 1980). Since erythrocytes comprise the vast majority of circulating blood cells ($5x10^{13}$/litre) in comparison with total leucocytes ($5x10^{9}$/litre), it has been estimated that 90-95% of CR1 in the circulation is present on the surface of erythrocytes. This observation led to the suggestion that erythrocytes may play an important role in certain immune reactions (Siegel et al, 1981).

CR1 bound C3b can be cleaved by factor I yielding a 140kD fragment, C3c, which is composed of intact β chain and the two fragments of α chain, 40kD and 60kD. This fragment is released to the fluid-phase while C3dg, 41kD remains attached to the carrier surface (Bokisch et al, 1975; Lachmann et al, 1982). Immune complexes which bear C3dg do not bind to CR1, and are therefore released from the receptor (Medof et al, 1982).

The binding and transport of IC in the circulation is another important function that can be fulfilled by CR1. *In vivo* studies have shown that the binding of opsonized IC to erythrocyte CR1 is a very rapid process in which maximum binding is achieved within 2-4 minutes, after which dissociation occurs (Medof and Oger, 1982). This binding is markedly delayed in the presence of Mg^{++}EGTA, indicating the importance of intact classical pathway, which is known to induce rapid generation of C3b (Jepsen et al, 1986). The dissociated complexes can rebind CR1 through the activation of the alternative pathway. Dissociation of IC from CR1 is a consequence of I-mediated cleavage of C3b, but there is sufficient C3b on the dissociated IC, that can interact with B for the generation of alternative pathway C3 convertase (Medof et al, 1982).

Following the injection of large preformed complement-treated IC intravenously into primates it was shown that rapid binding to erythrocytes occurred. As the

erythrocytes pass through the liver, the bound IC were removed and degraded (Cornacoff et al, 1983). The role of FcγIII (Kimberly et al, 1989) has been discussed above (section 1.6.2). IC formed by the infusion of BSA into pre-immunised primates resulted in the formation of IC that did not bind to erythrocytes (Cornacoff et al, 1983). *In vitro* studies have shown that BSA IC formed in serum do not bind to CR1 (Whaley, 1987; Varga et al, 1988). However, when IC are formed with a large antigen such as thyroglobulin, the IC remain relatively insoluble after complement activation and CR1 binding will occur (Whaley, 1987). However, even in this situation only a small portion of IC bind to erythrocytes. In contrast, solubilised immune precipitates bind readily to erythrocytes *in vitro* (Medof and Oger, 1982). This difference in IC behavior probably occurs because solubilised IC have more molecules of bound C3b to IgG (1:1, Fujita et al, 1981) than IC which have been kept soluble by PIP (2:5, Naama et al, 1985). As erythrocyte CR1 are clustered, multivalent attachment is important in promoting adhesion between the receptors and the C3b-coated particles. It is reasonable to conclude that erythrocyte transportation of IC, which have been formed in the circulation, only occurs when they remain relatively large and insoluble after complement activation. Such transportation prevents the deposition of large insoluble IC in the microvasculature. In most instances, it is probable that PIP will render IC sufficiently small and soluble to circulate without danger of their deposition in the microvasculature.

Recently it has been demonstrated that the addition of packed human erythrocytes to human or guinea pig serum, resulted in the inhibition of the capability of serum to induce solubilisation of immune complexes. CR1 did not appear to be involved in this process, as blockade with anti-CR1 mAb or the removal of CR1 by trypsinization of erythrocytes did not alter the ability of erythrocytes to inhibit solubilisation of IC (Dorval et al, 1989).

1.9. THE ROLE OF COMPLEMENT IN IMMUNE COMPLEX DISEASES

1.9.1 Introduction

It is agreed that the deposition or the formation of IC in tissues is associated with tissue injury and IC diseases. IC mediated tissue injury was classified into three processes, termed types I, II and III (Coombes and Gell, 1968). Type I hypersensitivity is an immediate hypersensitivity due to antigen binding with cell-bound IgE antibody. Type II hypersensitivity reactions are associated with host tissue-bound antigens, whereas type III reactions are produced by the binding of the antibodies with free-antigens which could be either exogenous in origin or host-derived. Type III reactions were considered to be important in the pathogenesis of immune complex diseases. However, it has been pointed out (Henson, 1982) that as the mediator systems involved in the production of tissue injury in both types II and III hypersensitivity reactions are the same, it serves no useful purpose to distinguish between them. This point is further emphasized when one considers that certain forms of glomerulonephritis, which were previously thought to be produced by the deposition of circulating IC, may well be due to antibody reacting with antigen which is part of the normal renal glomerular epithelial cell (eg Heymann's nephritis) or non-renal antigens which have become bound to normal glomerular structures (eg DNA binding to basement membrane collagen). In these situations, examination of the tissues by immunofluorescence microscopy would reveal appearances which were indistinguishable from those produced by the deposition of circulating IC.

The binding of antibody with exogenous antigens protects the individual because the active site of an enzyme or the binding site of a virus particle or bacterial exotoxin may be blocked. Possible mechanisms for this include: (1) the active site being the epitope recognised by the antibody, (2) steric blockade by antibody binding to an epitope adjacent to the active site, (3) a conformational change induced by antigen-antibody union may render the active site inaccessible, or (4) aggregation of the antigen

by antibody may also restrict the expression of the biological activity of the antigen. Although the consequence of antibody binding to antigen is for example the neutralization of viruses or exotoxins, as most naturally-occurring antigens are multivalent, and as antibody molecules possess at least two antigen-binding sites, extensive cross-linking may occur leading to the formation of large aggregates of reduced solubility. Such aggregates if formed in the tissues would be difficult to remove, and if formed in the circulation would tend to precipitate within the blood vessels. However, it is not certain whether IC on their own induce tissue injury without the participation of Fc-dependent mediator systems (complement activation, Fc receptors on platelets, neutrophils, macrophages etc.). Indeed most experimental data show that the acute inflammation, which occurs as a consequence of IC deposition or formation within tissues, was ameliorated by prior depletion of mediators such as complement or neutrophils (Henson and Cochrane, 1974). However, these experiments were all short term because of the difficulty in depleting complement and neutrophils over prolonged periods of time. Thus, the long term direct effects of the presence of insoluble IC in the tissues are unknown.

1.9.2. Experimental models of immune complex tissue injury

Arthus reaction: In 1903 Maurice Arthus showed that daily intracutaneous injections of horse serum into rabbits eventually began to produce inflammation at the injection site. On the fifth day he noted the transient occurrence of oedema, but as the injection continued the inflammatory response became more marked with the appearance of erythema, haemorrhage and occasionally necrosis (Arthus, 1903). The Arthus reaction has now been investigated in great detail (Cochrane and Janoff, 1973). The reaction is a local vasculitis which develops in and around the small blood vessels of the skin. When antigen is injected into the skin of actively immunised animals it diffuses towards antibody which is present within the vessel wall. The vasculitis is due to the formation of IC in the region of the vessel wall with the subsequent activation of

a number of mediator systems which are responsible for the inflammatory response (Cochrane and Janoff, 1973).

The time taken for the Arthus reaction to reach its peak intensity is between 4 and 10 hours, after which it wanes and has usually disappeared between 24 and 48 hours. However if sufficient antigen and antibody are available, haemorrhage and necrosis may occur, and the lesions are obvious for longer periods. Sequential immunofluorescence and histological studies have shown that following the deposition of IC and complement components in the vessel wall, neutrophils accumulate in the vessel wall and the perivascular tissues. Intravascular clumping of platelets results, and if severe, vascular occlusion and necrosis occurs. Neutrophils predominate in the early cellular infiltrate, but by 8 hours mononuclear cells appear, and by 24-48 hours they are predominant cell type.

Precipitating antibody is far more effective than non-precipitating antibody in the production of Arthus reaction (Cochrane and Weigle, 1958). Early studies showed that the Arthus reaction did not occur in animals which had been depleted of complement by cobra venom factor (Henson and Cochrane, 1974). Although complement is required, the observation that the reaction occurs in C4-deficient guinea pigs (Frank et al, 1973) suggests that, in this species at least, the classical pathway is not essential. These observations indicate (1) that insoluble complexes are of central importance in the production of immune complex-mediated tissue injury, and (2) the complement system plays an important role in the pathogenesis of the inflammatory response induced by insoluble complexes.

Human diseases which are thought to be examples of Arthus reactions, include:

1) Extrinsic allergic alveolitis, eg farmer's lung and pigeon fancier's lung where the antigen is inhaled. 2) Filariasis, where the antigen is present on, and may be released from parasites, which are found in the lymphatic vessels. 3) Erythema nodosum leprosum, and the Jarisch-Herxheimer reaction occurring in patients with leprosy and syphilis, respectively. Where patients who have high titres of antibody against the causative micro-organisms are treated with chemotherapy, antigen release

results in IC formation. 4) Rheumatoid arthritis is thought to be a variant of the Arthus reaction. The formation of IC within synovial joints produces an inflammatory response. 5) Certain types of glomerulonephritis might be due to the entrapment of circulating antigen within the glomerulus. ICs are also formed when antibody binds to the tissue-fixed antigen. Although this mechanism operates in an experimental murine system, at present there are no known naturally occurring counterparts.

Apart from their formation *in situ,* IC may be deposited within the tissues from the circulation.

Serum sickness: Acute serum sickness is the prototype of the circulating IC disease, and this can be produced experimentally by injecting a large dose of a soluble protein antigen, such as BSA, intravenously into rabbits. After a period of a few days (usually 7-8) in which the concentration of BSA in the blood gradually diminish (non-immune elimination) the rate of decline accelerates, corresponding to the production of antibody (immune elimination). Antigen-antibody complexes are formed in moderate antigen-excess, and are deposited in glomeruli, arteries and joints. Systemic hypocomplementaemia occurs and immunofluorescence studies reveal deposits of C3 as well as immunoglobulin and antigen in the tissue lesions (Germuth, 1953; Dixon et al, 1958). Depletion of complement does not influence the renal lesions, but the development of arteritic lesions are prevented by prior decomplementation or neutrophil depletion (Henson and Cochrane, 1971). Large IC (>19S) are detected in the circulation of these rabbits during the period of serum sickness (Cochrane and Hawkins, 1968). Pharmacological or haemodynamic manipulations, which promote or prevent the entry of circulating complexes to the microcirculation and the tissues, amplify or reduce tissue injury (Cochrane, 1971). Thus, in the acute serum sickness model, the evidence supports the view that circulating IC formed in antigen-excess produce glomerulonephritis. The histological appearances of this nephritis are similar to those present in human acute post-infectious glomerulonephritis (Germuth, 1953;

Dixon et al, 1958).

Chronic renal injury can be produced in rabbits by repeated immunisation with a foreign protein antigen (Dixon et al, 1961; Germuth et al, 1967), with a variety of different glomerular, histological and immunohistochemichal lesions induced in the same strain of rabbits. Electron microscopic studies showed the presence of mesangial, subendothelial and subepithelial electron dense deposits. Although these variations were dependent upon the antigen dosage schedule and the antibody response of the rabbits, they could be produced with many different antigens. Mesangial injury was usually associated with the presence of IC which were greater than 500 000 daltons, whereas capillary wall deposition was associated with smaller circulating IC (Germuth et al, 1972). It was suggested that these were models of chronic soluble IC diseases and that pathogenic complexes were only formed in antigen-excess. Variations in histopathology were explained by differences in the size of the complexes and the avidity of the antibody (Germuth et al, 1977; Scherzer and Ward, 1978). The results of these experiments showed that chronic glomerulonephritis could occur as the result of the deposition in the tissues of soluble circulating IC, containing antigens which had no affinity to the kidney. It is worth noting that rabbits which developed IC glomerulonephritis made precipitating antibodies, and that renal disease occurred only in those rabbits in which complexes were formed at equivalence (23 of 28) or in antibody-excess (22 of 95) (Dixon et al, 1961), whereas the rabbits permanently in antigen-excess did not develop renal disease. As large complexes are formed at equivalence, and as these are removed rapidly from the circulation by the liver following their injection intravenously (Haakenstad et al, 1975), it is rational to conclude that such complexes can only produce tissue damage *in vivo*, when their removal by the reticuloendothelial system is impaired. It has been proposed that large insoluble complexes could be formed within the microcirculation and deposited locally, and thus would not be detected systemically. When rabbits or pigs were pre-immunised with protein antigens, and small quantities of antigen, insufficient to saturate available

antibody, were injected directly into the renal artery, acute glomerulonephritis developed (Gabbiani et al, 1975; Shigemetsu et al, 1979; Clark et al, 1980) and repeated antigen injection gave rise to mesangiocapillary glomerulonephritis (Clark et al, 1980). In contrast, in a carefully performed study, rabbits maintained in permanent antigen-excess over prolonged periods never developed glomerulonephritis (Boyns et al, 1968). Thus, circulating large IC formed with precipitating antibodies formed at or around equivalence appear to be incriminated in the pathogenesis of some forms of experimental glomerulonephritis and vasculitis. There are also experiments which show that interstitial lung disease may be produced in rabbits by similar procedures (Brentjens et al, 1974). The role of circulating IC in the pathogenesis of membranous glomerulonephritis is controversial. In experimental immune complex nephritis, subepithelial electron-dense deposits are distinctly unusual. A popular alternative hypothesis which has experimental backing, is that antigen gets trapped in the glomerulus and the IC are formed in situ (Couser and Salant, 1980). However, there is as yet no evidence that this occur *in vivo*. Although it has been shown that small IC formed with low affinity antibody and antigen with low epitope density can localize in the sub-epithelial space following intravenous injection (Law et al, 1984), it is not known whether the antigen and antibody dissociate in the circulation and re-complex within the glomerulus.

Once IC have been deposited or formed within the kidney, they must undergo a process of aggregation (termed condensation) to give rise to the electron dense deposits which are the hallmark of IC diseases. Following the intravenous injection of complexes these electron dense deposits will only occur with precipitating antigen-antibody systems (Agodoa et al, 1983).

The role of complement in protecting the individual against immune complex disease, is related to its ability to render complexes small and soluble (see prevention of immune precipitation and solubilisation of IC, section 1.7). Thus, on the one hand complement is a mediator of immune complex mediated tissue injury, and on the other it protects against immune complex disease.

1.10. MEASUREMENT OF COMPLEMENT AND COMPLEMENT ACTIVATION

The extent of complement activation, *in vivo* or *in vitro* may be readily assessed by measuring functional activity of individual components in serum. Normal values of total complement or of a single component may result from compensatory synthesis or hypersynthesis which do occur in certain chronic inflammatory diseases. Also decreased complement activity does not distinguish between deficiency or consumption. Accordingly, from a clinical point of view, the use of a precise assay for the measurement of complement activation is very important for the diagnosis and management of patients with different immunopathological diseases which are associated with complement activation or deficiency for example, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), hereditary angioedema and so forth.

Many methods have been developed for the measurement of complement activation through the direct determination of the levels of complement activation split products or complexes in tissues or biological fluids. These cleavage products include the anaphylatoxins (C4a, C3a and C5a), the cleavage products of C4, C3 and B which include C4d, α2D (C3dg), Ba and Bb respectively. Also it includes the multimolecular complexes C1r:C1s:C1-INH C3bBbP and C5b-9.

Measurement of C4 activation cleavage products, C4d, has been performed by an electroimmunoassay (rocket immunoelectrophoresis) using polyclonal antibodies (Folkersen et al, 1985; Petersen et al, 1988). A mouse monoclonal antibody which recognizes neoantigenic determinant of C4d has been produced and used for the trapping of C4d fragment in an ELISA system where the bound C4 fragment was detected with polyclonal anti-C4d (Kolb et al, 1988).

C3 degradation fragments (C3dg and C3c) are the naturally occurring cleavage products of iC3b produced by the action of factor I and its cofactor CR1 (Medof et al, 1982). Since C3, C3b and iC3b are recognised by C3dg antibodies, they must be separated from C3dg prior to assay. Polyethyleneglycol (PEG; 11%w/v) can be used

to precipitate C3, C3b and iC3b (Perrin et al, 1975). The drawback of this method is that incomplete precipitation of and sometimes excessive precipitation of C3dg may occur. The non-precipitable C3dg which is soluble in 11% PEG, can be measured by radial immunodiffusion (Perrin et al, 1975; El-Ghobary and Whaley, 1980), electroimmunoassay (Pepys, 1985) or laser nephelometry (Senaldi et al, 1988) using antisera to C3dg. A double decker-electroimmunoassay has been suggested in order to avoid the problem of PEG precipitation, and included the electrophoresis of EDTA-plasma (test samples) through a gel containing anti-C3c, which removes C3, C3b, iC3b and C3c, and then into a second gel containing anti-C3d (C3dg). The height of the rocket is proportional to the concentration of C3dg (Brandslund et al, 1981). ELISA's have also been introduced for the measurement of C3dg, using monoclonal antibodies (mAb) that recognize neoantigenic determinants on the molecule. A monoclonal antibody that recognizes the C3d determinants (Nielsen et al, 1986) or C3g determinant (Mollnes and Lachmann, 1987) and another mAb (bH6) that recognizes the neoantigen on the C3b, iC3b and C3c fragments, have also been developed (Garred et al, 1988).

The measurement of factor B cleavage products has been introduced as a means of measuring alternative pathway activation. Quantitation of the Ba fragment by radial immunodiffusion (Götze and Müller-Eberhard, 1976) or laser nephelometry (Senaldi et al, 1988) can be performed following PEG precipitation of B and Bb. The problems of PEG precipitation have been discussed above. A double-decker electroimmunoassay has been suggested, where the first gel contains antiserum recognizing B and Bb, whilst the second gel contains antibody recognizing Bb fragment. The size of the rocket is proportional to the concentration of Ba (Teissner and Rasmussen, 1988).

Measurement of anaphylatoxins, C3a, C4a and C5a is performed using fluid-phase radioimmunoassays after the precipitation of native C3, C4 and C5 with PEG or saturated ammonium sulphate (Gorski, 1981; Chenoweth et al, 1981). The measurement of anaphylatoxins in clinical specimens (plasma) does not always reflect the ongoing complement activation; for instance, plasma levels of C5a are extremely

low during complement activation due to the binding of C5a and $C5a_{des\ Arg}$ to their receptor on leucocytes (Chenoweth and Hugli, 1978; Van Epps and Chenoweth, 1984), while this anaphylatoxin is readily detectable during *in vitro* activation of serum. Recently, a monoclonal antibody to a synthetic octapeptide C3a (69-76) has been produced. Since this mAb recognizes a neoantigenic determinant on C3a which is not expressed on native C3, it can be used for the measurement of $C3a_{desArg}$ in clinical samples without the need for precipitating C3 (Burger et al, 1988). Anaphylatoxin assay kits are available commercially but are extremely expensive.

The multimolecular complexes include; $(C1\text{-}INH)_2C1r$:C1s complex (Sim et al, 1979; Ziccardi and Cooper, 1979), C3bBbP complex (Fearon and Austen, 1975) and the SC5b-9 complex (Podack et al, 1977). C1r:C1s:C1-INH complex can be measured by electroimmunoassay (Laurell et al, 1979), solid phase radioimmunoassay, where the C1s moiety of the complex is trapped on the solid-phase using anti-C1s and the C1-INH is probed with radiolabled anti-C1-INH (Hack et al, 1981). ELISA procedures have been widely used for the detection of C1s:C1r:C1-INH complex applying the same principle of the radioimmunoassay already described, where instead of using radiolabelled of anti-C1-INH, the antibody is linked into an enzyme such as alkaline phosphatase (Inman and Harpel, 1983; Lockshin et al, 1986) or peroxidase (Nilsson and Bäck, 1985). The C3bBbP complex can also be measured by ELISA by trapping the properdin moiety of the complex with anti-properdin and probing with anti-C3 conjugated with alkaline phosphatase (Mayes et al, 1984) or peroxidase (Asghar et al, 1987). The SC5b-9 complex may be quantified with the use of antibodies that recognize the neoantigenic determinants that are generated on the complex. This can be performed using a radioimmunoassay (Bhakdi and Muhly, 1983), although several ELISA procedures have been introduced for SC5b-9 measurement in biological fluids. Gawryl et al (1986) has described an ELISA where the C9 moiety of the complex is trapped with anti-C9 and the C5 is detected using rabbit anti-human C5 followed by peroxidase conjugated goat anti-rabbit IgG.

The use of ELISA procedures to measure the levels of these multimolecular complement activation complexes should circumvent many of the problems encountered with the other methods described above. Antisera can be purchased from commercial sources, the IgG fraction purified and easily labeled with biotin. Thus, any immunology laboratory could introduce these tests.

1.11. PROPOSED STUDY

As complement activation plays a significant role in the immune complex diseases, it is not unreasonable to assume that the level of complement activation should parallel the degree of tissue injury. Thus, measurement of one or more activation products in plasma could be used to monitor the extent of tissue injury. The purpose of this study included:

1) To measure complement activation products in patients with IC diseases after the development of avidin-biotin ELISA procedures for C1s:C1r:C1-inhibitor (C1s:C1-INH), C3bBbP (C3:P) and SC5b-9 (C5b-9) complexes.

2) To use these assays to investigate the effect of variations in IC concentration, antigen:antibody ratio and the role of complexed antigen on IC-mediated complement activation.

3) To investigate the effect of erythrocytes on IC mediated complement activation. This study was undertaken as complement activation within the blood occurs in the presence of 50%(v/v) erythrocytes, expressing membrane proteins which regulate complement activation (eg. CR1 and DAF).

CHAPTER

TWO

MATERIALS AND METHODS

2.1. BUFFERS

Phosphate buffered saline (PBS 20x)

NaCl	320gm
K_2HPO_4	48.4gm
KH_2PO4	13.6gm

Dissolved in deionised water to a final volume of 2 litres.

PBS Tween:

20xPBS	50ml
Deionised water	950ml
Tween 20 (Sigma)	500ul

Isotonic veronal-buffered saline (5x VBS)

Solution A: Sodium chloride (85gm) and sodium barbitone, Na5,5-diethylbarbital, N.F. $NaC_8H_{11}N_2O_3$ (3.75gm) were dissolved in 1 litre of deionised water.

Solution B: Barbitone, Na5,5-diethylebarbituric acid, N.F. $C_8H_{12}N_2O_3$ (5.75gm) was dissolved in 600ml deionised water by heating and vigorous stirring.

Solution A was added to solution B and the final volume was adjusted to 2 litres with deionised water. The pH was between 7.4-7.6.

Isotonic EDTA (0.086M.L^{-1} pH 7.4)

Solution A: Disodium ethylenediaminetetracetic acid (EDTA, 0.12M.L^{-1}) was made by dissolving 89.338gm EDTA (BDH) in 1500ml deionised water and the volume was adjusted to 2 litres with deionised water. The pH of this solution is 4.5.

Solution B: Sodium hydroxide (300mM.L^{-1}) was made by dissolving 24gm NaOH pellets in 1500ml deionised water and the final volume was adjusted to 2 litres with deionised water.

Solution B was added to solution A, while stirring, until a pH of 7.4 was achieved. The molarity of EDTA in the buffer was calculated according the following formula:

$$\text{Final molarity of EDTA} = \frac{\text{Starting volume of EDTA}}{\text{Starting volume EDTA + volume of NaOH used}} \times 0.12$$

The concentration is usually 0.086M.L^{-1}.

EGTA (0.1M.L^{-1})

Solution A: Ethyleneglycol-bis-(B-amino-ethyl-ether) N,N'-tetracetic acid (9.51gm) was added to 125ml deionised water while stirring.

Solution B: Sodium hydroxide (10M.L^{-1}) was made by dissolving 80gm NaOH in 200ml deionised water.

Solution B was added to solution A, while stirring, until EGTA dissolves. The pH was adjusted to 7.4 and the final volume was adjusted to 250ml by addition of deionised water.

VBS.EDTA (0.01M.L^{-1})

5xVBS	100ml
Isotonic EDTA (0.086M.L^{-1})	58ml

The volume was adjusted to 1 litre with deionised water.

Gelatin (10%)

Ten grams gelatin (BDH) were dissolved in 80ml hot water and the final volume was adjusted to 100ml by the addition of deionised water. The solution was divided to 10 ml aliquots and kept at 4^{o}C until use. Each aliquot was melted by placing it in a boiling water bath.

$CaCl_2$ (30mM.L^{-1})

Calcium chloride (3.286gm) was dissolved in deionised water so that the final volume of the solution was 500ml. Calcium ions are required to keep C1 macromolecule intact.

$MgCl_2$ (100mM.L^{-1})

Magnesium chloride ($MgCl_2.6H_2O$, 10.165gm) was dissolved in deionised water so that the final volume of the solution was 500ml. Magnesium ions are required for the formation of the classical and alternative C3 and C5 convertases (C4b2a, C4b2a3b, C3bBbP, $(C3b)_nBb$).

Isotonic veronal-buffered saline containing gelatin and cations (GVB^{++})

5xVBS	200ml
$MgCl_2$ ($100mM.L^{-1}$)	10ml
$CaCl_2$ ($30mM.L^{-1}$)	5ml
Gelatin (10%)	10ml

Molten gelatin was added to 100ml deionised water before the addition of the other reagents. The volume was then adjusted to 1 litre by the addition of deionised water. The buffer was stored at 4°C and used within 1 week.

Isotonic veronal-buffered saline containing gelatin without cations ($GVB^{=}$)

5xVBS	200ml
Gelatin (10%)	10ml

Molten gelatin was added to 100ml deionised water before the addition of 5xVBS. The volume was then adjusted to 1 litre by the addition of deionised water. The buffer was stored at 4°C and used within 1 week.

$GVB^{=}$.EDTA ($10mM.L^{-1}$)

EDTA ($0.086mM.L^{-1}$)	58ml
$GVB^{=}$	442ml

Prepared weekly and stored at 4°C.

Phosphate Buffer ($0.5M.L^{-1}$)

Stock solutions:

A) NaH_2PO_4 ($0.5\ M.L^{-1}$)

NaH_2PO_4	60gm

Dissolved in deionised water to a final volume of 1 litre.

B) Na_2HPO_4 ($0.5M.L^{-1}$)

Na_2HPO_4	71gm

Dissolved in deionised water to a final volume of 1 litre.

Stock solution of $0.5\ M.L^{-1}$ phosphate buffer was made by the addition of solution A to solution B until the required pH was achieved. To make $0.01\ M.L^{-1}$ phosphate

buffer, the stock buffer was diluted 1:50 with deionised water.

Phosphate buffer ($0.5M.L^{-1}$ pH 8 containing cysteine $0.01M.L^{-1}$ and EDTA $0.002M.L^{-1}$)

Cysteine	1.21gm
EDTA	0.672gm

Dissolved in phosphate buffer ($0.5M.L^{-1}$ pH 8) to a final volume of 1 litre.

Acetate buffer ($0.1M.L^{-1}$ pH 4.5)

Sodium acetate	8.2gm

Sodium acetate was dissolved in 750ml deionised water, and the pH was adjusted to 4.5 by the drop-wise addition of glacial acetic acid. The final volume was adjusted to 1 litre by the addition of deionised water.

Glycine-HCl ($0.1M.L^{-1}$ pH 2.8)

Glycine	7.05gm

Deionised water up to 1 litre.

The pH was corrected to 2.8 with concentrated HCl prior to adjusting the volume to 1 litre.

2.2. MEASUREMENT OF pH

The pH of solutions was measured using a Pye Unicam pH meter (model 292). The pH meter was adjusted, before the pH of any solution was measured, using an appropriate reference pH standard. For each measurement the temperature of the solution was determined and the pH meter calibrated at that temperature.

2.3. MEASUREMENT OF CONDUCTIVITY

Conductivity was measured at $0^{o}C$, using a Radiometer Copenhagen conductivity meter (model CD M3).

2.4. MEASUREMENT OF OPTICAL DENSITY

The optical density, at different wave lengths, was measured by Schimadzu spectrophotometer, model No. UV 120-02.

2.5. PREPARATION OF DIALYSIS TUBING

Dialysis tubing was prepared by boiling 3 times in EDTA ($1mM.L^{-1}$) and then stored at 4^oC in the same solution.

2.6. PREPARATION OF NORMAL HUMAN SERUM

Venous blood was drawn from normal volunteers, and allowed to clot at room temperature, for 1 hour before being transferred to ice for 1 hour. The serum was separated by centrifugation at 2000g, for 15 minutes, at 4^oC. The recovered serum was divided into aliquots and stored at -70^oC.

2.7. CONCENTRATION OF PROTEIN SOLUTIONS

2.7.1 Vacuum dialysis

The fluid to be concentrated was placed in dialysis tube which was then suspended in a vacuum flask. The vacuum was generated by using an electric vacuum pump. Protein concentration was checked spectrophotometrically at OD_{280}, until the required concentration was achieved.

2.7.2 Lyophilization (Freeze-drying)

The material to be lyophilized was placed in a suitably sized lyophilization flask, and frozen with liquid nitrogen or a mixture of ethanol and dry ice. The frozen material was connected to the freeze-dryer until all the moisture had been removed.

2.8. TECHNIQUES USING AGAROSE

2.8.1 Double diffusion (Ouchterlony technique)

Materials and Buffers:

Agarose (BDH)

VBS.EDTA ($0.01M.L^{-1}$) (section 2.1)

Method

The agarose gel was prepared by melting agarose (1gm) in 100ml isotonic VBS.EDTA. The resulting molten agarose was placed in sterile universal containers and stored at 4^oC until required. Agarose gel was melted in a boiling water bath, and

9.6ml was poured onto 10x10cm glass plate which had previously been washed, swabbed with 70% alcohol, dried and placed on a level surface. After the agarose had set the gel was then cut using a template and gel punch.

These plates were used for measuring the titres of rabbit antisera (anti-BSA and anti-thyroglobulin). The antisera were titrated using serial doubling dilutions and each peripheral well was loaded with a single dilution. The antigen (1mg/ml) was loaded in the central well. The plate was then incubated at room temperature, overnight, in a humid chamber.

2.8.2 Immunoelectrophoresis

Materials and Buffers

Agarose (BDH)

Barbitone buffer (pH8.6):

Barbitone	4.605gm
Sodium barbitone	25.77gm
Sodium azide	2.5gm

Deionised water up to 2.5 litres

Method

One gram agarose was added to 100ml of hot barbitone buffer, and when dissolved 3gm of polyethyleneglycol 6000 was added. The resulting solution was divided into aliquots, each aliquot being stored in a sterile universal container and stored at $4^{o}C$. A glass plate (10x10cm) was washed, swabbed with 70% alcohol, dried and placed on a horizontal surface. Molten agarose (9.6ml) was poured on the plate and allowed to set, after which the sample wells and troughs were cut using a template. The samples were loaded and bromophenol blue was placed in one of the wells as indicator. Immunoelectrophoresis was performed for two hours (10 Volts/cm) in a standard immunoelectrophoresis tank (Shandon). After the bromophenol blue had migrated to the anodal end of the troughs, the current was switched off and the antisera were placed

in the appropriate troughs. The plate was then placed in a humified chamber, at room temperature overnight, to allow diffusion of the antigen and antibody to occur. The plates were then placed in a plastic box and covered with half-strength physiological saline (0.075M). The saline was changed three times over a 24 hour period, and then washed with deionised water for a further three hours. The gels were stained by covering them with the staining solution (section 2.9) for 15 minutes at room temperature. Destaining was performed by washing with destain solution (section 2.9) for 10 minutes. The plate was then allowed to air-dry at room temperature for one day.

2.9. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) (Laemmli, 1970)

Reagents

All reagents were of the 'electrophoresis' grade and were obtained from the following sources:

N,N,N',N'-Tetramethylethylendiamine (TEMED) (Sigma)

2-Mercaptoethanol (Sigma)

Coomassie brilliant blue (Sigma)

Bromophenol blue (Sigma)

Acrylamide (BDH)

bis-Acrylamide (BDH)

Sodium dodecyl sulphate (BDH)

Buffers and solutions:

Acrylamide 30% (w/v)

Acrylamide	30gm
bis-Acrylamide	0.8gm
Deionised water	100ml

Kept at 4°C and was used within 1 month.

SDS 10% (w/v)

SDS	10gm
Deionised water	100ml

Tris (0.75M.L.$^{-1}$)

Tris base	90.8gm
Deionised water up to 1 litre.	

Tris-HCl (0.75M.L^{-1} pH6.8)

Tris base	90.8gm
Deionised water up to 1 litre	

The pH was corrected to 6.8 with concentrated HCl prior to adjusting the final volume to 1 litre.

Tris-HCl (0.75M.L^{-1} pH8.8)

Tris base	90.8gm
Deionised water up to 1 litre	

The pH was corrected to 8.8 with concentrated HCl prior to adjusting the final volume to 1 litre.

Glycine (0.86M.L^{-1})

Glycine	64.4gm
Deionised water up to 1 litre	

Separation gel

Acrylamide 30% (w/v)	10ml
Deionised water	4.8ml
Tris HCl (0.75M.L^{-1} pH8.8)	15ml

SDS 10% (w/v)	0.3ml
Ammonium persulphate	30mg
TEMED (added just before pouring the gel)	15µl

Stacking gel

Acrylamide 30%	1.8ml
SDS 10%	0.1ml
Tris HCl (0.75M.L^{-1} pH6.8)	3ml
Ammonium persulphate	18mg
Deionised water	13ml
TEMED (added just before pouring the gel)	9µl

Running buffer

SDS 10% (w/v)	20ml
Tris (0.75M.L^{-1})	66ml
Glycine (0.86M.L^{-1})	440ml
Deionised water up to 2 litres.	

Sample buffer

SDS 10% (w/v)	30ml
Tris HCl (0.75M.L^{-1} pH6.8)	8.5ml
Glycerol	10ml
Deionised water	46.4ml
Mercaptoethanol	5µl

Staining solution

Methanol	5 parts
Acetic acid	1 part
Deionised water	4 parts

Coomassie brilliant blue 0.1% (w/v final concentration)

Destaining solution

Methanol	1 part
Acetic acid	1 part
Deionised water	8 parts

Methods

Two glass plates 20cmx20cm were washed thoroughly, swabbed with 70% alcohol and dried. The plates were then separated by 1mm spacers clamped and held vertically. The two sides and the bottom of the chamber were sealed by adding 15µl TEMED to 1.5 ml of running gel and allowing the mixture to run down the edges of the gel cavity. The separation gel was then poured in the sealed chamber and a layer of isopropyl alcohol was added, in order to ensure that the surface of the gel was perfectly level. After polymerization (1 hour at room temperature) the alcohol layer was removed and the surface of the gel was washed. The stacking gel was poured over the surface of the running gel to fill the surface of the cavity. A comb was placed in the gel to form the lanes in which the samples would be loaded. The gel was clamped to the electrophoresis tank which had already been filled with electrophoresis buffer. Once the stacking gel had set the comb was removed and the samples were loaded. Prior to loading the samples were mixed with sample buffer containing mercaptoethanol and boiled for three minutes. When the samples were to be electrophoresed under non-reducing conditions, the mercaptoethanol was omitted from the sample buffer.

Molecular weight standard curve construction

Molecular weight standards (Sigma) contain the following standards: Carbonic anhydrase (29kD), ovalbumin (45kD), BSA (66kD), phosphorylase B (97kD), β-galactosidase (116kD) and myosin (205kD), were 10µl reduced on the gel together with

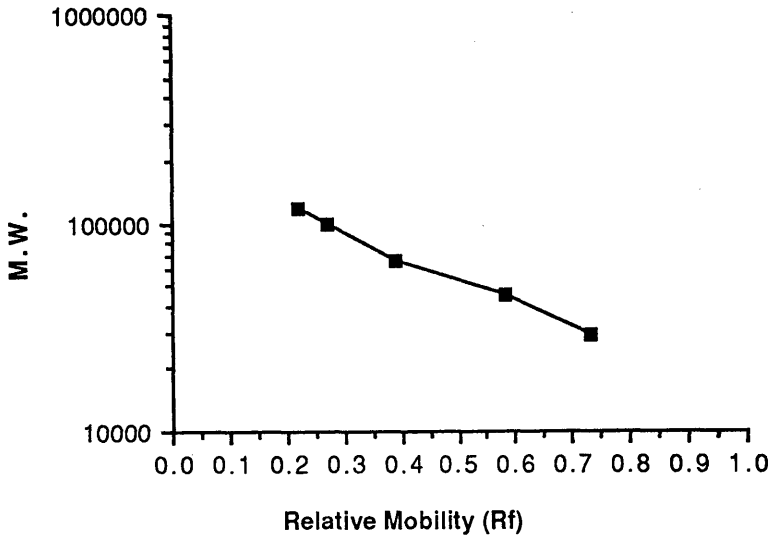


Figure 2.1: Plot of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) standard curve. The logarithm of the molecular weight of each standard protein is plotted against its relative mobility.

samples. After staining the relative mobility for each band was measured according to the following formula:

$$Rf = \frac{\text{The migration distance of protein}}{\text{The migration distance of the dye front}}$$

The standard curve was constructed on a semi-log paper by plotting the logarithm of the molecular weight of each standard against its relative mobility (Fig 2.1). The relative mobility of each protein band in the test gel was determined and their molecular weights determined directly from the standard curve.

2.10. PRODUCTION OF ANTIBODIES IN RABBITS

Materials

Bovine serum albumin (BSA) (Sigma)

Bovine thyroglobulin (Sigma)

Freund's complete adjuvant (Difco)

Freund's incomplete adjuvant (Difco)

Sterile isotonic saline (Hospital Pharmacy)

Method

The immunogen (BSA, or thyroglobulin) was dissolved in sterile isotonic saline to give a solution with a final concentration of 1mg/ml. This solution was then emulsified with equal volume of Freund's complete adjuvant. Each rabbit was injected subcutaneously with 0.5ml of emulsion at each of four sites. Four and six weeks after the first injection, booster injections were given. Each animal received 1ml of 1mg/ml immunogen in Freund's incomplete adjuvant at four sites. Ten days after the last boost, test bleeds (5ml) were taken from the marginal ear vein and tested for antibody content against specific antigen by using double diffusion. When reasonably high titres of precipitating antibodies were present, 50ml blood was drawn from the marginal ear vein of the animals at weekly intervals for 3 weeks.

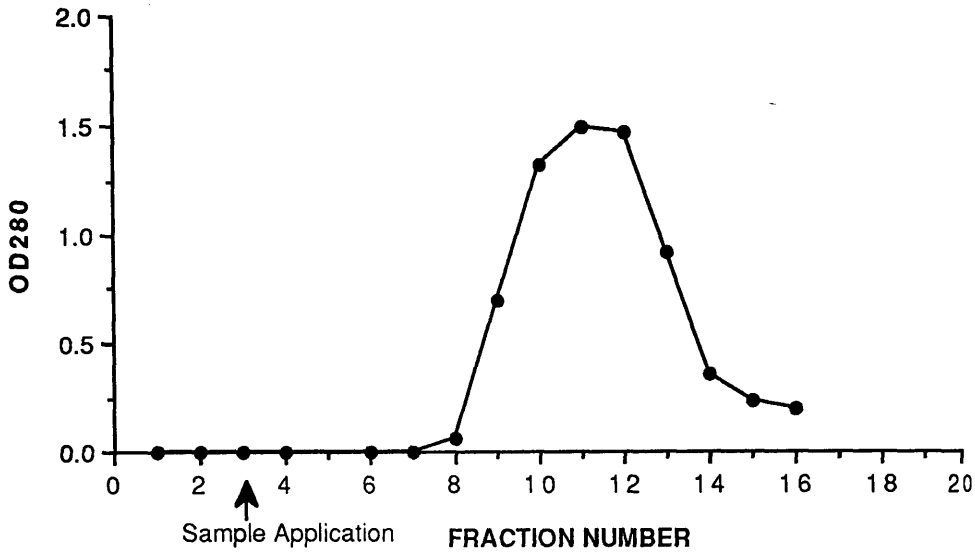


Figure 2.2: Elution profile of a DE52 column used for the purification of IgG. The purified IgG is excluded in a single peak.

The blood was collected in sterile glass universal containers and allowed to clot at 37°C for 30 minutes, and then transferred to ice for one hour to allow clot retraction to occur. The serum was then recovered by centrifugation, 2500g, at 4°C for 15 minutes, heat inactivated at 56°C for 1 hour, and then stored in aliquots at -20°C until required.

2.11 PURIFICATION OF IgG ON DIETHYLEAMINOETHYL CELLULOSE (DE52)

Materials and buffers:

DE52 (Whatman)	
Saturated ammonium sulphate (SAS)	(section 2.1)
VBS	(section 2.1)
PBS	(section 2.1)
Phosphate buffer ($0.01M.L^{-1}$ pH 7.6)	(section 2.1)

Method

The serum globulins were precipitated by placing the serum in an ice bath and adding an equal volume of SAS dropwise with continuous stirring. The serum was allowed to stand for 1/2 hour in the ice bath and the precipitated globulins were separated by centrifugation (2000g for 15 minutes at 2°C). The precipitate was dissolved in 5xVBS (half the volume of the original serum) and then dialysed extensively against phosphate buffer overnight at 4°C, before being applied to a small column of DE52 (1ml swollen gel per 10mg applied protein) which had been equilibrated with the same buffer. Following the application of the sample, the column was washed with the equilibration buffer and 5ml fractions were collected. The protein concentration (OD_{280}) of each fraction was measured and the peak of excluded protein (IgG) was pooled (Fig 2.2). The protein concentration of the pool was measured (OD_{280}) prior to its division into aliquots for storage at -20°C.

2.12. CONJUGATION OF BSA AND THYROGLOBULIN IN CNBR ACTIVATED SEPHAROSE-4B

Materials

CNBr activated Sepharose-4B	(Pharmacia)
BSA	(Sigma)
Bovine thyroglobulin	(Sigma)
Sodium azide	(BDH)

Reagents and Buffers

0.001M HCl

Deionised water	990 ml
0.1M HCl	10ml

Coupling buffer

$NaHCO_3$	8.4gm
NaCl	29.22gm

Dissolved in deionised water and made up to 1 litre final volume.

Blocking Buffer

Tris base	12.114gm

Dissolved in deionised water and pH adjusted to 8 with concentrated HCl prior to adjusting volume to 1 litre with deionised water.

Acetate buffer

Sodium acetate	8.203gm
NaCl	29.22gm

Dissolved deionised water and pH adjusted to 4 with glacial acetic acid prior to adjusting final volume to 1 litre with deionised water.

Tris-HCl ($0.1M.L^{-1}$.pH8). NaCl ($0.5M.L^{-1}$):

Tris base	12.114gm
NaCl	29.22gm

Dissolved in deionised water and pH adjusted to 8 with concentrated HCl prior to

adjusting volume to 1 litre with deionised water.

Phosphate Buffered saline (pH 7.2)

NaCl	8gm
K_2HPO_4	1.21gm
KH_2PO_4	0.34gm

Dissolved in 1 litre of deionised water.

Method

CNBr-activated Sepharose was washed with 0.001M.L^{-1} HCl (10 volumes per one volume swollen gel). The ligand which had been dissolved in coupling buffer was added to the swollen gel (10mg/ml swollen gel) and mixed end-over-end for 1 hour at room temperature. The mixture was centrifuged (1000g for 5 minutes at 2^{o}C), the supernatant removed and its protein concentration was measured spectrophotometrically (OD_{280}). The efficiency of binding of ligand to the gel was expressed as percent bound ligand and calculated as follows:

$$\%\text{Bound Ligand} = \frac{\text{Amount of non-bound ligand}}{\text{Amount of ligand applied}} \times 100$$

Usually the percent of binding was over 90%. The remaining active sites on the Sepharose beads were blocked by incubating the gel with 0.1M.L^{-1} Tris-HCl pH8 with end-over-end mixing for 1 hour at room temperature. The gel was then washed with three alternating cycles of acetate buffer (0.1M.L^{-1} pH4 containing 0.5M.L^{-1} NaCl) and Tris-HCl (0.1M.L^{-1} pH8 containing 0.5M.L^{-1} NaCl). The gel was resuspended in an equal volume of same buffer (Tris-HCl 0.1M.L^{-1} pH8 containing 0.5M.L^{-1} NaCl) and sodium azide was added to a final concentration of 0.1 % (w/v) and the product stored at 4^{o}C.

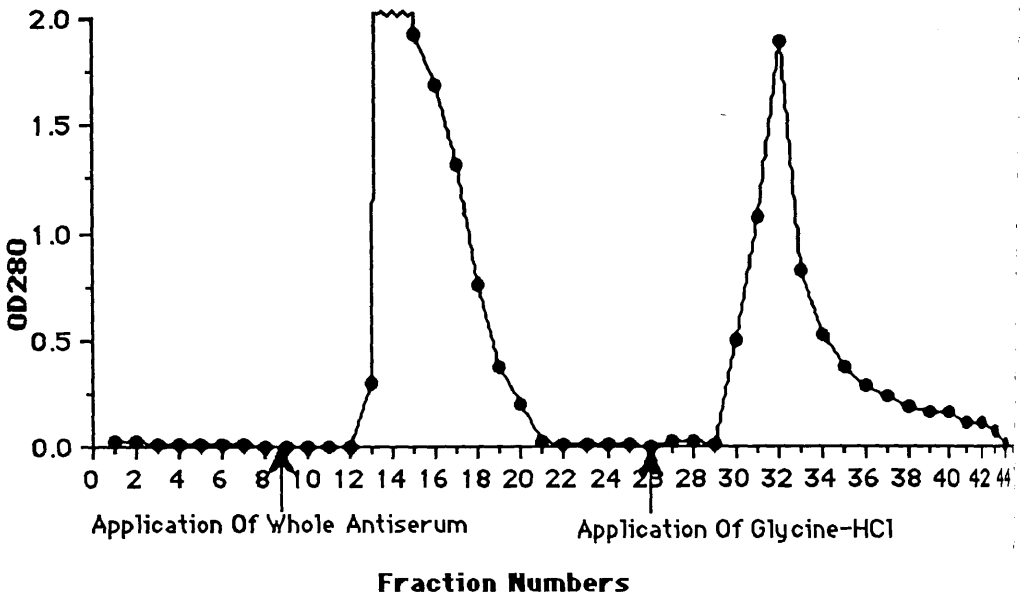


Figure 2.3: The elution profile of Sepharose-4B-thyroglobulin immunoadsorbent column. The unbound protein is excluded in the first peak, and the second peak represents the anti-thyroglobulin which eluted with glycine-HCl buffer.

2.13. AFFINITY PURIFICATION OF ANTIBODIES (IgG FRACTION)

Materials

Thyroglobulin-Sepharose 4B or BSA-Sepharose 4B (Section 2.1)

DE 52 (Whatman)

Anti-whole rabbit serum (donkey) (SAPU)

Anti-rabbit IgG (sheep) (SAPU)

Buffers and reagents

Glycine HCl ($0.1M.L^{-1}$ pH2.8) (section 2.1)

Phosphate Buffer ($0.01M.L^{-1}$ pH 7.6) (section 2.1)

Tris Base $1M.L^{-1}$

Tris base 12.1gm

Dissolved in deionised water to a final volume of 100ml.

Sodium hydroxide ($0.1\ M.L^{-1}$)

NaOH 0.4gm

Dissolved in deionised water to a final volume of 100ml.

Method

Affinity purification of anti-BSA or anti-thyroglobulin was performed by mixing equal volume of antiserum with the appropriate antigen-Sepharose 4B complex. After end-over-end mixing for 2 hours at room temperature the mixture was poured into a small column and washed extensively with PBS until the eluate contained no protein. The antibody was eluted by glycine-HCl ($0.1M.L^{-1}$ pH 2.8) and 5ml fractions were collected (Fig 2.3). Rapid neutralization of the fractions was achieved by the addition of 150µl Tris ($0.1M.L^{-1}$) to each tube. The protein concentration was measured spectrophotometrically. The fractions containing the peak of eluted protein were pooled and dialysed extensively against phosphate buffer ($0.01M.L^{-1}$ pH7.6), before being passed over a DE52 column which had been pre-equilibrated with the same buffer. The exclusion peak (purified IgG antibody) was dialysed against PBS, and the protein concentration was adjusted to 1mg/ml by vacuum dialysis. The preparation was

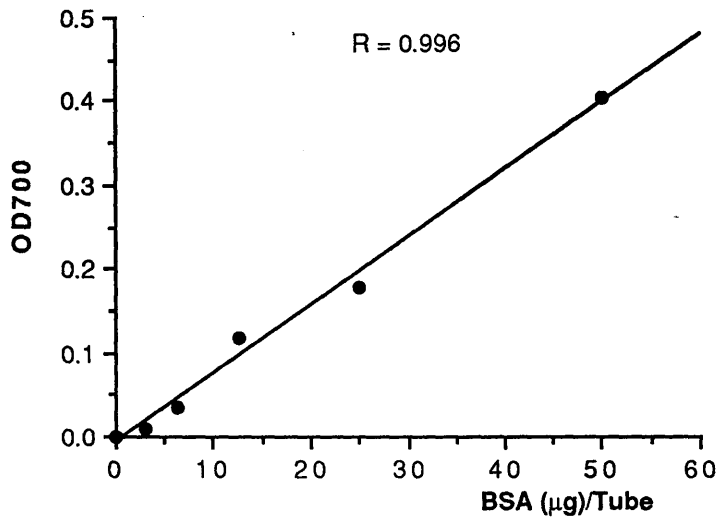


Figure 2.4: Plot of Folin-phenol assay standard curve. The amount of the standard protein (BSA) is plotted against its optical density (OD_{700}).

divided into aliquots and stored at -20°C until required.

The purity of the purified IgG was checked by SDS-PAGE which showed a single band (150kD) when run under non-reducing conditions and two bands (50kD and 25kD) when run under reducing conditions.

2.14. DETERMINATION OF PROTEIN CONCENTRATION BY MODIFIED FOLIN-PHENOL ASSAY

Buffers, reagents and stock solutions

Folin reagent (Sigma)

Solution A: Prepared by dissolving 10gm of sodium carbonate and 100mg of sodium potassium tartrate in 500ml of sodium hydroxide ($0.1M.L^{-1}$).

Solution B: 0.15% (w/v) aqueous solution of copper sulphate ($CuSO_4.5H_2O$).

Solution C: Folin reagent (diluted 1:1 with deionised water).

Solution D: is freshly made by mixing of 50 volumes of solution A with 1 volume of solution B. Two millilitres from this solution were added to each of two series of test tubes. One series was used for test samples and the other for the reference standard and negative control. Ten microlitres of the test sample or the standard, were added to each designated tube. Two hundred microlitres of Folin reagent was added to each tube. The tubes were vortexed and incubated at room temperature for 30 minutes.

The colour intensity was measured spectrophotometrically at 700nm, using the blank tube as a zero reference for all tubes. The standard curve (Fig 2.4) was constructed by plotting the OD_{700} values against the protein concentration of each standard, and test sample values were determined from the standard curve and multiplied by the dilution factor.

2.15 QUANTITATIVE PRECIPITIN CURVE AND DETERMINATION OF EQUIVALENCE POINT FOR ANTI-BSA AND ANTI-THYROGLOBULIN

Method

One hundred microlitres of each of a series of doubling dilutions (in PBS) of BSA

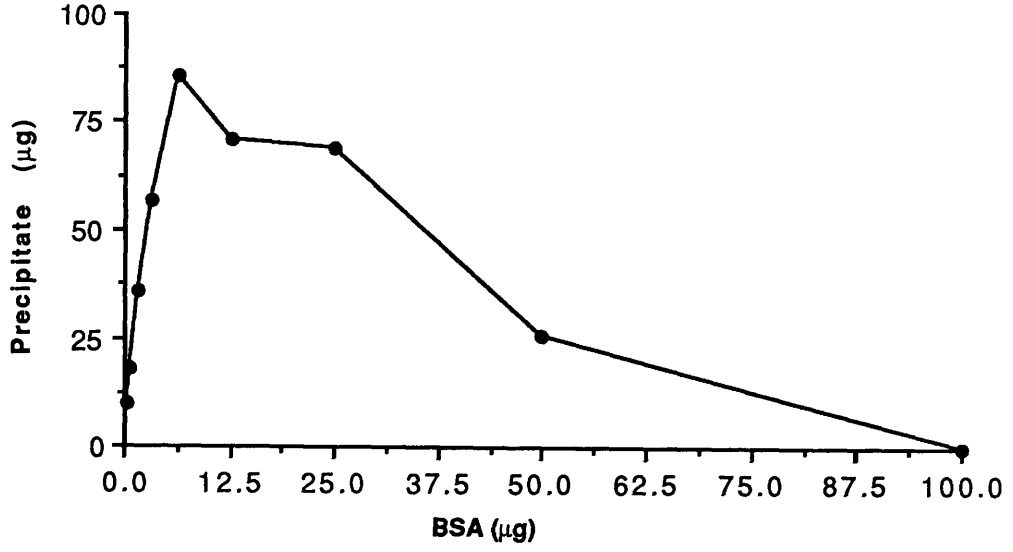
(starting from 8mg/ml) or thyroglobulin (starting from 10mg/ml) were added to a series of microcap centrifuge tubes. An additional tube in each series received 100µl PBS and was used as a negative control. An equal volume (100µl) of the appropriate purified antibody (1mg/ml) was added to each tube and after mixing they were incubated in a 37°C water bath for 1 hour, and then kept at 4°C overnight. Precipitated immune complexes were separated by centrifugation in a Beckman microfuge (10000rpm) for 10 minutes and the supernatant was aspirated. The pellet was washed three times with ice-cold PBS, and then dissolved in 100µl of NaOH ($0.1M.L^{-1}$). The amount of protein in each tube was measured by the Folin phenol assay (Table 2.1). The precipitin curve was constructed by plotting the quantity of the precipitate on the ordinate against the concentration of antigen in the same tube on the abscissa (Fig 2.5).

In order to validate the equivalence point, the supernatants from the quantitative precipitin curve tubes were screened for antigen and antibody by double diffusion (section 2.8.1).

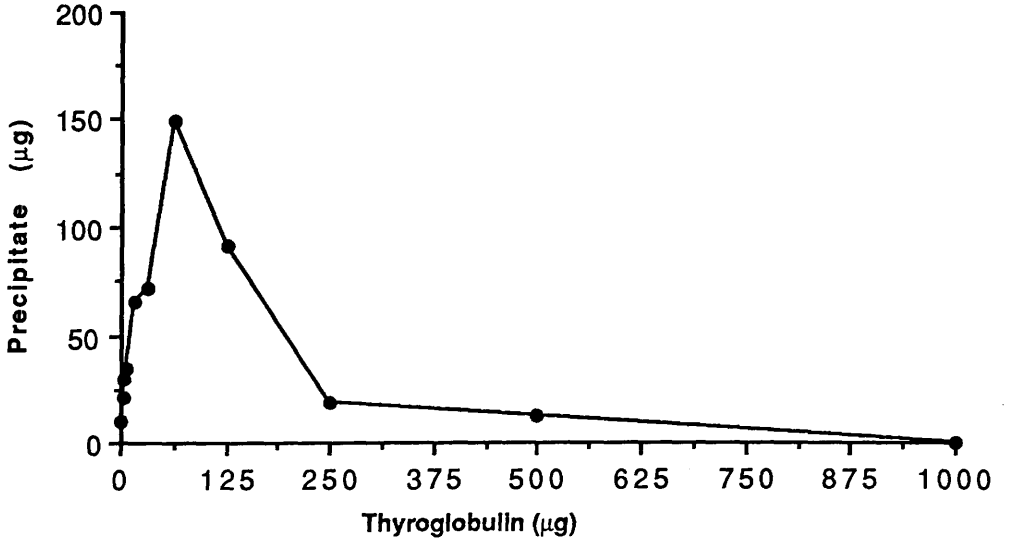
Table 2.1: Quantitative precipitation and equivalence points for BSA anti-BSA and thyroglobulin anti-thyroglobulin IC.

Tube No.	BSA* (μg)	OD_{700}	ppt (μg)	Thyroglobulin* (μg)	OD_{700}	ppt (μg)
1	800	000	000	1000	0.0001	00
2	400	000	000	500	0.013	12
3	200	000	000	250	0.021	19
4	100	0.0001	000	125	0.099	91
5	50	0.028	26	62.5	0.152	149
6	25	0.075	69	31.25	0.078	72
7	12.5	0.078	71	15.625	0.072	66
8	6.25	0.094	86	7.5	0.038	35
9	3.125	0.062	57	3.9	0.032	30
10	1.56	0.039	36	1.95	0.023	21
11	0.78	0.02	18	0.97	0.012	10
12	0.39	0.011	10			

(*) Each concentration of the antigen was mixed with 100μg of affinity purified antibody



(A)



(B)

Figure 2.5: Quantitative precipitin curve for anti-BSA (A) and anti-thyroglobulin (B) antibodies. The amount of antigen (BSA or thyroglobulin) is shown on the abscissa and the amount of the precipitated IC is shown on the ordinate. The equivalence point is that which showed the largest precipitate. The zone of antigen-excess and that of antibody-excess are to the right and to the left of the equivalence point respectively.

2.16 PREPARATION OF IgG FRAGMENTS

Materials and Buffers

Papain (Sigma)

Pepsin (Sigma)

Acetate buffer (0.1M.L^{-1} pH 4.5) (section 2.1)

Phosphate buffer (0.5M.L^{-1} cysteine 0.01M.L^{-1} EDTA 0.002M.L^{-1} pH8.0) (section 2.1)

Fab fragment (Papain digestion, Hudson and Hay, 1980)

The IgG concentration was adjusted to 20mg/ml by means of vacuum dialysis, and the concentrated material was dialysed overnight against phosphate buffer (0.5 M.L^{-1} pH8) containing cysteine (0.01M.L^{-1}) and EDTA (0.002M.L^{-1}). The dialysed material was placed in a small bijoux bottle, and papain was added (1mg/100mg IgG). The mixture was incubated at 37°C overnight in a shaking water bath. The mixture was then dialysed overnight against 5 litres PBS. Following centrifugation (1000g for 5 minutes at 2°C) to remove any insoluble material, the supernatant was passed over a small column of protein-G Sepharose to remove the Fc piece, while the Fab fragments, which did not bind to the column were collected in the exclusion peak. The purity of Fab fragments was determined by IEP (section 2.8) and SDS-PAGE (section 2.9) using 10 % gel, where samples were run in the reduced and non-reduced form.

Preparation of F(ab')$_2$ (pepsin digestion, Hudson and Hay, 1980)

The IgG concentration was adjusted at 20mg/ml, and dialysed against sodium acetate buffer (0.1M.L^{-1} pH4.5). Pepsin was added (1mg/50mg IgG) and the mixture was incubated at 37°C overnight in a shaking water bath. The reaction mixture was then dialysed overnight against 5 litres PBS. The dialysed material was centrifuged (1000g for 5 minutes at 2°C) to remove any insoluble material. The digested IgG was passed over a protein-G Sepharose column equilibrated with PBS and the F(ab')$_2$

fragments which did not bind to the column were collected in the exclusion peak (section 2.17). The purity of $F(ab)_2$ was checked by SDS-PAGE (section 2.9) using a 10% gel and samples were run in the reduced and non-reduced form.

2.17 PROTEIN-G SEPHAROSE CHROMATOGRAPHY

Materials and Buffers:

Protein-G Sepharose 4 Fast Flow (Pharmacia)

Glycine-HCl ($0.1M.L^{-1}$.pH2.8) (section 2.1)

PBS (section 2.1)

Method

The required quantity of protein-G Sepharose was reconstituted in PBS and the gel was then packed in a small column. The digested IgG was passed through the column which was then washed with PBS and the exclusion peak was collected in 1ml fractions. The purity of the recovered Fab or $F(ab)_2$ were checked on SDS-PAGE (section 2.9) using a 10% gel and samples were run in the reduced and non-reduced form.

The protein-G Sepharose column was recycled by washing with glycine-HCl buffer until no further protein was eluted. The column was then washed with PBS for re-equilibration. The column was then suspended in 20% ethanol, as a preservative as directed by the supplier, and kept at 4^oC until required.

2.18 BIOTINYLATION OF IgG (Jackson et al, 1982; Holmskov-Nielsen et al, 1986; Costa-Castro et al, 1987)

Materials and Buffers

Goat IgG anti-human C1-INH (ATAB)

Goat IgG anti-human C3 (ATAB)

Goat IgG anti-human C5 (ATAB)

Biotin N-hydroxy-succinimide ester (Sigma)

Dimethyl sulphoxide (DMSO) (BDH)

Merthiolate (Sigma)

Glycerine (BDH)

Phosphate buffered saline (PBS, pH 7.2) (section 2.1)

Phosphate buffered saline

(adjusted to pH8.5 with 0.5% w/v Na_2CO_3) (section 2.1)

Method

The protein concentration of IgG antibody was adjusted to 1 mg/ml, and 6ml were dialysed against PBS (pH 8.5) overnight at 4°C. Biotin was dissolved in DMSO (1mg/ml). Each of the dialysed antibodies were placed in small bijoux bottle and to each one 1ml of biotin solution was added and mixed. After incubation at room temperature for 4 hours with intermittent gentle shaking, the reaction mixtures were dialysed extensively against PBS, pH 7.2, to remove free biotin. Merthiolate and glycerine were added to the biotin-conjugated antibodies as preservatives to final concentration of 0.05% (w/v) and 5%(v/v) respectively. The conjugates were kept at 4°C until required.

2.19 MEASUREMENT OF COMPLEMENT COMPONENTS

Complement components C3, C4, factor B and C1-INH were measured by laser-nephelometry, using a Baker Instruments Nephelometer Type 420. Normal ranges established in this laboratory were: C4 (199-574µg/ml), factor B (149-421µg/ml), C3 (720-1800µg/ml) and C1-INH (160-370µg/ml).

2.20 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Materials:

Goat IgG anti-human C1s (ATAB)

Goat IgG anti-human Properdin (ATAB)

Sheep IgG anti-human C9 (ICN)

Goat IgG anti-human C1-INH (ATAB)

Goat IgG anti-human C3 (ATAB)

Goat IgG anti-human C5 (ATAB)

Biotin-conjugated goat IgG anti-human C3 (section 2.18)

Biotin-conjugated goat IgG anti-human C1-INH (section 2.18)

Biotin-conjugated goat IgG anti-human C5 (section 2.18)

H_2O_2 30%(v/v) (BDH)

O-phenylenediamine (OPD) (Sigma)

96 well flat bottom microelisa plates (Dynatech)

Avidin-horse radish peroxidase conjugate (Vector)

Buffers:

Coating Buffer (0.05M.L^{-1} Carbonate-Bicarbonate, pH 9.6)

Na_2CO_3	0.79g
$NaHCO_3$	1.46g

Deionised water to 500ml

Blocking Buffer (PBS, 0.1% Gelatin)

PBS	500ml
Gelatin	0.5g

Substrate Buffer (0.2M.L^{-1} phosphate, 0.1M.L^{-1} citrate buffer pH 5.6)

Solution A: Na_2HPO_4	14.196g

Deionised water to 500ml

Solution B: Citric acid	9.61g

Deionised Water to 500ml

Solution C: Solution A (28ml) was mixed with solution B (22ml). The final volume was completed to 100ml with deionised water and OPD (34mg) was added. Hydrogen peroxide (30%) was added (40µl) to the buffer immediately, before use.

4N H_2SO_4

H_2SO_4 (concentrated)	108ml

Deionised water was added to a final volume of 1 litre.

Dilution Buffer:

Isotonic EDTA (0.086M.L^{-1} pH7.4)	116ml
Gelatin (10% w/v)	10ml
Tween 20	0.5ml

PBS was added to final volume of 1 litre.

Method

Enzyme linked immunosorbent assays (ELISA) were developed to measure the multimolecular activation products C1r:C1s:C1-INH, C3bBbP, and C5b-9. The assays were performed by coating the first two rows of each plate with 100µl/well of anti-C1-INH (10µg/ml), anti-C3 (5µg/ml) or anti-C5 (5µg/ml) and the remaining 6 rows were coated with anti-C1s (10µg/ml), anti-properdin (5µg/ml) and anti-C9 (5µg/ml) respectively. The first two rows were used for the reference standard curve in each assay. Coated plates were kept in a humid chamber at 4°C overnight prior to use. Plates were then washed 5 times with PBS-Tween, using an automated ELISA washer (Dynatech), 250µl/well of the blocking buffer was applied for 1 hour and plates incubated in humid chamber at room temperature, followed by another 6 washes. Reference samples, test samples and internal controls (100µl/well) were added in duplicate. Dilution buffer (100µl/well) was added to two wells as a negative (background) control. The plates were incubated for a further hour at room temperature followed by a further 6 washes. The biotin conjugated antibodies (100µl/well) were applied as follows; biotin anti-C1-INH for C1s:C1-INH assay, biotin anti-C3 for C3:P assay and biotin anti-C5 for C5b-9 assay and the plates were incubated at room temperature for an hour followed by 6 washes. Avidin-horse radish

peroxidase conjugate was added (100µl/well) and the plates were incubated at room temperature for an hour. After a further 6 washes 100µl substrate was added to each well and the plates were then incubated at room temperature until the colour developed. Plates were read using a microelisa reader (Dynatech) at wave length of 490nm.

2.20.1 Titration of the coating antibodies

The antibodies used in the three assays for coating the ELISA plates were titrated using different concentrations (20µg/ml, 10µg/ml, 5µg/ml and 2.5µg/ml) with the other conditions fixed for each assay.

2.20.2 Titration of biotin-antibody probes

Different dilutions were used of each of biotin anti-C1-INH (1/250, 1/1000, 1/5000 and 1/25000), biotin anti-C3 (1/250, 1/1000, 1/5000 and 1/25000) and biotin anti-C5 (1/250, 1/750 and 1/1500). All other conditions were fixed for each assay.

2.20.3 Reference serum standard

Total C1-INH, C3 and C5 concentrations were measured in a standard pooled serum (from 40 individuals, stored in aliquots at -70^{o}C). The pool contained 191µg/ml C1-INH, 1100µg/ml C3 and 75µg/ml C5. Rows A and B of the ELISA plates contained the standard curves, which were linear from 0.75 to 24 ng/ml for C1-INH, 0.9 to 30 ng/ml for C3 and 1.1 to 38 ng/ml for C5. The optical density readings for the samples were read off the standard curve and expressed as ng/ml.

2.20.4 Development of optical density (time course)

The change of the optical density was studied at different intervals (2, 4, 6 and 8 minutes). For this purpose, four standard curves were set under similar conditions, in duplicates, in each ELISA. Each standard curve was assigned for one of the suggested colour development intervals.

2.20.5 Sensitivity of the assays

The sensitivity of the assays was defined as the linear part of the standard curve with the upper and the lower ends of the standard curve representing the upper and the lower limits of the assay respectively. The standard curves were constructed using optimal conditions for each ELISA.

2.20.6 Reproducibility of the assays

Internal controls: For C1s:C1-INH ELISA, fresh NHS was activated with BSA anti-BSA IC at equivalence (50μg of antibody and 3.125μg of antigen per 100μl of NHS) for 30 minutes at 37°C. For C3:P and C5b-9 ELISAs, fresh NHS was activated by incubation with zymosan (1mg/ml NHS) at 37°C for 30 minutes. In both activation processes, the reaction was stopped by the addition of EDTA ($0.086M.L^{-1}$) to a final concentration of $10mM.L^{-1}$. The activated sera were divided into aliquots and stored at -70oC.

The interassay variation was defined by calculating the coefficient of variation for the internal controls which were assayed in 10 different experiments for each ELISA.

Stability of samples: The stability of C1s:C1-INH, C3:P and C5b-9 complexes was tested by measuring the levels in samples of patient plasma and EDTA-treated IC zymosan activated sera (see above) after; a) two cycles of freeze-thawing, b) incubation at 20°C and 37°C for 1, 2 and 4 hours and c) long term storage at -70°C. These conditions caused no significant alteration in the levels of any of the three activation complexes.

2.20.7 Use of ELISA for the measurement of complement activation in patients with different immune complex diseases

Patients: Thirty patients with systemic lupus erythematosus (SLE) (Tan et al, 1982) and 254 with rheumatoid arthritis (RA) (Ropes et al, 1958) were included in this study. Rheumatoid patients were subdivided, on the basis of IgM-RF sero-positivity, into the sero-positive (134) and the sero-negative (120) subgroups. Venous blood was

collected by venesection and divided into two aliquots. One was allowed to clot at room temperature and the other anti-coagulated with EDTA (final concentration of 10mM.L^{-1}). Serum and plasma were obtained by centrifugation (2000g for 10 minutes at -2°C) aliquoted and stored at -70°C within 2 hours of venesection. Synovial fluid was collected from knee effusions of 17 RA patients simultaneously with blood sampling.

Normal controls: Serum and plasma from thirty laboratory personnel (13 males and 17 females) were collected and stored as above, and the analysis of these groups was used to determine the normal ranges.

2.20.8 Effect of IgM rheumatoid factor (IgM-RF) on the ELISA systems

NHS was activated with IC or zymosan (as described above). Further activation was stopped by the addition of EDTA (0.086M.L^{-1}, pH 7.4) to a final concentration of 10mM.L^{-1}. IgM-RF (gift from Dr J. Davidson) was then added to the activated serum to give a final concentrations of 250, 500, and 1000µg/ml (normal serum concentration of IgM-RF ≤40µg/ml). Activated serum levels of C1s:C1-INH, C3:P and C5b-9 were measured by ELISA. Controls included IgM-RF in buffer (same concentrations used with activated serum) and activated serum without IgM-RF.

2.21 ELISA FOR THE MEASUREMENT OF RHEUMATOID FACTOR IN PATIENTS SERA AND PLASMA

ELISA plates were coated with human IgG (for IgM-RF and IgA-RF) or rabbit IgG (for IgG-RF) at a concentration of 5µg/ml in carbonate-bicarbonate buffer (0.005M.L^{-1}, pH9.6, 100µl/well). Coated plates were kept in humid chamber at 4°C overnight prior to use. Plates were then washed four times with PBS-Tween (0.005%v/v) and blocked with 0.1% BSA in PBS (200µl/well) for 1 hour at room temperature. The excess blocking buffer was removed by a further four washings with PBS-Tween and following the application of samples and standards (100µl/well), the plates were

incubated for 2 hours at room temperature. In the IgG-RF ELISA, the standards and samples were processed by treatment with pepsin. This was performed by mixing the sample (5µl) with 250µl of pepsin solution (10µg pepsin/ml of 0.1M.L^{-1} acetate buffer pH 4.4) and the mixture was incubated at 37°C overnight. The pepsin was then inactivated by the addition of 250µl of disodium hydrogen phosphate solution (0.563M.L^{-1}). One hundred microlitres/well was taken from the resulting solution for the assay of IgG-RF. The plates were washed again four times and the bound RF was probed using anti-µ chain (Dakopatts), anti-α chain (Dakopatts) or anti-human IgG (Cappel) $F(ab)_2$ fragment conjugated to horse-radish peroxidase for the IgM-RF, IgA-RF and IgG-RF assays respectively. All conjugates were used at a dilution of 1/1000 and the plate incubated for 1 hour at room temperature. After four washes the bound enzyme was detected using the standard developing reaction (section 2.20).

2.22. STATISTICS

Non-parametric statistical tests (Spearman rank correlation test and Wilcoxon matched pairs test) were used for data analysis.

2.23 TITRATION OF OPTIMAL SENSITIZING DOSE OF RABBIT ANTI-SHEEP ERYTHROCYTES

Materials and buffers

Polyethylene 96 well round bottomed microtitre plate (Dynatech)

Rabbit anti-sheep erythrocytes antiserum (Haemolytic serum) (Serotec)

PBS (section 2.1)

Method

Rabbit haemolytic serum was heat-inactivated by incubation at 56°C for 30 minutes. Fifty microlitres PBS were added to a series of wells and the haemolytic serum was titrated by doubling dilutions. For each dilution 50µl of sheep erythrocytes

($1x10^9$/ml) was added to each well and after shaking, the plate was incubated at room temperature for 1 hour. The lowest antibody dilution which did not give agglutination was chosen as the optimal sensitizing dose. It was usually a 1/500 dilution of the antiserum.

2.24 PREPARATION OF ANTIBODY SENSITIZED SHEEP ERYTHROCYTES (EAs)

Materials

Sheep erythrocytes (Gibco)

Rabbit anti-sheep erythrocyte antiserum (Haemolytic serum) (Serotec)

PBS (section 2.1)

GVB^{++} (section 2.1)

GVB=EDTA (section 2.1)

Method

Sheep erythrocytes were washed three times with GVB=EDTA, taking care to remove the buffy coat. Between washes the cells were centrifuged (2000g for 10min at 4°C). After washing the cells were resuspended in GVB=EDTA and the cell concentration was determined by lysing 100µl of E in 2.9ml water and measuring the optical density at 541nm ($1x10^9$ E/ml at OD_{541} = 0.385). The cell count was adjusted to $1x10^9$/ml by the addition of GVB=EDTA containing the optimal dilution of sensitizing antibody and incubated in a shaking water bath at 37°C for 30 minutes. The cells were then washed in GVB^{++} and the concentration adjusted to $5x10^8$/ml in GVB^{++} kept at 4°C for use within 4 days.

2.25 DETERMINATION OF THE OPTIMAL DOSE OF IC FOR DEPLETION OF HUMAN COMPLEMENT

Materials

EA ($1x10^8$/ml GVB^{++}) (section 2.21)

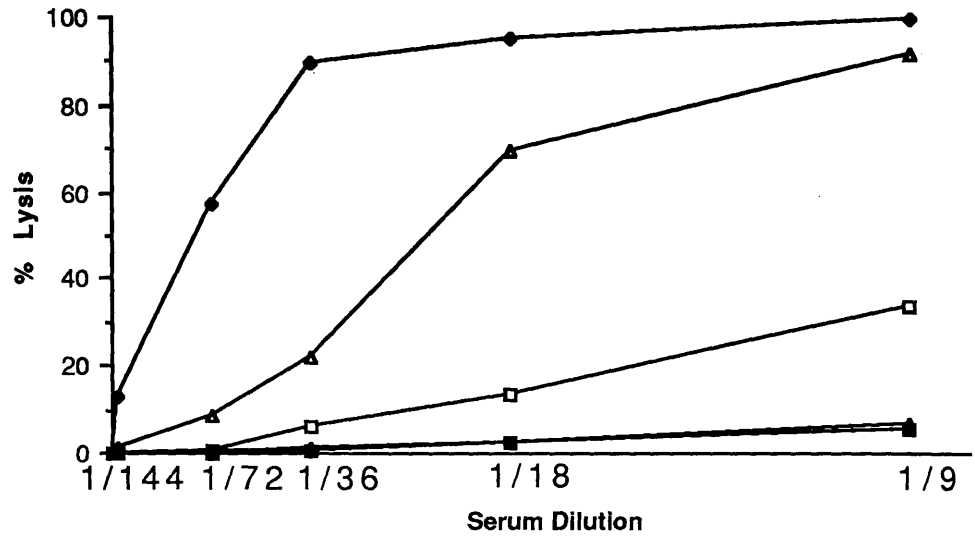


Figure 2.6: Haemolytic assay for the titration of the efficiency of different doses of IC, formed at equivalence, on the depletion of NHS complement. Four IgG antibody concentrations were used (per ml NHS): 1000µg (◆—◆), 500µg (△—△), 250µg (□—□), 125µg (■—■) and 62.5µg (▲—▲). Untreated NHS was used as a control (●—●).

GVB^{++} (section 2.1)

NHS (section 2.6)

Method

The ability of immune complexes to activate the complement system in human serum was assessed by examining the residual haemolytic activity of serum. Different concentrations of IC (100, 50, 25 and 12.5µg anti-thyroglobulin in 25µl GVB^{++}) were added to a series of four tubes containing 100µl serum. These tubes were incubated at 37°C for 30 minutes, after which time they were centrifuged (10000rpm for 5 minutes in a Beckman microfuge). The supernatant serum was then serially diluted (1/9, 1/18, 1/36, 1/72 and 1/144) in GVB^{++} and 100µl of each dilution was added to a series of test tubes. One hundred microlitres (100µl) of EA ($1x10^8$) were added to each tube and the mixtures were incubated in a shaking water bath at 37°C for 30 minutes. Control tubes included 100% lysis and a cell blank (100µl EA + 100µl GVB^{++}). After the addition of 2ml saline/tubes (2ml deionised water for the 100% lysis control) and centrifugation (2000rpm for 5 minutes at 2°C) the degree of lysis was determined spectrophotometrically (OD_{414}). The percent lysis was measured using the formula:

$$\%\text{lysis} = \frac{\text{OD414 (Sample) - OD414 (Cell Blank)}}{\text{OD414 (100\% lysis) -OD414 (Cell Blank)}}$$

The optimal dose of IC (Fig 2.6) that depleted more than 90% of NHS complement was selected (500µg IgG antibody/ml NHS) as the maximum IC dose to be used in all the experiments performed throughout this study.

2.26 ACTIVATION OF NORMAL HUMAN SERUM BY IMMUNE COMPLEXES (IC)

Materials

BSA (Sigma)

Thyroglobulin (Sigma)

IgG-fraction of affinity purified antibodies (anti-BSA and anti-thyroglobulin) (1mg/ml)	(section 2.13)
Normal human serum (NHS)	(section 2.6)
$MgCl_2$ ($0.1M.L^{-1}$)	(section 2.1)
EGTA ($0.1M.L^{-1}$)	(section 2.1)
EDTA ($0.086M.L^{-1}$)	(section 2.1)
PBS	(section 2.1)

The IC were prepared from two different antigens (BSA and thyroglobulin). Each antigen was studied at five antigen antibody ratios (16-times and 4-times antibody-excess, equivalence, 4-times and 16-times antigen-excess). Dose response curves for each antigen:antibody ratio were performed using four antibody doses (500, 250, 125 and 62.5µg IgG antibody/ml NHS). Kinetic experiments were performed using a single dose of BSA and thyroglobulin IC (250µg IgG antibody/ml NHS) for each of the five antigen:antibody ratios.

Dose response and kinetic experiments for IC formed with both antigens were performed using nascent and preformed IC (see below).

2.26.1 Activation by nascent IC (dose response)

The antibodies (starting concentration 1mg/ml in PBS) and the antigens (BSA or thyroglobulin) were doubly diluted in PBS, in a separate series of microcap tubes which were then warmed in a water bath at 37°C.

Normal human serum (100µl) was dispensed into two series, of four test tubes each, and placed in a water bath (37°C). EGTA ($0.1M.L^{-1}$) was added to the tubes of one series to give a final concentration of $0.01M.L^{-1}$ in NHS followed by $MgCl_2$ ($0.1M.L^{-1}$) to give a final concentration of $0.0025M.L^{-1}$. An equivalent volume of PBS was added to the tubes of the other series which had not received Mg^{++}EGTA. The appropriate concentrations of antigen (BSA or thyroglobulin) were then added (10µl/100µl NHS) to the duplicate sets of tubes, the appropriate concentration of pre-

warmed antibody was added in a constant volume of 50µl to give final concentration of 500, 250, 125 and 62.5µg IgG antibody/ml NHS. IC were formed at 16-times antibody-excess, 4-times antibody-excess, equivalence, 4-times antigen-excess and 16-times antigen-excess. Controls in this experiment included: NHS containing the highest IC dose (for each antigen:antibody ratio) with EDTA (final concentration of $0.01M.L^{-1}$), NHS/EDTA, NHS/Mg^{++}EGTA, NHS without IC, NHS in ice, NHS with highest does of antibody (500µg IgG antibody/ml NHS), NHS with the highest dose of antigen. After mixing their contents, the tubes were placed in a shaking water bath ($37^{o}C$) for half an hour. The reaction was stopped by the addition of an equal volume of ice-cold PBS containing EDTA ($0.02M.L^{-1}$). After mixing the tubes were centrifuged (10000rpm for 5 minutes at room temperature) using a Beckman microfuge and the supernatants were collected and the concentration of complement activation products were measured.

2.26.2 Activation by preformed IC (dose response)

Antibody (400µl at 1mg/ml in PBS) was added to a series of five microcap tubes and the required concentration of antigen (BSA or thyroglobulin) in 40µl was added to give the following antigen:antibody ratios: 16-times and 4-times antibody-excess, equivalence, 4-times and 16-times antigen-excess. The contents were mixed and the tubes incubated in water bath ($37^{o}C$) for 30 minutes before being transferred to an ice bath a further 30 minutes. At the end of this incubation period the tube contents were mixed thoroughly and IC at each antigen-antibody ratio were doubly diluted (3 times) in PBS, and 60µl of each dilution containing 50, 25, 12.5 and 6.25µg of antibody were added to 100µl of NHS at $37^{o}C$. The contents were mixed and incubated for 30 minutes in a shaking water bath at $37^{o}C$. The reaction was stopped by the addition of an equal volume of ice-cold PBS containing EDTA ($0.02M.L^{-1}$) to each tube and after mixing the tubes were centrifuged (10000 rpm for 5 minutes at room temperature) using a Beckman microfuge. The supernatants were collected for assaying for complement

activation products. The controls were identical to those used for the experiments on complement activation by nascent IC.

2.26.3 Kinetics of complement activation by IC

Nascent and preformed IC were studied at different antigen-antibody ratios but the concentration of antibody in the IC was kept constant at 250μg IgG/ml NHS. For the studies of complement activation by nascent thyroglobulin anti-thyroglobulin IC, antigen:antibody ratios of 16-times and 4-times antibody-excess, equivalence, 4-times and 16-times antigen-excess were used, and all experiments were performed at three temperatures (37°C, 30°C, 20°C). For all other experiments IC were studied at only three different antigen-antibody ratios (4-times antibody-excess, equivalence, 4-times antigen-excess) and incubations were performed only at 37°C.

Nascent IC: Normal human serum (NHS, 1ml) was dispensed into two series of tubes (5 tubes each) and EGTA ($0.1M.L^{-1}$) was added to one series to give a final NHS concentration of $0.01M.L^{-1}$ followed by $MgCl_2$ (final concentration of $0.005M.L^{-1}$). The tubes were placed in a shaking water bath. The pre-warmed antigen was added, mixed, followed by the addition of the pre-warmed antibody (250μg IgG antibody/ml NHS); finally the tubes were mixed well. The time of the addition of the antibody was considered to be time zero. Samples (175μl) were removed at designated time points and transferred into ice-cold PBS containing EDTA ($0.02M.L^{-1}$). The tubes were centrifuged (10000rpm for 5 minutes at room temperature) using a Beckman microfuge, and the supernatants were collected and assayed for complement activation products.

Preformed IC: IC were prepared as above and NHS was dispensed into two series of tubes (5 tubes each). EGTA and Mg^{++} were added to one set of tubes to give a final concentrations of $0.01M.L^{-1}$ and $0.0025M.L^{-1}$ respectively. The tubes were placed in a shaking water bath at 37°C. The preformed IC at different antigen:antibody ratios

were added to NHS to give 250μg IgG antibody/ml NHS. The time of the addition of preformed IC was considered as the time zero, and samples (175μl) were removed from the reaction mixture at designated time points and transferred to tubes containing ice-cold PBS and EDTA ($0.02M.L^{-1}$). The tubes were then centrifuged (10000rpm for 5 minutes at room temperature) using a Beckman microfuge, and the supernatant collected and assayed for complement activation products.

2.27. ACTIVATION OF COMPLEMENT IN THE PRESENCE OF HUMAN ERYTHROCYTES

These studies were undertaken to study the effect of erythrocytes on complement activation in NHS, as complement activation occurring in whole blood occurs in the presence of 50%(v/v) erythrocytes (approximately) and as erythrocytes express at least two membrane proteins which regulate complement activation (DAF and CR1).

These experiments were performed at four IC concentrations (500, 250, 125 and 62.5μg IgG antibody/ml NHS) and at five antigen:antibody ratios (16-times and 4-times antibody-excess, equivalence, 4-times and 16-times antigen-excess), in the presence or absence of an equal volume of packed autologus erythrocytes. Activation of complement in the presence of human erythrocytes was studied using the same procedures as described in section 2.24. The exception being in the addition of an equal volume of packed human erythrocytes to the NHS. At the end of the experiments the erythrocytes were removed by centrifugation and the supernatants were assayed for complement activation products.

2.28. PREPARATION OF HUMAN ERYTHROCYTES

Blood was withdrawn from normal volunteers and anti-coagulated with EDTA ($10mM.L^{-1}$). The plasma and buffy coat were removed after centrifugation at 2000g for 10 minutes at $4^{o}C$. The erythrocytes were washed 5 times with GVB=EDTA, taking care to remove residual buffy coats after each centrifugation. A 50% suspension of erythrocytes in GVB^{++} was prepared. The cells were used at the same day of

preparation. For the complement activation experiments, in which human erythrocytes were included, autologus NHS was used.

2.29. TRYPSINIZATION OF HUMAN ERYTHROCYTES

Materials and Buffers

Trypsin (Sigma)	
Isotonic VBS (1x)	section 2.1
GVB^{++}	section 2.1
Human erythrocytes	section 2.26

Method

In an attempt to investigate the role of CR1, erythrocytes were trypsinised (Lay and Nussenzweig, 1968; Dorval et al, 1989) prior their addition to the complement activation mixture.

Purified human erythrocytes were centrifuged (2000g for 5 minutes at 4°C) resuspended in an equal volume of VBS and placed in a glass tube and warmed to 37°C. Trypsin (2.5ml at 2mg/ml in VBS) was added to 5ml of 50% erythrocyte suspension. The mixture was incubated at 37°C for 1 hour in a shaking water bath. Trypsin was removed by washing the cells 5 times with GVB^{++} and centrifuging the cells (2000g x 5mins at 4°C) between each wash. The efficiency of trypsinization was determined using an immune adherence assay. In a preliminary experiment it was shown that trypsinised erythrocytes did not activate complement.

2.30. IMMUNE ADHERENCE ASSAY

Materials and buffers

Microtitre plate (96 well) (Dynatech)

Immune adherence buffer (IAB) (Isotonic VBS (1x) containing 1% BSA and 3.2% dextrose)

EAC43b ($1x10^8$/ml IAB) (Section 2.27)

Human erythrocytes (E^{hum} $1x10^9$/ml IAB)

Method

Fifty microlitres IAB were added to every well of a microtitre plate and 50µl of control or trypsinised E^{hum} ($1x10^9$/ml IAB) were dispensed into the wells in row one. The cells were doubly diluted across six wells. The indicator cells, EAC43b ($1x10^8$), were added (50µl) to all wells, the cells mixed and the plates incubated for 1 hour at room temperature. The pattern of agglutination was then read. Controls included control and trypsinised E^{hum} alone and EAC43b alone. Agglutination was observed with control E^{hum} but not with trypsinised E^{hum}.

2.31. BLOCKING OF ERYTHROCYTE CR1 AND TITRATION OF OPTIMAL BLOCKING CONCENTRATION OF ANTI-CR1 FAB FRAGMENTS

Materials

Anti-CR1 (Fab fragment) section 2.16

Normal human erythrocytes (E^{hum}) section 2.26

GVB^{++} Section 2.1

Method

In a further attempt to investigate the role of CR1, erythrocytes were pre-incubated with Fab anti-CR1 or normal rabbit IgG Fab which had been pre-absorbed against NHS-Sepharose (half an hour at room temperature), prior to their incubation with erythrocytes.

Two hundred microlitres of E^{hum} ($1x10^9$/ml GVB^{++}) were placed in each of six microcap tubes. Anti-CR1 Fab was doubly diluted (0.6mg/ml down to 0.0187mg/ml) and 200µl was added to each tube containing the cells. The normal rabbit IgG Fab was added to a second series of tubes. The tube contents were mixed end-over-end at room

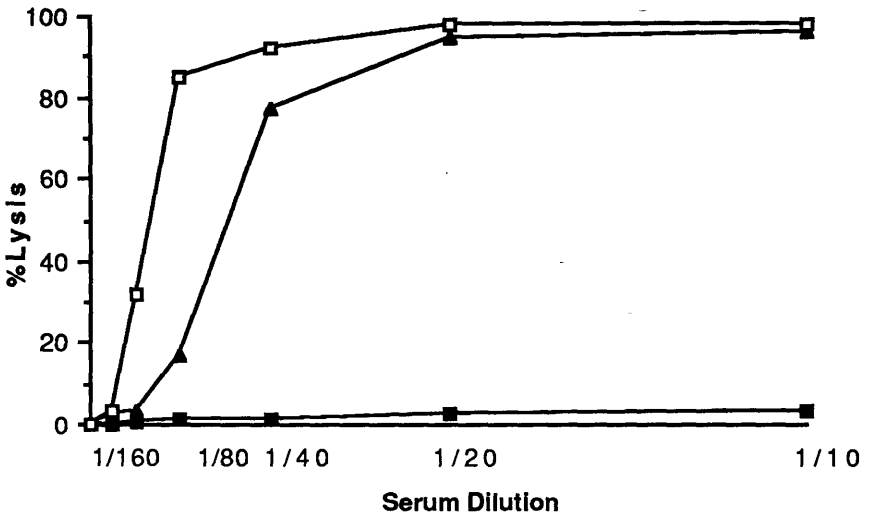


Figure 2.7: Titration of C3 depleted serum (R3 reagent). Zymosan was used for the depletion of serum C3 in two doses: 1mg/ml (■—■) and 0.5mg/ml (▲—▲). Untreated NHS (□—□) was used as control.

temperature for 1 hour. The unbound Fab fragments were removed by washing the cells two times with GVB^{++}. CR1 blockade was determined using the immune adherence assay (section 2.8). The lowest dilution of Fab anti-CR1 which blocked immune adherence completely was 0.0374mg/ml. Normal rabbit IgG Fab did not inhibit immune adherence. In a preliminary experiment it was shown that Fab treated erythrocytes did not activate complement.

2.32. PREPARATION OF C3-DEPLETED SERUM (R3)

Zymosan was mixed with freshly drawn NHS to give final concentrations of 0.5mg/ml and 1mg/ml. The zymosan-serum mixture was incubated in a shaking water bath at 37°C for 1 hour, after which the mixture was centrifuged (2000g for 10 minutes at 4°C) and the zymosan treated serum (R3-reagent) recovered and divided into aliquots and stored at -70°C.

Titration of R3-reagent

Titration of the R3-reagent was performed by haemolytic assay. The R3 preparations were serially diluted with GVB^{++} from 1/10 to 1/320. One hundred microlitres of each dilution was transferred to a duplicate series of test tubes to which 100 µl of EAs ($1x10^8$ cells/ml) were added. Controls included NHS diluted in the same manner of R3, cell blank and 100% lysis. The tubes were incubated in a shaking water bath at 37°C, for 1 hour and the reaction was stopped by addition of 2ml isotonic saline (except the 100% lysis to which 2ml deionised water were added). All tubes were centrifuged at 2000g for 5 minutes at 4°C. The extent of haemolysis was determined spectrophotometrically at OD_{414} (Fig 2.7). The optimal dilution of R3 selected was the lowest dilution which produced less than 5% haemolysis.

2.33. PREPARATION OF EAC43B INTERMEDIATE

Materials and buffers

EA section 2.21

R3-reagent	section 2.30
Suramin (Antrypol, 10mg/ml) (Bayer)	
GVB^{++}	section 2 1
GVB=EDTA	section 2.1
$DGVB^{++}$	section 2.1
NHS	section 2.6

Method

Nine millilitres of EA adjusted at $1x10^8$ cells/ml in GVB^{++} were pre-warmed and mixed with 1ml of pre-warmed R3-reagent, with continuous shaking for 75 seconds, after which suramin was added to a final concentration of 1mg/ml. After a further 2 minutes incubation at 37°C, the EAC43b were diluted in an equal volume of ice-cold GVB=EDTA, centrifuged (2000g for 5 minutes at 4°C) then washed three times in GVB=EDTA and finally resuspended in 5ml GVB=EDTA and incubated at 37°C for 2 hours in a shaking water bath in order to decay C2a. The EAC43b were washed with $DGVB^{++}$and standardized at $1x10^8$ /ml $DGVB^{++}$, stored at 4°C and used within 4 days.

2.34. ASSAYS FOR SOLUBILISATION AND PREVENTION OF IMMUNE PRECIPITATION

Radiolabeling of BSA and thyroglobulin with ^{125}I: The procedure was performed. as described by McConahey and Dixon (1966). Briefly, the protein solution (1mg/ml PBS) was placed in a bijoux containing one millicurie (mCi) of carrier-free ^{125}I, 100µl of fresh solution of chloramine T (1mg/ml) was added and after incubation at room temperature for four minutes, the reaction was stopped by the addition of 100µl of (2mg/ml) sodium metabisulphite. Protein-bound ^{125}I was separated from free ^{125}I by passing the solution through a 25x1cm column of Sephadex G25 Superfine (Pharmacia) pre-equilibrated with PBS, and one ml fractions were collected. The

radioactivity of each fraction was measured by counting 5µl from each fraction using a Packard gamma counter (model 300C). The radioactivity was represented by two peaks. The first peak contained the protein-bound ^{125}I, as determined by precipitation with 10% trichloracetic acid, and the second one represented the protein free ^{125}I. The protein concentration of the first peak was measured spectrophotometrically at OD_{280} and determined as cpm/µg of protein.

The prevention of immune precipitation and solubilisation assays were performed to determine the solubility of nascent and preformed BSA anti-BSA IC and thyroglobulin anti-thyroglobulin IC at the concentrations used in complement activation experiments. This was performed as an attempt to investigate relation of IC solubility with the concentration of C3:P complex produced by these complexes.

Prevention of immune precipitation assay was performed by mixing 5µl antigen (^{125}I-BSA or ^{125}I-thyroglobulin), 10µl antibody and 50µl NHS and the volume was completed to 115µl with GVB^{++} where the concentrations of IC at each antigen-antibody ratios were proportional to that used in complement activation experiments. The reaction mixture, excluding the antibody, was prepared on ice and then incubated at 37°C, and the pre-warmed antibody was added at time zero. Incubation continued for 30 minutes when 50µl sample was taken from each tube into 1ml ice-cold PBS, in a microcap tube, and the IC precipitate removed by centrifugation (Bechman microfuge).

Controls included: (A) precipitable radioactivity by antibody in the absence of serum (GVB^{++}), (B) total radioactivity which is represented by the antigen input alone, for each antigen-antibody ratio, in buffer and (C) TCA precipitable counts: 50µl antigen (BSA or thyroglobulin, 5mg/ml) plus 5µl ^{125}I-antigen plus 60µl 20% TCA.

The percentage of soluble IC was calculated according to the following formula:

$$\% \text{ soluble} = \frac{\text{cpm in supernatant (serum)} - \text{cpm in supernatant (buffer)}}{\text{total cpm/tube} - \text{cpm in buffer supernatant}} \times 100$$

In case of solubilisation, all the experimental conditions were similar to that of the

PIP assay, with the exception that the IC were preformed prior to their addition to the pre-warmed serum.

2.35. RADIOIMMUNOASSAY FOR THE MEASUREMENT OF C3a, C4a AND C5a

Materials and buffers

All reagents were obtained from Amersham International plc, and include the following:

1. Precipitating reagent
2. Assay buffer
3. Tracer (^{125}IC3a des-Arg, ^{125}IC4a des-Arg or ^{125}IC5a des-Arg)
4. Rabbit anti-C3a des-Arg, anti-C4a des-Arg or anti-C5a des-Arg. (First antibody)
5. Standards containing different concentrations of C3a des-Arg, C4a des-Arg and C5a des-Arg (Table 2.2).
6. Goat anti-rabbit antiserum (second antibody).

Microcap centrifuge tubes

Conical bottom assay tubes (70x12mm)

Method

Because of the expense of commercially available anaphylatoxin immunoassay kits, concentrations of C4a, C3a and C5a were only measured in certain studies of complement activation.

The principle of all the assays was the same and include the following steps;

Sample preparation:

The sample (15µl) and the precipitating buffer (75µl) were mixed in microcap tubes. After 30 minutes at room temperature the tubes were centrifuged for 5 minutes in a Beckman microfuge (10000rpm for 5 minutes). The supernatants which were free

of native C4, C3 or C5, were aspirated for assay.

Assay procedure

Each sample was assayed in duplicate and each assay included the following series of controls; total radioactivity (TC), non-specific binding (NSB), blank (Bo) and the five standards containing known concentrations of C4a, C3a or C5a (Table 2.2).

Fifty microlitres of assay buffer were pipetted into all assay tubes, the content of which were then vortex mixed. The standards and samples were pipetted (50µl)into the appropriate tubes, after which the assay tracer (50µl) was added. The TC tubes were then covered with clingfilm and set aside for counting. The antiserum (rabbit anti-C4a, C3a or C5a des Arg) was pipetted (50µl) into all the tubes except NSB to which 50µl isotonic saline was added. The tube contents were then vortex mixed, covered with cling-film and incubated at room temperature for 3 hours. The second antibody (goat anti-rabbit IgG) was added into all tubes except TC tubes, and the contents were re-mixed and incubated at room temperature for 30 minutes. At the end of this incubation period 2ml isotonic saline was added to all tubes except TC tubes. The tubes were centrifuged (2500g for 15 minutes at 4°C) and the supernatant was carefully aspirated. The radioactivity present in each assay tube was counted using a Packard automatic gammacounter (Model 300C).

Calculation of results

The average counts per minutes (cpm) for each set of replicate tubes was calculated and the average NSB cpm was subtracted from all the tubes except TC. The percentage Bo/TC was determined using the following formula:

$$\%Bo/TC = \frac{\text{Bo cpm - NSB cpm}}{\text{TC cpm}} \times 100$$

The percent B/Bo for standards and samples was calculated using the following formula:

$$\frac{\text{Standard or/ Sample cpm - NSB cpm}}{\text{Bo cpm - NSB cpm}} \times 100$$

The standard curve was constructed by plotting the percent B/Bo as a function of the log C3a des-Arg or C5a des-Arg (Fig 2.8) concentration on a log-logit graph paper, while for C4a des-Arg assay the log concentration of C4a was constructed against the %B/Bo of those standards (according to the instruction of the manufacturer). The concentration of anaphylatoxins, in nanograms, per tube were determined directly from the standard curve, and the total concentrations of original samples was calculated by multiplying by the dilution factor.

Table 2.2. Concentrations of C4a des-Arg, C3a des-Arg and C5a des-Arg in the standard curves of the radioimmunoassays used for the determination of C4a, C3a and C5a respectively.

Standard	C4a (ng/50µl)	C3a (ng/50µl)	C5a (ng/50µl)
A	25	65	10
B	10	30	4
C	5	12	1.6
D	2.5	5	0.64
E	1	2	0.25

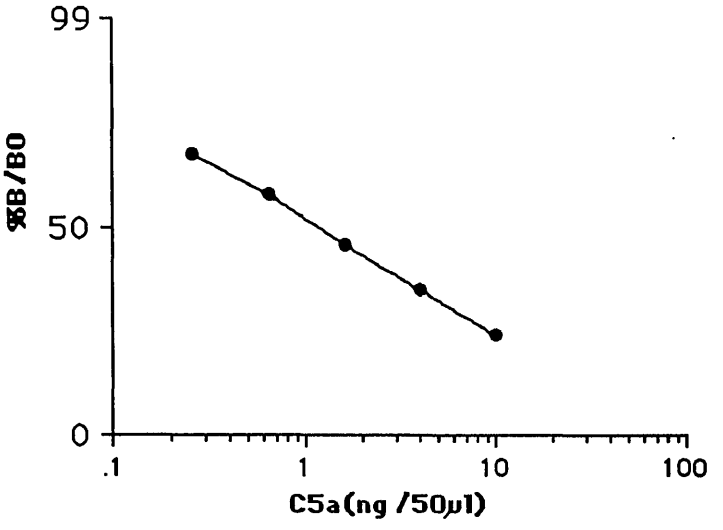


Figure 2.8: Plot of the standard curve of the radioimmunoassay used for the determination of C5a. The concentrations of C5a are plotted on the abscissa (log scale) against their B/Bo% which is shown on the ordinate (logit scale).

CHAPTER

THREE

RESULTS

3.1. DEVELOPMENT OF ELISA PROCEDURES FOR THE MEASUREMENT OF C1s:C1-INH, C3:P AND C5b-9 COMPLEXES

3.1.1. Titration of the coating antibodies

Titration of coating antibodies revealed that antibody concentrations of 10μg/ml, 5μg/ml and 5μg/ml for C1s:C1-INH, C3:P and C5b-9 assays respectively gave good standard curves in terms of sensitivity range, and in terms of the amount of antibody required (Fig 3.1.1).

3.1.2. Titration of biotin-conjugated antibody probes

The optimal biotin-conjugated antibody probe dilution which gave the widest range of sensitivity and best linear fit was found to be with 1/1600, 1/1000 and 1/750 for C1s:C1-INH, C3:P and C5b-9 assays respectively (Fig 3.1.2).

3.1.3. Sensitivity of the assays

The sensitivity limits of the assays were 24-0.75, 29.5-0.92 and 30-0.9 ng/ml for C1-INH (in the C1s:C1-INH assay), C3 (in the C3:P assay) and C5 (in the C5b-9 assay) respectively (Fig 3.1.3). The optical density readings for the samples were read off the standard curve and expressed as ng/ml.

3.1.4. Development of OD

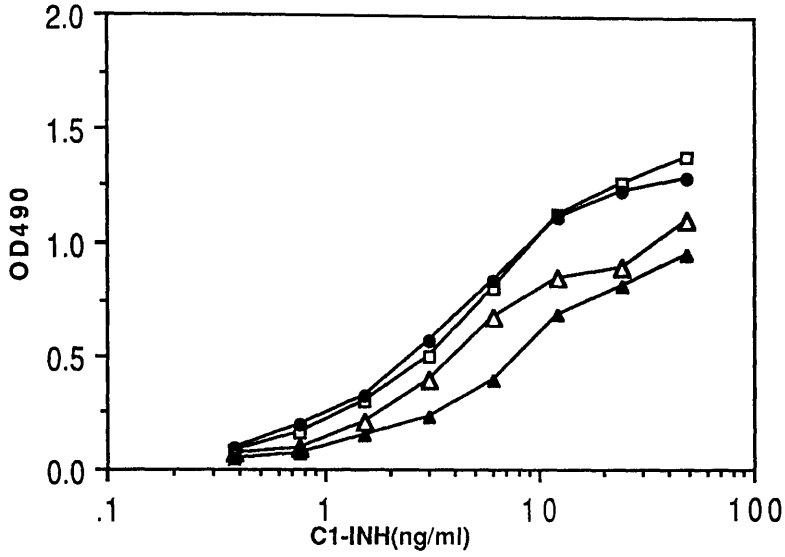
The time course of OD_{490} development showed that the OD_{490} increased with time for each of the standard curves of the three ELISAs (Fig 3.1.4).

3.1.5. Reproducibility of the assays

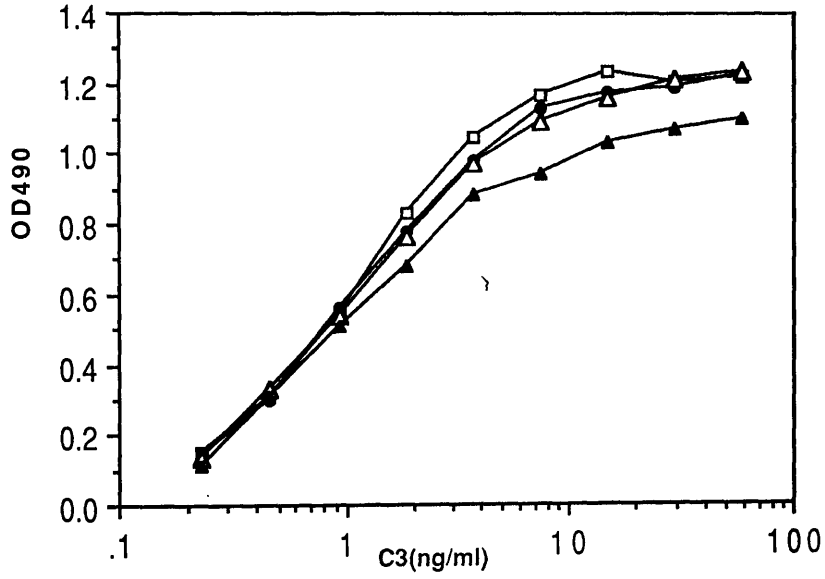
In the three ELISAs all samples were assayed in duplicate. When the difference between the duplicate determinations was greater than 5% of the mean, the sample was retested.

The interassay coefficient of variations of the internal controls, which were tested on ten consecutive experiments, were ±10%, ±3% and ±5% for the C1s:C1-INH, C3:P

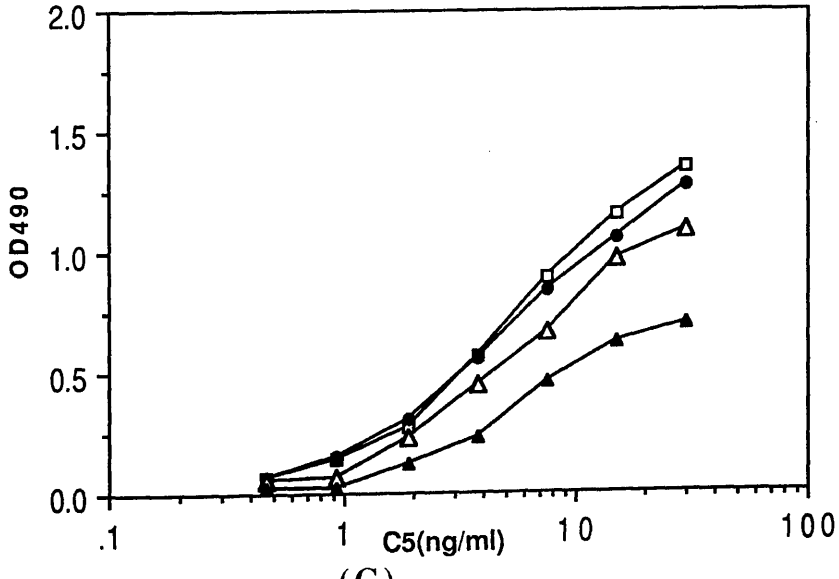
Figure.3.1.1 (A, B and C): Titration of coating antibodies for (A) C1s:C1-INH assay, (B) C3:P assay and (C) C5b-9 assay. The concentrations used are 20µg/ml (□—□), 10µg/ml (●—●), 5µg/ml (△—△) and 2.5µg/ml (▲—▲). The concentration of the antigen was plotted on the abscissa, and the optical density (OD_{490}) on the ordinate.


2.0
1.5
1.0
0.5
0.0
OD490
.1
1
10
100
C1-INH(ng/ml)

(A)

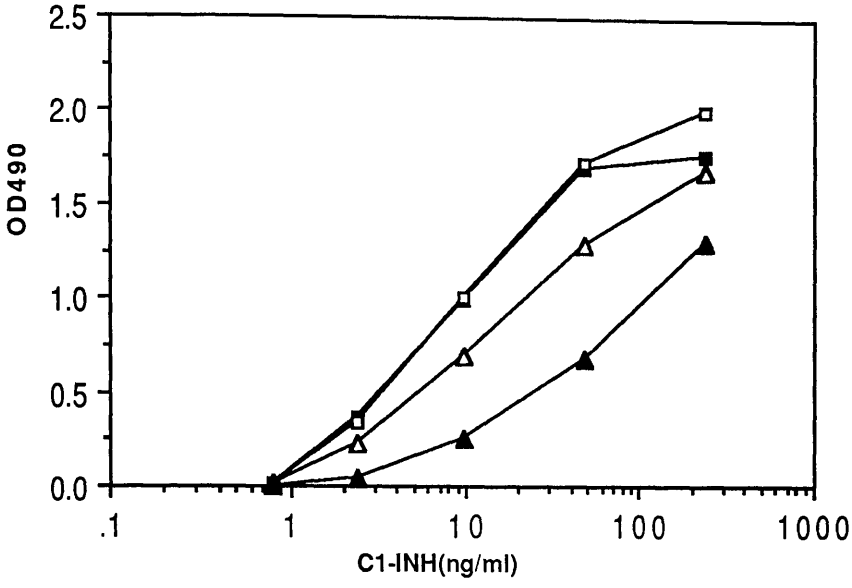

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD490
.1
1
C3(ng/ml)
10
100

(B)

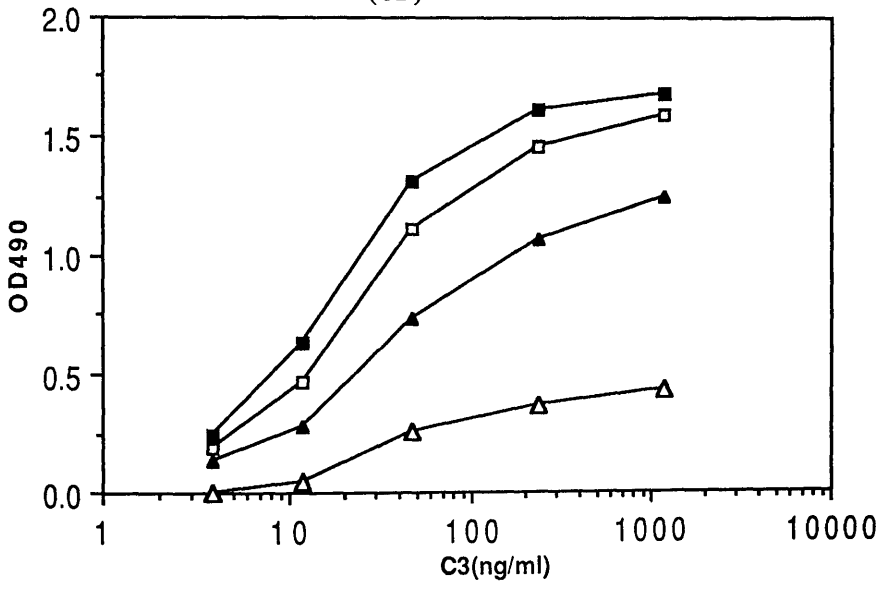

2.0
1.5
1.0
0.5
0.0
OD490
.1
1
C5(ng/ml)
10
100

(C)

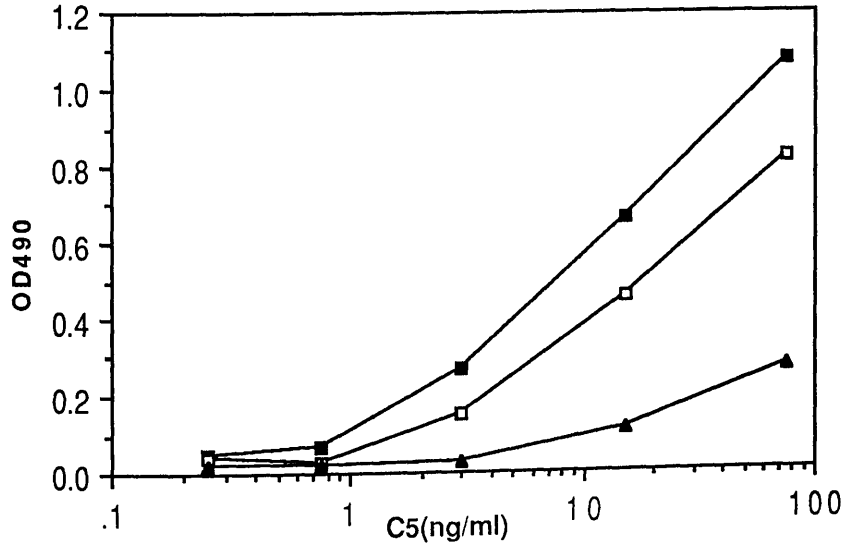
Figure 3.1.2 (A, B and C): Titration of biotin-second antibody conjugates for (A) C1s:C1-INH assay, (B) C3:P assay and (C) C5b-9 assay. The conjugate dilutions for biotin-anti-C1-INH were 1/250 (■—■), 1/1000 (□—□), 1/5000 (▲—▲) and 1/25000 (△—△). The dilutions for biotin-anti-C3 were 1/250 (■—■), 1/1000 (▲—▲), 1/5000 (▲—▲) and 1/25000 (△—△). For the biotin-anti-C5 the dilutions were 1/250 (■—■), 1/750 (□—□) and 1/1500 (▲—▲). The concentration of the antigen was plotted, on the abscissa, against the optical density (OD_{490}) on the ordinate.


2.5
2.0
1.5
1.0
0.5
0.0
OD490
.1
1
10
100
1000
C1-INH(ng/ml)

(A)

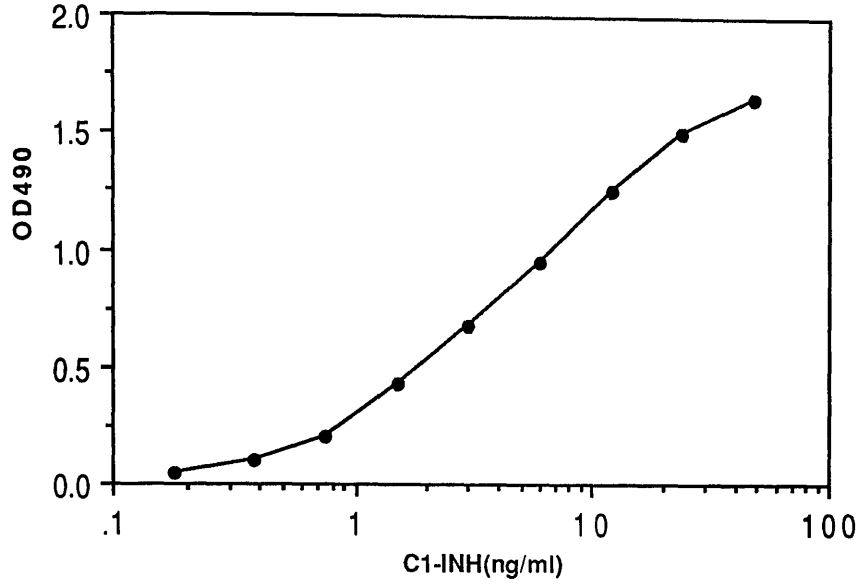

2.0
1.5
1.0
0.5
0.0
OD490
1
10
100
1000
10000
C3(ng/ml)

(B)

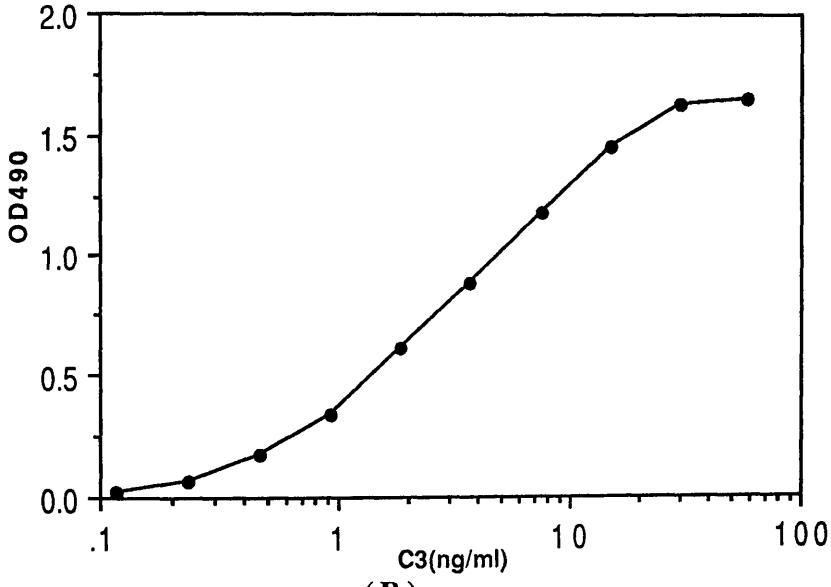

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD490
.1
1
10
100
C5(ng/ml)

(C)

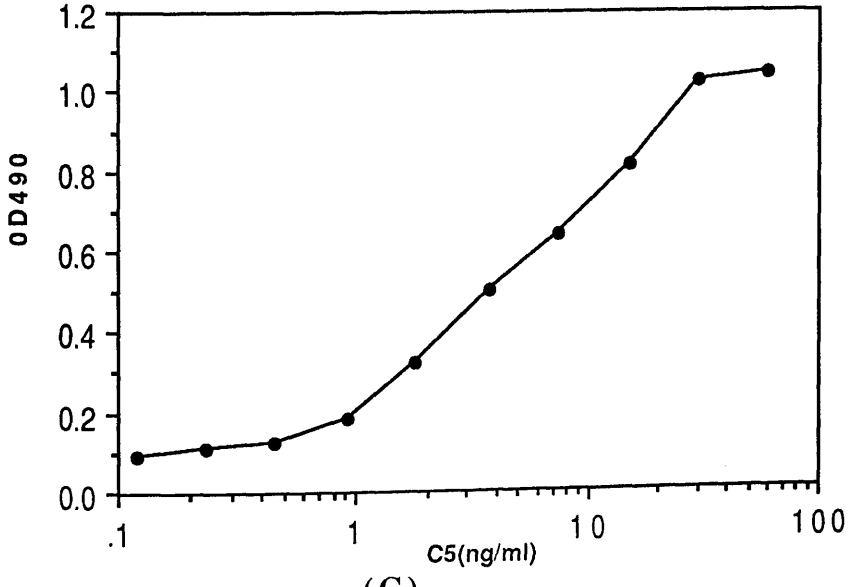
Figure 3.1.3 (A, B and C): The sensitivity limits of the (A) C1s:C1-INH assay, (B) C3:P assay and (C) C5b-9 assay. The concentration of the antigen was plotted, on the abscissa, against the optical density (OD_{490}) on the ordinate.


2.0
1.5
1.0
0.5
0.0
OD490
.1
1
10
100
C1-INH(ng/ml)

(A)

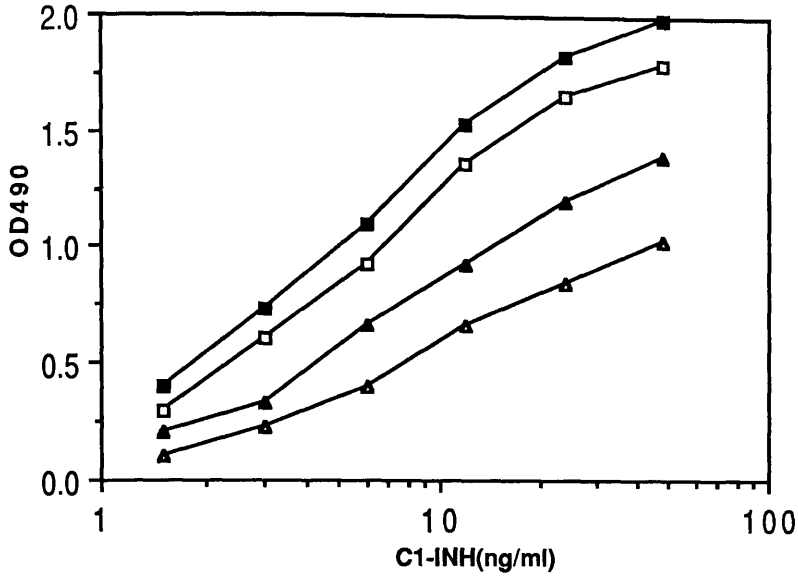

2.0
1.5
1.0
0.5
0.0
OD490
.1
1
10
100
C3(ng/ml)

(B)

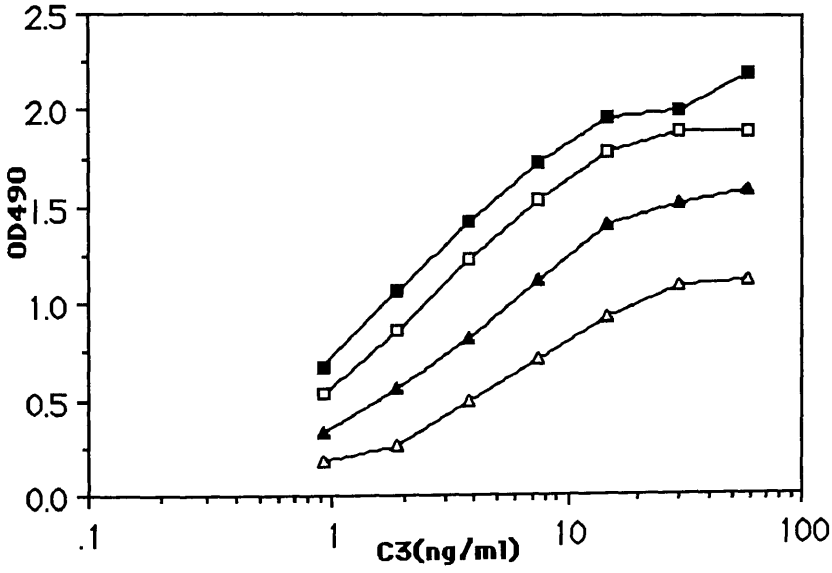

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD490
.1
1
10
100
C5(ng/ml)

(C)

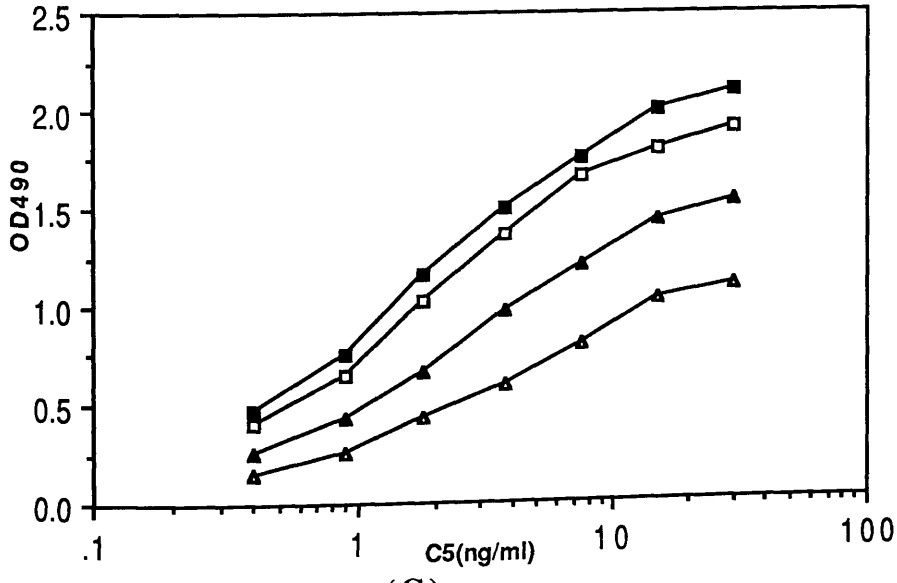
Figure 3.1.4 (A, B and C): Kinetics of OD_{490} development in (A) C1s:C1-INH assay, (B) C3:P assay and (C) C5b-9 assay. The colour development was stopped at 2 minutes (△—△), 4 minutes (▲—▲), 6 minutes (□—□) and 8 minutes (■—■). The concentration of the antigen was plotted, on the abscissa against the optical density (OD_{490}) on the ordinate.


2.0
1.5
1.0
0.5
0.0
OD490
1
10
100
C1-INH(ng/ml)

(A)


2.5
2.0
1.5
1.0
0.5
0.0
OD490
.1
1
10
100
C3(ng/ml)

(B)


2.5
2.0
1.5
1.0
0.5
0.0
OD490
.1
1
10
100
C5(ng/ml)

(C)

and C5b-9 assays respectively.

3.1.6. Stability of samples

Incubation of the multimolecular complexes C1s:C1-INH, C3:P and C5b-9 at 20°C and 37°C for 1, 2 and 4 hours and storage at -70°C for one month did not alter the levels of any of the three multimolecular complexes.

3.2. MEASUREMENT OF COMPLEMENT ACTIVATION COMPLEXES IN PATIENTS AND NORMAL INDIVIDUALS

3.2.1. Levels of complement activation complexes

Level of C1s:C1-INH, C3:P and C5b-9 were measured in normal individuals, SLE and RA patients (Table 3.1). The mean level of complement activation complexes in sero-negative RA patients (mean ± SE of mean) were C1s:C1-INH (80.8ng/ml ± 25), C3:P (44.4ng/ml ± 2.6) and C5b-9 (18ng/ml ± 0.7); in sero-positive RA patients the levels were C1s:C1-INH (271ng/ml ± 51.9), C3:P (90.2ng/ml ± 8.6) and C5b-9 (70ng/ml ± 19.7) and in SLE patients were C1s:C1-INH (77.8ng/ml ± 8.5), C3:P (56ng/ml ± 7.9) and C5b-9 (27ng/ml ± 2.4) (Table 3.1).

3.2.2. Comparison of the levels of complement activation complexes in serum and plasma

Comparison of the levels of the complement activation complexes C1s:C1-INH, C3:P and C5b-9 in normal controls sera and plasma showed no significant difference. However, the RA patients had significantly higher serum levels of C3:P (mean 88ng/ml, median 58ng/ml) compared to plasma (mean 68ng/ml, median 45ng/ml; $p<0.001$ by Wilcoxon pairs test). No significant difference was observed between serum and plasma levels of C1s:C1-INH or C5b-9 in RA patients. However, for the remainder of the study measurements of complement activation products were performed on samples of EDTA-plasma.

3.2.3. Comparison of the levels of the complement activation complexes in normals and SLE and RA patients

In both RA and SLE patients plasma levels of all three activation products were higher than those in normals ($p<0.0001$ for all three, Table 3.1, Fig 3.2.1).

In normal individuals there was a significant correlation (Spearman rank test) between C1s:C1-INH and C3:P and between C3:P and C5b-9 but not between C1s:C1-INH and C5b-9 (Table 3.2). In the RA patients plasma levels of all activation products correlated with each other, while in SLE patients levels of C1s:C1-INH and C3:P correlated with those of C5b-9 (Table 3.2).

C1s:C1INH C3:P C5b-9

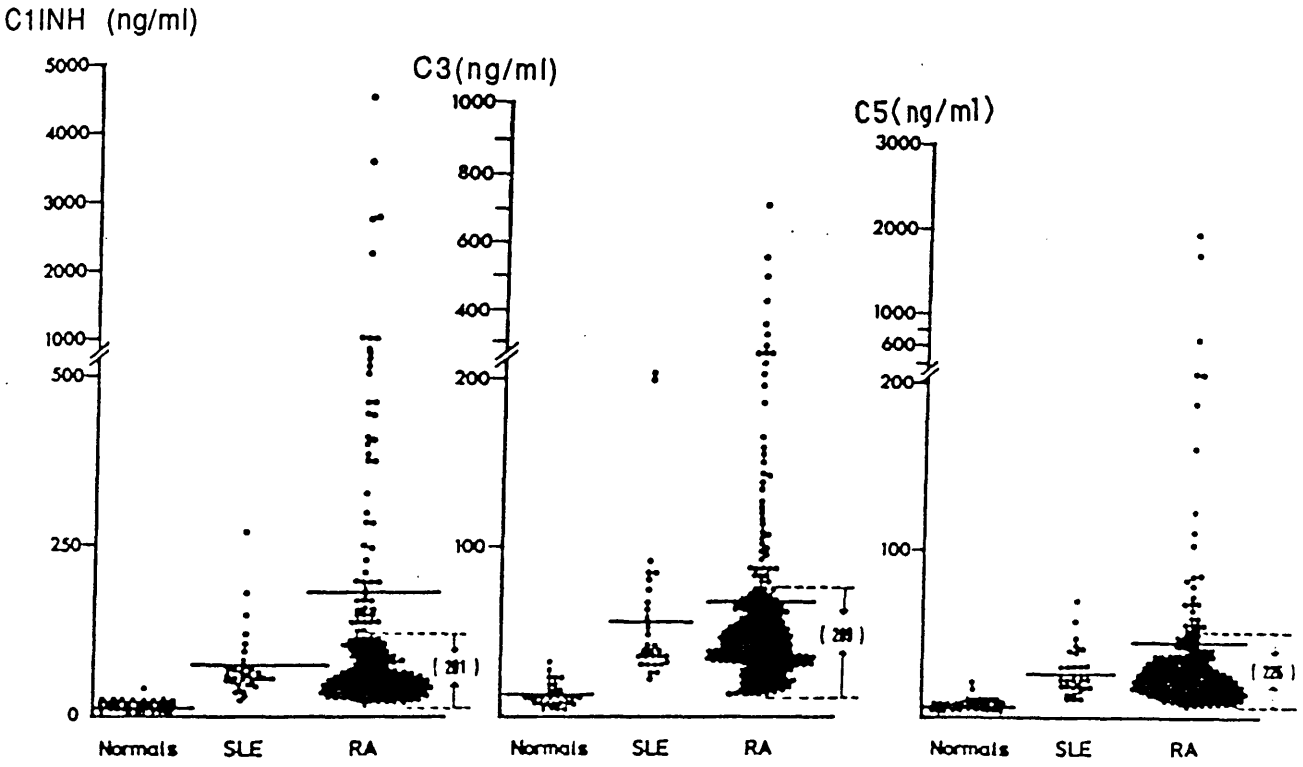


Figure 3.2.1: Levels of the multimolecular complement activation complexes in plasma of normals, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients. Solid lines represent the mean of each group.

Table 3.1. Plasma levels of C1s:C1-INH, C3:P and C5b-9 (ng/ml) in normals and patients with SLE and RA.

Group		C1s:C1-INH	C3:P	C5b-9
Normals	mean	16	13	5
	median	15	12	5
	range	12-40	5-40	3-14
SLE	mean	77.8	56	27
	median	62	36	25
	range	30-265	26-200	9-71
% in normal range		7%	53%	13%
		p<0.0001	p<0.0001	p<0.0001*
RA	mean	182	68.2	45
(total group)	median	68	45	21
	range	27-4600	19-710	5-1950
% in normal range		7%	43%	24%
		p<0.0001	p<0.0001	p<0.0001*
RA	mean	271	90.2	70
(IgM-RF	median	102	58	27
positive)	range	44-4600	21-710	6-1950
% in normal range		0%	26%	12%
		p<0.0001	p<0.0001	p<0.0001*
RA	mean	80.8	44.4	18
(IgM-RF	median	53	34	17
negative)	range	31-2816	20-258	6-51
% in normal range		14%	52%	34%
		p<0.0001	p<0.0001	p<0.0001*

(*) Wilcoxon pairs test

Table 3.2 : Correlations of the three complement activation complexes in plasma samples of normal individuals (n=30) and SLE (n=30) and RA (n=254) by Spearman rank correlation.

GROUP	C1s:C1-INH *versus* C3:P	C1s:C1-INH *versus* C5b-9	C3:P *versus* C5b-9
Normals	r=0.39	r=-0.14	r=-0.038
	p<0.01	NS*	p<0.01
RA	r=0.44	r=0.49	r=0.62
	p<0.0001	p<0.0001	p<0.0001
SLE	r=0.16	r=0.5	r=0.75
	NS*	p<0.001	p<0.0001

(*) not significant

Table 3.3: Correlations between plasma levels of complement activation complexes and the rheumatoid factor isotypes in sero-positive RA patients (Spearman rank correlations).

RF Isotype	C1s:C1-INH	C3:P	C5b-9
IgM-RF	$p<0.0001$	$p<0.01$	$p<0.001$
IgG-RF	$p<0.0001$	$p<0.001$	$p<0.0001$
IgA-RF	$p<0.0001$	$p<0.01$	$p<0.001$

3.2.4. Correlation of rheumatoid factors with complement activation complexes

Division of the RA patients on the basis of IgM-RF sero-positivity, showed that the levels of all three activation complexes were significantly higher in the sero-positive than in sero-negative ($P<0.0001$ for all three complement activation complexes, Wilcoxon pairs test), compared to the normal controls (Table 3.1).

In those patients who were sero-positive, levels of C1s:C1-INH, C3:P and C5b-9 correlated with IgM-RF ($p<0.0001$, $p<0.01$ and $p<0.001$ respectively), IgG-RF ($p<0.0001$, $p< 0.001$ and $p<0.0001$ respectively) and IgA-RF ($p<0.0001$, $p< 0.01$ and $p<0.001$ respectively) (Table 3.3).

3.2.5. Correlation of the complement activation complexes with ESR

In the total RA group ESR levels correlated with C1s:C1-INH ($r= 0.21$, $p<0.001$) and with C5b-9 ($r= 0.18$, $p<0.005$). These correlations persisted when the sero-positive RA group was studied ($r=0.306$, $p<0.001$ and $r=0.18$, $p<0.01$ respectively), but no correlation with ESR was observed in the sero-negative group (Table 3.4).

3.2.6. Levels of complement activation complexes in paired plasma and and synovial fluids:

Synovial fluid levels of C1s:C1-INH, C3:P and C5b-9 were not significantly different from those in paired plasma. The synovial fluid level of C1s:C1-INH and C5b-9 correlated with their respective plasma levels ($r=0.75$, $p<0.005$ and $r= 0.5$, $p<0.0001$ respectively, Wlcoxon pairs test, Fig 3.2.2). However, C3:P levels in synovial fluid did not correlate with those in plasma ($p>0.1$).

3.2.7. IgG versus $F(ab')_2$ fragment of anti-C1-INH, anti-P and anti-C9 as coating antibodies

ELISA plates were coated with whole IgG or the $F(ab')_2$ fragments of the appropriate trapping antibodies and the levels of C1s:C1-INH, C3:P and C5b-9 were measured in 65 samples. Results were analysed by Wilcoxon pair test and Spearman

rank correlation. There was no significant difference in the observed mean or rank of C1s:C1-INH, C3:P or C5b-9 levels, when samples were measured using IgG or $F(ab')_2$ as coating antibody.

3.2.8. Effect of IgM-RF on the levels of complement activation complexes in ELISA

Increasing concentrations of purified IgM-RF (250, 500, and 1000μg/ml) had no effect on the levels of formed C1s:C1-INH, C3:P and C5b-9 measured in EDTA treated unactivated and activated NHS (Fig 3.2.3)

3.2.9. Correlation between levels of complement activation complexes and complement components

Analysis of the correlation between the levels of C3, C4, factor B and C1-INH as measured by nephelometry and levels of C1s:C1-INH, C3:P and C5b-9, by Spearman rank test showed no correlation in SLE patients (Table 3.5). In RA patients C1-INH and factor B correlated with C1s:C1-INH ($r= 0.32$, $p<0.0001$ and $r=0.18$, $p<0.005$ respectively), C1-INH and factor B correlated with C3:P ($r=0.21$, $p<0.001$ and $r=0.13$, $p<0.01$ respectively) and C1-INH correlated with C5b-9 ($r=0.25$, $p<0.0001$). No correlations were observed with C3 or C4 (Table 3.6).

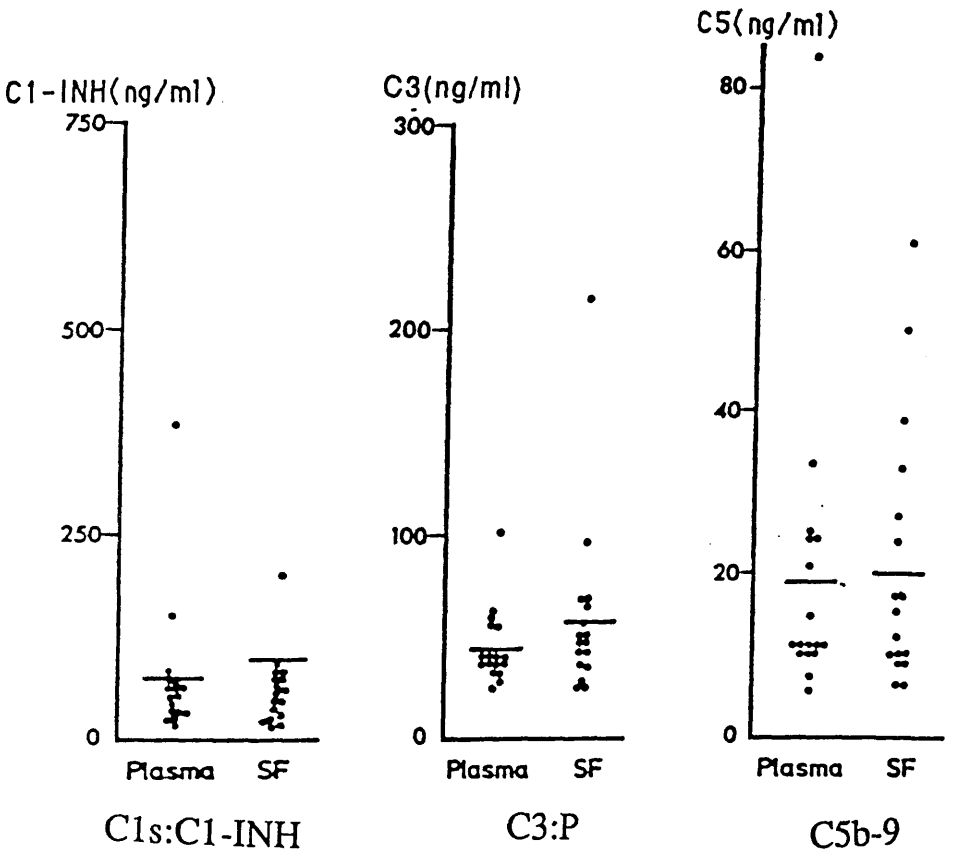


Figure 3.2.2: Distribution of multimolecular complement activation complexes in plasma and synovial fluid of RA patients.

Table 3.4 **Spearman rank correlations of the complement activation complexes with ESR in different RA patients.**

CAC*	RA Group/Subgroup RA (All)	RA (IgM-RF sero +ve)	RA (IgM-RF sero -ve)
C1s:C1-INH	0.2061 (257) 0.001	0.3096 (134) 0.001	-0.0077 (110) 0.5
C3:P	0.1157 (223) 0.1	0.1469 (135) 0.1	-0.0433 (107) 0.5
C5b-9	0.1826 (257) 0.005	0.1875 (135) 0.01	0.0649 (109) 0.5

(Number order: Correlation coefficient, variable number, significance)

(*) Complement activation complexes

Table 3.5 **Spearman rank correlations of the complement activation complexes with level of complement components in SLE patients.**

	Complement Components			
CAC*	**C1-INH**	**C4**	**C3**	**B**
C1s:C1-INH	-0.0232	-0.1515	-0.1875	-0.2254
	(30)	(30)	(30)	(30)
	0.5	0.5	0.1	0.1
C3:P	-0.1385	-0.1472	0.0747	-0.1801
	(30)	(30)	(30)	(30)
	0.5	0.5	0.5	0.1
C5b-9	0.0398	-0.1922	0.0228	-0.1401
	(30)	(30)	(30)	(30)
	0.5	0.1	0.5	0.5

(Number order: Correlation coefficient, variable number, significance)

(*) Complement activation complexes

Table 3.6 **Spearman rank correlations of the complement activation complexes with levels of complement components in RA patients.**

	Complement Components			
CAC*	**C1-INH**	**C4**	**C3**	**B**
C1s:C1-INH	0.3513	-0.0957	0.0855	0.0813
	(267)	(267)	(269)	(269)
	0.0001	0.1	0.1	0.005
C3:P	0.2079	-0.0536	0.0246	0.1296
	(246)	(246)	(248)	(248)
	0.001	0.1	0.5	0.1
C5b-9	0.2477	-0.0803	-0.0197	0.0915
	(267)	(267)	(269)	(269)
	0.001	0.1	0.5	0.1

(Number order: Correlation coefficient, variable number, significance)

(*) Complement activation complexes

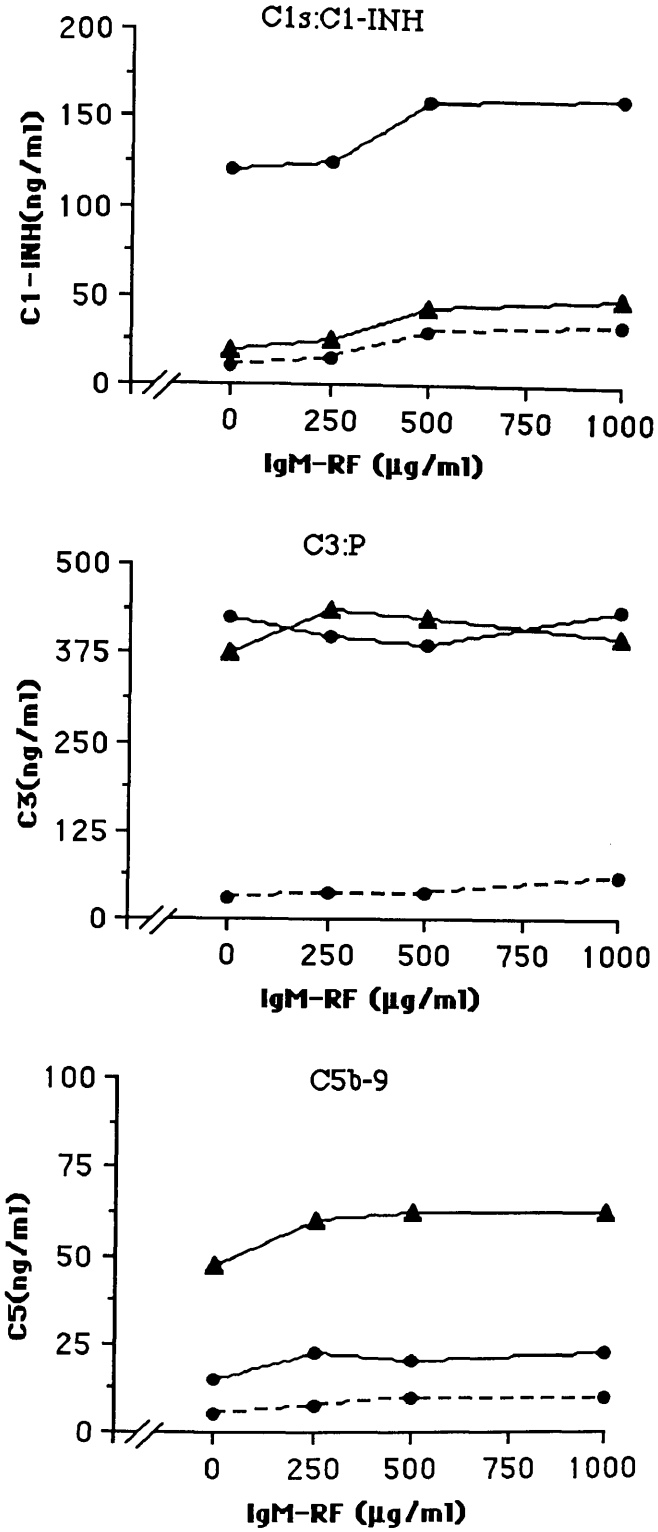


Figure 3.2.3: Effect of IgM-rheumatoid factor on C1s:C1-INH, C3:P and C5b-9 ELISA systems. IgM-RF was added into EDTA-containing IC-activated (●—●), and zymosan-activated (▲—▲) NHS and to buffer (●···●). The level of the multimolecular complement activation complexes (ng/ml) were plotted against the RF concentration (µg/ml).

3.3. COMPLEMENT ACTIVATION BY IMMUNE COMPLEXES

3.3.1. Nascent thyroglobubin anti-thyroglobulin IC

a) Effect of IC concentration

The extent of C1 activation, as assessed by the serum level of the C1s:C1-INH complex and C4a, was dependent upon the concentration of IC for all antigen-antibody ratios (Fig 3.3.1 A).

The amount of C3 turnover, as assessed by C3a generation was dependent on the concentration of IC for all antigen-antibody ratios, with the exception of IC formed at 16 times antigen-excess where the highest concentration of IC (500μg antibody/ml) resulted in less C3a generation than was observed at the second highest concentration of IC (250μg IgG antibody/ml NHS) (Fig 3.3.1 B right panel). In contrast when turnover of C3 was assessed by levels of C3:P complex, although there was evidence of dose dependent effect at antigen-antibody ratios of 16-times antibody-excess, and four and 16-times antigen-excess, the level of C3:P decreased at the highest IC concentrations (Fig 3.3.1 B right panel). In order to explain this finding, the possibility that the C3:P assay could detect both the fluid-phase C3b and properdin (± Bb) complex and C3:P bound to soluble IC was considered. Thyroglobulin containing IC are relatively insoluble and at higher concentrations, a greater proportion would be insoluble. If some of the C3:P complex is bound to IC then these would also be precipitated at these higher IC concentrations. This point is discussed later (section 3.3.8).

C5 activation as assessed by the formation of C5b-9 complex formation and C5a generation was related to IC concentration at antigen-antibody ratios of 4-times and 16-times antibody-excess, but did not show any clear dose dependent effect when IC were formed at equivalence or in antigen-excess (Fig 3.3.1 C).

b) Effect of antigen-antibody ratio

C1 activation as measured by C1s:C1-INH generation, occurred most efficiently when IC were formed in antibody-excess (particularly at 4-times antibody-excess) and

at equivalence (Fig 3.3.2 A left panel), while little activation occurred in antigen-excess. These results were confirmed by the results of C4a assays, although there was a consistent, but unexplained increase in C4a when IC were formed at 16-times antigen-excess (Fig 3.2 2 A right panel).

Activation of C3 as shown by C3a formation was again efficient in antibody-excess and at equivalence with little C3a generated when IC were formed in antigen-excess (Fig 3.3.2 B right panel).

At the two lowest IC concentrations studied, C3:P levels were greatest at 4-times antibody-excess and at equivalence and was lower when IC were formed in 16-times antibody-excess and in antigen-excess (Fig 3.3.2 B left panel). At the higher concentrations of IC, less C3:P was detected at 4-times antibody-excess and at equivalence than at the other antigen-antibody ratios (Fig 3.3.2 B left panel). The possibility that this lower concentration of C3:P at 4-times antibody-excess and equivalence is due to precipitation of IC-bound C3:P is considered later (Section 3.3.8).

The effect of antigen-antibody ratio on C5 activation as assessed by formation of C5a was the same as that seen for C1 activation and C3a generation, with most activation occurring when IC were formed at 4-times antibody-excess and least activation occurring in antigen-excess (Fig 3.3.2 C). In contrast, the greatest level of the C5b-9 complex was produced at 16-times antibody-excess, with very little being generated when IC formed at equivalence or in antigen-excess (Fig 3.3.2 C).

c) Role of classical and alternative pathways

When IC were formed in Mg^{++}EGTA-treated serum, no evidence of C1-activation, as assessed by measuring C1s:C1-INH and C4a levels, was detected.

At all antigen-antibody ratios less C3a was produced in Mg^{++}EGTA-treated serum than in NHS, but as in NHS, in Mg^{++}EGTA the greatest formation of C3a occurred in antibody-excess and at equivalence (Fig 3.3.3 A right panel).

With lower concentrations of IC (62,5µg IgG/ml and 125µg IgG/ml) the greatest amounts of C3:P complex were formed at 4-times antibody-excess and at equivalence, but the amount of these complexes which was formed in Mg^{++}EGTA was less than that formed in NHS (Fig 3.3.3 A left panel). When the highest concentration of IC was studied in Mg^{++}EGTA treated serum there was no clear effect of antigen-antibody ratio on C3:P formation (Fig 3.3.4A left panel). In this case the amount of C3:P present was the same as that formed when IC were formed at equivalence or 4-times antibody-excess in normal serum.

C5a generation in the presence of Mg^{++}EGTA was reduced when the IC were formed in 16-times antibody-excess, 4-times antibody-excess and equivalence, while in IC formed at antigen-excess the C5a generation was similar to that in the presence of normal serum (Fig 3.3.4 B right panel). The dose-response studies showed a reduction in C5a generation at the higher concentrations of IC (250 and 500µg IgG antibody/ml NHS) (Fig 3.3.3 B right panel).

C5b-9 formation was only reduced very slightly in Mg^{++}EGTA treated serum (Fig 3.3.3 B, 3.3.4 B left panels).

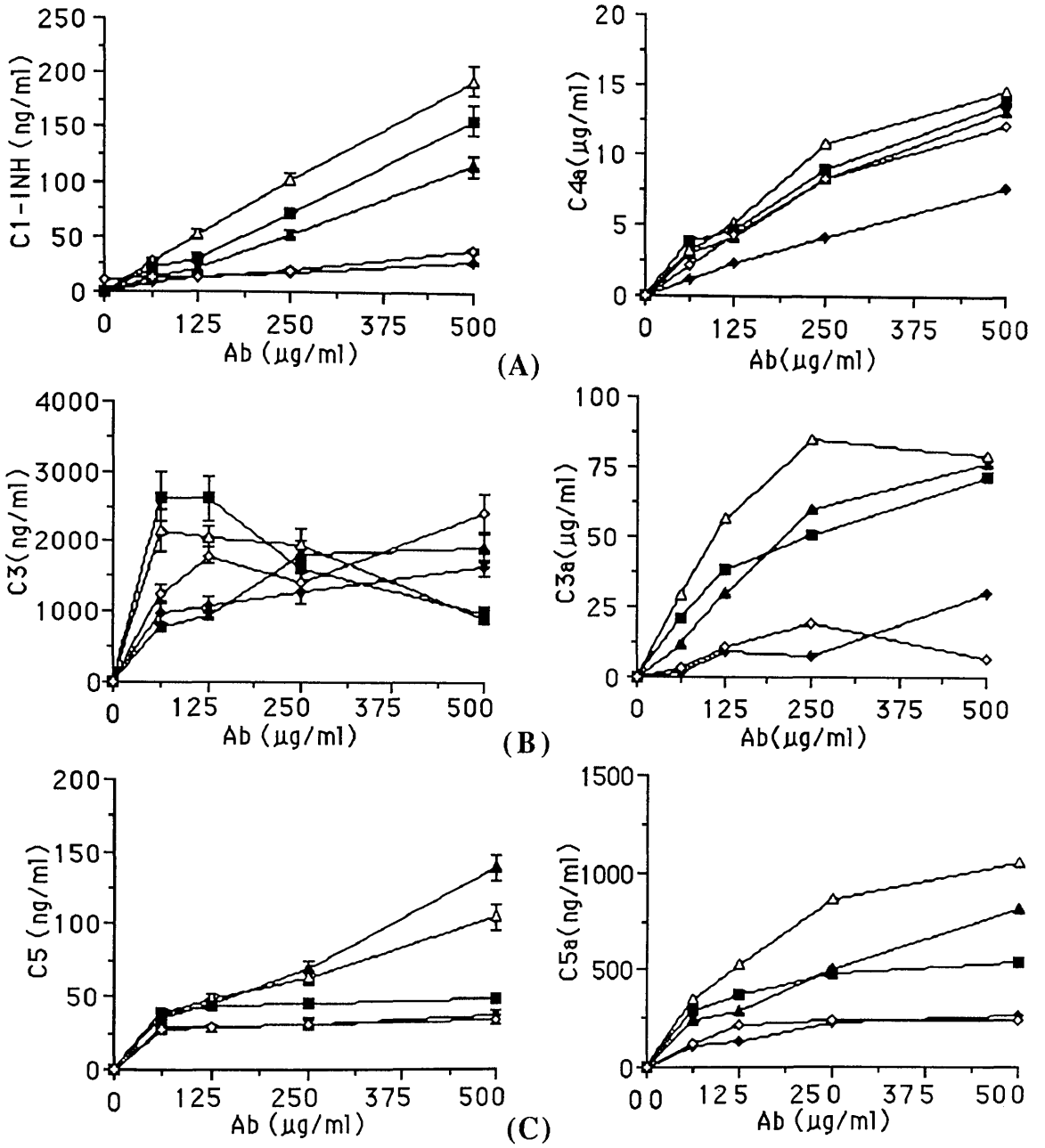


Figure 3.3.1 (A, B and C): Effect of of nascent thyroglobulin anti-thyroglobulin IC concentration on complement activation. Five antigen-antibody ratios were used, 16-times antibody excess (▲—▲), 4-times antibody excess (△—△), equivalence (■—■), 4-times antigen excess (◆—◆) and 16-times antigen excess (◇—◇). (A) C1 activation as assessed by generation of C1s:C1-INH (left panel) and C4a (right panel),(B) C3 activation as assessed by C3:P formation (left panel) and C3a generation (right panel) and (C) C5 activation as assessed by assembly of C5b-9 (left panel) and C5a (right panel). Bars represent the standard error of the mean of three determinations. Anaphylatoxin measurements are the mean of duplicate determinations. Concentration of C1s:C1-INH expressed as ng/ml C1-INH, C3:P as ng/ml C3 and C5b-9 as ng/ml C5.

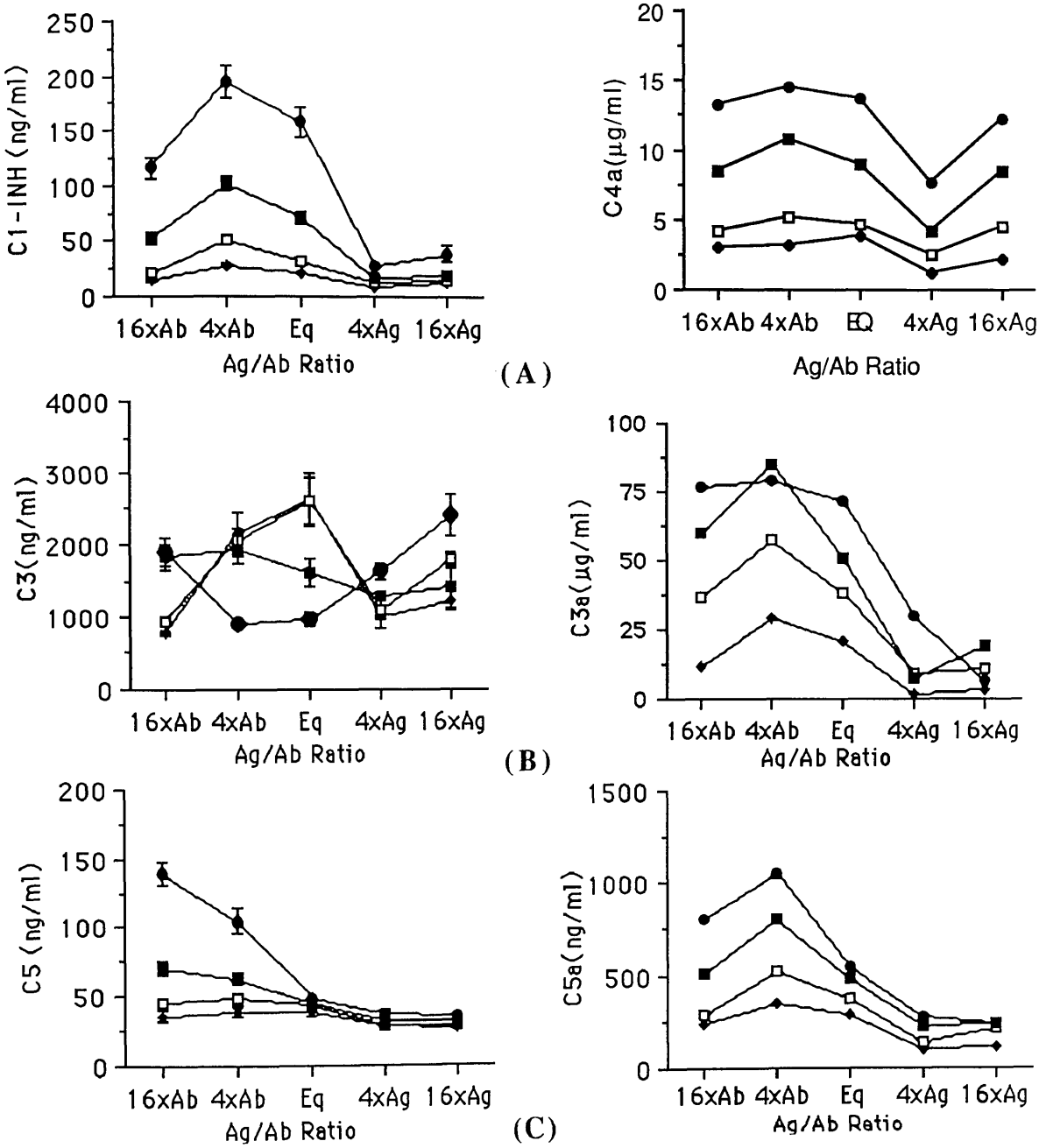


Figure 3.3.2 (A, B and C): Effect of antigen-antibody ratio of nascent thyroglobulin anti-thyroglobulin IC on complement activation. Four differet IC concentrations were used; 62.5 (◆—◆), 125 (□—□), 250 (■—■) and 500µg (●—●) IgG antibody/ml NHS. (A) C1 activation as assessed by generation of C1s:C1-INH (left panel) and C4a (right panel), (B) C3 activation as assessed by C3:P formation (left panel) and C3a generation (right panel) and (C) C5 activation as assessed by assembly of C5b-9 (left panel) and C5a (right panel). Bars represent the standard error of the mean of three determinations. Anaphylatoxin measurements are the mean of duplicate determinations. Concentration of C1s:C1-INH expressed as ng/ml C1-INH, C3:P as ng/ml C3 and C5b-9 as ng/ml C5.

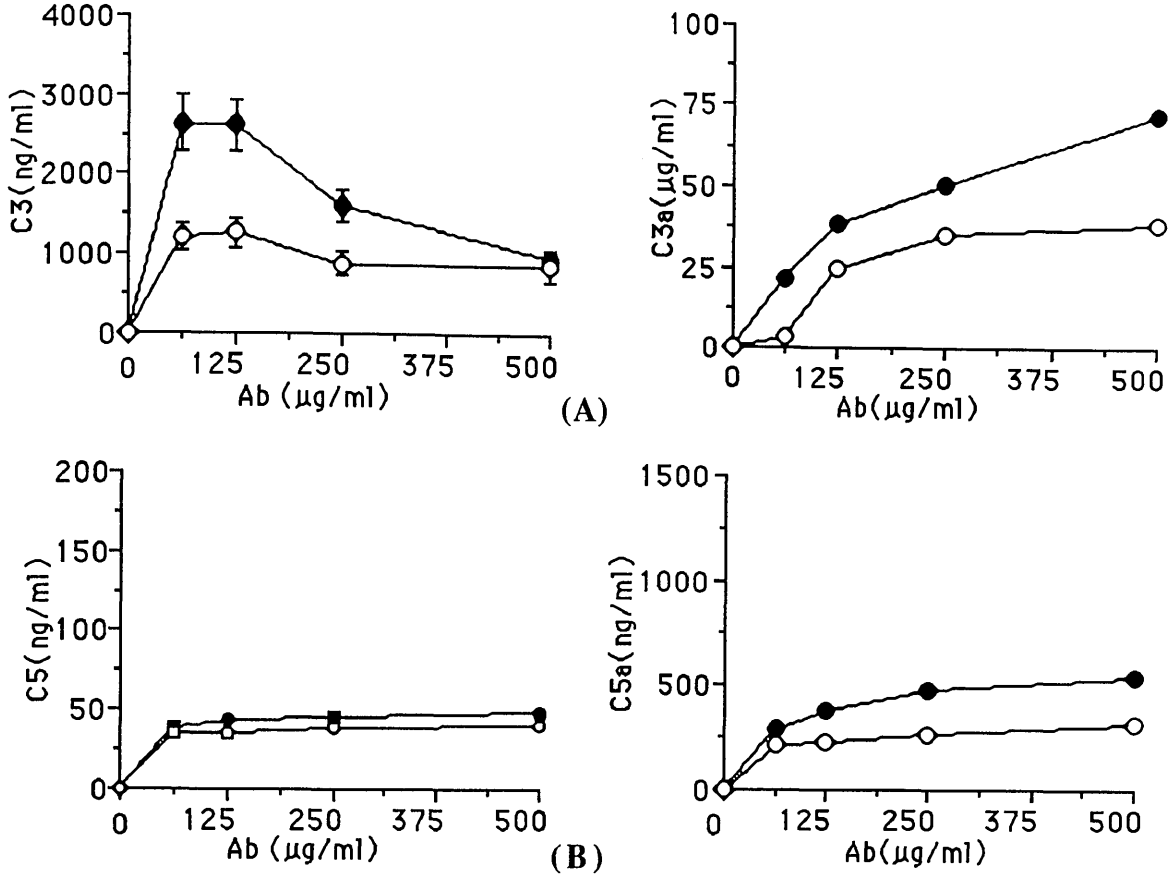


Figure 3.3.3 (A and B): Role of the classical and alternative pathways on activation of C3 and C5 by nascent thyroglobulin anti-thyroglobulin IC at equivalence (dose response curves) in the absence (●—●) or the presence (○—○) of Mg^{++}EGTA. (A) Generation of C3:P (left panel) and C3a (right panel). (B) Generation of C5b-9 (left panel) and C5a (right panel). Bars represent the standard error of the mean of three determinations. Anaphylatoxin measurements are the mean of duplicate determinations. Concentration of C3:P expressed as ng/ml C3 and C5b-9 as ng/ml C5.

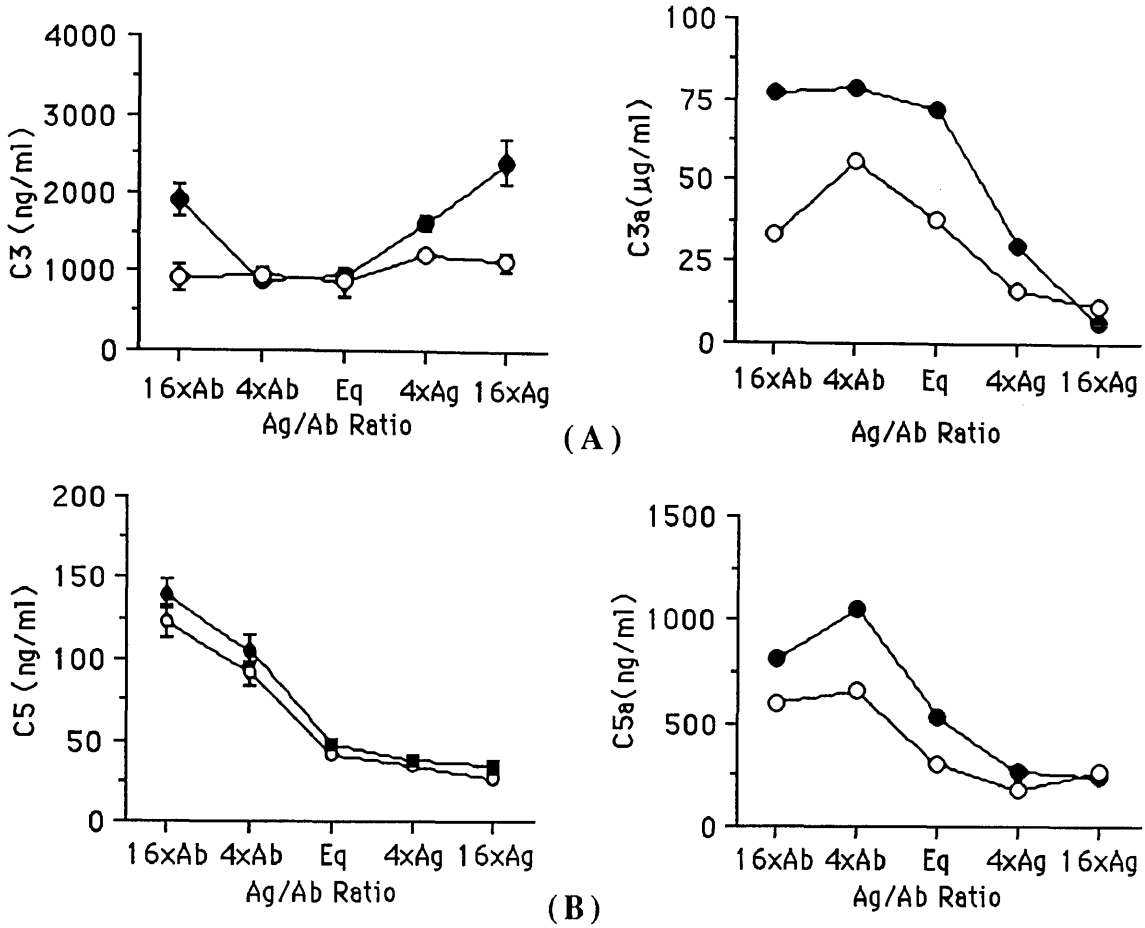


Figure 3.3.4 (A and B): Role of the classical and the alternative pathways on C3 and C5 activation by nascent thyroglobulin anti-thyroglobulin IC at equivalence (Effect of antigen-antibody ratios) at IC dose of 500µg IgG antibody/ml.in the absence (●—●) or the presence (○—○) of MgEGTA. (A) Generation of C3:P (left panel) and C3a (right panel). (B) Generation of C5b-9 (left panel) and C5a (right panel). Bars represent the standard error of the mean of three determinations. Anaphylatoxin measurements are the mean of duplicate determinations. Concentration of C3:P expressed as ng/ml C3 and C5b-9 as ng/ml C5.

3.3.2 Preformed thyroglobulin anti-thyroglobulin IC

a) Effect of IC concentration

Activation of C1, as assessed by C1s:C1-INH and C4a levels was dependent upon the concentration of IC for all antigen-antibody ratios (Fig 3.2 5 A).

The amount of C3 turnover, as assessed by C3a generation, was also dependent upon the concentration of IC added to the serum (Fig 3.3.5 B right panel). However the concentration of C3:P complex was related to the concentration of IC only at the extremes of antigen or antibody-excess (16-times antibody-excess and 16-times antigen-excess) (Fig 3.3.5 B left panel).

Levels of C5a increased with IC concentration at all antigen-antibody ratios (Fig 3.3.5 C right panel). When IC formed at 16-times and 4-times antibody-excess were incubated with serum, the amount of C5b-9 generation was proportional to the concentration of IC. There was no clear dose-response relation at the other antigen-antibody ratios (Fig 3.3.5 C left panel).

b) Effect of antigen-antibody ratio

C1 activation, as assessed by C1s:C1-INH and C4a concentrations, was dependent upon antigen-antibody ratio. IC formed at 4-times antibody-excess were the most effective activators of C1 while those formed at 4-times antigen-excess and 16-times antigen-excess were the least effective, when assessed by C1s:C1-INH formation (Fig 3.3.6 A left panel). The overall picture was similar when C4a was measured, although, in this assay IC formed in 16-times antibody-excess appeared to be about as effective as those formed in 4-times antigen-excess and 16-times antigen-excess (Fig 3.3.6 A right panel).

C3 activation, as assessed by C3a generation was also dependent upon antigen-antibody ratio. IC formed at 4-times antibody-excess and at equivalence were the most effective (Fig 3.3.6 B right panel).

At the lowest concentration of IC, the generation of C3:P was most efficient with

IC formed at equivalence or 4-times antibody-excess, but this pattern was lost as the concentration of IC was increased (Fig 3.3.6 B left panel). At the two highest IC concentrations, the highest levels of C3:P were produced at the extremes of antibody (16-times) and antigen-excess (16-times). This is similar to the pattern observed with nascent IC.

C5a generation was most efficient with IC formed at 4-times antibody-excess whereas IC formed in antigen-excess were least effective (Fig 3.3.6 C right panel). When C5 activation was measured by C5b-9 production, IC formed at 16-times antibody-excess were most effective, with the efficiency of activation decreasing as the proportion of antigen in the IC increased (Fig 3.3.6 C left panel).

c) Role of classical and alternative pathways

C1 activation did not occur in Mg^{++}EGTA-treated serum.

C3a generation was reduced by approximately 50% at all antigen-antibody ratios in the presence of Mg^{++}EGTA (Figs. 3.3.7 B; 3.3.8 B right panels). Similarly, in Mg^{++}EGTA-treated serum, levels of the C3:P complex were reduced by 50% for IC formed in antigen-excess. In antibody-excess the reduction was less pronounced and no reduction was seen at equivalence (Figs. 3.3.7 A; 3.3.8 A left panels).

C5a generation by IC formed at 4-times antibody-excess, 4-times antigen-excess and at equivalence was reduced by 30-40% by Mg^{++}EGTA (Figs. 3.3.7 B; 3.3.8 B right panels). A reduction of about 25% was found at the higher concentrations of IC formed at 4-times antibody-excess, but no reduction was seen at the lower concentrations and at this ratio or for any of the ratios seen at 16-times antibody-excess. There was little difference or only a small reduction in the amounts of C5b-9 generated by IC in antibody-excess in Mg^{++}EGTA-treated serum (Figs. 3.3.7 B; 3.3.8 B left panels). IC formed at equivalence or in antigen-excess produced as much C5b-9 in the presence of Mg^{++}EGTA as they did in NHS.

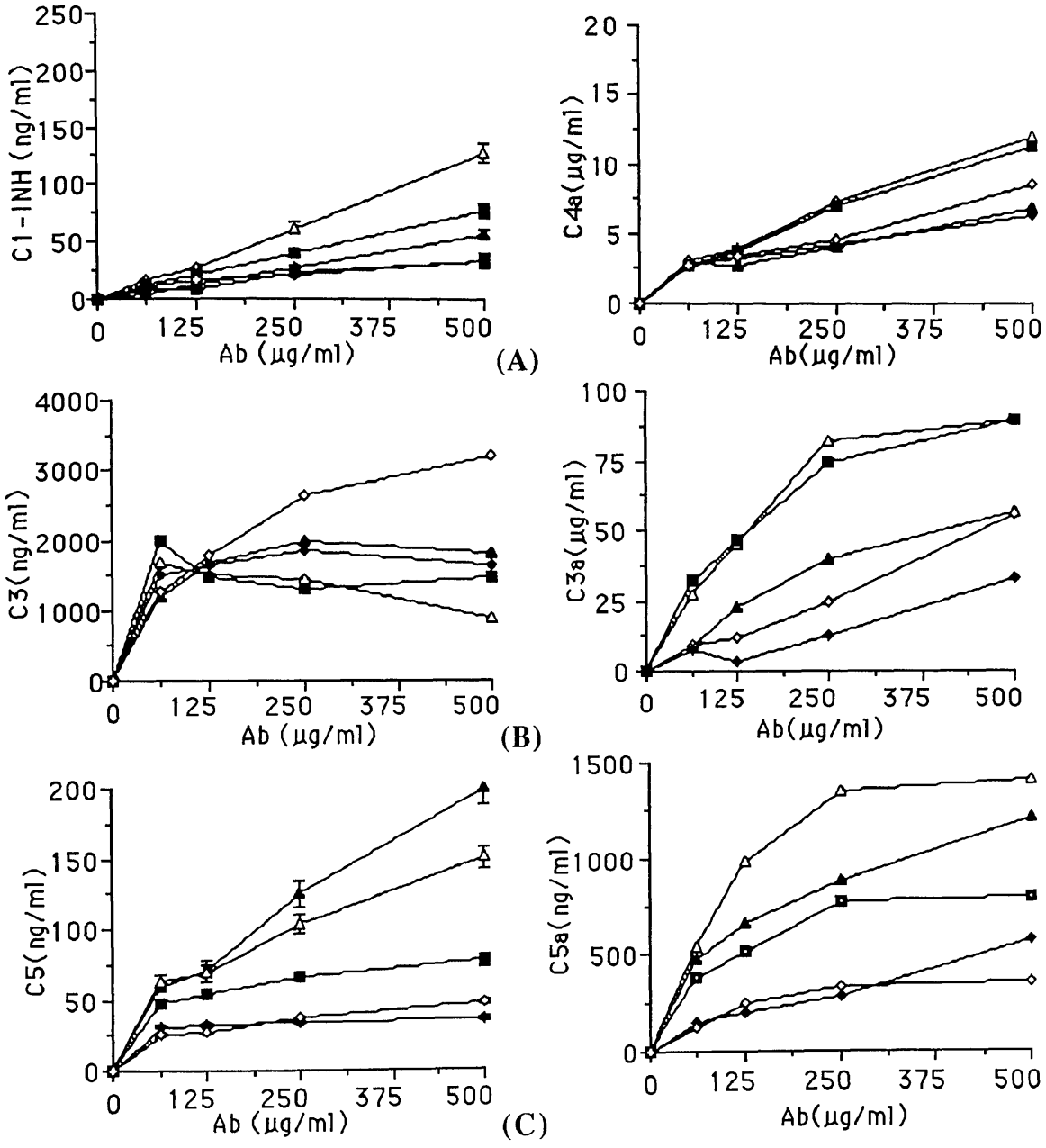


Figure 3.3.5 (A, B and C): Effect of IC concentration of preformed thyroglobulin anti-thyroglobulin IC on complement activation at five antigen-antibody ratios, 16-times antibody excess (▲—▲), 4-times antibody excess (△—△), equivalence (■—■), 4-times antigen excess (◆—◆) and 16-times antigen excess (◇—◇). (A) C1 activation as assessed by generation of C1s:C1-INH (left panel) and C4a (right panel), (B) C3 activation as assessed by C3:P formation (left panel) and C3a generation (right panel) and (C) C5 activation as assessed by assembly of C5b-9 (left panel) and C5a (right panel). Bars represent the standard error of the mean of three determinations. Anaphylatoxin measurements are the mean of duplicate determinations. Concentration of C1s:C1-INH expressed as ng/ml C1-INH, C3:P as ng/ml C3 and C5b-9 as ng/ml C5.

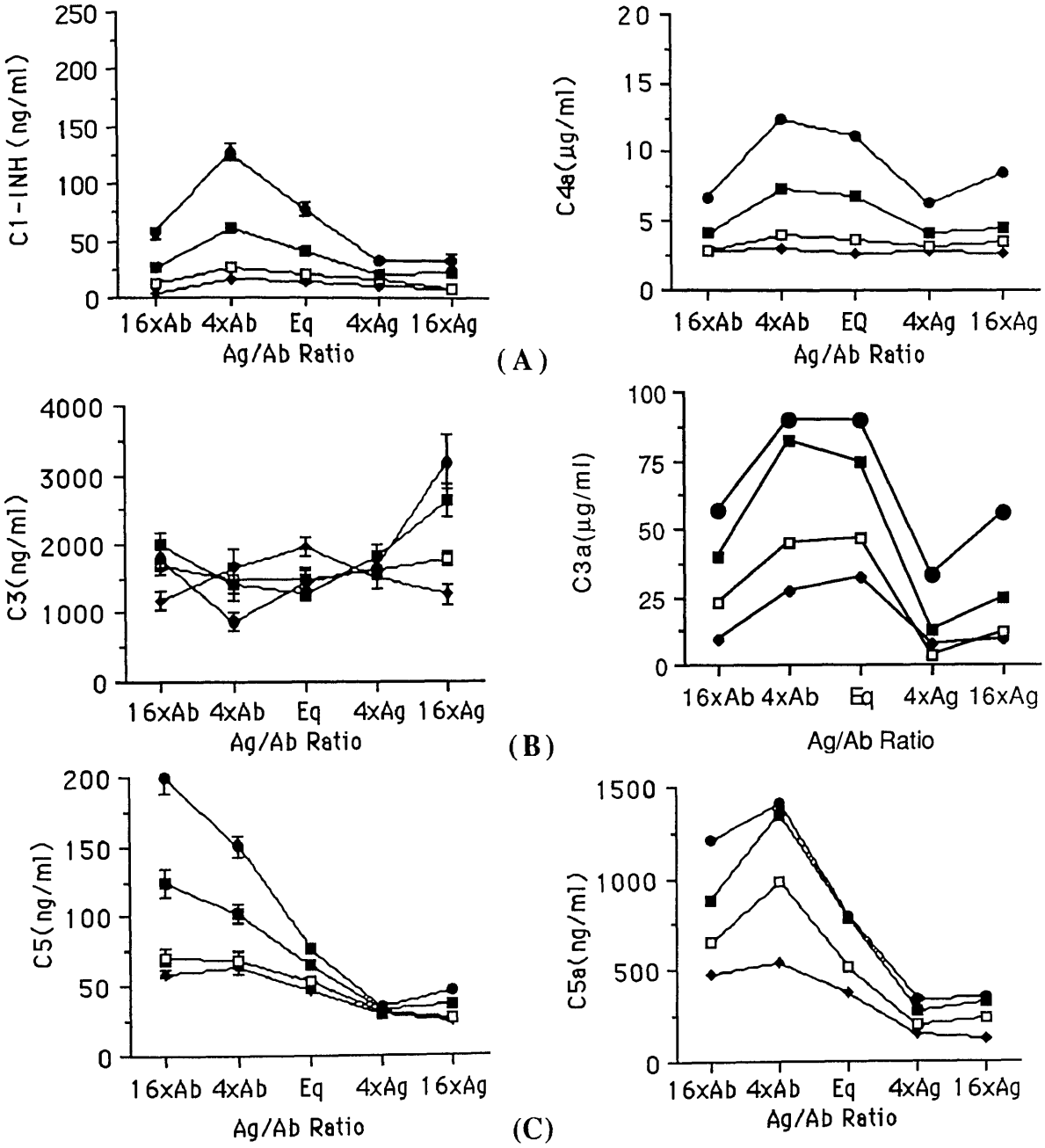


Figure 3.3.6 (A, B and C): Effect of antigen-antibody ratio of preformed thyroglobulin anti-thyroglobulin IC on complement activation. Four different IC concentrations were used, 62.5 (◆—◆), 125 (□—□), 250 (■—■) and 500µg IgG antibody/ml NHS (●—●). (A) C1 activation as assessed by generation of C1s:C1-INH (left panel) and C4a (right panel),(B) C3 activation as assessed by C3:P formation (left panel) and C3a generation (right panel) and (C) C5 activation as assessed by assembly of C5b-9 (left panel) and C5a (right panel). Bars represent the standard error of the mean of three determinations. Anaphylatoxin measurements are the mean of duplicate determinations. Concentration of C1s:C1-INH expressed as ng/ml C1-INH, C3:P as ng/ml C3 and C5b-9 as ng/ml C5.

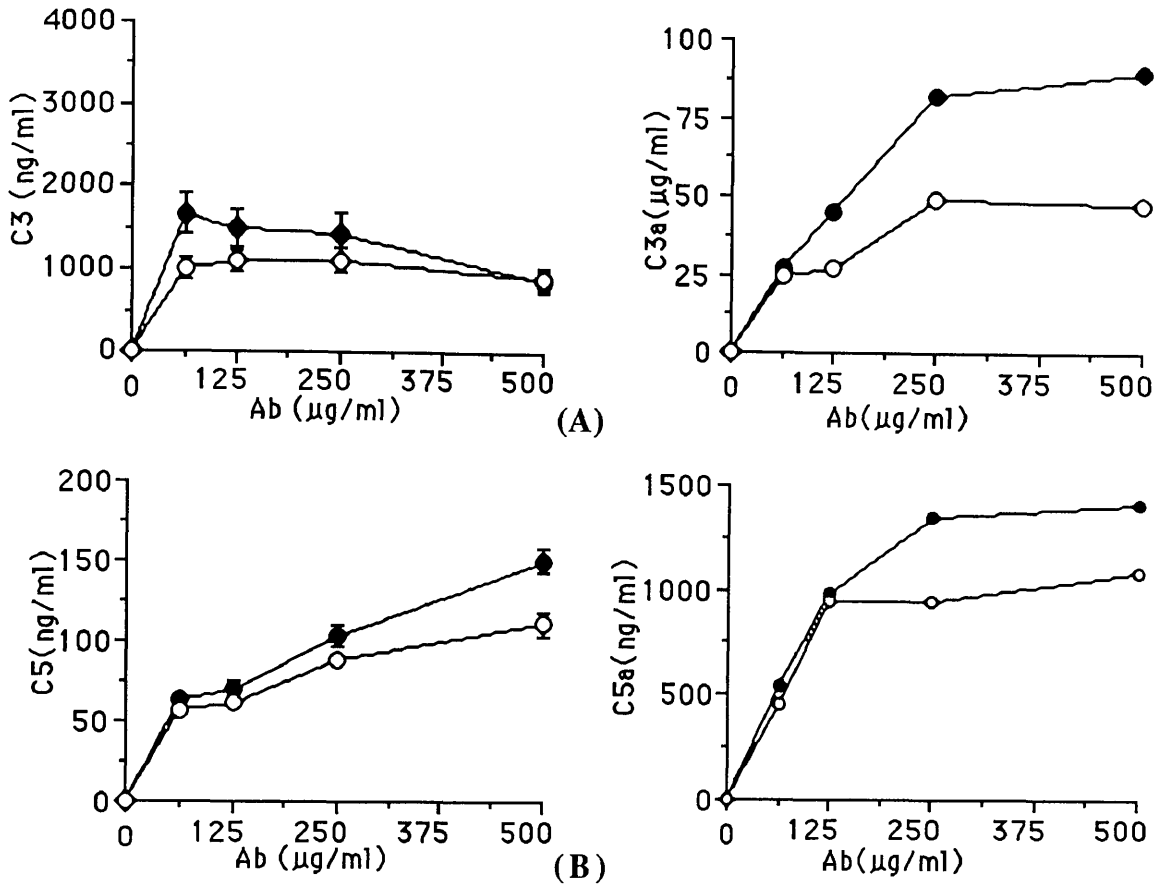


Figure 3.3.7 (A and B): Role of the classical and the alternative pathways on activation of C3 and C5 by preformed thyroglobulin anti-thyroglobulin IC at 4-times antibody-excess (effect of IC concentration) in the absence (●—●) or the presence (○—○) of $Mg^{++}EGTA$. (A) Generation of C3:P (left panel) and C3a (right panel). (B) Generation of C5b-9 (left panel) and C5a (right panel). Bars represent the standard error of the mean of three determinations. Anaphylatoxin measurements are the mean of duplicate determinations. Concentration of C3:P expressed as ng/ml C3 and C5b-9 as ng/ml C5.

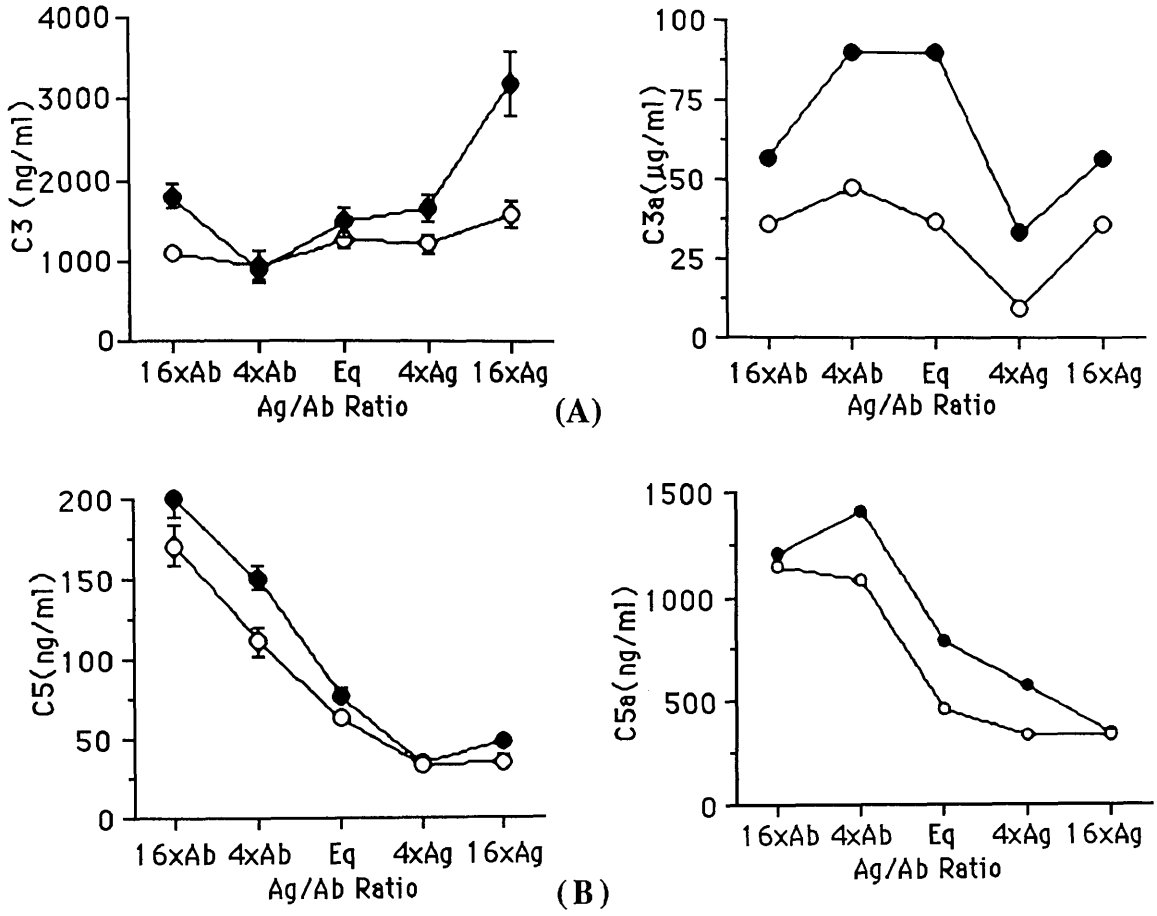


Figure 3.3.8 (A and B): Role of the classical and the alternative pathways on activation of C3 and C5 by preformed thyroglobulin anti-thyroglobulin IC at five antigen-antibody ratios (effect of antigen-antibody ratios), at IC dose of 500µg IgG antibody/ml NHS, in the absence (●—●) or the presence (○—○) of Mg^{++}EGTA. (A) Generation of C3:P (left panel) and C3a (right panel). (B) Generation of C5b-9 (left panel) and C5a (right panel). Bars represent the standard error of the mean of three determinations. Anaphylatoxin measurements are the mean of duplicate determinations. Concentration of C1s:C1-INH expressed as ng/ml C1-INH, C3:P as ng/ml C3 and C5b-9 as ng/ml C5.

3.3.3. Comparison of complement activation by nascent and preformed thyroglobulin IC

As a generalization, IC formed in serum produced more C1 activation than preformed IC (Fig 3.3.9). At the extremes of antigen and antibody-excess there was either little difference or slightly less C1 activation with preformed IC.

C3a generation was higher with preformed IC than with nascent IC (Fig 3.3.9 B). The studies of C3:P complex formation showed that in antigen-excess more generation occurred with preformed IC.

Preformed IC at all antigen-antibody ratios produced more C5a than IC which were formed in the presence of serum. C5b-9 generation was greater with preformed IC in antibody-excess or at equivalence than with IC formed in serum at the same antigen-antibody ratio (Fig 3.3.9 C).

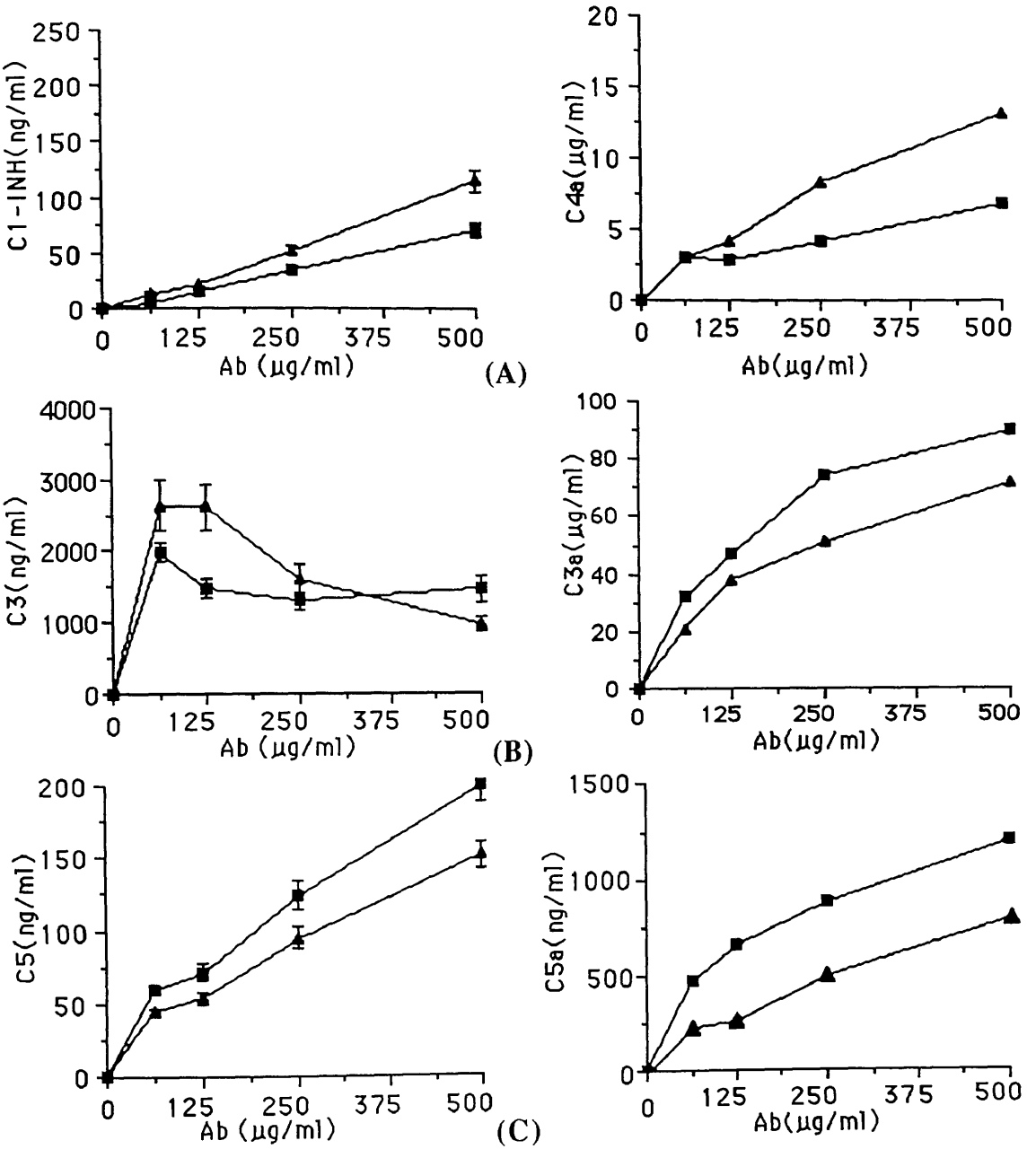


Figure 3.3.9 (A, B and C): Comparison of complement activation by nascent (▲—▲) and preformed (■—■) thyroglobulin anti-thyroglobulin IC at 16-times antibody excess (A and C) and at equivalence (B). A) C1 activation as assessed by the generation of C1s:C1-INH complex (left panel) and C4a generation (right panel), B) C3 activation as assessed by the generation of C3:P complex (left panel) and C3a generation (right panel) and C) C5 activation as assessed by the generation of C5b-9 complex (left panel) and C5a generation (right panel). Bars represent standard error of the mean of three determinations. Anaphylatoxin measurements are the mean of duplicate determinations. Concentration of C1s:C1-INH expressed as ng/ml C1-INH, C3:P as ng/ml C3 and C5b-9 as ng/ml C5.

Comparison of C4a and C1s:C1-INH as measures of C1 activation

There was a good correlation between levels of C4a and C1s:C1-INH at all antigen-antibody ratios for IC formed in the presence of serum and for preformed IC (Fig 3.3.10 A). When IC were formed in serum at antigen-excess there appeared to be disproportionately more C4a formed than C1s:C1-INH.

Comparison of C3a and C3:P as measures of C3 convertase activity

There was correlation between the generation of C3:P and C3a at extreme antigen and antibody ratios in both nascent and preformed IC. In contrast, with IC formed at equivalence or at four times antibody-excess there was no correlation (Fig 3.3.10 B).

Comparison of C5a and C5b-9 as measures of C5 convertase activity

There was a good correlation between the concentration of C5a and C5b-9 at all antigen-antibody ratios for IC formed in the presence of serum and for preformed IC (Fig 3.3.10 C).

When the number of C5a (12kD) molecules generated during complement activation by thyroglobulin anti-thyroglobulin IC was compared with the number of C5b molecules (178kD) which had been incorporated to the C5b-9 complex, it was found that the C5a:C5b ratio was 166±8. This suggests that only a very small proportion of C5b molecules are incorporated to the C5b-9 complex when complement is activated by IC containing soluble protein antigen.

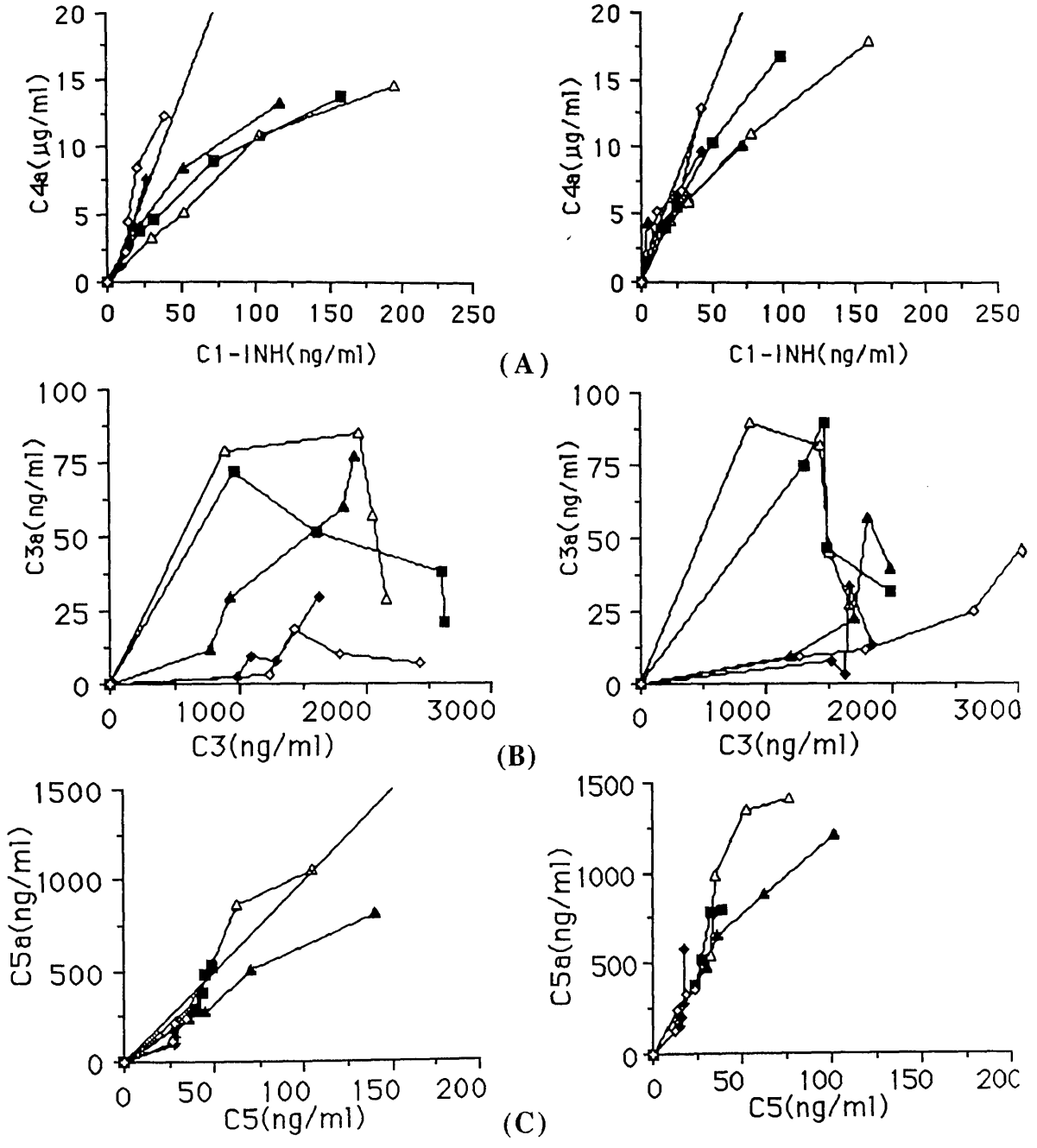


Figure 3.3.10 (A, B and C): Relationship between the generation of multimolecular complement activation complexes and anaphylatoxin generation by thyroglobulin anti-thyroglobulin IC at five antigen antibody ratios, 16-times antibody excess (▲—▲), 4-times antibody excess (△—△), equivalence (■—■), 4-times antigen excess (◆—◆) and 16-times antigen excess (◇—◇). A) C1s:C1-INH v. C4a by nascent IC (left panel) and preformed IC (right panel), B) C3:P v. C3a by nascent IC (left panel) and preformed IC (right panel) and C) C5b-9 v. C5a by nascent IC (left panel) and preformed IC (right panel).

3.3.4. Nascent BSA anti-BSA IC

a) Effect of IC concentration

IC formed in serum at five different antigen-antibody ratios produced dose dependent C1 activation as assessed by the serum level of the C1s:C1-INH complex (Fig 3.3.11 A left panel).

Activation of C3, as shown by the generation of the C3:P complex was found to be dose-dependent except when higher concentration of ICs were formed at equivalence or in antibody-excess (Fig 3.3.11 B left panel). Studies of the cause of this decreased concentration of the C3:P complex at the highest IC concentration will be presented in section 3.3.7.

Activation of C5, as measured by the generation of the C5b-9 complex was dose-dependent when IC were formed in antibody-excess or at equivalence, but when IC were formed in antigen-excess the amount of C5b-9 formed was maximal with an antibody concentration of 12 5μg IgG /ml (Fig 3.3.11 C left panel).

b) Effect of antigen-antibody ratio

C1 activation occurred most efficiently with IC formed at equivalence, followed by those formed at 4-times antibody-excess (Fig 3.3.11 A right panel). IC formed at 16-times antibody-excess or in antigen-excess produced far-less C1 activation.

At the two lowest IC concentrations, C3 activation was greatest at equivalence, with far less activation occurring when IC were formed in either antigen or antibody-excess (Fig 3.3.11 B). However, when the two highest concentrations of IC were used, the concentration of C3:P at equivalence was reduced markedly (Fig 3.3.11 B).

C5 activation was greatest in antibody-excess (Fig 3.3.11 C right panel).

c) Role of the classical and alternative pathways

C1 activation did not occur in serum treated with Mg^{++}EGTA. C3 activation was markedly reduced at all antigen-antibody ratios when classical pathway activation had

been blocked by Mg^{++}EGTA (Fig 3.3.12 A, B). In contrast C5b-9 generation was almost identical in NHS and in Mg^{++}EGTA-treated serum at all antigen-antibody ratios.(Fig 3.3.12 A, B).

3.3.5. Preformed BSA anti-BSA IC

a) Effect of IC concentration

The extent of C1 activation was dependent on IC concentration at all antigen-antibody ratios (Fig 3.3.13 A left panel).

In contrast the amount of C3:P complex formed did not show any clear relationship to the concentration of IC (Fig 3.3.13 B left panel).

A dose-dependent increase in the formation of the C5b-9 complex was observed with IC formed in antibody-excess and equivalence (Fig 3.3.13 C left panel), but the effect of IC concentration was not so obvious with IC formed at 4 and 16-times antigen-excess (Fig 3.3.13 C left panel).

b) Effect of antigen-antibody ratio

Activation of C1 was pronounced with IC formed at equivalence, but was much less marked when IC were formed in either antigen or antibody-excess (Fig 3.3.13 A right panel).

The concentration of the C3:P complex at different antigen-antibody ratios was dependent upon the IC concentration. At the lowest concentration (62.5µg antibody/ml) C3:P formation was greatest at equivalence and least at 16-times antigen-excess (Fig 3.3.13 B right panel). At higher IC concentrations less C3:P was detected at equivalence than when IC were formed in either antigen or antibody-excess (Fig 3.3.13 B right panel).

C5b-9 generation was greatest in antibody-excess and least in antigen-excess (Fig 3.3.13.C right panel).

c) Role of classical and alternative pathways

C1 activation did not occur in Mg^{++}EGTA-treated serum.

In most cases, the concentrations of C3:P complex in Mg^{++}EGTA-treated serum were lower than those in NHS (Fig 3.3.14 A and B). At the three lower IC concentrations, the level of C5b-9 generated in Mg^{++}EGTA-treated serum were almost the same as those produced in NHS (Fig 3.3.14 A and B). At the higher concentration, the C5b-9 concentration was slightly lower in Mg^{++}EGTA-treated serum.

3.3.6. Comparison of complement activation by nascent and preformed BSA anti-BSA IC

Nascent IC at all antigen-antibody ratios (Fig 3.3.11 A) produced more C1 activation than preformed IC (Fig 3.3.13 A).

Production of the C3:P complex was also greater with nascent IC (Fig 3.3. 11 B and 3.3.13 B), but C5b-9 generation was greater with preformed IC (Fig 3.3.11 C and 3.3.13 C).

There was little difference in the effect of Mg^{++}EGTA on the generation of C5b-9 by nascent or preformed IC.

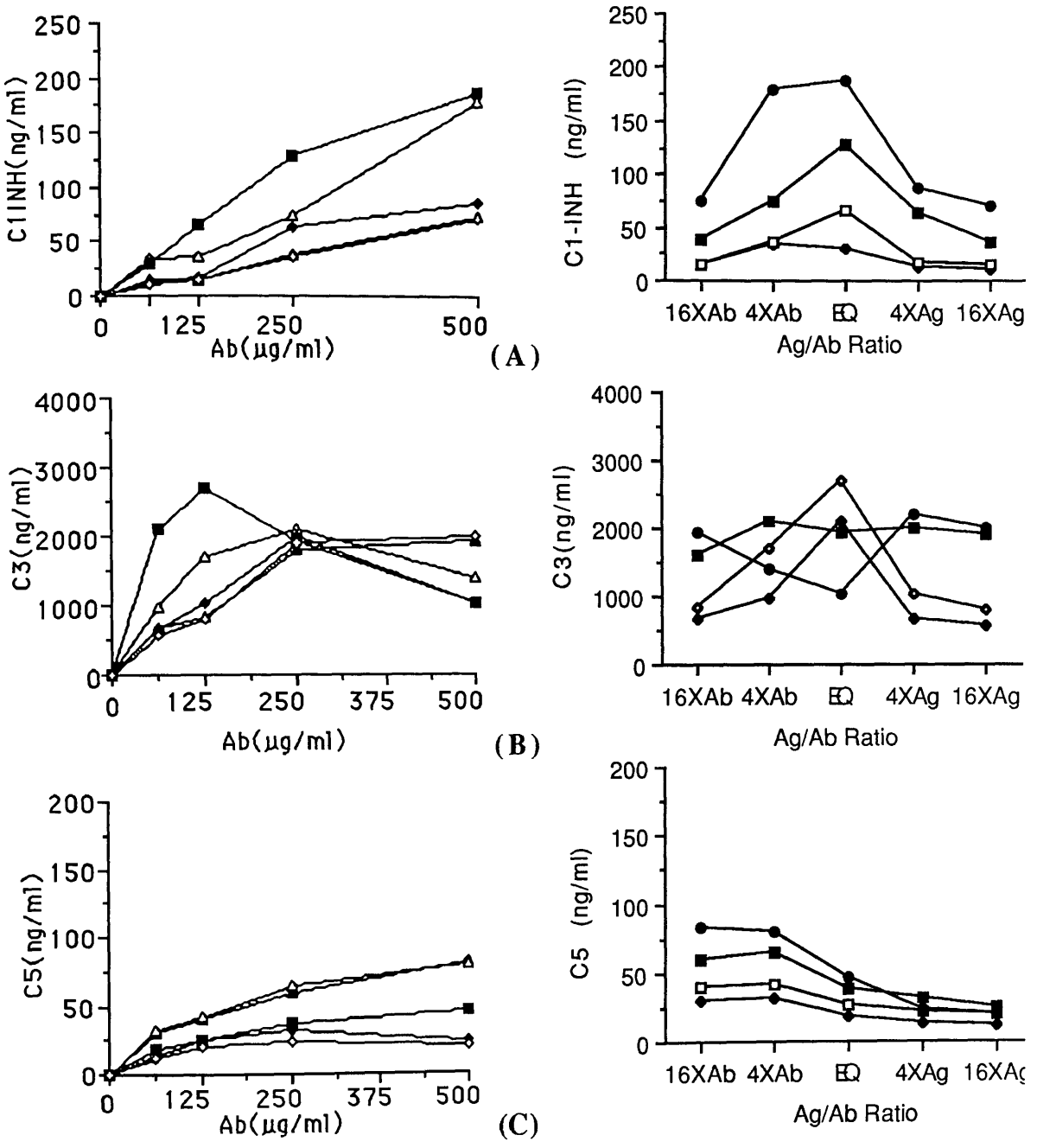


Figure 3.3.11 (A, B and C): Complement activation by nascent BSA anti-BSA IC. (A) C1 activation as assessed by C1s:C1-INH generation, (B) C3 activation as assessed by C3:P complex formation and (C) C5 activation as assessed by the formation of C5b-9 complex. Left panel represents the effect of IC concentration for the five antigen-antibody ratios; 16-times antibody-excess (▲—▲), 4-times antibody-excess (△—△), equivalence (■—■), 4-times antigen-excess (◆—◆) and 16-times antigen-excess (◇—◇). The right hand panels represent the effect of antigen-antibody ratio at four IC concentrations: 62.5 (◆—◆), 125 (□—□), 250 (■—■) and 500µg IgG antibody/ml NHS (●—●). Concentration of C1s:C1-INH expressed as ng/ml C1-INH, C3:P as ng/ml C3 and C5b-9 as ng/ml C5. Each point represents the mean of duplicate determinations.

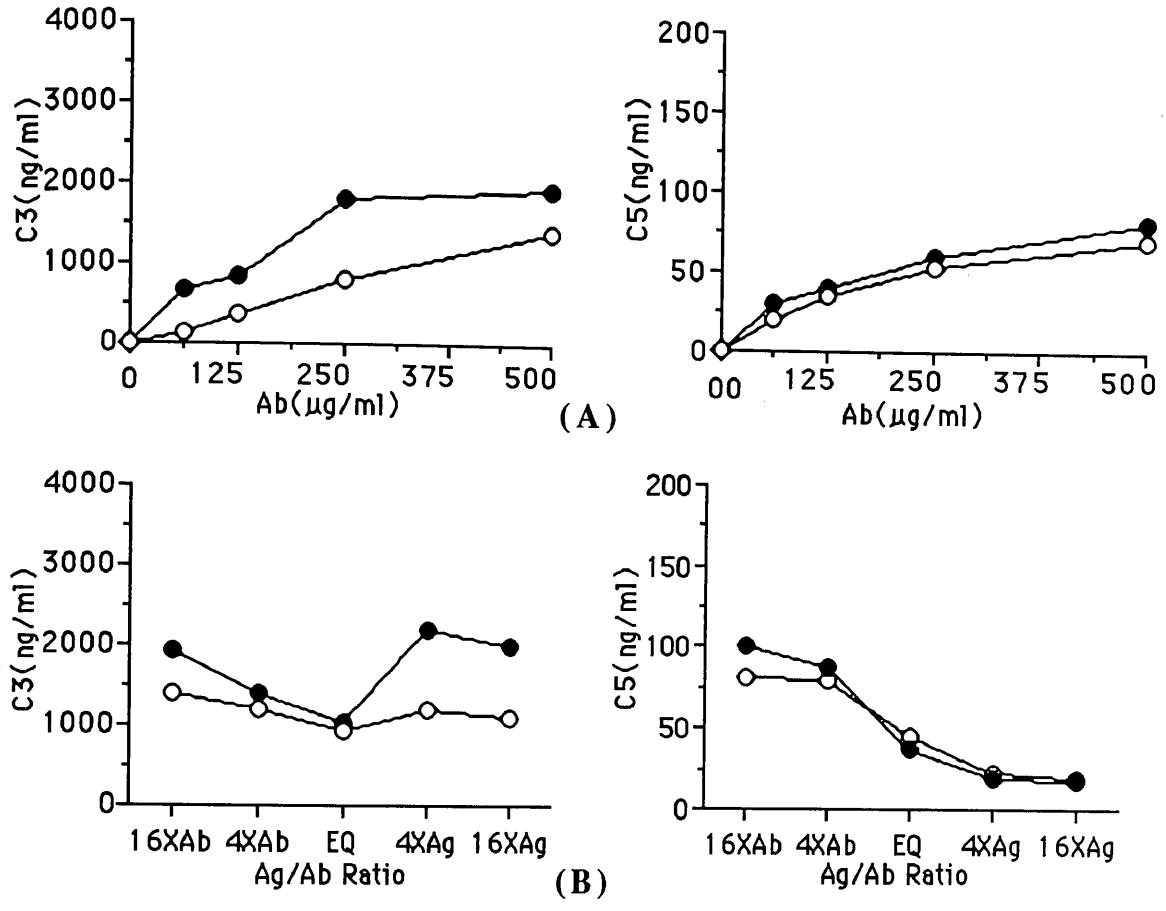


Figure 3.3.12 (A and B): Complement activation by nascent BSA anti-BSA IC in the absence (•—•) or the presence (○—○) of $Mg^{++}EGTA$ to investigate the role of the classical and the alternative pathways. A) Effect of IC concentration: C3:P generation (left) and C5b-9 formation (right) by IC at 16-times antibody-excess. B) Effect of antigen-antyibody ratio: C3:P generation (left) and C5b-9 formation (right) at an IC concentration of 500 µg IgG antibody/ml NHS Concentration of C3:P expressed as ng/ml C3 and C5b-9 as ng/ml C5. Each point represents the mean of duplicate determinations.

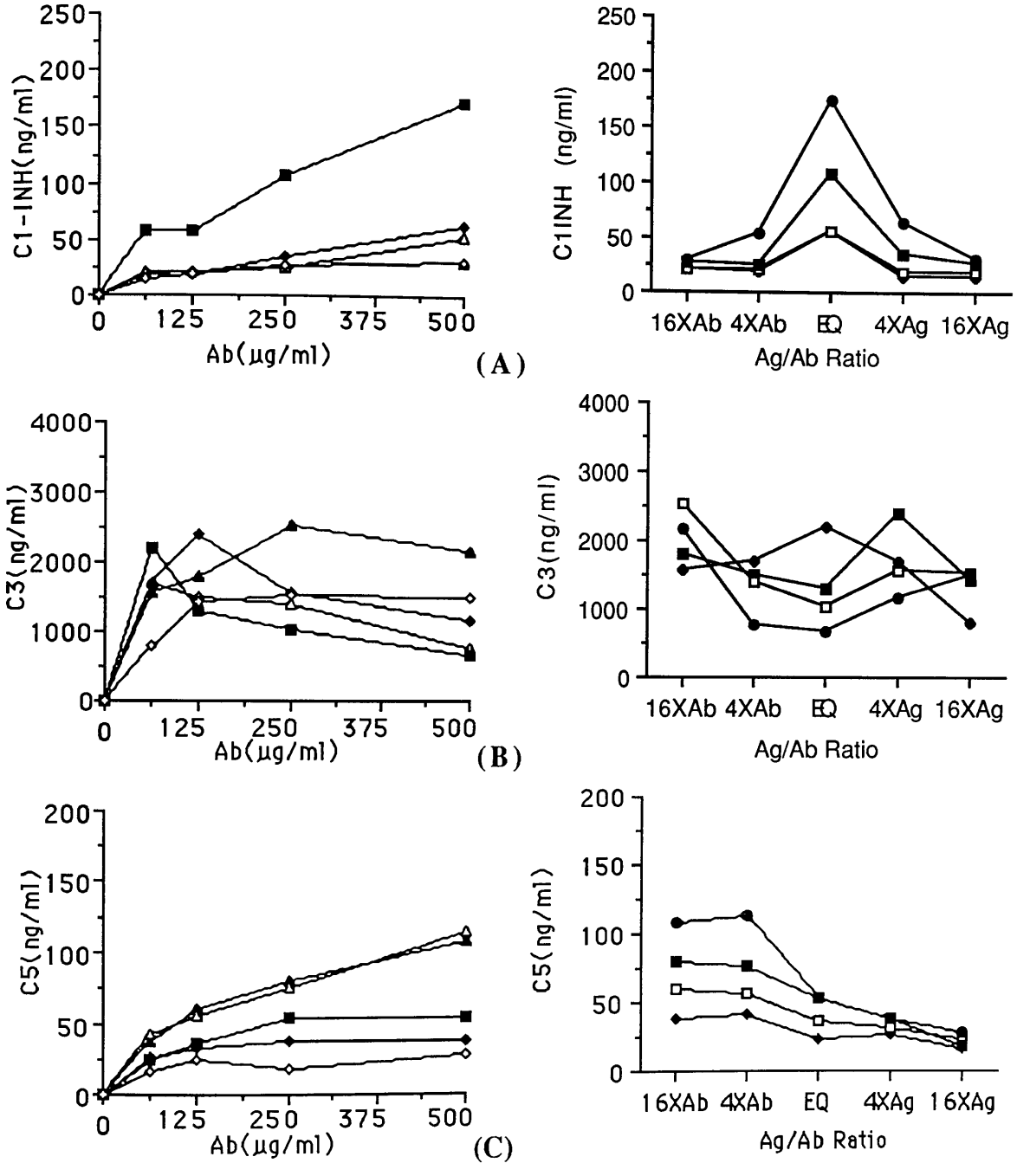


Figure 3.3.13 (A, B and C): Complement activation by preformed BSA anti-BSA IC. (A) C1 activation as assessed by C1s:C1-INH generation (B) C3 activation as assessed by C3:P complex formation and (C) C5 activation as assessed by the formation of C5b-9 complex. Left hand panel represent the effect of IC concentrations for IC at five antigen-antibody ratios; 16-times antibody-excess (▲—▲), 4-times antibody-excess (△—△), equivalence (■—■), 4-times antigen-excess (◆—◆) and 16-times antigen-excess (◇—◇). The right panels show the effect of antigen-antibody ratio at four IC doses: 62.5 (◆—◆), 125 (□—□), 250 (■—■) and 500µg IgG antibody/ml NHS (●—●). Concentration of C1s:C1-INH expressed as ng/ml C1-INH, C3:P as ng/ml C3 and C5b-9 as ng/ml C5. Each point represents the mean of duplicate determinations.

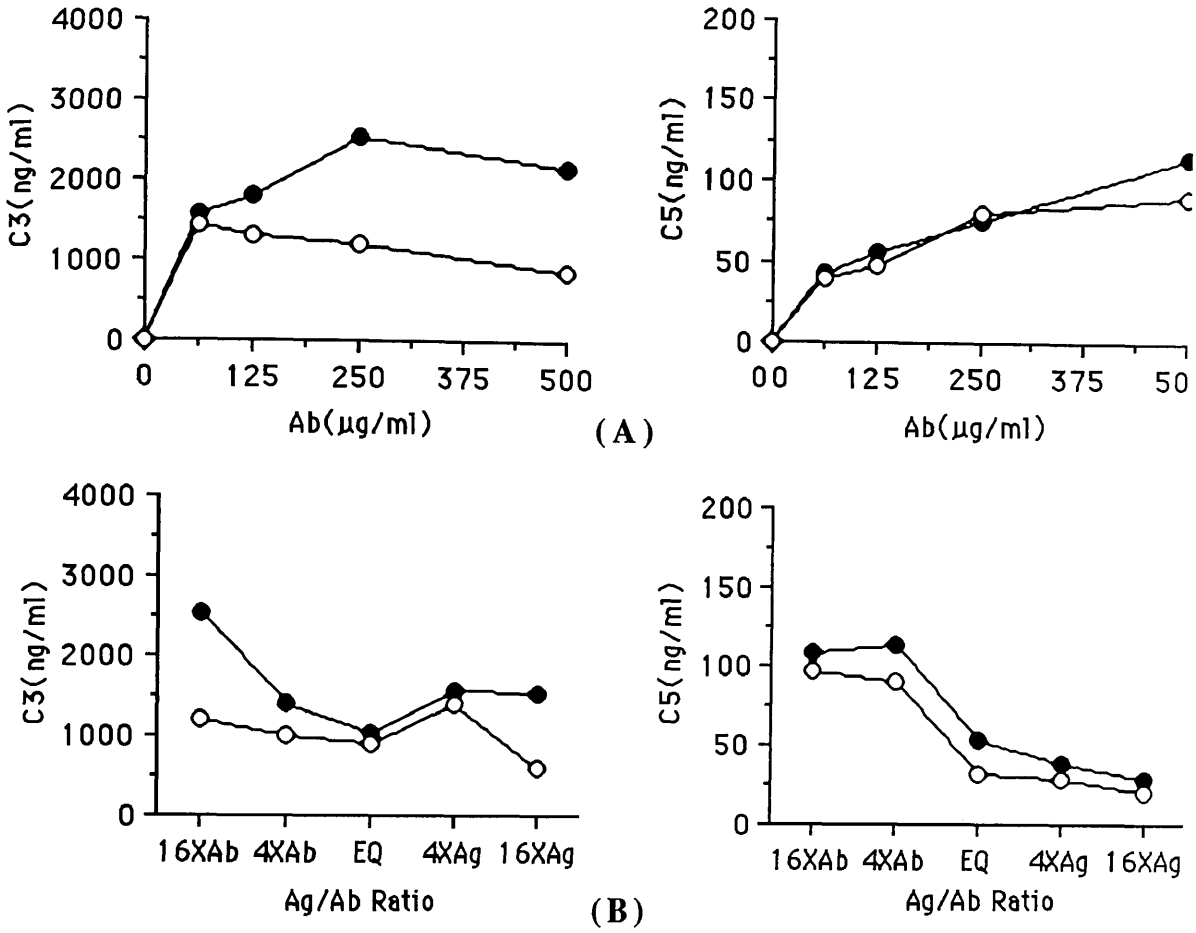


Figure 3.3.14 (A and B): Complement activation by preformed BSA anti-BSA IC in the absence (●—●) or the presence (○—○) of Mg^{++}EGTA to study the roles of the classical and the alternative pathways. A) Effect of IC concentration: C3:P generation (left) and C5b-9 formation (right) by IC at 16-times antibody-excess. B) Effect of antigen-antibody ratio: C3:P generation (left) and C5b-9 formation (right) at an IC concentration of 500 µg IgG antibody/ml NHS. Concentration of C1s:C1-INH expressed as ng/ml C1-INH, C3:P as ng/ml C3 and C5b-9 as ng/ml C5. Each point represents the mean of duplicate determinations.

3.3.7 Comparison of complement activation by thyroglobulin anti-thyroglobulin IC and BSA anti-BSA IC

C1 activation occurred more efficiently with thyroglobulin anti-thyroglobulin IC formed in 4-times antibody-excess whereas in the BSA anti-BSA system IC formed at equivalence were most effective. For both types of IC, C1 activation was greater with nascent IC than with preformed IC (Fig 3.3.2, 5 A and 3.3.11, 13 A).

Generation of the C3:P complex was clearly most effective with nascent BSA anti-BSA IC at equivalence at the three lower IC concentrations. In contrast with nascent thyroglobulin anti-thyroglobulin IC there were two distinct differences: 1) At the two lower IC concentrations IC formed at 4-times antibody-excess stimulated the production of almost as much C3:P as IC formed at equivalence, 2) At higher IC concentration less C3:P was formed at 4-times antibody-excess and at equivalence than at other antigen-antibody ratios (Fig 3.3.2 B and 3.3.11 B).

The patterns of C3:P generation were similar when both types of IC were preformed at all antigen-antibody ratios. However, greater levels of C3:P were produced with thyroglobulin anti-thyroglobulin IC (Fig: 3.3.5 B and 3.3.13 B).

C5b-9 complex formation was greater when both types of IC were formed in antibody-excess, and with preformed IC rather than with nascent IC. Thyroglobulin anti-thyroglobulin IC produced more C5b-9 than BSA anti-BSA IC (Fig 3.3.2, 5 C and 3.3.11, 13 C). C5b-9 formation was not significantly reduced when either type of IC was formed in Mg^{++}EGTA (Fig 3.2.4 B, 3.3.8 B.and 3.3.12 B, 3.3.14 B right panel.).

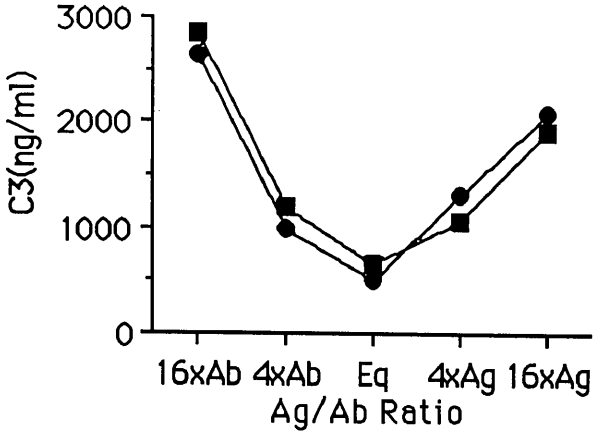
3.3.8. Investigation into the reduced C3:P concentrations

Concentrations of C3:P were reduced when nascent thyroglobulin anti-thyroglobulin IC were formed at equivalence and when nascent BSA anti-BSA IC were formed at equivalence or at 4-times antigen or 4-times antibody-excess.

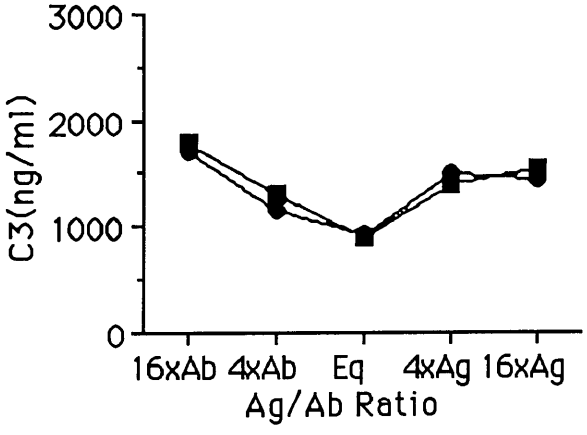
a) Measurement of C3:P levels was undertaken before and after centrifugation (Beckman Microfuge 10000rpm for 5 minutes) at room temperature, in order to investigate the possibility that at least some of the C3:P was bound to IC, and if these IC were insoluble, bound C3:P complexes would co-sediment and not be detected in the assay. However, the levels of C3:P were the same in centrifuged and non-centrifuged samples (Fig 3.3.15).

b) This problem was investigated further by measuring the solubility of nascent thyroglobulin anti-thyroglobulin IC and BSA anti-BSA IC after incubation in serum for 30 minutes at 37^{o}C. The results of the experiment showed that nascent thyroglobulin anti-thyroglobulin IC were extremely insoluble at equivalence or at 4-times antigen or 4-times antibody-excess (Fig 3.3.16 A, B left panel) whereas C3:P levels were only markedly reduced at equivalence. In contrast all nascent BSA anti-BSA IC, with the exception of a small proportion of those formed in 16-times antibody-excess, remained soluble (Fig 3.3.16 A, B right panel), while C3:P levels were markedly reduced when IC were formed at equivalence or in 4-times antigen or 4-times antibody-excess.

Likewise the pattern of solubilisation of preformed thyroglobulin anti-thyroglobulin and BSA anti-BSA IC (Fig 3.3.17 A, B) were not related to the pattern of C3:P formation with either types of preformed IC.



(A)



(B)

Figure: 3.3.15 (A and B): Relationship between C3:P levels and insolubility of IC, as detected by ELISA. C3P was generated during complement activation by (A) nascent thyroglobulin anti-thyroglobulin IC and (B) nascent BSA anti-BSA IC at five antigen-antibody ratios at an IC dose of 500µg IgG antibody/ml NHS in centrifuged (●—●) and uncentrifuged (■—■) samples. Concentration of C3:P expressed as ng/ml C3. Each point represents the mean of duplicate determinations.

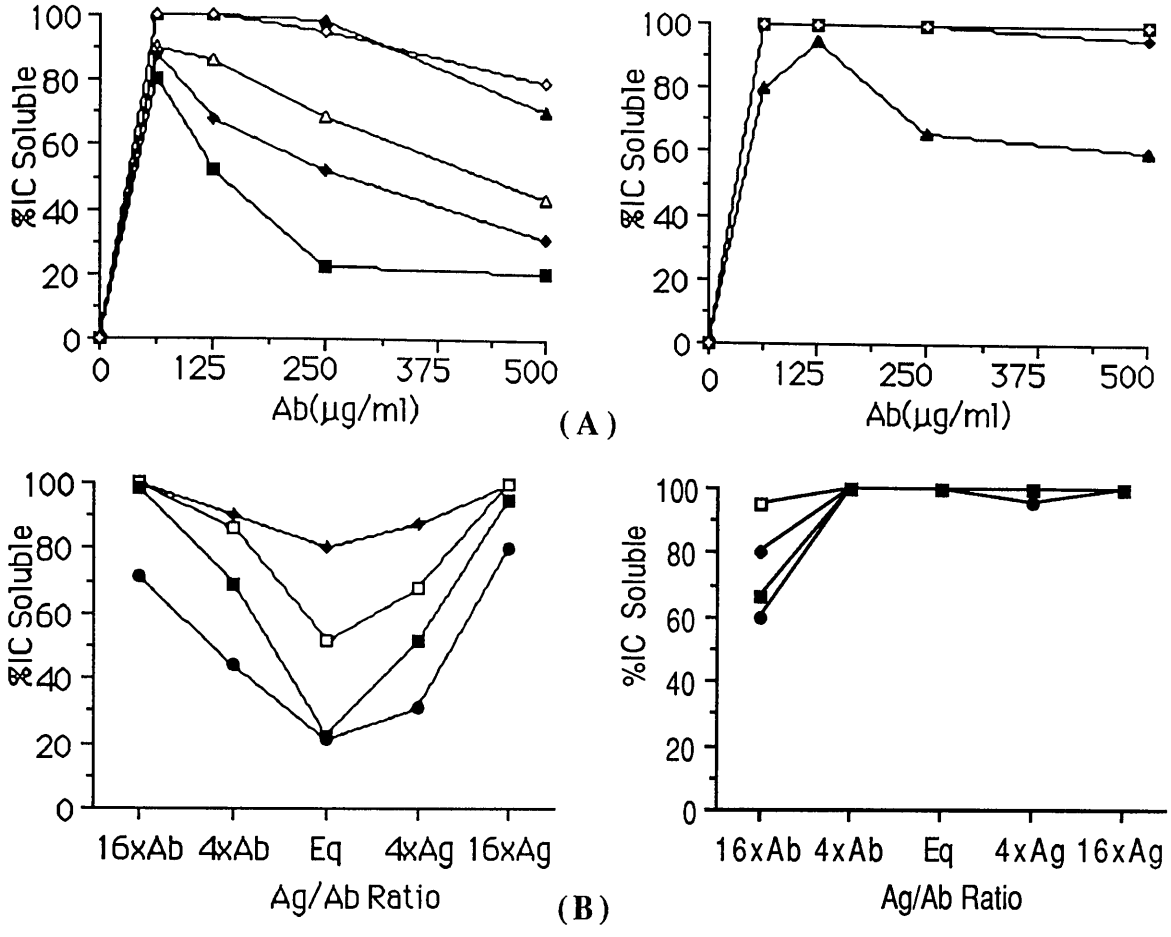


Figure 3.3.16 (A and B): Solubility of nascent thyroglobulin anti-thyroglobulin IC (left) and nascent BSA anti-BSA IC (right) A) Effect of IC concentration at five antigen-antibody ratios, 16-times antibody excess (▲—▲), 4-times antibody excess (△—△), equivalence (■—■), 4-times antigen excess (◆—◆) and 16-times antigen excess (◇—◇) and B) Effect of antigen antibody ratio on the solubility of nascent thyroglobulin anti-thyroglobulin IC (left) and nascent BSA anti-BSA IC (right) at four IC dose: 62.5 (◆—◆), 125 (□—□), 250 (■—■) and 500µg (●—●) IgG antibody/ ml NHS.

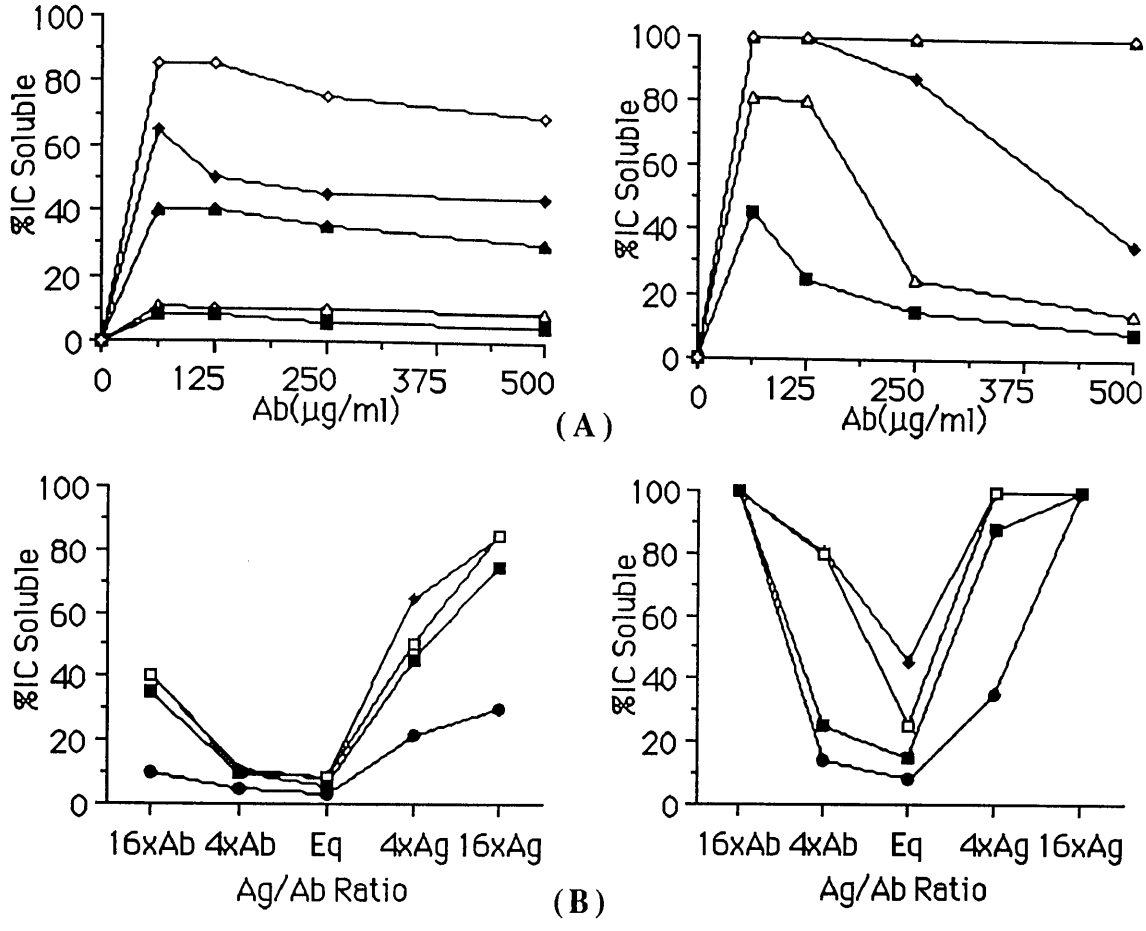


Figure 3.3.17 (A and B): Solubility of preformed thyroglobulin anti-thyroglobulin IC (left) and preformed BSA anti-BSA IC (right) A) Effect of IC concentration at five antigen-antibody ratios: 16-times antibody excess (▲—▲), 4-times antibody excess (△—△), equivalence (■—■), 4-times antigen excess (◆—◆) and 16-times antigen excess (◇—◇) and B) Effect of antigen antibody ratio on the solubility of nascent thyroglobulin anti-thyroglobulin IC (left) and nascent BSA anti-BSA IC (right) in four IC doses 62.5 (◆—◆), 125 (□—□), 250 (■—■) and 500µg (●—●) IgG antibody/ ml NHS.

3.4. KINETICS OF COMPLEMENT ACTIVATION BY IC

3.4.1 Nascent thyroglobulin anti-thyroglobulin IC

a) C1 activation

In the experiments performed at 37°C, increased levels of the C1s:C1-INH complex were detected within one minute of the addition of antigen and antibody to serum (Fig 3.4.1 A left panel). For IC formed at 4-times antibody-excess the level of C1s:C1-INH complex increased to reach a plateau after 15 minutes, whereas the plateau was reached somewhat earlier with IC formed at all other antigen-antibody ratios. In the case of IC formed at 16-times antibody-excess the level of C1s:C1-INH was maximal within 2.5 minutes, suggesting that at extremes of antibody-excess, C1 activation is completed extremely rapidly. C4a generation was studied at only 3 antigen-antibody ratios (Fig 3.4.1 A right panel.). After an extremely rapid increase in C4a levels, the maximal rate of increase and highest levels were achieved between 10 and 15 minutes. Disproportionately more C4a than C1s:C1-INH complex was generated at 4-times antigen-excess.

Comparison of the results of the two assays of C1 activation showed that C4a levels tended to rise earlier than those of C1s:C1-INH (Fig 3.4.1. A).

When C1 activation was studied at the three different temperatures it was shown that the rate and extent of formation of the C1s:C1-INH complex were lower at 30°C and 20°C (Fig 3.4.2 A). Indeed very little C1 activation occurred at 20°C.

b) C3 activation

In the experiments performed at 37°C, levels of C3:P complex increased from time 0, with all IC except for those formed at 16-times antigen-excess, and reached a peak after 15 minutes (Fig 3.4.1 B left panel.). For IC formed at 16-times antigen-excess there appeared to be a lag period of 5 minutes before the C3:P level increased. At 30 minutes C3:P concentrations were similar for IC formed at all antigen-antibody ratios.

In contrast, there was a marked dependence on antigen-antibody ratios with the initial rate of C3a generation and the final concentration was greater for IC formed at

equivalence and 4-times antibody-excess than for those formed at 4-times antigen-excess (Fig 3.4.1 B right panel). When IC were formed in antibody-excess or in antigen-excess, the peak concentration of C3a was produced within 7.5 minutes whereas when IC were formed at equivalence, the peak level was not reached until after 15 minutes (Fig 3.4.1 B right panel).

C3:P complex formation in response to the formation of IC in serum, was found to be temperature dependent. In contrast to the experiments described above, in this experiment when IC were incubated at 37^{o}C, there appeared to be two phases of C3:P complex formation (Fig 3.4.2 B left panel). For all antigen-antibody ratios there was phase with a slow rate of formation which was followed by a second phase when C3:P complex levels increased rapidly. When IC were formed at 16-times antigen-excess there was a clear lag phase of 7.5 minutes before levels of the C3:P complex began to rise. When IC were incubated at 30^{o}C, the initial-phase of slowly increasing C3:P levels was prolonged and the final concentration of the C3:P complex was reduced. When the experiments were performed at 20^{o}C little, or no C3:P complex was formed (Fig 3.4.2 B left panel).

c) C5 activation

There was a lag-period of 7.5 minutes before levels of the C5b-9 complex were detected (Fig 3.4.1.C left panel). When IC were formed at equivalence or in antigen-excess, the concentration of the C5b-9 complex reached their maximum after 15 minutes whereas when IC were formed in antibody-excess the level continued to rise throughout the 30 minutes incubation period (Fig 3.4.1. C left panel).

In contrast to C5b-9 formation, C5a levels increased immediately after the formation of IC (Fig 3.4.1. C right panel), and levels were maximal within 10 minutes at all antigen-antibody ratios studied. Although the peak level of C5a was higher for IC formed at 4-times antibody-excess than those formed at equivalence, the difference between the levels was far less than the difference in C5b-9 complex levels formed at

these two antigen-antibody ratios. IC formed at 4-times antigen-excess resulted in the generation of far lower levels of C5a than with IC formed at the other two antigen-antibody ratios. Levels of C5b-9 complex were reduced when IC were formed at 30°C and the lag-phase tended to be longer than when IC were formed at 37°C (Fig 3.4.2 C left panel). When the incubation temperature was reduced to 20°C, levels of C5b-9 were even lower and in antigen-excess virtually no C5b-9 complexes were formed.

d) Role of classical and alternative pathways

C1s:C1-INH complexes and C4a were not formed in Mg^{++}EGTA-treated serum.

A pronounced lag-phase prior to the formation of the C3:P complex occurred when IC at all 5 antigen-antibody ratios were formed in serum containing Mg^{++}EGTA. Furthermore the rate of formation and the final concentration of the C3:P complex were reduced under these conditions. When IC were formed in Mg^{++}EGTA-treated serum at 30°C or 20°C, very little C3:P complex was formed (Fig 3.4.2 B left).

The rate and extent of C3a generation was also reduced when IC were formed in Mg^{++}EGTA-treated serum. The reduction was most marked for IC formed at equivalence (Fig 3.4.2 B right panel).

The lag-phase for C5b-9 complex formation was the same in normal serum as in Mg^{++}EGTA-treated serum (Fig 3.4.2 C left). However, the level of C5b-9 was slightly higher in normal serum than in Mg^{++}EGTA-treated serum. In contrast, the lag-phase was not apparent when C5a generation was studied in antibody-excess or at equivalence (Fig 3.4.2 C right panel), while in antigen-excess there was a lag-phase.

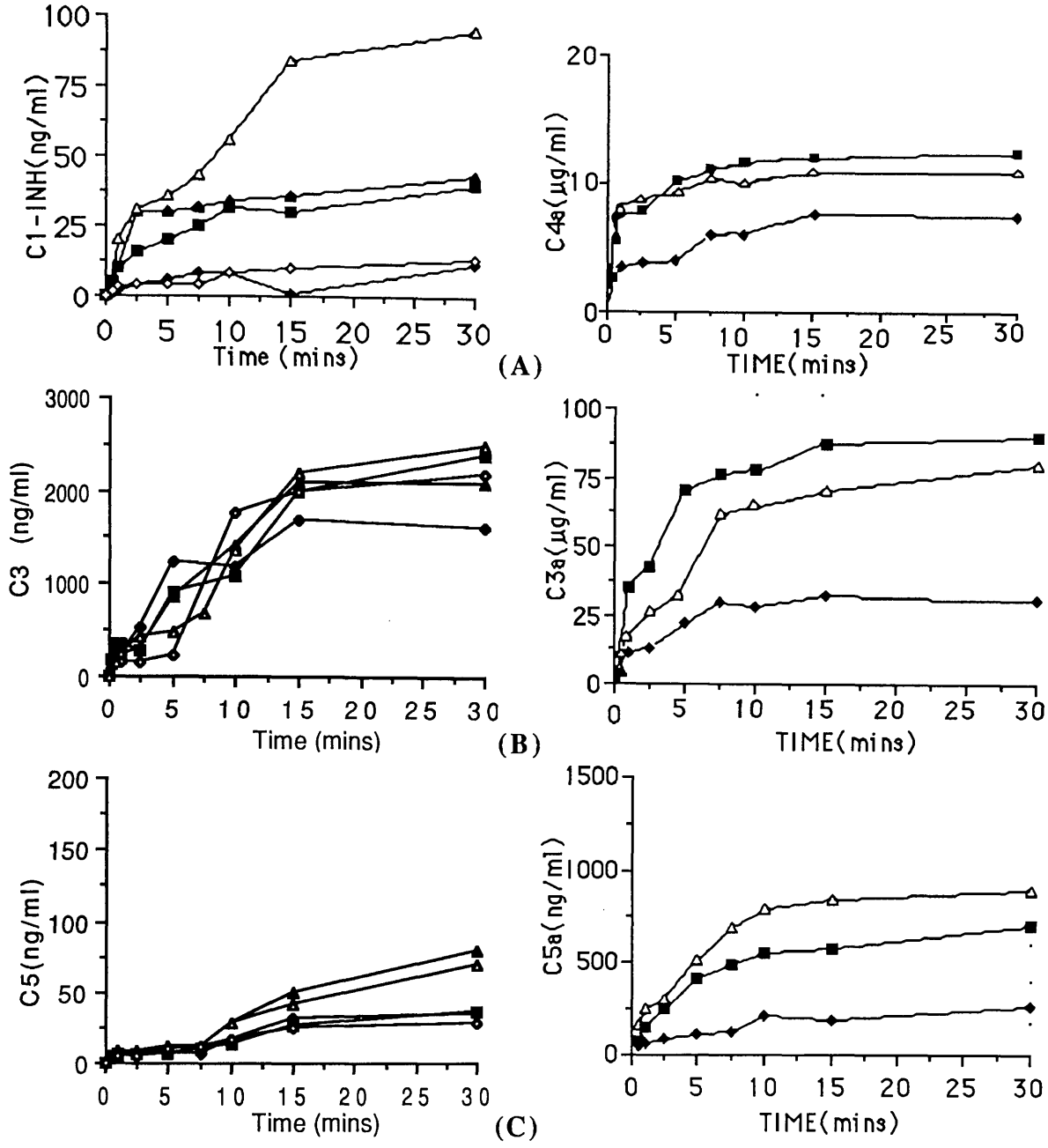


Figure 3.4.1 (A, B and C): Kinetics of complement activation by nascent thyroglobulin anti-thyroglobulin IC: (A) C1 activation as assessed by C1s:C1-INH complex (left) and C4a (right), (B) C3 activation as assessed by C3:P complex (left) and C3a (right) and (C) C5 activation as assessed by C5b-9 complex (left) and C5a (right) at five antigen-antibody ratios at 37°C, 16-times antibody-excess (▲—▲), 4-times antibody-excess (△—△), equivalence (■—■), 4-times antigen-excess (◆—◆) and 16-times antigen-excess (◇—◇). Concentration of IC used was 250µg IgG antibody/ml serum . Each point represents the mean of duplicate determinations.

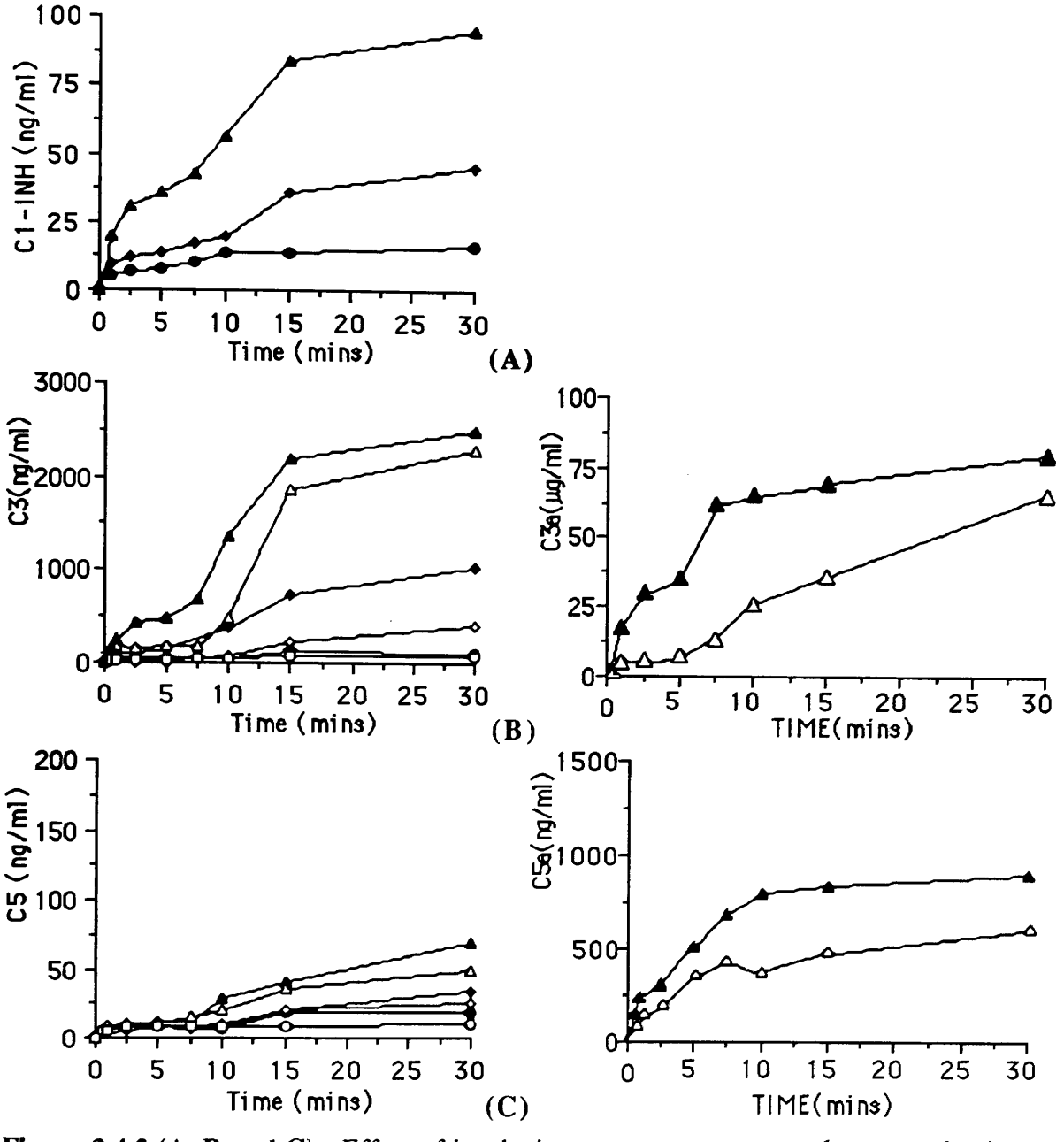


Figure 3.4.2.(A, B and C): Effect of incubation temperature on complement activation by nascent thyroglobulin anti-thyroglobulin IC at 4-times antibody-excess, in the absence (solid symbols) or the presence (empty symbols) of Mg^{++}EGTA (A) C1 activation as assessed by C1s:C1-INH, (B) C3 activation as assessed by C3:P complex and C3a and (C) C5 activation as assessed by C5b-9 and C5a. The incubation temperatures were 37°C (▲—▲), 30°C (◆—◆) and 20°C.(●—●). Each point represents the mean of duplicate determinations.

3.4.2. Preformed thyroglobulin anti-thyroglobulin IC

a) C1 activation

Levels of the C1s:C1-INH complex rose rapidly when preformed IC formed at 4-times antibody-excess and at equivalence were incubated in serum (Fig 3.4.3 A left panel). For IC formed at equivalence the peak level was reached after 2.5 minutes whereas for IC formed at 4-times antibody-excess the rate of increase slowed after 0.5 minutes to reach a maximum at 10 minutes. In contrast to the rapid increase in C1s:C1-INH levels seen with IC formed in antibody-excess or at equivalence, a much slower rise in C1s:C1-INH levels was seen with IC formed at 4-times antigen-excess. Maximum levels were reached after 7.5 minutes (Fig 3.4.3 A left panel).

Levels of C4a also rose rapidly and maximal levels were achieved between 5 and 10 minutes. The generation of C4a was proportionally similar to that of C1s:C1-INH complex (Fig 3.4.3 A right panel).

b) C3 activation

Serum levels of the C3:P complex increased rapidly when preformed IC were incubated with serum. When IC were formed at equivalence the highest level of the C3:P complex was reached within 2.5 minutes, whereas when IC were formed at either 4-times antibody-excess or 4-times antigen-excess, the rate of increase slowed after 1 minute to reach their maximum levels between 7.5 and 10 minutes (Fig 3.4.3 B left panel).

Serum levels of C3a rose rapidly on exposure to IC, with maximum levels being achieved by 5 minutes for IC formed at equivalence or 4-times antibody-excess (Fig 3.4.3 B right panel). In contrast, when IC were formed at 4-times antigen-excess, the rate of increase was slower and the maximum concentration was much lower than those formed with IC formed at the two other antigen-antibody ratios (Fig 3.4.3 B right panel).

c) C5 activation :

Within 30 seconds of the onset of the incubation, levels of C5b-9 increased rapidly for IC formed at 4-times antibody-excess and at equivalence. This phase was followed by a second phase in which the level of C5b-9 complex increased more slowly (up to 10 minutes for IC formed at 4-times antibody-excess and up to 7.5 minutes for IC formed at equivalence). Levels of C5b-9 were much lower when IC formed at 4-times antigen-excess were incubated in serum. In this case the levels increased slowly from time 0. The rate of increase was so small that it is difficult to say whether a significant increase in C5b-9 occurred between 15 and 30 minutes.(Fig 3.4.3 C left panel).

When IC formed at 4-times antibody-excess were studied, the C5a levels reached its peak after 10 minutes, whereas when IC were formed at equivalence the level of C5a continued to rise throughout the 30 minutes incubation period (Fig 3.4.3 C right panel). With IC formed at 4-times antigen-excess, very little C5a was formed and the rate of increase was extremely low (Fig 3.4.3 C right panel).

d) Role of classical and alternative pathways:

Formation of C1s:C1-INH complex and C4a did not occur in Mg^{++}EGTA-treated serum.

Formation of C3:P complex was delayed when IC were incubated with Mg^{++}EGTA-treated serum. When the IC were formed at 4-times antigen-excess, the concentration of C3:P complex was much lower in Mg^{++}EGTA-treated serum than in NHS (Fig 3.4.4 A left). The rate of formation of C3a was markedly lower in Mg^{++}EGTA-treated serum than in normal serum, particularly when IC were formed at 4-times antibody-excess and at equivalence (Fig 3.4.4 A right). Far less C3a was formed when IC were formed at 4-times antigen-excess, making it difficult to conclude that the rate of formation of C3a was lower in Mg^{++}EGTA-treated serum.

For IC formed at 4-times antibody-excess or at equivalence, the rate of formation of the C5b-9 complex occurred in two phases, an initial rapid phase followed by a slower secondary phase. In Mg^{++}EGTA these rates were similar to those in normal serum

(Fig 3.4.4 B left panel), although the amount of C5b-9 formed during both phases was lower. When IC were formed at 4-times antigen-excess, very little C5b-9 complex was formed, and Mg^{++}EGTA did not appear to influence its rate of formation or the amount formed. The rate of C5a generation was lower in the presence of Mg^{++}EGTA at all three antigen-antibody ratios. In 4-times antibody-excess and equivalence the C5a levels continued to rise throughout the 30 minutes incubation period (Fig 3.4.4 B right panel) whereas when IC were formed in 4-times antigen-excess very little C5a was formed.

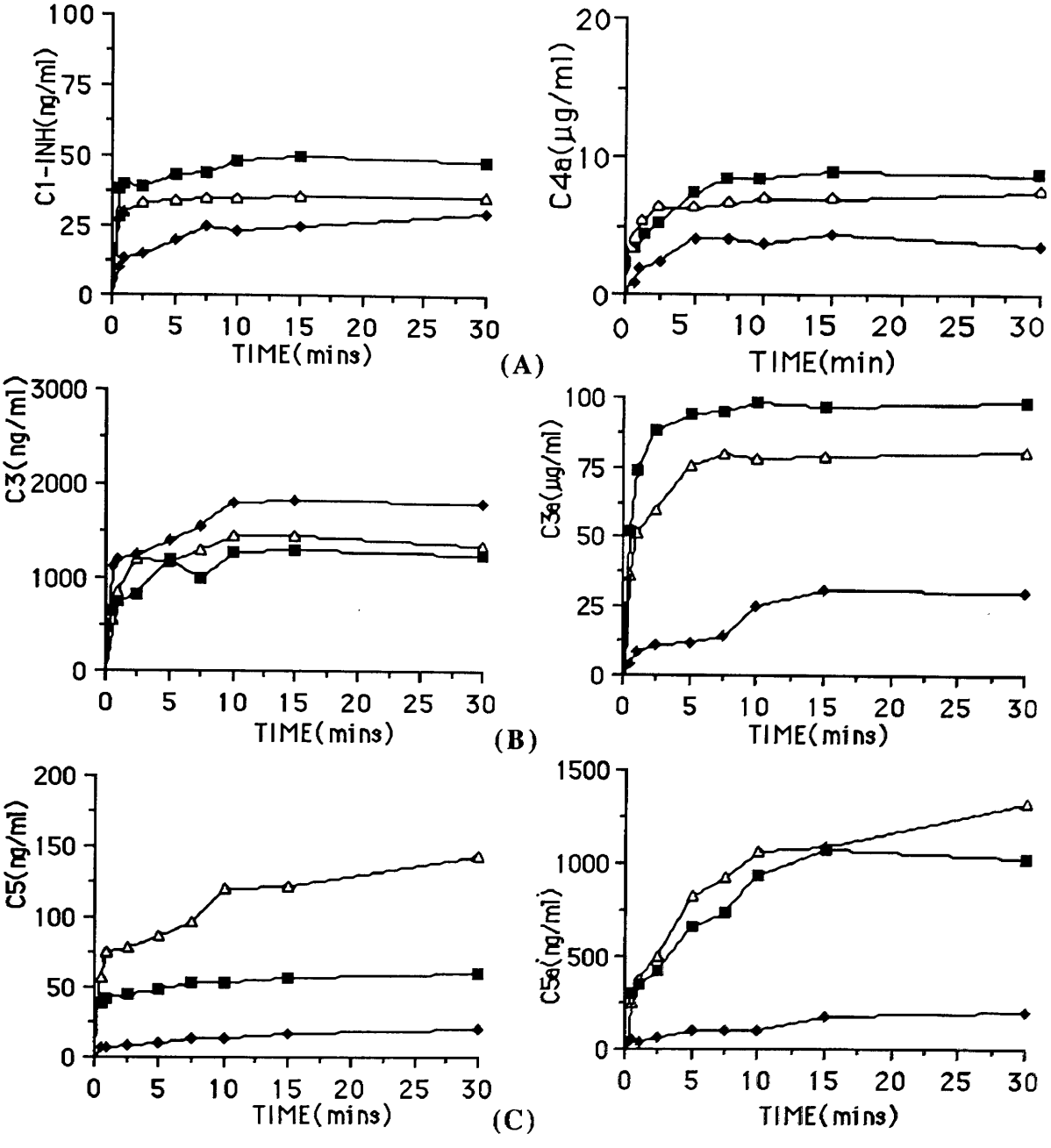


Figure 3.4.3 (A, B and C): Kinetics of complement activation by preformed thyroglobulin anti-thyroglobulin IC at three antigen-antibody ratios, (A) C1 activation as assessed by C1s:C1-INH complex (left) and C4a (right), (B) C3 activation as assessed by the generation of C3:P complex (left) and C3a (right) and (C) C5 activation as assessed by the generation of C5b-9 complex (left) and C5a (right), at 37°C: 4-times antibody-excess (△—△), equivalence (■—■) and 4-times antigen-excess (◆—◆). Concentration of IC used was 250µg IgG antibody/ml serum. Each point represents the mean of duplicate determinations.

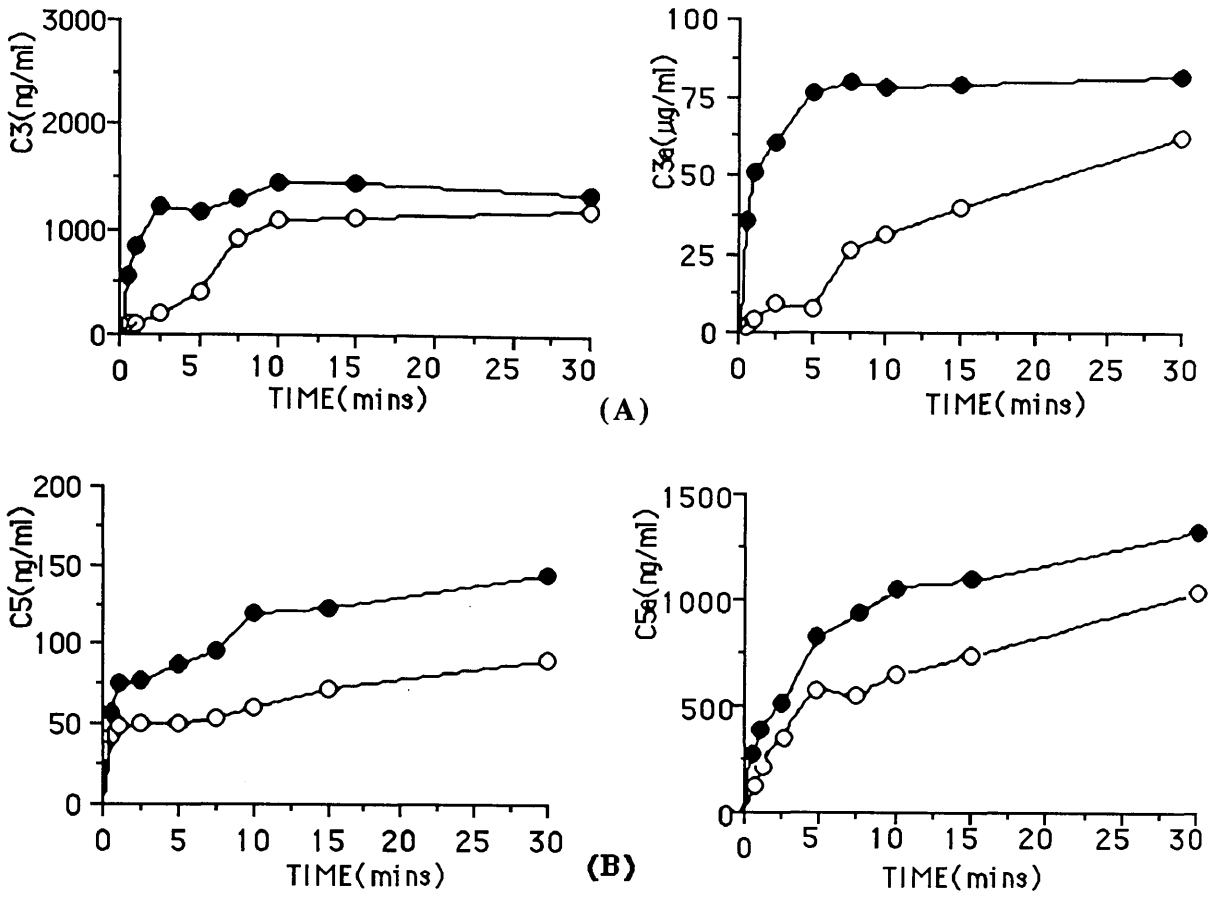


Figure 3.4.4 (A and B): Role of classical and alternative pathways on the kinetics of complement activation by preformed thyroglobulin anti-thyroglobulin IC at an antigen-antibody ratio of 4-times-antibody-excess. (A) C3 activation as assessed by the generation of C3:P complex (left panel) and C3a (right) and (B) C5 activation as assessed by the generation of C5b-9 complex (left panel) and C5a (right). The incubation temperature was 37°C and the experiment was performed in the absence (●—●) or the presence (○—○) of Mg^{++}EGTA. Each point represents the mean of duplicate determinations.

3.4.3. Comparison of kinetics of complement activation by nascent and preformed thyroglobulin anti-thyroglobulin IC

a) C1 activation

The rate of formation of C1s:C1-INH complex was slower when nascent IC formed in 4-times antibody-excess were studied. However the level of C1s:C1-INH complex reached a plateau after 2.5 minutes with preformed IC whereas the level increased for 15 minutes to a far higher level with nascent IC (Fig 3.4.5 A left panel).

b) C3 activation

The initial rate of formation of the C3:P complex was faster for preformed IC than for nascent IC, although the final concentration of C3:P complex was higher for nascent IC (Fig 3.4.5 B left panel). In the presence of Mg^{++}EGTA the same pattern was seen although there was a distinct lag-phase of 1 minute for preformed IC and 7.5 minutes for nascent IC (Fig 3.4.5 B left panel).

The initial rates of formation of C3a with preformed IC were faster than those with nascent IC at 4-times antibody-excess and at equivalence, whereas when IC were formed at 4-times antigen-excess, the initial rate of C3a production for nascent and preformed IC was similar (Fig 3.4.5 B right panel). In the presence of EGTA both the rate and extent of C3a production were reduced. The rate and extent of C3a formation for IC in antibody-excess or antigen-excess was the same in normal and Mg^{++}EGTA-treated serum (Fig 3.4.5 B right panel). In contrast, when IC were formed at equivalence, Mg^{++}EGTA appeared to produce a somewhat greater reduction on C3a formation by nascent IC (Fig 3.4.5 B right panel).

c) C5 activation

C5b-9 formation occurred almost immediately for preformed IC formed at equivalence or in antibody-excess. Whereas a significant delay was observed with nascent IC. In the presence of Mg^{++}EGTA, C5b-9 formation by preformed IC was

reduced, whereas little effect was observed on C5b-9 formation by nascent IC (Fig 3.4.5 C left panel).

C5a was formed more rapidly and to a greater extent when preformed IC at 4-times antibody-excess and at equivalence were incubated with serum. Mg^{++}EGTA reduced the rate of C5a production by both types of IC (Fig 3.4.5 C right panel). At 4-times antigen-excess neither preformed nor nascent IC stimulated the production of much C5a and Mg^{++}EGTA had no effect on this.

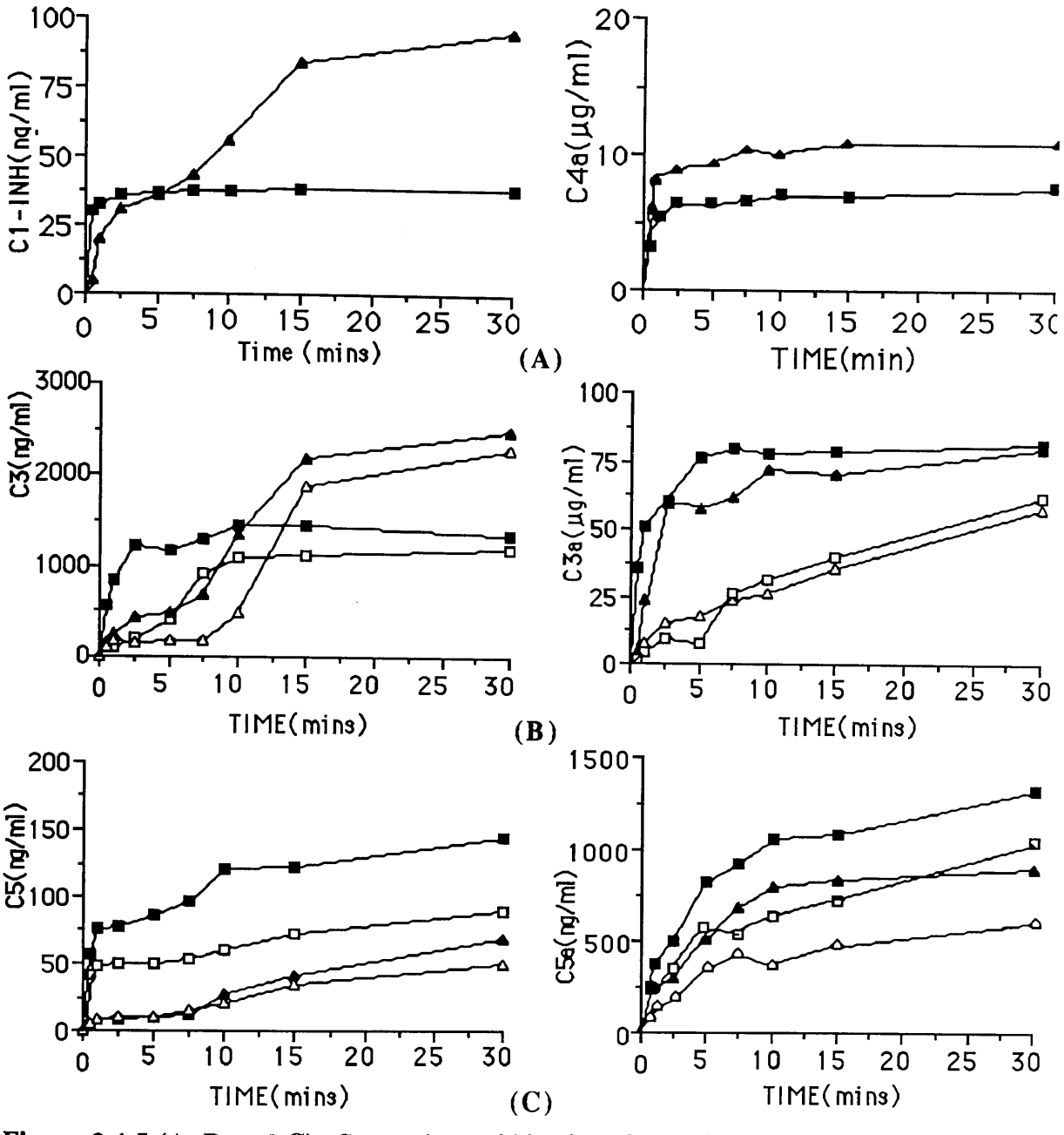


Figure 3.4.5 (A, B and C): Comparison of kinetics of complement activation between nascent thyroglobulin anti-thyroglobulin IC (▲—▲) and preformed thyroglobulin anti-thyroglobulin IC (■—■) at an antigen-antibody ratio of 4-times antibody-excess in the presence (empty symbols) or the absence (solid symbols) of Mg^{++}EGTA (A) C1 activation as assessed by the generation of C1s:C1-INH complex (left panel) and C4a (right panel), (B) C3 activation as assessed by the generation of C3:P complex (left panel) and C3a (right panel) and (C) C5 activation as assessed by the generation of C5b-9 complex (left panel) and C5a (right panel). Incubation temperature 37°C. Concentration of IC was 250µg IgG antibody/ml serum. Each point represents the mean of duplicate determinations.

3.4.4. Nascent BSA anti-BSA IC

a) C1 activation

Increased levels of C1s:C1-INH complex were detected within 1 minute of formation of the IC (Fig 3.4.6 A). When IC were formed at 4-times antibody-excess levels of C1s:C1-INH complex continued to rise rapidly over the 30 minutes whereas when IC were formed at equivalence or 4-times antigen-excess, the rate of increase fell markedly after 2.5 minutes.

b) C3 activation

The rates of C3:P generation were virtually identical at all three antigen-antibody ratios studied (Fig 3.4.6 B left panel). There was no lag-phase prior to the appearance of the C3:P complex and the maximum levels were achieved after 15 minutes. In contrast, antigen-antibody ratio had a pronounced influence on the rate and extent of formation of C3a. For IC formed at equivalence or 4-times antibody-excess, there was an initial phase during which C3a levels increased slowly, followed by a second phase in which levels increased more rapidly. For IC formed at equivalence this second phase of C3a generation was faster and peak level was higher and was reached by 7.5 minutes (Fig 3.4.6 B right panel). The peak C3a level was reached after 15 minutes when IC were formed at 4-times antibody-excess. When IC were formed at 4-times antigen-excess little C3a was formed and the rate of formation was low.

c) C5 activation

Very little C5b-9 complex was formed when nascent BSA anti-BSA IC were incubated with serum (Fig 3.4.6 C). The level generated during incubation with IC formed at 4-times antibody-excess and at equivalence was maximal within 0.5-1 minutes, whereas the rate of increase was lower for IC formed at 4-times antigen-excess, where the maximum level was achieved after 15 minutes.

d) The role of the classical and alternative pathways

C1s:C1-INH complexes were not formed in Mg^{++}EGTA-treated serum.

At all three antigen-antibody ratios studied, the rate of increase and the peak level of the C3:P complex was reduced in Mg^{++}EGTA-treated serum (Fig 3.4.7 A).

C5b-9 formation was not altered when IC prepared at 4-times antigen-excess or at equivalence were formed in Mg^{++}EGTA-treated serum (Fig 3.4.7 B). However, when IC prepared at 4-times antibody-excess both the rate of the formation and the final concentration of C5b-9 complex were reduced by Mg^{++}EGTA.

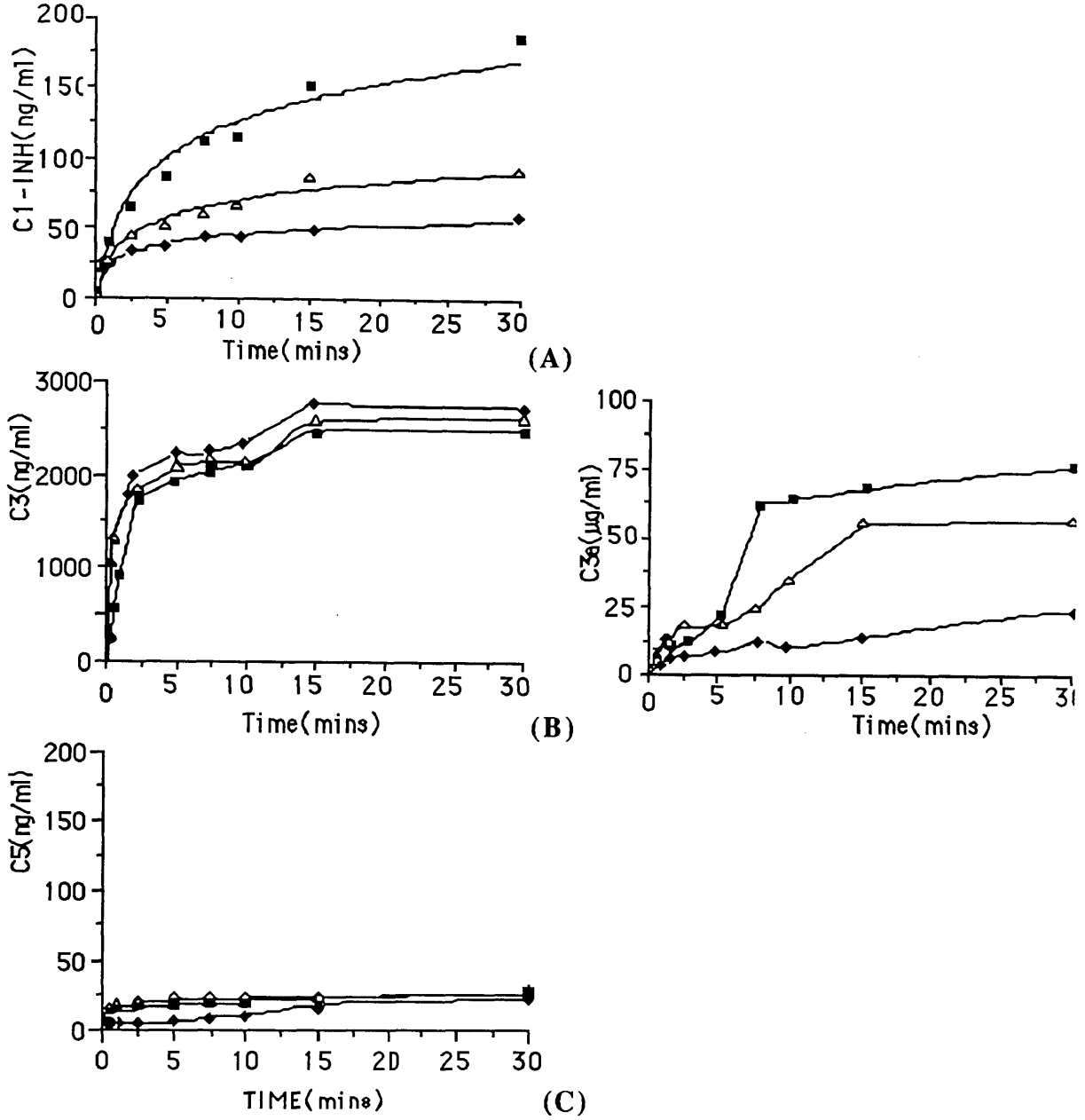


Figure 3.4.6 (A, B and C): Kinetics of Complement activation by nascent BSA anti-BSA IC, (A) C1 activation as assessed by C1s:C1-INH complex, (B) C3 activation as assessed by C3:P complex (left) and C3a (right) and (C) C5 activation as assessed by C5b-9 complex. Three antigen-antibody ratios were used, 4-times antibody-excess (△—△), equivalence (■—■) and 4-times antigen-excess (◆—◆). The concentration of IC was 250µg IgG antibody/ml serum. Incubation temperature was 37°C. Each point represents the mean of duplicate determinations.

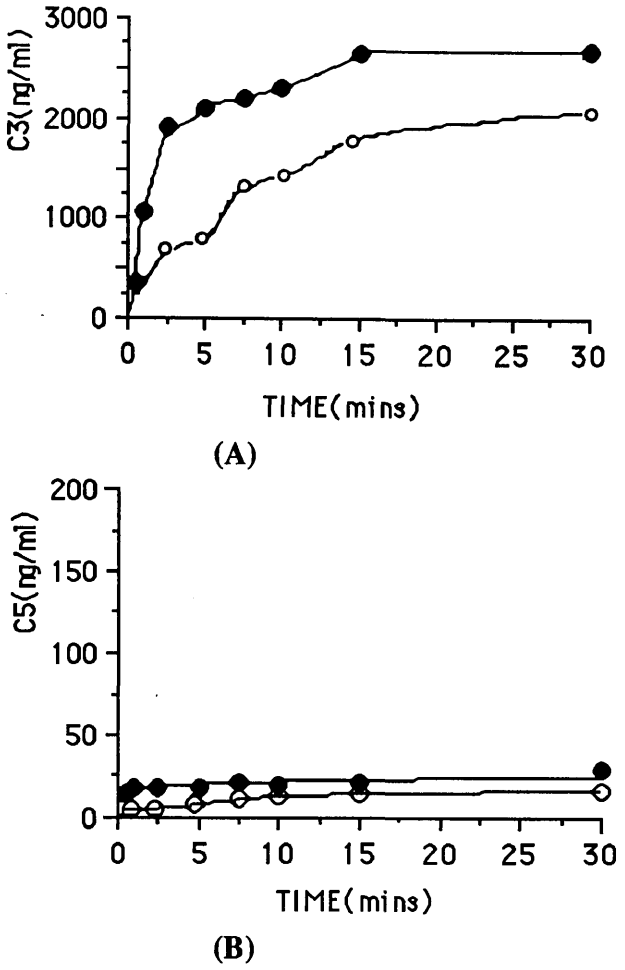


Figure 3.4.7 (A and B): Role of classical and alternative pathways on the kinetics of complement activation by nascent BSA anti-BSA IC formed at equivalence. (A) C3 activation as assessed by the generation of C3:P complex and (B) C5 activation as assessed by the generation of C5b-9 complex. The incubation temperature was 37°C and the experiment was performed in the absence (●—●) or the presence (○—○) of Mg^{++}EGTA. The concentration of IC used was 250μg IgG antibody/ml serum. Each point represents the mean of duplicate determinations.

3.4.5 Preformed BSA anti-BSA IC

a) C1 activation

Very low levels of C1s:C1-INH complex were formed at all three antigen-antibody ratios studied. The lowest levels were produced by IC formed at 4-times antibody-excess. A small increase occurred within 1 minute for IC formed at 4-times antibody-excess or at equivalence (Fig 3.4.8 A). There also appeared to be a 5 minutes period while levels of C1s:C1-INH increased slowly when using IC formed at 4-times antigen-excess.

b) C3 activation

IC formed at 4-times antigen-excess produced the fastest rate of increase in levels of the C3:P complex and the plateau level was reached by 5 minutes. In contrast, the rate of increase for IC formed at equivalence and 4-times antibody-excess were somewhat slower and peak levels occurred after 15 minutes (Fig 3.4.8 B left panel). The rate of increase of C3a was greater with IC formed at equivalence (Fig 3.4.8 B right panel). The maximum levels of C3a were reached after 15 minutes for IC formed at 4-times antibody-excess and at equivalence, and after 5 minutes for IC formed at 4-times antigen-excess.

c) C5 activation

When IC were formed at 4-times antibody-excess the level of C5b-9 complex increased rapidly. The rate of increase was slightly less for IC formed at equivalence. The peak levels were reached after 15 minutes for IC at 4-times antibody-excess and at equivalence (Fig 3.4.8 C). The rate and extent of formation of C5b-9 were both much lower when IC were formed at 4-times antigen-excess.

d) Role of the classical and alternative pathways

The C1s:C1-INH complex was not formed in Mg^{++}EGTA-treated serum.

When IC were prepared at 4-times antibody-excess, the initial rate of production of C3:P complex was probably identical in normal and Mg^{++}EGTA-treated serum. However, the peak level, which was lower in the presence of Mg^{++}EGTA, was reached within 5 minutes, whereas in normal serum it was reached after 15 minutes (Fig 3.4.9 A). When IC were formed at equivalence or at 4-times antigen-excess, the rate of C3:P complex formation was reduced by Mg^{++}EGTA. The final concentration of C3:P complex for IC prepared at equivalence was the same in normal and Mg^{++}EGTA-treated serum. However, Mg^{++}EGTA reduced the concentration of C3:P formed in response to IC prepared at 4-times antigen-excess.

The initial rate of formation and final concentration of the C5b-9 complex were reduced in the presence of Mg^{++}EGTA at all antigen-antibody ratios (Fig 3.4.9 B).

3.4.6. Comparison of complement activation by preformed and nascent BSA anti-BSA IC

a) C1 activation

The rate and extent of production of the C1s:C1-INH complex were greater for nascent than for preformed IC (Fig 3.4.6 A, Fig 3.4.8 A). C4a levels were not measured for BSA IC.

b) C3 activation

The rate and extent of formation of the C3:P complex were greater for nascent than for preformed IC (Fig 3.4.10 B left panel). On the contrary, the rate and extent of C3a formation were greater for preformed than for nascent IC (Fig 3.4.10 B right panel).

c) C5 activation

After a short initial increase (0.5 minutes) in the level of C5b-9 with both types of IC, the level did not increase further with nascent IC, whereas with preformed IC the concentration increased slowly up to 15 minutes (Fig 3.4.10 C).

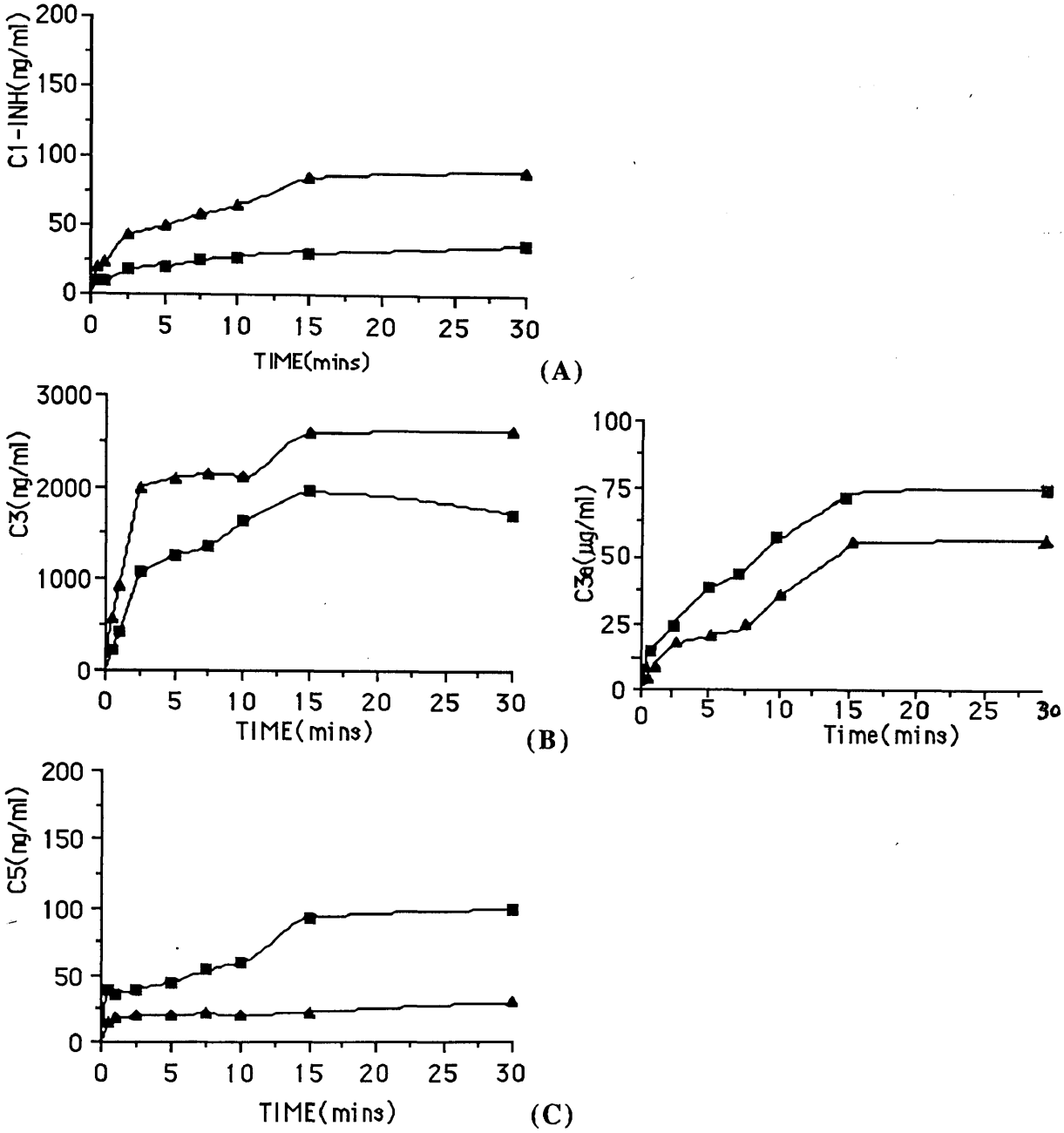


Figure 3.4.10 (A, B and C): Comparison of kinetics of complement activation between nascent BSA anti-BSA IC (▲—▲) and preformed BSA anti-BSA IC (■—■) at an antigen:antibody ratio of 4-times antibody-excess, (A) C1 activation as assessed by the generation of C1s:C1-INH complex, (B) C3 activation as assessed by C3:P complex and C3a formation and (C) C5 activation as assessed by C5b-9. The incubation temperature was 37°C. The concentration of IC was 250µg IgG antibody/ml serum. Each point represents the mean of duplicate determinations.

3.4.7. Comparison of kinetics of complement activation by thyroglobulin anti-thyroglobulin IC and BSA anti-BSA IC:

a) C1 activation

i) Nascent IC: The rate of formation of the C1s:C1-INH complex was higher for BSA anti-BSA prepared at 4-times antibody-excess, equivalence and 4-times antigen-excess than thyroglobulin: anti-thyroglobulin IC (Fig 3.4.1 A, Fig 3.4.6 A).

ii) Preformed IC: In contrast to the situation with nascent IC, the rate and extent of C1s:C1-INH formation were higher with thyroglobulin anti-thyroglobulin IC than BSA anti-BSA IC (Fig 3.4.3 A, Fig 3.4.8 A).

b) C3 activation

i) Nascent IC: The rate and extent of formation of the C3:P complex were higher with BSA anti-BSA IC than with thyroglobulin anti-thyroglobulin IC (Fig 3.4.1 B, Fig 3.4.6 B). There were no obvious effects of differences in antigen-antibody ratio for either type of IC.

C3a production occurred rapidly following the formation of thyroglobulin anti-thyroglobulin IC whereas there appeared to be an initial slow increase which was followed by a second phase of faster accumulation when BSA anti-BSA IC were studied (Fig 3.4.6 B). For both types of IC, those formed at 4-times antigen-excess resulted in the production of less C3a than IC formed at the other two antigen-antibody ratios. BSA anti-BSA IC formed at equivalence resulted in the production of more C3a than those formed at 4-times antibody-excess. This difference was not observed when thyroglobulin anti-thyroglobulin IC were studied.

ii) Preformed IC: The rates of formation of the C3:P complex were similar for thyroglobulin anti-thyroglobulin IC and BSA anti-BSA IC formed at all three antigen-antibody ratios. However the final C3:P levels were higher when BSA anti-BSA IC were used (Fig 3.4.3 B, Fig 3.4.8 B).

The initial rate of C3a generation was higher for thyroglobulin anti-thyroglobulin IC

formed at 4-times antibody-excess and at equivalence, than for BSA anti-BSA IC (Fig 3.4.4 B, Fig 3.4.8 B). In contrast, the initial production rate of C3a was higher for BSA anti-BSA formed at 4-times antigen-excess than for thyroglobulin anti-thyroglobulin IC.

c) C5 activation

i) Nascent IC: C5b-9 generation occurred after a lag-phase of 7.5 minutes for thyroglobulin anti-thyroglobulin IC. After the lag-phase the rate of increase of C5b-9 was greater for IC formed at 4-times antibody-excess and lowest for those formed at 4-times antigen-excess (Fig 3.4.1 C, Fig 3.4.6 C). When IC were formed in antibody-excess the level of C5b-9 continued to rise until the end of the experiment, whereas when IC were formed at equivalence or in antigen-excess the maximum level was reached after 15 minutes. In contrast, although little C5b-9 formation was stimulated by BSA anti-BSA IC, the levels were maximal within 5-7.5 minutes for IC formed at 4-times antibody-excess and at equivalence and after 15 minutes by those formed at 4-times antigen-excess.

ii) Preformed IC: Both types of IC formed at 4-times antibody-excess or at equivalence produced an initial rapid increase in levels of the C5b-9 complex. In both cases C5b-9 formation was slightly faster with IC formed at 4-times antibody-excess. When IC were prepared at 4-times antigen-excess the rate of production of C5b-9 was reduced markedly. The amount of C5b-9 complex formed with thyroglobulin anti-thyroglobulin IC prepared at 4-times antibody-excess was far greater than that formed when IC were prepared at equivalence. This effect of antigen-antibody ratio was not seen with BSA anti-BSA IC (Fig 3.4.3 C, Fig 3.4.8 C).

d) Role of classical and alternative pathways

i) Nascent IC: Although the rate of generation of C3:P complex was reduced by Mg^{++}EGTA for both types of IC, a distinct lag-phase occurred prior to the appearance of the C3:P complex in response to thyroglobulin anti-thyroglobulin IC which was not

seen with BSA anti-BSA IC (Fig 3.4.2 B, Fig 3.4.7 A).

The rate of production and the amount of C5b-9 complex produced in response to either type of complexes was not markedly affected by Mg^{++}EGTA (Fig 3.4.2 C, Fig 3.4.7 B).

ii) Preformed IC: The rate of C3:P complex formation by both types of IC was reduced in the presence of Mg^{++}EGTA (Fig 3.4.4 A, Fig 3.4.9 A).

For both types of IC, the initial rate of C5b-9 complex formation was not reduced, but the slower secondary phase and the final levels were reduced by Mg^{++}EGTA (Fig 3.4.4 B, Fig 3.4.9 B).

3.5. Effect of erythrocytes on IC-mediated complement activation

3.5.1.Nascent thyroglobulin anti-thyroglobulin IC

a) Effect of IC concentration

C1 activation, as assessed by the concentration of C1s:C1-INH, was increased in the presence of erythrocytes, at all IC concentration (Fig 3.5.1 A). Levels of C4a were not measured.

C3 activation, as assessed by the concentration of C3:P, was generally less in the presence of erythrocytes (Fig 3.5.1 B). Levels of C3a were not measured.

Formation of the C5b-9 appeared to be increased slightly when complement activation occurred in the presence of erythrocytes, particularly when IC were formed at equivalence or in antibody-excess (Fig 3.5.1 C). Levels of C5a were not measured.

b) Effect of antigen-antibody ratio

C1 activation was greater when IC were formed at 4-times antibody-excess and least when IC were formed in antigen-excess. At all the antigen-antibody ratios studied, greater levels of C1s:C1-INH were produced when complement activation occurred in the presence of erythrocytes (Fig 3.5.1 A right panel).

At the lowest IC concentration (62.5μg IgG antibody/ml NHS), serum levels of C3:P were greater for IC formed at equivalence, in the absence of erythrocytes. When erythrocytes were present, levels of C3:P were reduced except when complement activation was stimulated by IC formed at 16-times antigen-excess. In contrast, at the highest concentration of IC (500μg IgG antibody/ml NHS), C3:P levels were highest at the extremes of antigen and antibody-excess and lowest when IC were formed at equivalence or at 4-times antibody-excess. When complement activation occurred in the presence of erythrocytes, C3:P levels were reduced (Fig 3.5.1 B right panel).

Generation of C5b-9 was greater when IC were formed at 16-times antibody-excess and least when IC were formed at 16-times antigen-excess. In the presence of erythrocytes, C5b-9 levels were increased slightly (Fig 3.5.1 C right panel).

c) Effect of trypsinization of erythrocytes

As the presence of erythrocytes reduced the concentration of C3:P complex formed during IC-mediated complement activation, the possibility that C3:P complexes were bound to erythrocyte CR1 or that C3b degradation to iC3b had occurred with CR1 acting as a cofactor to factor I was considered. In a preliminary attempt to investigate this problem, the effect of trypsinized erythrocytes on complement activation was compared with the effect of normal erythrocytes. It has been shown previously that trypsin destroys CR1 (Lay and Nussenzweig, 1968; Dorval et al, 1989). In a preliminary study it was shown that neither normal nor trypsinized erythrocytes activated complement when incubated with autologous serum at 37°C.

In the experiments described in this study a single concentration of nascent thyroglobulin anti-thyroglobulin IC (500μg IgG antibody/ml NHS) at five different antigen-antibody ratios (16-times and 4-times antibody-excess, equivalence, 4-times and 16-times antigen-excess) were formed in serum in the absence or the presence of autologus normal or trypsinized erythrocytes.

The amount of C3:P formed was reduced when normal erythrocytes were present, whereas when trypsinized erythrocytes were used, the level of C3:P was not less than that produced in the absence of erythrocytes (Fig 3.5.2 B).

Indeed, when complement activation was produced at 16-times antigen-excess more C3:P was formed in the presence of trypsinized erythrocytes.

The concentrations of C1s:C1-INH and C5b-9 complexes produced in the presence of normal erythrocytes were similar to that produced in the presence of trypsinized erythrocytes (Fig 3.5.2 A, Fig 3.5.2 C).

d) Effect of CR1 blockade

In a further attempt to investigate the role of CR1, erythrocytes were pre-incubated with Fab anti-CR1 or normal rabbit IgG Fab which had been pre-absorbed against NHS-Sepharose, prior to their incubation with erythrocytes. In a preliminary

experiment it was shown that Fab treated erythrocytes did not activate complement.

C1s:C1-INH formation was increased in all five antigen-antibody ratios when complement activation occurred in the presence of erythrocytes, and the effect of erythrocytes was not altered when they had been pre-incubated with Fab anti-CR1 or normal rabbit Fab (Fig 3.5.3 A).

The concentration of C3:P was reduced when complement activation occurred in the presence of normal erythrocytes. After the erythrocytes had been pre-incubated with Fab anti-CR1, the erythrocyte mediated reduction of C3:P levels was not observed. Normal rabbit Fab did not abrogate the effect of erythrocytes (Fig 3.5.3 B).

The slight increase in C5b-9 levels observed when complement activation occurred in the presence of erythrocytes was not altered when the erythrocytes had been pre-incubated with Fab anti-CR1 or normal rabbit Fab (Fig 3.5.3 C).

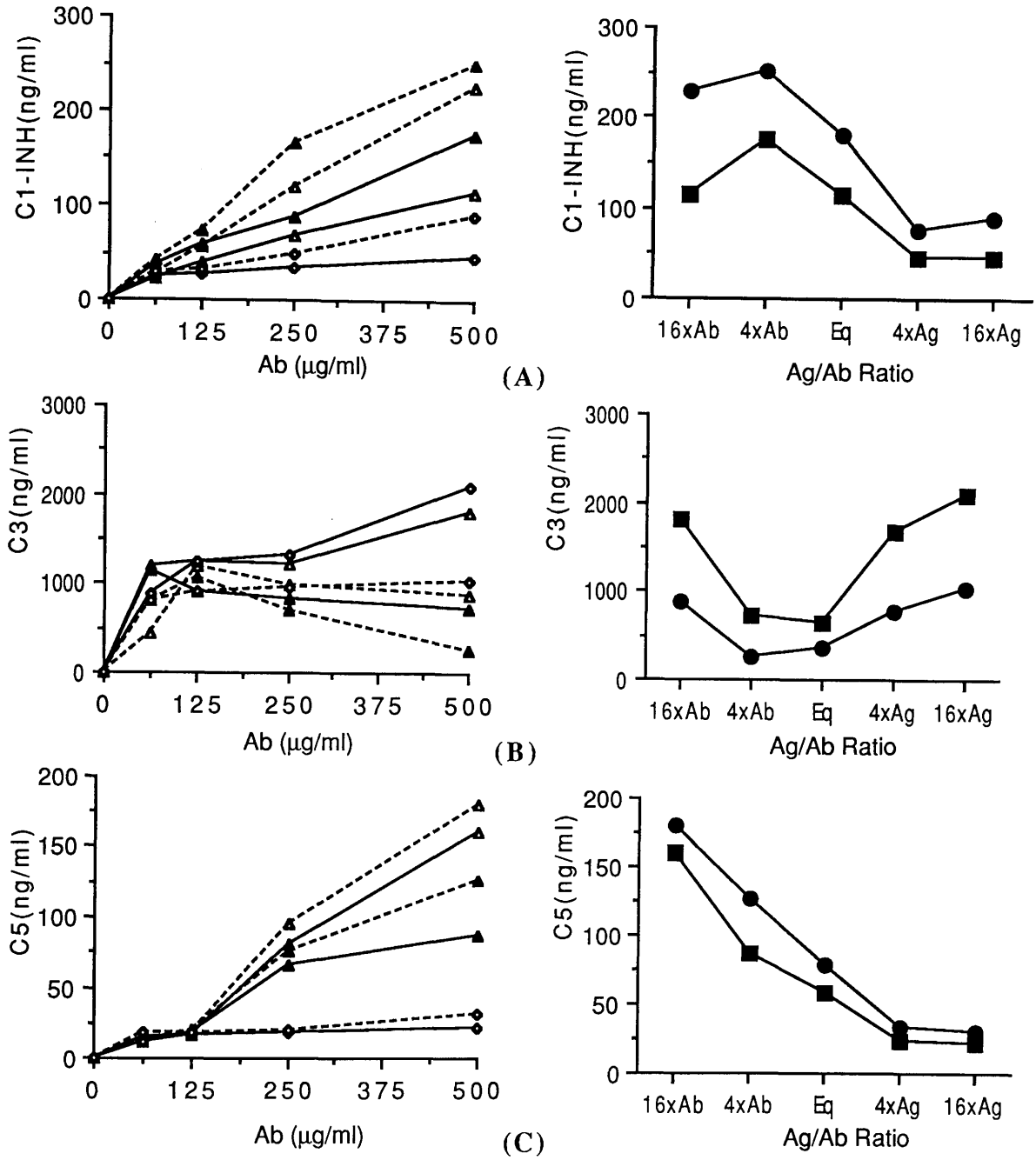
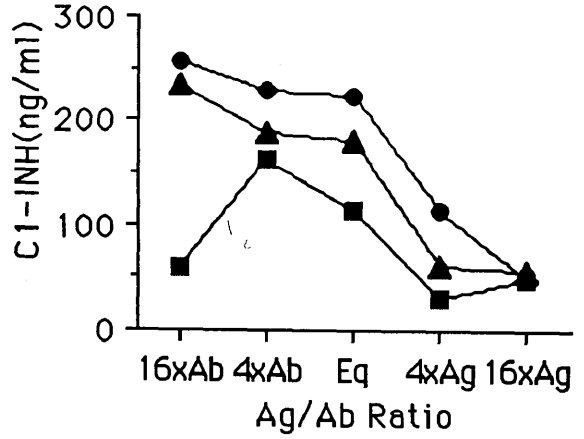
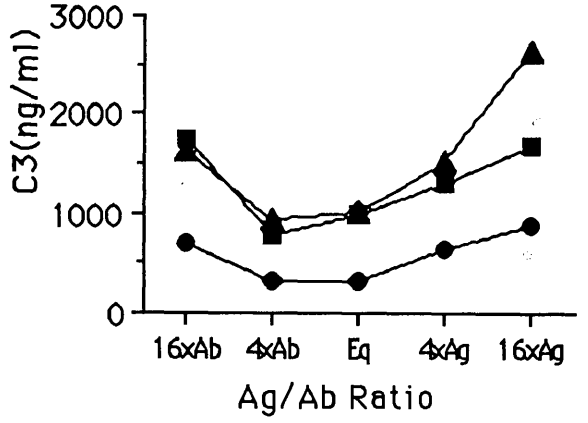


Figure 3.5.1 (A, B and C): Complement activation by nascent thyroglobulin anti-thyroglobulin IC in the presence of human erythrocytes. Left panel: effect of IC concentration at 16-times antibody-excess (▲—▲), 4-times antibody-excess (△—△) and 16-times antigen-excess (◇—◇) in the presence (- - -) or the absence (——) of erythrocytes. Right panel: Effect of antigen-antibody ratio, on complement activation, in the presence (●—●) or absence (■—■) of erythrocytes. (A) C1 activation as assessed by the generation of C1s:C1-INH complex, (B) C3 activation as assessed by C3:P complex and (C) C5 activation as assessed by C5b-9 complex. Concentration of IC was 500µg IgG antibody/ml serum. Each point represents the mean of duplicate determinations.



(A)



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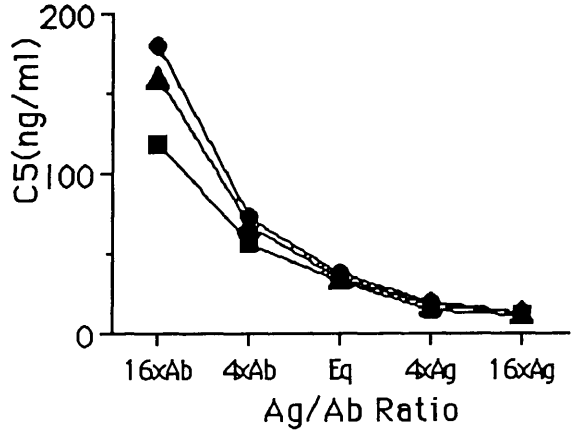
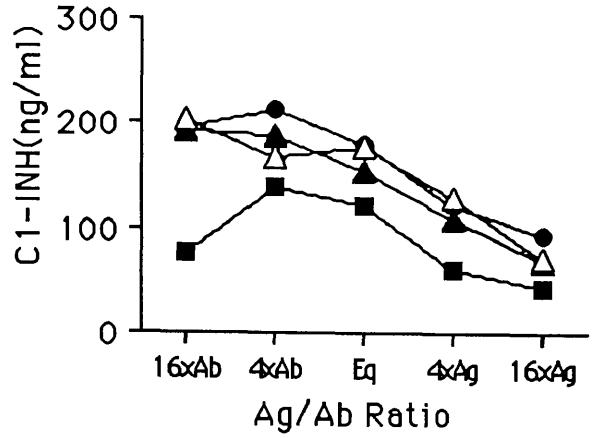
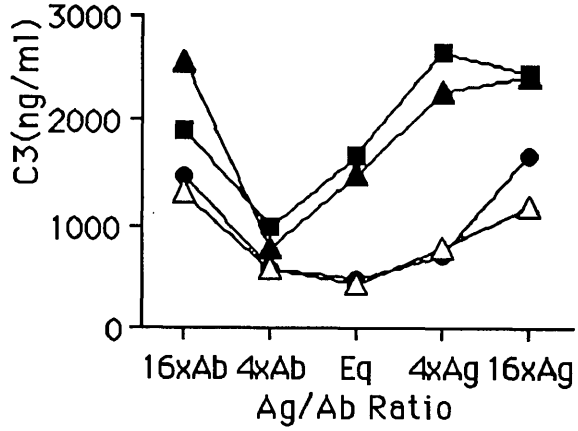


(C)

Figure 3.5.2 (A, B and C): Effect of trypsinised human erythrocytes on complement activation by nascent thyroglobulin anti-thyroglobulin IC, formed at five antigen-antibody ratios, at an IC concentration of 500μg IgG antibody/ml NHS, in the absence (■—■) or the presence of normal human erythrocytes (●—●) or trypsinised human erythrocytes (▲—▲). (A) C1 activation as assessed by the generation of C1s:C1-INH, (B) C3 activation as assessed by the generation of C3:P and (C) C5 activation as assessed by the generation of C5b-9. Each point represents the mean of duplicate determinations.



(A)



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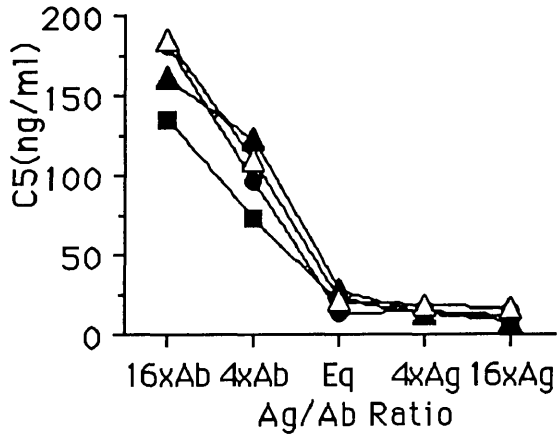

(C)

Figure 3.5.3 (A, B and C): Effect of erythrocyte CR1 blockade on complement activation by nascent thyroglobulin anti-thyroglobulin IC at different antigen-antibody ratios, at an IC concentration of 500μg IgG antibody /ml NHS, in the absence (■—■), the presence (●—●) of normal human erythrocytes or treated human erythrocytes with anti-CR1 Fab fragment (▲—▲) and with normal rabbit Fab (△—△). Each point represents the mean of duplicate determinations.

3.5.2. Preformed thyroglobulin anti-thyroglobulin IC

Levels of C1s:C1-INH and C5b-9 were only increased slightly when complement activation (produced by IC containing 500μg IgG antibody/ml) occurred in the presence of erythrocytes (Fig 3.5.4 A, Fig 3 5.4 C). The changes in C1s:C1-INH and C5b-9 levels were not affected by trypsinization or pre-incubation of erythrocytes with Fab anti-CR1. In contrast, trypsinized erythrocytes and Fab anti-CR1 treated erythrocytes abrogated the effect of erythrocytes on C3:P levels.

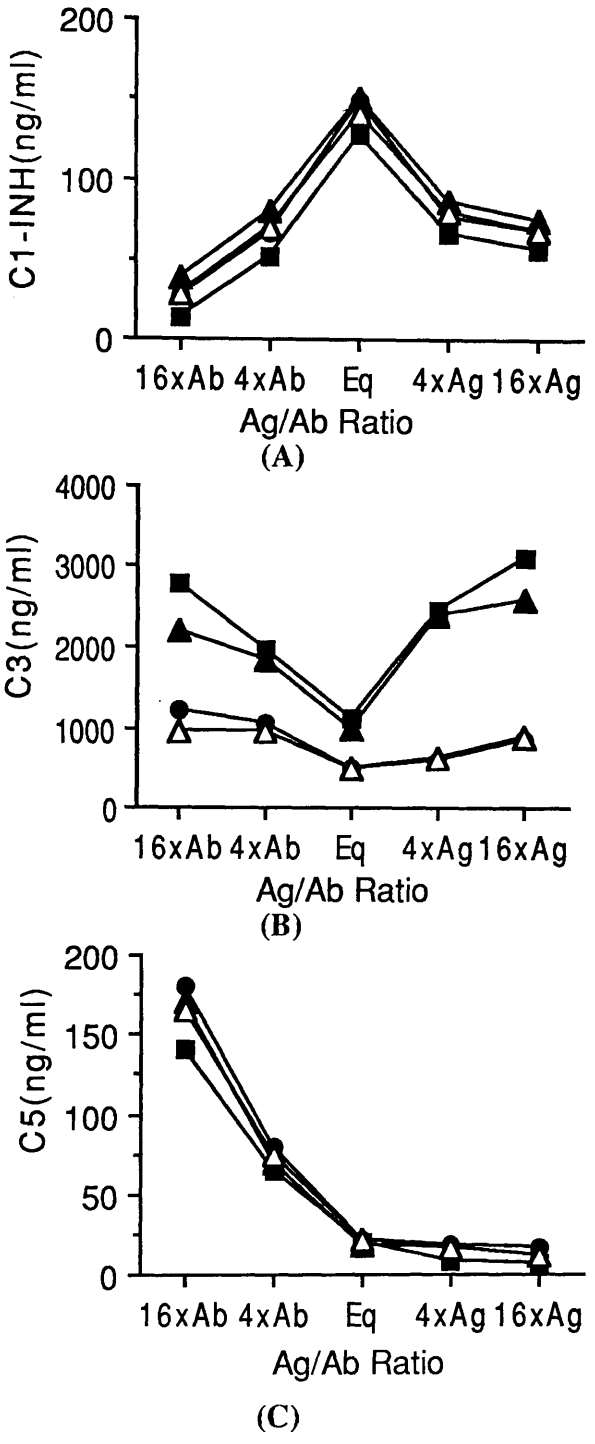

Figure 3.5.4 (A, B and C): Effect of erythrocyte CR1 blockade on complement activation by preformed thyroglobulin anti-thyroglobulin IC at five antigen-antibody ratios and an IC concentration of 500μg IgG antibody/ml NHS, in the absence (■—■) or the presence of normal human erythrocytes (●—●), human erythrocytes treated with anti-CR1 Fab (▲—▲) or with normal rabbit Fab (△—△) (A) C1 activation as assessed by the generation of C1s:C1-INH, (B) C3 activation as assessed by C3:P and (C) C5 activation as assessed by the generation of C5b-9. Each point represents the mean of duplicate determinations.

3.5.3. Activation of complement by nascent BSA anti-BSA IC in the presence and absence of erythrocytes

a) Effect of antigen-antibody ratio

Levels of C1s:C1-INH were higher when complement activation by IC (500μg IgG antibody/ml) at all five antigen-antibody ratios occurred in the presence of trypsinized or normal erythrocytes. Trypsinized erythrocytes behave in an identical manner to normal erythrocytes (Fig 3.5.5. A).

The concentration of C3:P complex was reduced when IC were formed in the presence of normal erythrocytes but when trypsinized erythrocytes were used there was little difference to that formed in the absence of erythrocytes (Fig 3.5.5 B).

The level of C5b-9 complex was increased when IC were formed at 16-times antibody-excess in the presence of erythrocytes (Fig 3.5.5 C). Trypsinized erythrocytes had the same effect as normal erythrocytes.

b) Effect of CR1 blockade

The increased levels of C1s:C1-INH and C5b-9 which were found when complement activation occurred in the presence of normal erythrocytes were not observed when Fab anti-CR1 treated erythrocytes were used (Fig 3.5.6 A, Fig 3.5.6 C).

C3:P level were lower when erythrocytes were present during complement activation, but this effect was not observed when they had been pre-incubated with Fab anti-CR1. Normal rabbit Fab had no effect on the erythrocytes ability to reduce C3:P (Fig 3.5.6 B).

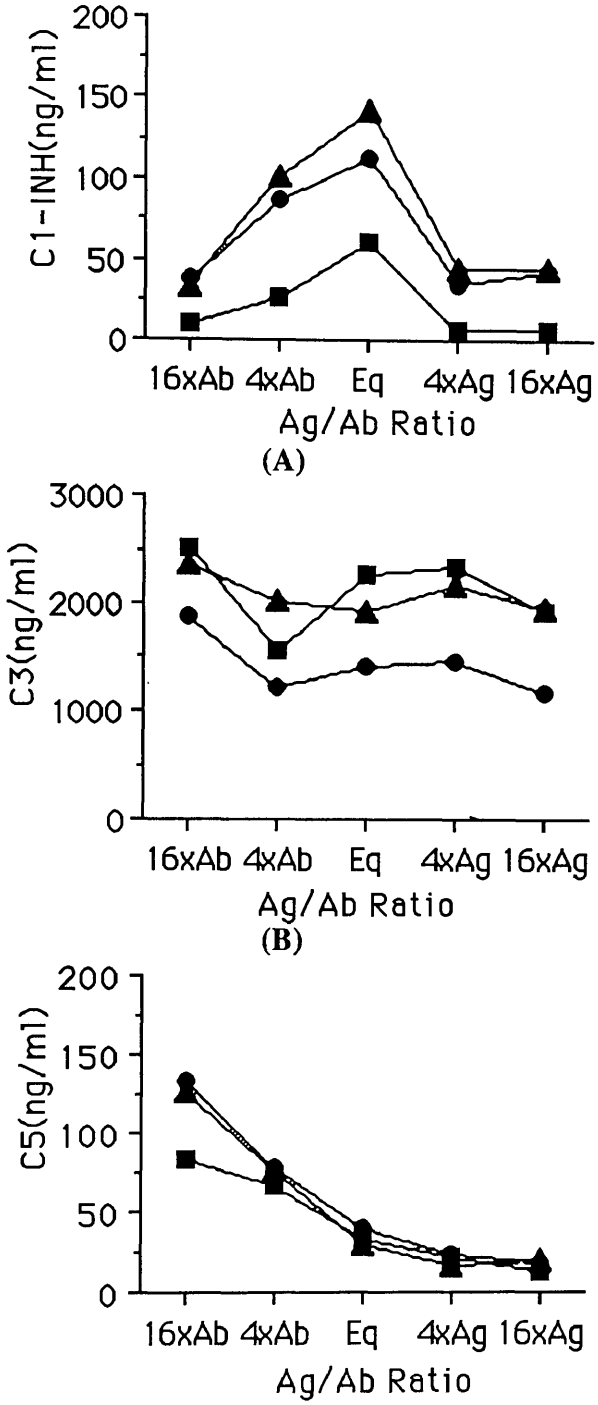

Figure 3.5.5 (A, B and C): Effect of human erythrocytes on complement activation by nascent BSA anti-BSA IC at different antigen-antibody ratios, at an IC dose of 500µg IgG antibody/ml NHS, in the absence (■—■) or the presence of normal human erythrocytes (●—●) or trypsinised human erthrocytes (▲—▲). (A) C1 activation as assessed by the generation of C1s:C1-INH, (B) C3 activation as assessed by the generation of C3:P and (C) C5 activation as assessed by the generation of C5b-9. Each point represents the mean of duplicate determinations.

3.5.4. Effect of erythrocytes on the kinetics of complement activation by IC

In these experiments nascent and preformed IC were studied at three antigen-antibody ratios (4-times antibody-excess, equivalence, 4-times antigen-excess), at a concentration of 250μg IgG antibody/ml NHS.

Nascent thyroglobulin anti-thyroglobulin IC

a) C1 activation

C1s:C1-INH complexes were detectable within one minute of incubation. Peak levels were higher in an incubation mixture containing erythrocytes. For IC formed at 4-times antibody-excess, the initial rates of C1s:C1-INH formation were similar, but after 5 minutes the rate of increase decreased in the reaction mixture which did not contain erythrocytes. The peak level was achieved between 10-15 minutes and 15-30 minutes in the absence and presence of erythrocytes respectively. When IC were formed at equivalence the initial rates of formation of C1s:C1-INH were similar in both reaction mixtures for the first 5 minutes, then as before, there was a reduction in the rate of increase in the absence of erythrocytes and a plateau was reached by 10 minutes (Fig 3.5 7 A left panel). When erythrocytes were present the rate of increase in C1s:C1-INH concentration continued to increase until it reached a plateau level at 10 minutes. For IC formed at 4-times antigen-excess, very little C1s:C1-INH was formed, particularly in the absence of erythrocytes.

The initial rate of the production of C4a was rapid over the first minute, after which time the rate of production decreased and reached a plateau by 15 minutes. The rate and extent of C4a formation were not altered by the presence of erythrocytes (Fig 3.4 7 A left panel). As in the studies of C1s:C1-INH formation, C4a production was greater when IC were formed in antibody-excess or at equivalence and lower when they were formed in antigen-excess.

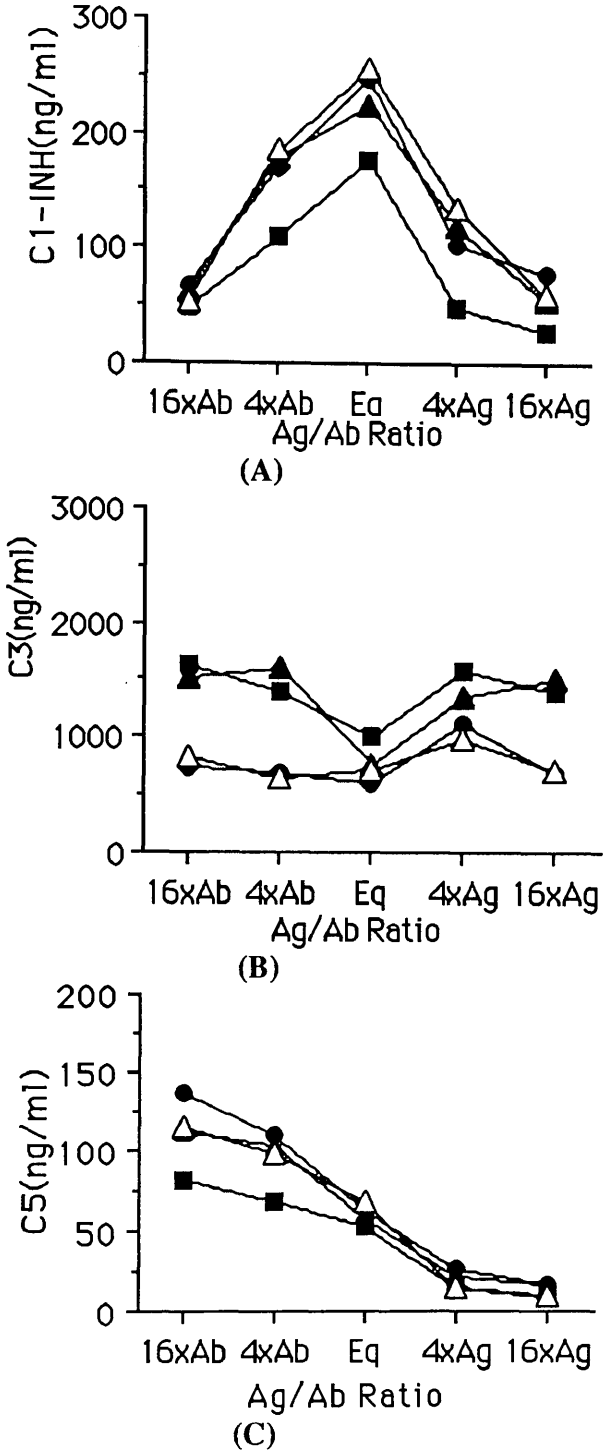

Figure 3.5.6 (A, B and C): Effect of CR1 blockade on complement activation by nascent BSA anti-BSA IC at different antigen-antibody ratios, at an IC dose of 500µg IgG antibody/ml NHS in the absence (■—■) or the presence of normal human erythrocytes (●—●) or treated human erythrocytes with anti-CR1 Fab fragment (▲—▲) or with normal rabbit Fab fragment (△—△). A) C1 activation as assessed by the generation of the C1s:C1-INH complex, B) C3 activation as assessed by the generation of the C3:P complex and C) C5 activation as assessed by the generation of C5b-9 complex. Each point represents the mean of duplicate determinations.

b) C3 activation

The initial rate of increase in the level of C3:P was greater when IC were formed in antibody-excess and lower when they were formed in antigen-excess. For IC formed at 4-times antibody-excess, the initial rate fell after 2.5 minutes, whereas the change in rate occurred later for IC formed at equivalence (5 minutes) or at 4-times antigen-excess (5-10 minutes). The peak C3:P level was similar at all three antigen-antibody ratios. In the presence of erythrocytes the initial rate of increase of C3:P and the final concentration of C3:P were reduced (Fig 3.5.7 B left panel). The effect of erythrocytes was most marked when IC were formed in antigen-excess, and least for IC formed in antibody-excess.

The rates of formation of C3a were rapid for IC formed in antibody-excess or at equivalence, but lower for IC formed in antigen-excess. The final concentration of C3a was slightly higher for IC formed in antibody-excess. In contrast, the level of C3a was much lower when IC were formed in antigen-excess. This is in contrast to the level of C3:P in these reaction mixtures. The presence of erythrocytes did not affect the rate or extent of C3a formation (Fig 3.5.7 B right panel).

c) C5 activation

C5 activation, as assessed by measuring the C5b-9 levels, occurred rapidly and was maximal within 7.5-10 minutes when IC were formed in antibody-excess. The rate and extent of C5b-9 formation were lower for IC formed at equivalence, and were minimal when IC were formed in antigen-excess. More C5b-9 was formed in the presence of erythrocytes (Fig 3.5.7 C left panel), and it appeared that the initial rate of C5b-9 formation was also greater when erythrocytes were present at best when IC were formed in antibody-excess.

The initial rate of increase of C5a was faster than that for C5b-9, particularly when IC were formed in antibody-excess and at equivalence. Although C5a levels were lower when IC were formed at equivalence than in antibody-excess, the reduction was

not as pronounced as the reduction in levels of C5b-9.

From the data it was difficult to determine whether the erythrocytes affected the rate and extent of C5a accumulation (Fig 3.5.7 C right panel).

d) Role of classical and alternative pathways

When complement activation occurred in the presence of Mg^{++}EGTA, there was a lag-phase of 2.5-5 minutes before C3:P levels began to increase. The rate of increase and the final concentration of C3:P and C3a were reduced in Mg^{++}EGTA-treated serum. Although levels of C3:P were lower when erythrocytes were present in the reaction mixture, C3a levels were almost identical in the presence and absence of erythrocytes.

The rate and extent of C5b-9 formation at all three antigen-antibody ratios were similar or slightly reduced in Mg^{++}EGTA-treated serum. The effect of erythrocytes was not affected by Mg^{++}EGTA (Fig 3.5.7 C).

Preformed thyroglobulin anti-thyroglobulin IC

a) C1 activation

Very little formation of C1s:C1-INH occurred at any antigen-antibody ratios, although there was slightly more produced with IC formed at equivalence. The initial rate of formation was extremely rapid with IC formed at equivalence or in antibody-excess and was little slower for IC formed in antigen-excess. Slightly more C1s:C1-INH was formed when erythrocytes were present (Fig 3.5.8 A).

C4a formation occurred rapidly for the first 30 seconds and then slowed until the plateau level was achieved (5 minutes for IC formed at equivalence or in antigen-excess and 7.5 minutes for IC formed in antibody-excess). When IC at equivalence were studied, C4a was formed slightly more quickly when erythrocytes were present. However, the increase was quite small and was not seen at the other antigen-antibody ratios (Fig 3.5.8 A).

b) C3 activation

In the absence of erythrocytes, the initial rate of increase in the level of C3:P was rapid at all three antigen-antibody ratios. The rate slowed after one minute for IC formed at equivalence or in antibody-excess, and after 2.5 minutes for IC formed in antigen-excess. A plateau level of C3:P was reached within 5-10 minutes at all antigen-antibody ratios. The maximum level was greatest for IC in antigen-excess. When erythrocytes were present the maximum level of C3:P was lower than that seen in their absence, and there was an obvious decrease in C3:P levels after 30 minutes incubation. This decrease was particularly marked for IC in antigen-excess (Fig 3.5.8 B left panel).

C3a production was extremely rapid, whether erythrocytes were present or not. Plateau levels were achieved by 5 minutes for IC in antibody-excess and 2.5 minutes for IC at equivalence (Fig 3.5.8 B right panel).

C3a levels were higher when IC at equivalence were used. In contrast, when IC were formed in antigen- excess far less C3a was formed, and the rate of production was lower than that occurring in response to IC at equivalence or in antibody-excess. Erythrocytes probably did not affect the rate of C3a production.

c) C5 activation

For IC formed at equivalence or in antibody-excess, the initial rate of C5b-9 formation was the same in the presence or absence of erythrocytes. However, the second phase of formation was greater when erythrocytes were present (Fig 3.5.8 C left panel). More C5b-9 was formed when IC were formed in antibody-excess than at equivalence. IC in antigen-excess stimulated little C5b-9 formation, and the presence of erythrocytes did not affect this.

A similar effect of the antigen-antibody ratio was observed on the kinetics of C5a production (Fig 3.5.8 C right panel). The greatest amount of C5a was produced with IC in antibody-excess and the lowest with IC in antigen-excess. At all antigen-antibody ratios, the rate and extent of C5a production were unaltered by the presence of erythrocytes.

d) Role of the classical and alternative pathways

i) C3 activation

In Mg^{++}EGTA there was a distinct lag-phase before C3:P levels began to increase slowly at all three antigen-antibody ratios. For IC in antibody-excess or at equivalence the plateau level was reached at 7.5 and 10 minutes whereas for IC in antigen-excess it was reached between 10 and 15 minutes. The maximum levels of C3:P were similar in normal serum and Mg^{++}EGTA-treated serum for IC formed in antibody-excess and at equivalence (Fig 3.5.8 B left panel). The level of C3:P formed in antigen-excess was reduced in the presence of Mg^{++}EGTA. Levels of C3:P were slightly reduced when erythrocytes were present, but the rate of C3:P increase was unaltered.

The rate of formation of C3a was reduced in Mg^{++}EGTA and a definite lag-phase occurred with IC formed in antibody-excess, although this was less obvious for IC at equivalence (Fig 3.5.8 B right panel). The final level of C3a was the same in normal serum and in Mg^{++}EGTA-treated serum at all three antigen-antibody ratios. Erythrocytes did not affect the rate or extent of formation of C3a in Mg^{++}EGTA-treated serum.

ii) C5 activation

The rate and extent of C5b-9 formation were reduced in Mg^{++}EGTA-treated serum. In the presence of erythrocytes the final C5b-9 formation was increased when complement activation was produced by IC formed at antibody-excess or at equivalence (Fig 3.5.8 C left panel). However, erythrocytes did not affect C5b-9 levels when IC in antigen-excess were used.

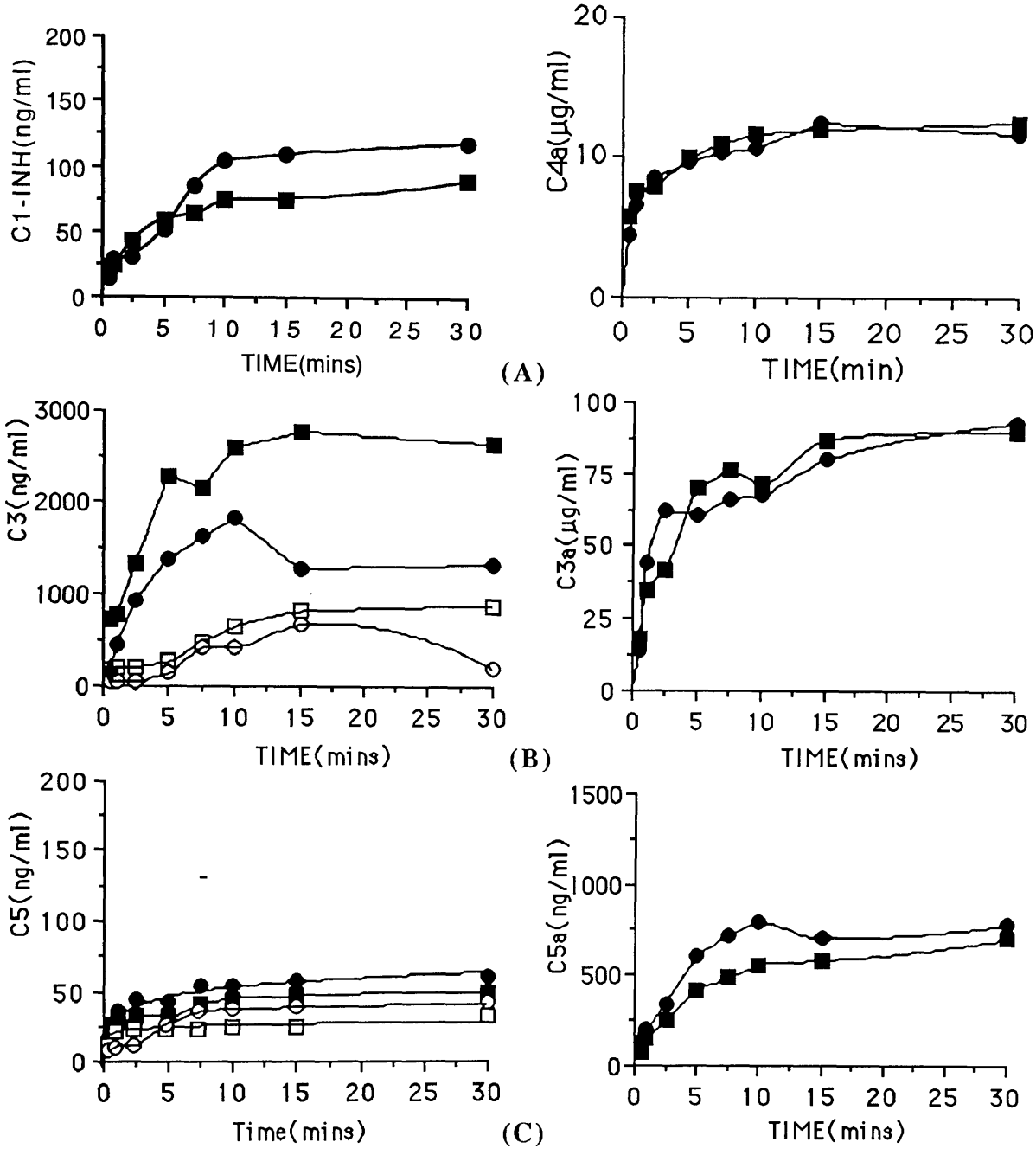

Figure 3.5.7. (A, B and C): Kinetics of complement activation by nascent thyroglobulin anti-thyroglobulin IC (250µg IgG antibody/ml NHS) at equivalence in the presence (●—●) or the absence (■—■) of human erythrocytes. (A) C1 activation as assessed by the generation of C1s:C1-INH complex (left) and C4a (right), (B) C3 activation as assessed by the generation of C3:P complex (left) and C3a (right) and (C) C5 activation as assessed by the generation of C5b-9 complex (left) and C5a (right). The experiment was performed in the presence (empty symbols) or the absence (solid symbols) of $Mg^{++}EGTA$, C4a and C5a levels were not measured in the $Mg^{++}EGTA$ experiments Each point represents the mean of duplicate determinations.

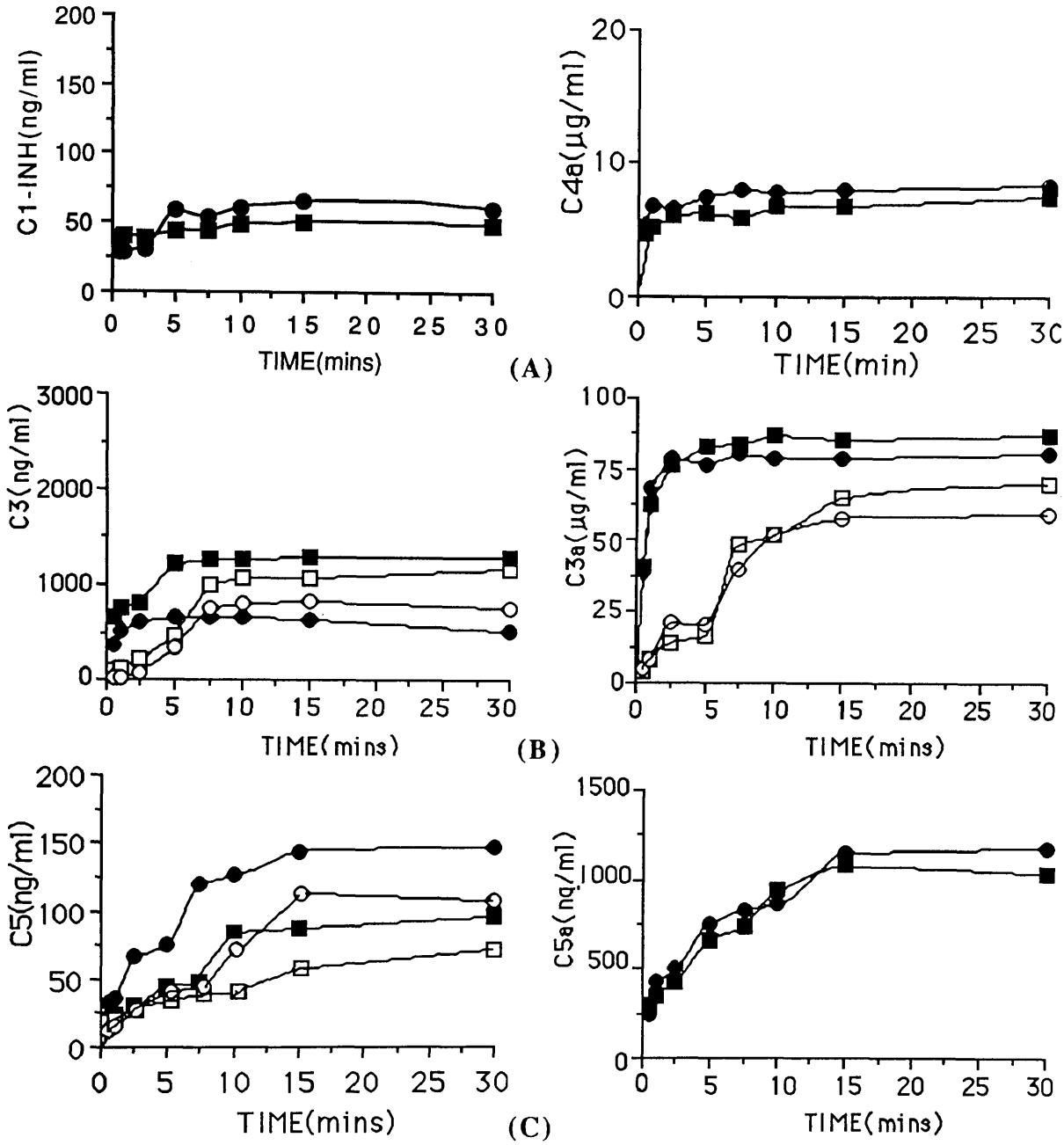

Figure 3.5.8. (A, B and C): Kinetics of complement activation by preformed thyroglobulin anti-thyroglobulin IC (250µg IgG antibody/ml NHS) at equivalence in the presence (●—●) or the absence (■—■) of human erythrocytes. (A) C1 activation as assessed by the generation of C1s:C1-INH complex (left) and C4a (right), (B) C3 activation as assessed by the generation of C3:P complex (left) and C3a (right) and (C) C5 activation as assessed by the generation of C5b-9 complex (left) and C5a (right). The experiment was performed in the presence (empty symbols) or the absence (solid symbols) of Mg^{++}EGTA. C4a and C5a levels were not measured in Mg^{++}EGTA experiments. Each point represents the mean of duplicate determinations.

3.5.6 Comparison of nascent and preformed thyroglobulin anti-thyroglobulin IC

a) C1 activation

Although the initial rate of increase in C1s:C1-INH level was slower with nascent IC, they produced a larger final concentration than preformed IC. In the presence of erythrocytes C1s:C1-INH levels were increased markedly with nascent IC, whereas with preformed IC the erythrocyte effect was less pronounced (Fig 3.5.7 A left panel, Fig 3.5.8 A left panel).

The rate of C4a production was similar for nascent and preformed IC. The peak levels of C4a were higher when nascent IC were used, particularly at equivalence or in antigen-excess. Erythrocytes did not affect C4a production with either type of IC (Fig 3.5.7 A right panel, Fig 3.5.8 A right panel).

b) C3 activation

When erythrocytes were present C3:P levels were reduced with both types of IC. The peak levels of C3:P were lower and were reached earlier when preformed IC were used. The largest reduction was seen when nascent IC were formed in antigen-excess.

C3a generation increased more rapidly when preformed IC were used, although the peak levels were higher with nascent IC. Erythrocytes did not affect the rate or extent of C3a generation for either type of IC (Fig 3.5.7 B right panel, Fig 3.5.8 B right panel).

c) C5 activation

Greater amounts of C5b-9 were formed by preformed IC (Fig 3.5.8 C left panel). When erythrocytes were present with preformed IC in antibody-excess or at equivalence, more C5b-9 was formed than with nascent IC. The initial rate of production and final concentrations of C5a were higher for preformed IC formed in antibody-excess or at equivalence than for nascent IC.

Kinetics of complement activation by nascent BSA anti-BSA IC

a) C1 activation

Serum levels of C1s:C1-INH increased rapidly at all three antigen-antibody ratios. The initial rate of increase was greatest for IC formed at equivalence (Fig 3.5.9 A). In the presence of erythrocytes the rate of increase remained higher for longer at all three antigen-antibody ratios.

b) C3 activation

C3:P levels increased rapidly over the first 2.5 minutes at all three antigen-antibody ratios (Fig 3.5.9 B left panel). The presence of erythrocytes did not affect the initial rate of increase in C3:P, although the plateau level was generally achieved earlier and was lower than was seen in the absence of erythrocytes.

C3a levels increased more slowly than C3:P levels and the presence of erythrocytes did not alter the rate of increase or the final concentration. The rate of increase and the final concentration were lowest for IC formed in antigen-excess and greater for IC formed at equivalence (Fig 3.5.9 B right).

c) C5 activation

Little C5b-9 formation occurred, and rate and extent of formation were not affected by the presence of erythrocytes. The initial rate of formation was higher for IC formed in antibody-excess or at equivalence than for IC formed in antigen-excess (Fig 3.5.9 C).

d) Role of the classical and alternative pathways

In the presence of Mg^{++}EGTA the rate of increase of C3:P was markedly reduced, although the final concentration was only reduced with IC in antigen-excess. When complement activation occurred in the presence of erythrocytes, the final concentration of C3:P was reduced with IC in antibody-excess and in antigen-excess but not with IC

at equivalence (Fig 3.5.9 C).

The rate of formation of C5b-9 was reduced in Mg^{++}EGTA-treated serum, but the final level for C5b-9 was unchanged. Erythrocytes had no effect on the rate of formation or the final concentration of C5b-9 (Fig 3.5.9 C).

Preformed BSA anti-BSA IC

a) C1 activation

In the absence of erythrocytes, the rate of increase and the final concentration of C1s:C1-INH were low. Both were increased slightly when complement activation occurred in the presence of erythrocytes, but their effect was mainly seen when complement activation was produced by IC at equivalence (Fig 3.5.10 A).

b) C3 activation

The rate of increase in C3:P was rapid, particularly with IC in antigen-excess. In the presence of erythrocytes, the rate of increase slowed earlier and the final concentration was reduced (Fig 3.5.10 B left panel).

The rate of of increase of C3a was slower than C3:P. For IC formed in antibody-excess or at equivalence the plateau level was not achieved until 15 minutes had elapsed, whereas with IC in antigen-excess, the plateau was reached within 5 minutes. Erythrocytes did not affect the rate or extent of C3a formation (Fig 3.5.10 B right panel).

c) C5 activation

For IC formed in antibody-excess or in antigen-excess the rates of formation of C5b-9 were lower than for IC formed at equivalence. The rate of C5b-9 formation was increased when erythrocytes were present in the reaction mixtures containing IC at equivalence (Fig 3.5.10 C), and to a lesser extent in antibody-excess, but not with IC in antigen-excess.

d) The role of the classical and alternative pathways

The rate of increase in C3:P was reduced in Mg^{++}EGTA-treated serum, particularly with IC formed at equivalence or in antigen-excess. The final concentration of C3:P was reduced with IC formed in antibody-excess or in antigen-excess, but not with IC at equivalence (Fig 3.5.10 B left panel). In the presence of erythrocytes the rate of increase and the final concentration of C3:P were reduced.

The rate of production and the final concentration of C5b-9 were reduced in Mg^{++}EGTA-treated serum. When complement activation occurred in the presence of erythrocytes, increased C5b-9 formation was only seen with IC in antibody-excess (Fig 3.5.10 C).

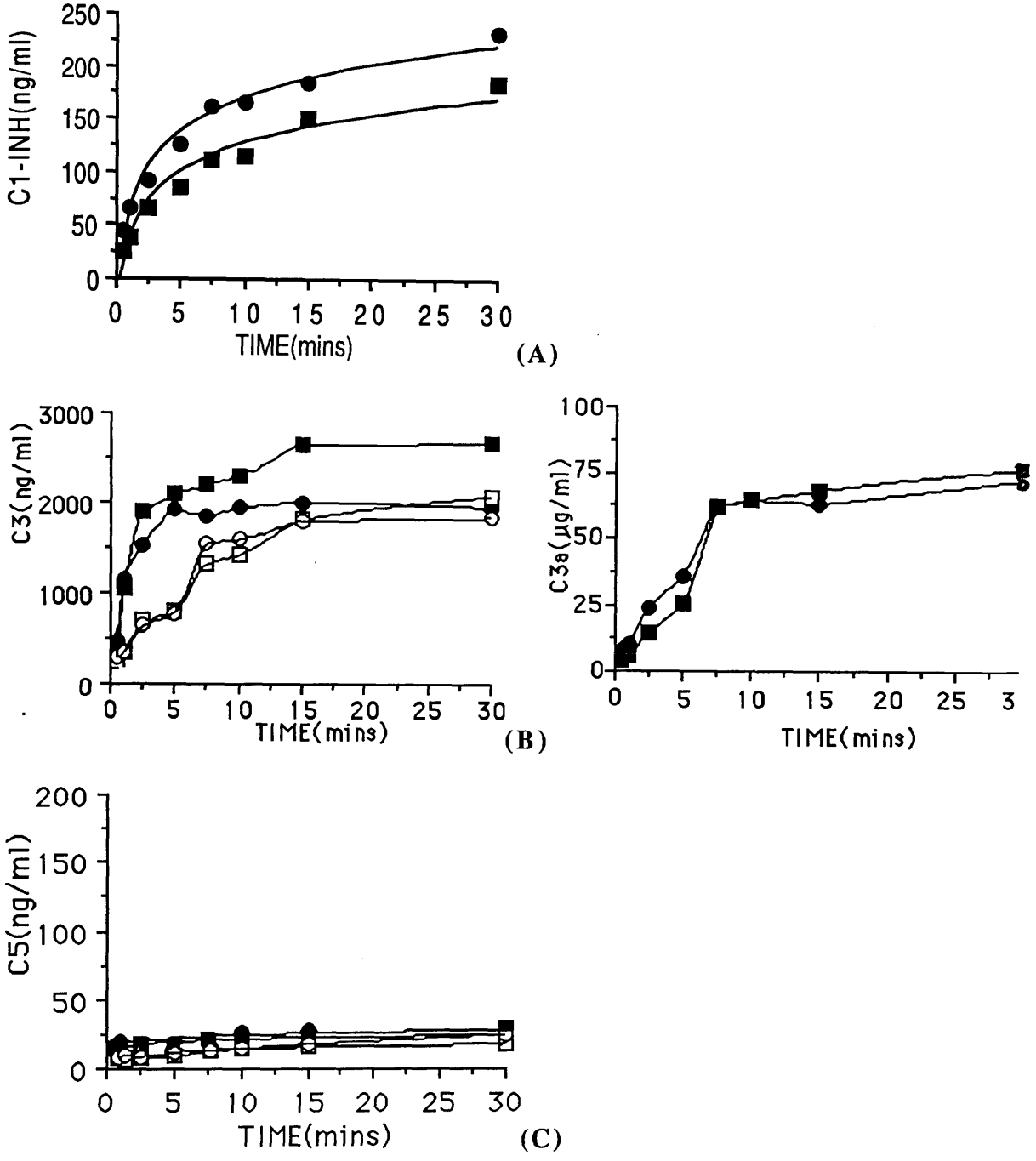

Figure 3.5.9 (A, B and C): Kinetics of complement activation by nascent BSA anti-BSA IC (250µg IgG antibody/ml NHS) at equivalence in the presence (●—●) or the absence (■—■) of human erythrocytes. (A) C1 activation as assessed by the generation of C1s:C1-INH complex, (B) C3 activation as assessed by the generation of C3:P complex (left) and C3a (right) and (C) C5 activation as assessed by the generation of C5b-9 complex. The experiment was performed in the presence (empty symbols) or the absence (solid symbols) of $Mg^{++}EGTA$. Each point represents the mean of duplicate determinations.

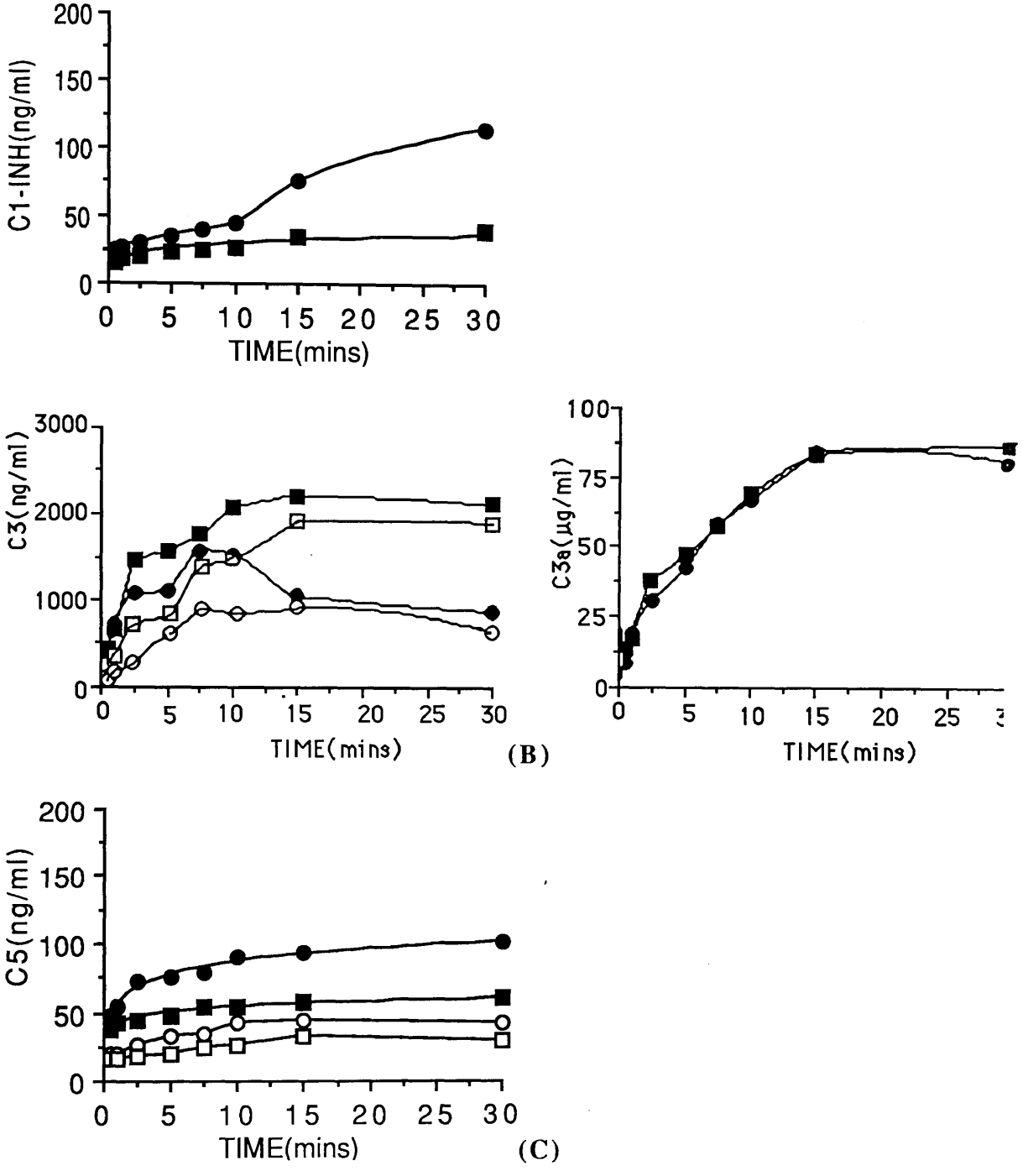

Figure 3.5.10 (A, B and C): Kinetics of complement activation by preformed BSA anti-BSA IC (250µg IgG antibody/ml NHS) at equivalence in the presence (●—●) or the absence (■—■) of human erythrocytes: (A) C1 activation as assessed by the generation of C1s:C1-INH complex, (B) C3 activation as assessed by the generation of C3:P complex (left) and C3a (right) and (C) C5 activation as assessed by the generation of C5b-9 complex. The experiment was performed in the presence (empty symbols) or the absence (solid symbols) of $Mg^{++}EGTA$. Each point represents the mean of duplicate determinations.

Comparison of nascent and preformed BSA anti-BSA IC

a) C1 activation

The rate and the extent of increase of C1s:C1-INH were greater with nascent than with preformed IC (Fig 3.5.9 A, Fig 3.5.10 A). Erythrocytes had a more pronounced effect on the rate and extent of the increase of C1s:C1-INH with nascent IC.

b) C3 activation

The rate of increase of C3:P over the first 2.5 minutes was higher with nascent IC, with the possible exception of preformed IC formed in antigen-excess (Fig 3.5.9 B left panel, Fig 3.5.10 B left panel). Erythrocytes appeared to slow the rate of increase in C3:P to a greater extent with preformed IC.

The rate and the extent of C3a production were greater with preformed IC (Fig 3.5.9 B right panel, Fig 3.5.10 B right panel). Erythrocytes had no effect on C3a formation with either types of IC.

c) C5 activation

The rate and extent of C5b-9 formation were higher with preformed IC. Erythrocytes increased the rate and extent of C5b-9 formation only with preformed IC (Fig 3.5.9 C, Fig 3.5.10 C).

d) Role of classical and alternative pathways

The rate of increase in C3:P formation with the nascent and preformed IC were reduced in Mg^{++}EGTA-treated serum (Fig 3.5.9 B left panel, Fig 3.5.10 B left panel). The effect of erythrocytes was more marked with preformed IC.

In Mg^{++}EGTA the rate of formation of C5b-9 was reduced, for both preformed and nascent IC, but the extent of formation was reduced only for preformed IC (Fig 3.5.9 C, Fig 3.5.10 C). Erythrocytes in normal serum only increased C5b-9 formation for preformed IC at equivalence or in antibody-excess, and in Mg^{++}EGTA-treated serum was only observed with IC in antibody-excess.

CHAPTER

FOUR

DISCUSSION

The measurement of the degree of complement activation is an important constituent of monitoring the activity of diseases such as rheumatoid arthritis (Inman and Harpel, 1983) and systemic lupus erythematosus (Lockshin et al, 1986; Horigome et al, 1987). Currently the degree of complement activation has been assessed by measurement of the antigenic levels of components and residual haemolytic activity by the classical and alternative haemolytic assays (CH50 and APH50). These results however, may be of limited value since some patients have normal or elevated levels due to hypersynthesis of complement components which compensates for the increased catabolism caused by complement activation (Ruddy et al, 1975). In addition, there are technical problems associated with the measurement of complement components, these are namely: 1) Antigenic levels may not correspond with functional activity. For instance, C4 is the product of two genetic loci, C4A and C4B (O'Neill et al, 1978). Null alleles are common in the normal population (Awdeh et al, 1979), accordingly a finding of a low total C4 in these people is not necessarily as a result of C4 activation (Holme et al, 1988). 2) Measurement of complement activation by haemolytic assays only shows the residual functional activity of the classical (CH50) or the alternative (APH50) pathway and does not necessarily indicate ongoing complement activation. These methods also cannot distinguish between genuine deficiency of a complement component and activation of the serum resulting in reduced complement levels. 3) Certain methods are accompanied with specific problems, for example, since C3dg shares antigenic epitopes with C3, C3b and iC3b they must be separated from C3dg prior to its measurement. Polyethyleneglycol (PEG; 11%w/v) can be used to precipitate C3, C3b and iC3b (Perrin et al, 1975) but precipitation of these components may be either incomplete or sometimes excessive C3dg precipitation may occur. Measurement of anaphylatoxins is believed to be a good indicator of activation in patients with IC diseases (Belmont et al, 1986), however, levels in clinical specimens (plasma) do not always reflect the ongoing complement activation. For instance, plasma levels of C5a are extremely low during complement activation due to the binding of C5a and $C5a_{dcs\text{-}Arg}$

to their receptors on leucocytes (Chenoweth and Hugli, 1978; Van Epps and Chenoweth, 1984), although this anaphylatoxin is readily detectable during *in vitro* activation of serum. Furthermore, the methods for the measurement of cleavage products of C4 (C4d), C3 (C3dg) and factor B (Ba) involve technical procedures which either limit their value in clinical service laboratories or their use to certain specialist centres. For instance, electro-immunoassays (Laurell et al, 1979; Brandslund et al, 1981) and radial immunodiffusion (Perrin et al, 1975; El-Ghobary and Whaley, 1980) are of limited value as only a few samples can be assayed in each procedure and immunoprecipitation may take more than 24 hours, which may be an unacceptable delay in a clinical service laboratory. Radioimmunoassays (Hack et al, 1981), although very sensitive, have the inherent potential health hazards which occur with the use of radioisotopes. ELISAs have been described based on the use of monoclonal antibodies against the neoantigenic determinants expressed on the activation products such as C3 activation products iC3b and C3d (Aguado et al, 1985; Mollnes, 1985), and C3dg (Mollnes and Lachmann, 1987), Similarly, monoclonal antibodies have been described for the neoantigens expressed on the membrane attack complex (Mollnes and Harboe, 1987; Falk et al, 1988). However these monoclonal antibodies are not available from commercial sources.

These problems have led to the development of ELISAs which detect soluble complement activation complexes (C1s:C1-INH, C3:P and C5b-9) in biological fluids, using polyclonal antibodies. Detection of C1s:C1-INH indicates classical pathway activation (Laurell et al, 1976; Inman and Harpel, 1983; Hack et al, 1984) and C5b-9 complexes the assembly of the membrane attack complex (Gawryl et el, 1986). Although the presence of the C3:P complex has been thought to measure alternative pathway activation (Asghar et al, 1987; Langlois and Gawryl, 1988), this has not been formally proven.

C1r:C1s:C1-INH complex is generated when C1-inhibitor binds to the active site of C1r and C1s in the activated C1 macromolecule, leading to their dissociation from IC-bound C1q. The resulting complex contains two molecules of C1-inhibitor and one

molecule each of C1r and C1s (Sim et al, 1979; Ziccardi and Cooper, 1979). Since the formation of this complex is independent of calcium ions, it remains stable even in the presence of EDTA which may be used to stop any further complement activation.

The C3:P complex has been detected using anti-human properdin and anti-human C3. This ELISA should detect a C3bBbP complex, since under physiological conditions, B binds C3b but not iC3b (Ross et al, 1983) and under the same conditions, properdin will not bind to C3b in the absence of bound Bb (Mayes et al, 1984; Asgher et al, 1987; Farries et al, 1988a).

The assembly of the membrane attack complex is initiated by the enzymatic cleavage of C5 molecules by the classical pathway (Goldlust et al, 1973) and/or the alternative pathway (Schreiber et al, 1978) C5 convertases. This step is followed by the non-enzymatic assembly of the late complement components (C6-9). The formation of the hydrophilic SC5b-9 complex results from the binding of the regulatory protein, S protein, to the hydrophobic binding site of C5b67 complex. It is composed of one molecule of each of C5b, C6, C7, C8, three molecules of C9 and three molecules of S protein (Podack et al, 1977).

The ELISA systems which I have developed to measure the C1s:C1-INH, C3:P and C5b-9 complexes are based on trapping respectively the C1s, properdin and C9 components of these complexes, using the IgG fraction of the appropriate goat antiserum. The bound complexes are then detected with biotin-conjugated goat IgG anti-human C1-INH, anti-human C3 and anti-human C5 for the three assays respectively. Each trapping antibody was selected as it recognised the component which was present in serum at the lowest molar concentration in order to allow maximum binding of the complex to the ELISA plate wells and minimising competition from the free component in the serum. Anti-C1r cannot be used to detect the C1r:C1s:$(C1\text{-}INH)_2$ complex as C1r loses most of its antigenic determinants when complexed to C1-INH (Laurell et al, 1976). The trapping of C9 and probing for C5 moieties in the C5b-9 complex was used for the measurement of C5b-9 complex but

Horigome et al (1987) showed that trapping of C7 instead of C9 and probing with anti-C5 gave similar results. In these assays, the use of coating and probing antibodies from the same animal species reduces the possibility of a high background which normally results from interactions between IgG fractions of different species. The biotin-avidin system has previously been shown to amplify the sensitivity of immunoassays (Guesdon et al, 1979; Sutton et al, 1985) and give rapid colour development. This technique was used throughout this study, and although developing rapidly, the colour intensity showed a linear relationship with time (Fig 3.1.4) as reported previously (Costa-Castro et al, 1987). Colour development has been reported to take a few hours (Nakane and Kawaoi, 1974; Nilsson and Bäck, 1985), however, in the ELISAs described in this study, colour development occurred within 15 minutes (Fig 3.1.4). Apart from its increased sensitivity, the most important advantage derived from using the avidin-biotin system is the greater recovery of conjugated antibodies after the conjugation procedure, which reduced the cost of materials. This method of signal amplification has not been used in previously published work in this field.

Commercially available antisera were found to be satisfactory (at low concentrations, Fig 3.1.1) for all three ELISA procedures.

Using the procedures described, all these assays were reproducible with intra-assay and interassay variations of less than 10%.

The standard curves used in the ELISA described in this study were referred to pooled standard serum from 40 normal volunteers. This type of reference curve is easier for use in routine laboratories, as purification of complement activation complexes for the standard curve requires specialized equipment and expertise. The standard curves were constructed by coating some wells with anti-human C1-INH, C3 or C5 for the C1s:C1-INH, C3:P and C5b-9 assays respectively. The concentrations of these complexes were then expressed as the amount of C1-INH, C3 and C5 (ng/ml) respectively. The biotin-conjugated antibodies which probe for antigenic epitopes of C1-INH, C3 and C5 are expected to show a similar proportional binding to those

determinants expressed on the native components (standard curve) as well as those on the complexed components (the samples) depending on the concentration of the antigenic epitopes. This procedure has been used for the calculation of concentration of the C3:P complex (Mayes et al, 1984; Asghar et al, 1987).

Comparison of the sensitivity of C1s:C1-INH and C5b-9 assays with those of other workers is difficult, as different standard curves were used. For instance, in case of the C1s:C1-INH ELISA the standard curve was either referred to purified C1s:C1-INH complex (Nilsson and Bäck, 1985), or by expressing the amounts of C1s into either equivalent C1s:C1-INH complex (μg/ml) (Inman and Harpel, 1983) or as nanomole C1s:C1-INH per ml (Lockshin et al, 1986). In case of the C5b-9 assay, the reference standard curves were either performed by using purified membrane attack complex (Gawryl et al, 1986) or expressed as an arbitrary units per ml (Mollnes and Paus, 1986; Horigome et al, 1987). The standard curves of C1s:C1-INH and C5b-9 assays, described in this study, were expressed in terms of complexed C1-INH and C5 (ng/ml) respectively. The C3:P ELISA described in this study was more sensitive (lower limit 0.45ng C3/ml; OD=0.25, Fig 3.1.3.B) than that described by Mayes et al (1984) (lower limit was 10ng C3/ml, OD=0.1).

The three ELISAs were used to measure the levels of complement activation in normals and patients with RA and SLE. The relationship between individual activation complexes and chronic rheumatic diseases has been studied by several groups (Nilsson and Bäck, 1985; Lockshin et al, 1986; Mayes et al, 1984; Morgan et al, 1988). However to date no other group has measured the levels of the three complexes in the same sample. The data presented here, examine the three activation complexes C1s:C1-INH, C3:P and C5b-9 in serum and plasma of normal individuals and patients with RA and SLE and synovial fluid of patients with RA.

Levels of C1s:C1-INH, C3:P and C5b-9 were similar in the serum and plasma of normal individuals, however, in the RA patients a significant difference was observed between serum and plasma levels of C3:P ($p<0.001$). Increased levels of activation products in serum with respect to plasma have been reported previously (Mollnes et al,

1985; Nilsson and Bäck, 1985; Lockshin et al, 1986) and it is thought to occur as a result of complement activation via activation of the clotting system and/or *in vitro* complement activation due to the presence of such substances as immune complexes or bacterial endotoxins. The use of EDTA-plasma overcomes the potential problems of *in vitro* complement activation. Thus statistical analysis was confined to this group.

Significantly elevated levels of all three complement activation complexes were found in SLE and RA patients compared to normals (Table 3.1). Elevated C1s:C1-INH levels have been described in SLE patients (Lockshin et al, 1986) and Gawryl et al (1986) found elevated C5-9 complexes in RA and SLE patients. A single study on C3:P levels in SLE patients showed only 3 out of 29 had increased levels (Mayes et al, 1984). This is a much lower frequency than that found in this study in which 53% of patients had levels above the upper limit of the normal range (Table 3.1). However, this discrepancy may be due to differences in the patient populations, levels of disease activity or both.

As levels of C1s:C1-INH and C5b-9 in RA plasma ranked significantly with those in the paired synovial fluid samples ($p<0.001$ and $p<0.0001$ respectively, Spearman rank correlation; Fig 3.2.2) and the mean levels of each of these activation products in synovial fluid were not significantly different from those in plasma, it is possible that these complexes can diffuse freely between the synovial cavity and the intravascular compartment and vice versa. However, it is also possible that the degree of systemic complement activation is similar to that occurring within the joint. The failure of synovial fluid C3:P levels to correlate with those in plasma suggests that the production of this complex within the joint may occur independently of that in the systemic circulation.

Other workers have found increased synovial fluid levels of C1s:C1-INH (Inman and Harpel, 1983) and C5b-9 (Gawryl et al, 1986; Mollnes et al, 1986; Morgan et al, 1988), supporting increased local complement activation in the synovial cavity.

RA patients were divided, according to the presence of rheumatoid factors, into

sero-positive and sero-negative groups. This grouping was performed in order to see if there is any effect of rheumatoid factor (IgM-RF, IgG-RF and IgA-RF) on the levels of complement activation complexes. Rheumatoid factors are known to bind IgG molecules leading to the formation of IC that may be involved in complement activation in rheumatoid patients and related to disease activity (Franklin et al, 1957; Hay et al, 1979; Sabharwal, et al, 1982; Hack et al, 1984). RA patients with RF had significantly higher levels of the three complement activation complexes compared to normals and sero-negative group (Table 3.1). In sero-positive patients the levels of C1s:C1-INH, C3:P and C5b-9 correlated significantly with the levels of IgM-RF, IgG-RF and IgA-RF (Table 3.3). This relationship did not result from the interaction of IgM-RF with the ELISA system, as similar levels of activation complexes were obtained when $F(ab')_2$ fragments or intact IgG was used to coat the ELISA plates. Furthermore, addition of purified IgM-RF at concentrations seen in RA patients did not alter the levels of complement activation products (Fig 3.2.3.). The findings of this study differ from those of other workers. Inman and Harpel (1983) found no relationship between RF and C1s:C1-INH levels while other workers found no correlations between RF and C5b-9 levels in patients with RA (Mollnes et al, 1986) or juvenile RA (Mollnes and Paus, 1986). This discrepancy, however, may be related to the small number of individuals examined in these earlier studies, to differences in the disease activity of the patients, or to the use of semi-quantitative agglutination tests for the measurement of RF levels.

In this large group the relationship between antigenic levels of the commonly measured complement components (C3, C4, B and C1-INH) and the complement activation complexes has been analysed. This was performed in order to study the relationship between *in vivo* complement activation and the levels of these complement components. There was no relationship between the serum levels of C1-INH, C4, C3 or factor B with levels of the three complement activation complexes in SLE patients (Table 3.5). However, in RA patients serum levels of C1-INH correlated with

C1s:C1-INH, C3:P and C5b-9, and factor B with C1s:C1-INH and C3:P (Table 3.6). This is the converse of what one would expect if complement activation led to increased catabolism of cleavage products. These correlations suggest that during *in vivo* complement activation the rate of synthesis of these complement components is in some way regulated by the level of complement activation. The lack of any correlation between levels of complement activation complexes and levels of C3 and C4 may have occurred as a result of the use of immunochemical measurements which detect inactivated components, because compensatory hypersynthesis may have masked complement activation or because the synthesis of these two components are not regulated by the level of ongoing complement activation.

Complement activation is thought to have a significant role in mediating the inflammatory responses and it is assumed that the level of complement activation should parallel the degree of tissue injury. Therefore, monitoring the levels of of complement activation products may provide additional information and allow prediction of the clinical status. One prospective study of the levels of the MAC in patients with adult respiratory distress syndrome (ARDS) has shown the predictive value of this test, with levels of MAC increasing prior to onset of ARDS (Laglois and Gawryl, 1988). In this situation it has been suggested that measurement of C5b-9 may provide a more sensitive indicator than measurement of CH50, C3a, C4a or C5a.

The result of this study suggest that all the three assays may be necessary for monitoring the disease activity and to provide an indication of which complement pathway is activated.

The observed correlations between the individual activation products in normals, RA and SLE patient groups clearly suggests that a well defined prospective study including serial studies is required to establish the role of these ELISA procedures as indices of disease activity.

C1 activation

a) Effect of IC concentration

As might be expected, increasing concentrations of both types of IC at all five antigen-antibody ratios resulted in dose-dependent increase in the generation of the C1s:C1-INH complex and in the case of thyroglobulin anti-thyroglobulin IC, C4a generation. Thus, as the maximum concentration of IC used in this study (500μg IgG antibody/ml) was probably far in excess of any IC formed normally, there must be a massive excess of C1 available in the plasma. This conclusion is supported by the observation that serum concentrations of C1, when measured by haemolytic assay, are between $2x10^5$ units/ml to $1.6x10^6$ units/ml (Whaley, 1985). The possibility that C1 transfer (that is C1 binds to one IC, activates C4 and C2 to form C3 convertase and then is transfered to another IC to repeat the process, Borsos et al, 1968) could explain this finding is untenable, as in whole serum C1 is inactivated rapidly after its activation (Ziccardi, 1982b) with dissociation of the C1 macromolecule (Sim et al, 1979).

With the possible exception of BSA anti-BSA IC formed at equivalence, all concentrations of nascent IC produced greater concentrations of C1s:C1-INH at all antigen antibody ratios than preformed IC. Similar results were obtained when C4a was used to quantify C1 activation. This more efficient activation of C1 by nascent as opposed to preformed IC probably reflects differing availability of the Fc regions of the antibody molecules in the two types of IC. As soon as IC are formed (nascent IC) and two IgG molecules are adjacent to each other, C1 binding and activation can occur (Borsos and Rapp, 1965; Cohen, 1968). In contrast, in the case of preformed IC a large lattice has developed which not only depends upon antigen-antibody cross-linking, but also on interactions between the Fc pieces of the different IgG molecules within the IC (Nisonoff and Pressman, 1958; Moller, 1979; Rodwell et al, 1980). As a result of these Fc-Fc interactions, less Fc regions would be available to bind and activate C1.

b) Effect of antigen-antibody ratio

Differences in the antigen-antibody ratio of IC had a significant effect on C1s:C1-INH formation. For thyroglobulin anti-thyroglobulin IC, C1s:C1-INH formation was maximal at 4-times antibody-excess, whereas in BSA anti-BSA IC it was maximal at equivalence. With thyroglobulin anti-thyroglobulin IC, marked C1s:C1-INH formation occurred with IC formed at 16-times antibody-excess and at equivalence, while little activation produced in antigen-excess. In contrast, when BSA anti-BSA IC were used the range of antigen-antibody ratio which produced high levels of C1s:C1-INH was even more restricted, particularly with preformed BSA anti-BSA IC. Previous studies with IC formed with soluble antigens have shown that consumption of haemolytic complement was greater in antibody-excess (Lundqvist and Norden, 1960). However, the data presented in this thesis show that only in the case of thyroglobulin anti-thyroglobulin IC was C1s:C1-INH generation and C4a generation greater in antibody-excess. In contrast to the result of this study, Ishizaka et al (1959) showed that only soluble IC formed in slight antigen-excess would consume complement. However, in that study IC were formed with a variety of different antigens (diphtheria toxoid, haemocyanin, chicken γ globulin, type II and III pneumococcal polysaccharides and the hapten, sodium arsanilate) at different antigen-antibody ratios. Prior to performing experiments, the IC mixtures were centrifuged to remove insoluble IC. Thus, the majority of any IC at equivalence or in slight antibody-excess would be removed and only those IC in far antibody-excess or in antigen-excess would remain in solution. In this way the only complement consuming IC left in solution would be those formed in slight antigen-excess. This conclusion is supported by other data from this laboratory which showed that only soluble BSA anti-BSA IC formed at 4-times antigen-excess would bind solid-phase C1q (Scullion et al, 1979).

An earlier study showed that the antigen in IC influenced the effect of antigen-antibody ratio on C1 activation (Füst et al, 1978). Although both BSA anti-BSA and ovalbumin anti-ovalbumin IC activated C1 (measured by C4 consumption) more effectively at equivalence, C1 activation by the former IC was much reduced when they

were formed at 2-times antigen-excess and 2-times antibody-excess. In contrast, ovalbumin anti-ovalbumin IC formed at these two antigen-antibody ratios produced almost as much C1 activation as those formed at equivalence. Thus, my data and those of Füst et al (1978) show that the antigen incorporated into an IC influences complement activation. The way in which this influence is exerted is by no means clear. However, one important factor must be epitope density on each antigen molecule (Garred et al, 1989). The more epitopes per molecule, the more likely one is to have two IgG antibody molecules in sufficiently close proximity to allow two of the globular C1q heads of a C1 molecule to bind separate IgG Fc CH2 domains so that C1 activation can occur (Burton et al, 1980). Such binding could occur as a result of the attachment of two IgG molecules to a single antigen molecule, or more likely by the cross-linking of two or three antigen molecules by at least two IgG molecules. The observation that IC formed in antigen-excess did not activate C1 terribly effectively can be explained on the basis that the greater the number of molecules of the antigen per IgG antibody molecule, the less likely one is to have antigen molecules cross-linked by IgG antibody. The epitope densities of thyroglobulin (650kD) and BSA (66kD) are approximately 40/molecule and 10/molecule respectively (Heidelberger, 1938). The molar antigen-antibody ratio in thyroglobulin anti-thyroglobulin IC at 4-times antibody-excess (maximum C1 activation) was 1:24, while that for BSA anti-BSA IC at equivalence (maximum C1 activation) was 1:6. When one considers the differences of number of epitopes per molecule of both antigens, it is clear that the average distribution of IgG molecules on thyroglobulin anti-thyroglobulin IC at 4-times antibody-excess approximates to that for BSA anti-BSA IC formed at equivalence.

Although C1s:C1-INH formation was clearly greater with thyroglobulin anti-thyroglobulin IC formed at antibody-excess, and little was formed with IC formed in antigen-excess, the effect of antigen-antibody ratio was far less pronounced when C4a production was measured. The explanation of the increased generation of C4a when IC were formed at 16-times antigen-excess compared with that occurring with IC formed at

4-times antigen-excess is not obvious. Because of the expense of the anaphylatoxin assays kits, these results were obtained from duplicate determinations of a single samples and could therefore be due to a technical error. However, the observation that it occurred with nascent as well as preformed IC and with C3a generation suggests that this may not be the case.

c) Kinetics of C1s:C1-INH formation

The rate and extent of C1s:C1-INH complex formation were clearly temperature dependent, as shown by the reduced formation at 30°C and 20°C compared with 37°C. Previous studies have shown that the initial step of C1 activation the binding of C1 to IC is temperature independent as it can occur at 0°C, but for activation to occur the IC-C1 complex must be incubated at higher temperature (Borsos et al, 1964). The initial rate of the C1s:C1-INH complex was greater for preformed IC than for nascent IC and for BSA anti-BSA IC it was greater at equivalence than at other antigen-antibody ratios whereas with thyroglobulin anti-thyroglobulin IC it was greater when IC were formed at 4-times antibody-excess. The more rapid initial rate of formation of C1s:C1-INH complex for preformed IC is due to the already assembled lattice to which C1 can bind whereas the slower rate of formation in the case of nascent IC reflects the time required for antigen and antibody to complex before C1 can bind and be activated.

C1s:C1-INH formation reached a plateau very rapidly with preformed IC because the IC was fully formed at the time of exposure to serum. In contrast, the continued increase in C1s:C1-INH during the incubation period with nascent IC probably reflects the continued formation of the IC throughout this time.

The effect of antigen-antibody ratio on complement activation has been observed previously (Hill and Osler, 1955). These authors, using IC containing the antigen type III pneumococcal polysacchride, stated that IC formed at equivalence consumed complement slightly more rapidly than IC formed in antibody-excess, while IC formed in antigen-excess consumed complement much more slowly. Their results are similar

to those described here on the effects of IC on C1s:C1-INH formation.

Interestingly, the rate of formation of C4a was greater than that of the C1s:C1-INH complex with nascent IC, whereas when preformed IC were used the rate of C4a formation was slower than that of the C1s:C1-INH complex. These differences emphasize that formation of C4a and C1s:C1-INH occur independently and as a result of two distinct events. C4a is formed as a result of the activation of C4 by C1 (Budzko and Müller-Eberhard, 1970), whereas the C1s:C1-INH complex is formed after activated C1 has been inactivated by C1-INH (Laurell et al, 1978; Sim et al, 1979). Thus, with nascent IC one can conclude that C1 activation occurs quickly while inactivation occurs more slowly, whereas with preformed IC the more rapid inactivation of C1 results in a slower rate of C4a generation.

d) Effect of erythrocytes on C1s:C1-INH generation

The finding that C1s:C1-INH formation was increased when complement activation occurred in the presence of erythrocytes was unexpected. The increase did not reflect increased C1 activation as C4a levels were unaffected by the presence of erythrocytes. Thus in the presence of erythrocytes, IC-mediated C1 activation occurs normally but activated C1 appears to be inactivated more readily.

Although the results of many of the kinetic assays from this series of experiments do not give clear-cut results, the results for nascent BSA anti-BSA IC (Fig 3.5.9) demonstrates clearly that both the rate and extent of formation of the C1s:C1-INH complex are increased in the presence of erythrocytes. It is interesting to note that C1s:C1-INH formation was only increased significantly when nascent IC were used. This observation suggests that the properties of erythrocytes endow C1 which has bound and been activated by IC to be inactivated more rapidly. The data obtained using trypsinized erythrocytes and Fab anti-CR1 show clearly that CR1 (the C3b receptor) is not involved in this phenomenon. There are data which show that IC can bind to human erythrocytes in the absence of complement by an Fc-dependent interaction (Virella et al, 1983). Because the effect of erythrocytes was more pronounced with

(Virella et al, 1983). Because the effect of erythrocytes was more pronounced with nascent IC, and as it is probable that Fc regions are more exposed on nascent IC (see above), an interaction between Fc region of the IgG molecules and a structure on the erythrocyte cell membrane could account for the effect of erythrocytes on C1s:C1-INH formation.

e) Relationship between C1s:C1-INH and C4a formation

During the study of C1 activation on a number of occasions it was noted that the change in the C1s:C1-INH complex levels did not mirror those seen in C4a concentrations. These differences are listed below

1) Although the IC dose response curves for C1s:C1-INH complex formation were similar to those for C4a generation, they were not always identical.

2) Although the changes seen in C4a levels with antigen-antibody ratio were similar to that for C1s:C1-INH significant differences were sometimes observed.

3) Although the amount of C1s:C1-INH complex produced correlated with the amount of C4a produced when IC were formed, in antigen-excess disproportionally more C4a than C1s:C1-INH complex was formed.

4) The kinetic studies showed that levels of C4a were produced at different rates to those of the C1s:C1-INH complex.

5) Erythrocytes did not affect C4a generation despite the increased rate and extent of C1s:C1-INH formation when complement activation was triggered with nascent IC.

These findings clearly show that measurement of C4a gives different information to that of the C1s:C1-INH complex. It has already been stated (see above) that C4a is a measure of C1 activation while C1s:C1-INH formation is a measure of C1 inactivation. This distinction is important when applying them to measure complement activation in the experimental situation. Whether it is important to draw this distinction in the service laboratory remains to be clarified.

C3 activation

a) Effect of IC concentration

Although there was a clear dose-dependent relationship between C3:P complex formation and IC concentration with preformed thyroglobulin anti-thyroglobulin IC formed at 16-times antigen-excess, this was not observed at other antigen-antibody ratios with nascent thyroglobulin anti-thyroglobulin IC or preformed BSA anti-BSA IC. Although some evidence of dose-dependent increase in C3:P was obtained with nascent BSA anti-BSA IC, this was only seen at the lower end of the dose response curve.

Usually after the peak value was reached, the level of C3:P stayed constant or decreased as higher concentrations of IC were used. This was in marked contrast to the clear dose-dependent relationship between IC concentration and the levels of C3a produced (with the exception of nascent thyroglobulin anti-thyroglobulin IC formed at 16-times antigen-excess). The discrepancy between the dose-response curves for C3:P and C3a suggests that the level of C3:P in serum cannot be used to quantify C3 activation. Further evidence will be discussed below.

b) Effect of antigen-antibody ratio

The antigen-antibody ratio influenced the amount of C3:P formed for both thyroglobulin anti-thyroglobulin IC and BSA anti-BSA IC. However, the effect of antigen-antibody ratio was dependent upon the concentration of IC used. The lowest concentrations of IC at equivalence stimulated the greatest production of C3:P, whereas at higher concentrations the lowest C3:P levels were formed with IC at equivalence or at 4-times antibody-excess. In the case of BSA anti-BSA IC, the former observation is in agreement with the observations on the effect of antigen-antibody ratio on C1s:C1-INH formation which suggests that C1 activation is related to C3:P formation. However, with thyroglobulin anti-thyroglobulin IC, the former observation is not in keeping with C1s:C1-INH or C4a formation, both of which were greater with IC formed at 4-times antibody-excess. Interestingly, in the case of nascent thyroglobulin anti-thyroglobulin

IC, the amount of C3a formed was greatest with IC formed at 4-times antibody-excess, which suggests that in this situation C3 turnover, but not C3:P formation, parallels activation of C1. However, for preformed thyroglobulin anti-thyroglobulin IC, slightly greater quantities of C3a were usually produced in response to IC formed at equivalence than at 4-times antibody-excess whereas the converse was true for C4a production. This observation suggests that turnover of C3, as measured by C3a generation, is not totally dependent of the extent of C4 turnover. One factor which influences the turnover of C3 is the amount of C3 convertase (C4b2a) generated. Variations in the amount of C4 cleaved and the site of C4b binding to IC formed at different antigen-antibody ratios would influence the number of molecules of C4b2a produced. It is also possible that the site of C4b binding could influence the efficiency of any resulting convertase molecules; conceivably in some locations, access of C3 to the active site of the convertase could be impaired. The amount of C4a formed was only approximately one fifth that of C3a. Using a haemolytic system, it has been calculated that only 5% of C4b molecules bind to IC after C4 activation and only 20% of those that bind are involved in C3 convertase formation; therefore only 1% of activated C4 molecules participate in C4b2a generation (Hughes-Jones, 1986). As C4a has a molecular weight of 6kD (Budzko and Müller-Eberhard, 1970) compared with 9kD for C3a (Sim et al, 1981; Tack et al, 1979) it can be calculated that in the system used in my studies, one molecule of C3 convertase activates 333 molecules of C3, whereas it has been calculated that 200 molecules of C3 are activated by one C3 convertase on haemolytic intermediates (Hughes-Jones, 1986). Considering the approximations used in my calculations and the different IC systems used, the two values are in reasonable agreement.

The reduced concentrations of C3:P at high IC concentrations formed at equivalence or in antibody-excess could be due to the following causes:

1) If C3:P was not bound to the IC, perhaps it might be less stable at higher IC concentrations.

2) At higher concentrations less C3:P formation occurs.

3) Binding of C3:P to IC, which at higher concentrations might be insoluble. Thus much of the C3:P formed would pellet with the insoluble IC.

4) If C3:P is bound to soluble IC perhaps at higher concentrations bound C3 antigens are masked by properdin.

The first explanation is unlikely as the C3:P complex was shown to be stable during preliminary studies. It is also extremely improbable that the presence of a greater IC concentration would alter the stability of the C3:P complex.

The levels of all other measurements of complement activation showed a dose-related relationship with IC concentration. As the C3:P complex is thought to be the alternative pathway C3 convertase (C3bBbP) (Mayes et al, 1984; Asghar et al, 1987) the level should parallel complement activation. Thus, reduced complement activation can not account for reduced level of C3:P complex.

It is distinctly possible that the C3:P complex is bound to IC. C3b could bind covalently to IC and properdin then bind to the C3bBb complex (Farries et al, 1988a). However, the precise constituents of the C3:P complex have not been defined. The possibility that IC-bound C3:P were precipitated as part of insoluble IC was made less likely as the levels of the C3:P complex were the same before and after centrifugation to remove insoluble IC. Furthermore although the amount of C3:P formed in response to the highest concentrations of nascent BSA anti-BSA IC was reduced, the IC remained totally soluble. Thus, the latter possibility appears most likely to be correct. This possibility could be investigated by firstly defining precisely the constituents of the C3:P complex, and secondly by investigating the stoichiometry of the binding of C3b to antibody and antigen and that of properdin to C3b.

c) The role of the alternative pathway on C3:P formation

The reduced formation of C3:P in Mg^{++}EGTA when IC were formed in 16-times antibody-excess and 4-times and 16-times antigen-excess, shows that the alternative pathway is partly responsible for C3:P formation under these conditions. In contrast,

as C3:P levels were the same in Mg^{++}EGTA-treated serum and normal serum when IC were formed at equivalence or 4-times antibody-excess, alternative pathway activation alone must be responsible for all the C3:P formation.

C3:P formation did not occur in EDTA-treated serum; thus the formation of this complex requires complement activation. As properdin binds to C3b in the presence of EDTA (Fearon and Austen, 1975b) the requirement for Mg^{++} supports the view that C3bBb formation (which requires Mg^{++}) is a prerequisite for C3:P formation.

In comparison with the effect on C3:P formation, Mg^{++}EGTA reduced C3a formation by approximately 50% at almost all IC concentrations and almost all antigen-antibody ratios. These data show that under most conditions the alternative and classical pathways usually contribute equally to C3 turnover by IC. They also confirm the results of the previous studies which showed that C3:P concentrations do not always parallel C3a levels.

d) Kinetics of C3:P formation

The rate and extent of C3:P formation were temperature dependent, which supports the previous conclusion (see above) that the formation of the complex is an active process involving the activation of C3 and the proteolysis of B by D prior to the binding of P. This conclusion is also supported by the observations of a distinct lag-phase prior to the formation of C3:P, particularly when thyroglobulin anti-thyroglobulin IC were used to activate Mg^{++}EGTA-treated serum. This delay is due to the initial slow assembly of the alternative pathway C3 convertase prior to the formation of IC-bound C3 convertase which produces more rapid C3 activation. In normal serum the rapid formation of C3:P demonstrates the importance of the classical pathway for the early triggering of the alternative pathway. The studies of the kinetics of C3a formation with thyroglobulin anti-thyroglobulin IC support this hypothesis. The results show that although in normal serum the initial rate of increase is similar to that for C3:P in Mg^{++}EGTA-treated serum, C3a levels increase while C3:P levels remain unchanged.

Thus, in this situation C3 turnover must occur before C3:P formation.

Although C3:P and C3a production occurred rapidly at 37°C in normal serum when complement activation was stimulated with thyroglobulin anti-thyroglobulin IC, this was not the case when BSA anti-BSA IC were studied. In the latter situation C3:P production was rapid, whereas C3a production occurred more slowly. These observations suggest that C3 activation varies with different types of IC. In the case of BSA anti-BSA IC, alternative pathway activation occurs reasonably rapidly, as confirmed by the much shorter lag-phase in Mg^{++}EGTA-treated serum, and most of the C3 turnover is due to alternative pathway C3 convertase activity. Unfortunately, I was unable to measure C3a levels in the experiments in which complement activation was stimulated using BSA anti-BSA IC in Mg^{++}EGTA-treated serum. As discussed above with thyroglobulin anti-thyroglobulin IC the converse situation appeared to be the case, as classical pathway activation seemed to be responsible for most of the initial C3 turnover with the alternative pathway playing a minor role.

e) Effect of erythrocytes

When complement activation occurred in the presence of human erythrocytes C3:P levels were reduced. The observations that trypsinization of erythrocytes or blockade of CR1 by Fab anti-CR1 showed that CR1 was responsible for this effect. As IC were shown to bind to erythrocytes at least part of the reduction could be explained by the binding of IC to erythrocyte CR1. Thus, IC-bound C3:P would also be removed from the fluid-phase. The alternative possibility that erythrocyte DAF and CR1 regulated the activity of fluid-phase and IC-bound C3-convertase is unlikely as C3a generation was unaffected by the presence of erythrocytes. This finding is contradictory to those of Gronski et al (1986) who found that spontaneous complement activation occurring in C1-INH depleted serum was reduced slightly by the presence of erythrocytes. This discrepancy could be due to the two different methods for activating the complement system. In my experiments IC were used and both the classical and alternative

pathways were activated whereas in the study of Gronski et al (1986) only the classical pathway was activated by depletion of C1-INH.

f) Relationship between C3:P and C3a formation

In these experiments it was seen that C3:P levels did not mirror those seen in C3a concentrations. These differences were seen in all the following experiments:

1) Dose-response curves

2) Studies of antigen-antibody ratios

3) Comparing complement activation in normal serum and Mg^{++}EGTA-treated serum

4) Kinetic studies

5) Effect of erythrocytes

The levels of C3:P and C3a were not related in any of the experiments

It was originally suggested that the C3:P complex was a measure of alternative pathway activation (Mayes et al, 1984). In the narrow sense this is true as it probably contains Bb in addition to properdin. However, it appears that with thyroglobulin anti-thyroglobulin IC the major stimulus to C3:P complex formation was activation of the classical pathway. Thus, in the absence of an intact classical pathway very little C3:P complex was formed when complement was activated by these IC. Thus, the use of C3:P as a measure of alternative pathway C3 turnover is not strictly correct. Although C3 turnover is required for C3:P formation, once the alternative pathway C3 convertase has been formed it can activate more C3. Furthermore, we do not know what proportion of C3b is incorporated to C3b:P. Further studies on the nature of the C3:P complex are clearly necessary and must include: a) investigations into the constituents of the complex and b) why the complex is so stable. In the presence of factors H and I the convertase should be destabilised and C3b converted to iC3b which does not bind properdin (Farries et al, 1988a). The relationships between the C3:P complex and the multiple sedimented species of properdin described by Minta and Lepow (1974) and Smith et al (1984) must also be determined. These different species of properdin were

complexed with C3, and appeared to occur spontaneously, in serum. Recent work has shown that complexes of C3 and properdin may be involved in the early stages of activation of the alternative pathway (Purkall and Ruddy; unpublished observations) but this has yet to be confirmed. It is unlikely that the C3:P complexes detected in this study were the same as those described earlier, as those detected by my techniques did not occur spontaneously. However, this can be proved only when the C3:P complexes are a) isolated and characterized and b) assembled *in vitro* using purified complement components and IC.

C5 activation

a) Effect of IC concentration

The dose-dependent increase in the concentration of C5b-9 and C5a were more pronounced with preformed than with nascent IC and with IC formed in antibody-excess. The highest concentration of C5a produced in these experiments was 1.5μg/ml. As C5a (12kD) comprises 6% w/w of C5 (200kD), 25μg of C5 must be activated to generate this quantity of C5a. This represents approximately 33% of serum C5 (concentration 75μg/ml). The amount of C5a produced was approximately 10% of the amount of C3a produced. Thus, for every 10μg of C3a generated, 1μg of C5a would be produced. As the molecular weight of C3a and C5a are 9kD and 12kD respectively, it appears that 13 molecules of C3 are required to cleave one molecule of C5. However, only those molecules of C3b which bind to C4b in C4b2a can participate in C5 convertase activity (Kinoshita et al, 1987). As one molecule of C3b binds to a single C4b in the C5 convertase, and as one molecule of C3/C5 convertase will activate 333 molecules of C3 (see above), one molecule of convertase will activate 26 molecules of C5. This figure is far higher than published figures which suggest that each molecule of convertase will only activate 2-3 molecules of C5 (Hughes-Jones, 1986). The more likely explanation for this difference is that the earlier studies were performed using haemolytic intermediates and purified complement components,

whereas in the present study complement activation occurred in whole serum and the alternative pathway activation would occur, with the formation of the alternative pathway C5 convertase. Evidence to support this argument is presented below.

b) Effect of antigen-antibody ratios

Although with thyroglobulin anti-thyroglobulin IC the formation of both C5b-9 and C5a was greatest in antibody-excess and minimal in antigen-excess, C5b-9 formation was greater at 16-times antibody-excess, whereas C5a production was greatest at 4-times antibody-excess. Thus, it would appear that the conditions which are optimal for C5 activation (C5a generation) are not optimal for assembly of the C5b-9 membrane attack complex. However, it is possible that more C5b-9 complex is formed at 4-times antibody-excess but some is bound to the IC, a greater proportion of which are insoluble at this antigen-antibody ratio than at 16-times antibody-excess. Although it has been stated that C5b-7 cannot bind to IC formed with soluble proteins (Podack, 1986), thyroglobulin, a large antigen, has regions of surface hydrophobicity which could permit binding of C5b-7. It is also possible that other proteins (eg lipoproteins, S-protein), bind to thyroglobulin anti-thyroglobulin IC, and so indirectly promote binding of C5b-7 or C5b-9 to IC.

If one accepts the observation that C5b-9 formation is greater at 16-times antibody-excess, one must postulate that IC formed at this ratio are in some way able to promote the interactions of C5b with the later terminal components or, alternatively, when IC are formed at 4-times antibody-excess these interactions are inhibited. Although the newly generated labile C5b is capable of forming a stable, hydrophilic, bimolecular complex with C6 (Podack et al, 1978), there is no good reason to believe that antigen-antibody ratio could influence this reaction.

Interestingly with BSA anti-BSA IC, although the greater C5b-9 formation occurred in antibody-excess, there was little difference between IC formed at 16-times antibody-excess and those formed at 4-times antibody-excess. These data emphasize

the point that the antigen in an IC can influence complement activation. Unfortunately, due to lack of anaphylatoxin assay kits, C5a assays were not performed in these experiments.

c) Role of classical and alternative pathways

The amount of C5b-9 formed in Mg^{++}EGTA-treated serum and normal serum were almost the same for all types of IC. Although there was a reduction in C5a production in Mg^{++}EGTA-treated serum, this was never greater than 25-30% with IC formed in antibody-excess. Thus, the alternative pathway must be responsible for most of the C5 convertase activity which is generated when complement activation is produced by IC. As fluid-phase alternative pathway activation does not permit efficient C5 convertase formation (Pangburn, 1986), the C5 convertase formed in my experiments must be bound to the IC. As one molecule of C4b2a activates many (333 according to my calculations) molecules of C3, a number of C3b molecules could bind to both antibody and antigen moieties of IC. Once bound, each could bind factor B to form C3bBbP to cleave more C3, some of which would bind to the C3 convertase to form C5 convertase (Kinoshita et al, 1987). Alternatively, as classical pathway activation would still be proceeding a second C3b molecule could bind to original bound C3b molecules. The subsequent binding of B and P would lead to the direct formation of an alternative pathway C5 convertase.

d) Kinetic studies of C5 activation

The rate and extent of C5b-9 and C5a formation were temperature dependent, showing that C5 activation is an active process. The kinetic studies with thyroglobulin anti-thyroglobulin IC showed that C5a was generated more slowly than C3a. This delay is probably due to the time required for conversion of C3 convertase to C5 convertase. With nascent thyroglobulin anti-thyroglobulin IC C5a levels increased earlier than C5b-9. This delay probably reflects the time for assembly of the multimolecular C5b-9 complex following C5b formation. In contrast C5b-9 formation

with preformed thyroglobulin anti-thyroglobulin IC occurred more rapidly. It is difficult to explain this discrepancy but it might be argued that preformed thyroglobulin anti-thyroglobulin IC allow the transient binding of C5b to promote its interaction with the later components. One would need to propose that although nascent IC form C5 convertase and activate C5, they may not have acquired the ability to promote the interaction of C5b with the later components. Perhaps as IC increase in size this ability is acquired.

The kinetic studies of C5b-9 formation with BSA anti-BSA IC gave results which were intermediate between those of nascent and preformed thyroglobulin anti-thyroglobulin IC, providing evidence that the antigen plays a role in the generation of C5b-9 complex. Unfortunately C5a levels were not measured.

e) Effect of erythrocytes

When complement activation occurred in the presence of erythrocytes, the rate and extent of C5b-9 formation were increased. In contrast, the rate and extent of C5a generation was unchanged. Thus, erythrocytes do not affect C5 convertase formation or activity but do appear to promote the assembly of the C5b-9 complex.

f) Comparison of C5b-9 and C5a formation

Investigation of C5 activation had showed a linear relationship between the generation of C5a and C5b-9 (Fig 3.3.10). In spite of that correlation, some discrepancies were observed:

1) The dose response curves in the case of nascent and preformed thyroglobulin anti-thyroglobulin IC showed that C5b-9 generation was almost straight line, while that for C5a a plateau was observed during the two highest IC doses.

2) In thyroglobulin anti-thyroglobulin IC, the generation of C5a was always higher at 4-times antibody-excess, while that for C5b-9 was higher at 16-times antibody-excess.

3) Kinetic studies showed that C5b-9 generation exhibited a lag-phase with nascent thyroglobulin anti-thyroglobulin IC, while C5a generation starts up much earlier. In case of preformed thyroglobulin anti-thyroglobulin IC levels of C5b-9 rose abruptly, while C5a was generated at a slower rate.

4) Erythrocytes did not affect the generation of C5a, despite the increased rate of C5b-9 generation

These findings reflect that in some situations, the measurement of C5a provides different information than that of C5b-9. Measurement of C5a expresses the activity of the classical and alternative pathway C5 convertases (Hugli and Müller-Eberhard 1978; Hammerschmidt et al, 1980; Chenoweth et al, 1981; Gawryl et al, 1986), while the C5b-9 complex measured in this study is the fluid-phase form of the membrane attack complex which has incorporated S-protein (SC5b-9). The other fact is that the concentration of the C5b-9 complex does not necessarily reflect the total number of activated C5 molecules, as it is possible that some C5b molecules become hydrated with the C6 binding site being lost. It is also possible that some of the complexes are present at different stages of formation such as C5b6, C5b67 or C5b678, and all these complexes are undetectable by the ELISA used.

Further studies

The results of the studies described in this thesis show that the measurement of multimolecular complement activation complexes in biological fluids is easily performed and could be undertaken in routine service laboratories. However, before being undertaken more information regarding their relationship to disease activity must be obtained by carefully performed prospective studies. In addition it is important to establish in each disease whether it is necessary to measure levels of one complex or a combination of two or more complexes.

At the research level it is important to define the composition of C3:P complex and to try explain its relationship to the spontaneously occurring serum C3:P complexes described earlier (Asghar et al, 1987) and its possible role in the initiation of alternative pathway activation. These studies would involve more complex ELISA assays using probes other than C3 and immunoblotting, to determine the constituents of the complex which could then be confirmed by assembling the complex *in vitro* using purified complement components.

Finally, the role of different antigens in complement activation could be investigated by extending the range of soluble antigens and investigating the composition (eg. protein, polysaccharide) and the molecular weight of antigens. Comparison between IC formed with soluble and cellular antigens could also be investigated.

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