MONOCYTE C1-INHIBITOR SYNTHESIS

by

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COMPLEMENT NOMENCLATURE

The components of the classical and terminal pathways of complement are denoted by the letter C followed by a number e.g. C1, C4, C2, C3, C5, C6, C7, C8, and C9. The alternative pathway components are termed factors and are denoted by a letter e.g. Factor B, factor D and properdin. These factors are abbreviated B, D and P respectively. The complement control proteins are referred to by their trival names C1-inhibitor (C1-inh), C4 binding protein (C4 bp), I (formerly called C3b inactivator), H (formerly called β1H globulin) and anaphylatoxin inactivator (AI).

Enzymatically active components are denoted by a bar above the component e.g. $C\bar{1}$, $C\bar{1}r$, $C\bar{1}s$. Conformational changes are denoted e.g. $C\tilde{1}q$. Cleavage fragments of complement components are suffixed by letters e.g. C3a, C3b, C3d, etc. The polypeptide chain composition of components is denoted by the Greek letters α, β, γ in descending molecular weight chain size e.g. C4α, C4β, C4γ.

The full names and abbreviated symbols are used throughout the thesis.

ABBREVIATIONS

BHK - Baby Hamster Kidney

BSA - Bovine serum albumin

^{o}C - ^{o} Centigrade

CB - Cell blank

CC - Complement colour

CoVF - Cobra venom factor

CPS - Counts per second

CR1,2 or 3 - Complement receptor 1, 2 or 3

D^2 - Diameter squared

DαRIgG - Donkey anti-rabbit IgG

DMEM - Dulbecco's minimal essential medium

E - erythrocyte

EA - Antibody coated erythrocyte

EDTA - Ethylene diaminotetra acetic acid

ELISA - Enzyme linked immunosorbent assay

em - effective molecules

FCS - foetal calf serum

f-met-leu-phe - N-formyl-L-methionyl-L-leucyl-L-phenylamine

gp - guinea pig

HAE - hereditary angio-oedema

HBSS - Hanks balanced salt solution

HMWK - High molecular weight kininogen

hr - hour

HRP - horse raddish peroxidase

IBMX - 3-isobutyl-1-methylxanthine

IEP - immunoelectrophoresis

Ifn - interferon

kD - thousand Daltons

LPS - lipopolysaccharide

mA - milli Amps

Mabs - monoclonal antibodies

min - minutes

mS - milli Siemens

ND - not determined

NHS - normal human serum

NRS - normal rabbit serum

OD - optical density

OPD - o-phenyldiamine

PBS - phosphate buffered saline

PDA - phorbol 12,13-diacetate

PDD - phorbol 12,13-didecanoate

PEG - polyethylene glycol

PG - prostaglandin

PMN - polymorphonuclear leukocyte

PMSF - phenylmethylsulphonyl fluoride

RA - radioactivity

RB - reagent blank

RIA - radioimmunoassay

RID - radialimmunodiffusion

SDS - sodium dodecyl sulphate

SDS-PAGE	-	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	-	trichloroacetic acid
TEMED	-	N,N,N',N'-tetramethylenediamine
TPA	-	12-O-tetradecanoyl phorbol 13-acetate
TXB_2	-	Thromboxane B_2
v	-	volume
VBS	-	veronal buffered saline
w	-	weight

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PUBLICATIONS

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

Yeung-Laiwah, A.C., Jones, L., Hamilton, A.O. and Whaley, K. (1985) Complement-subcomponent-$C\bar{1}$-inhibitor synthesis by human monocytes.
Biochemical Journal, 226, 199-205.

Hamilton, A.O., Jones, L., Morrison, L. and Whaley, K. (1987) Modulation of Monocyte Complement Synthesis by Interferons.
Biochemical Journal, 242, 809-815.

SUMMARY

C1-inhibitor is the major control protein of the classical pathway of complement. In man, its hereditary deficiency results in the clinical condition of hereditary angio-oedema (HAE). The disease is characterised by recurrent episodes of acute, circumscribed, non-inflammatory oedema involving the subcutaneous tissues and mucous membranes. Acquired C1-inhibitor deficiency can also occur without any previous family history, at a later stage in life and is usually associated with a lymphoproliferative disorder or paraproteinaemia. C1-inhibitor deficiency is treated by the administration of anabolic steroids, such as danazol or stanozolol, which are known to increase hepatic synthesis of C1-inhibitor, resulting in an increase in plasma C1-inhibitor.

Monocytes are known to synthesise some of the components of the classical and alternative pathways of complement. The synthesis of C1-inhibitor by monocytes isolated from normal donors was investigated in an attempt to develop a model to study C1-inhibitor synthesis in patients with C1-inhibitor deficiency. It was proposed to use this model system to study the effects of anabolic steroids and other steroids on monocyte complement synthesis. In particular in an attempt to understand the mechanisms by which anabolic steroids correct C1-inhibitor deficiency, and to help elucidate the underlying genetic defect in HAE.

C1-inhibitor was purified from human plasma and used to raise a polyclonal antiserum in rabbits, which was then utilised to develop a radioimmunoassay (RIA) to quantitate the levels of C1-inhibitor in monocyte culture supernatants. Other methods used in the study of complement component synthesis by monocytes, included enzyme-linked immunosorbent assay (ELISA), haemolytic assay, and pulse-chase studies using ^{35}S-methionine.

Monocytes isolated from normal individuals were shown to synthesise and secrete C1-inhibitor using the following techniques. Accumulation of C1-inhibitor in monocyte culture supernatants with time, as detected using an RIA. Cycloheximide was shown to reversibly block monocyte C1-inhibitor synthesis, demonstrating that this protein was synthesised and not merely stored and secreted during culture. Under serum-free culture conditions, the C1-inhibitor synthesised by monocytes was shown to possess functional haemolytic activity, of a magnitude similar to that reported for serum C1-inhibitor. Following pulse-chase experiments, immunoprecipitation using specific antiserum, SDS-PAGE and fluorography, C1-inhibitor could be detected in monocyte supernatants, with a molecular weight ranging from 93 kD to 103 kD. The intracellular precursor (pro-C1-inhibitor) showed a lower range of molecular weights from 80 kD to 85 kD, possibly due to incomplete glycosylation.

Monocytes from normal individuals respond to gluco-corticoids by increasing synthesis of C2, C1-inhibitor

and properdin, and reducing synthesis of C3 and lysozyme. There appeared to be a relative order of potency similar to that observed for the anti-inflammatory effects of glucocorticoids: Hydrocortisone < prednisolone < dexamethasone (< Org 6632). Androgens, anabolic steroids, oestrogens, progesterones, and mineralocorticoids were shown to have no effect on the synthesis of monocyte C1-inhibitor or C2.

Normal monocytes in culture were shown to respond to γ interferon by increasing C2 and B synthesis (2 to 4 fold), increasing C1-inhibitor synthesis (up to 890 fold), decreasing C3 synthesis (up to 84% inhibition) and having no effect on lysozyme synthesis.

The investigation of complement synthesis by monocytes isolated from patients with C1-inhibitor deficiency (Type I, Type II and acquired) demonstrated that these patients' monocytes synthesise similar levels of C1-inhibitor, C2 and C3 as monocytes isolated from normal individuals. Monocytes isolated from patients with Type I HAE, synthesised C1-inhibitor with a similar molecular weight and specific functional activity as that synthesised by normal monocytes. The monocytes from these patients respond as well as normal monocytes to γ interferon and glucocorticoids.

It was observed that anabolic steroids did not affect monocyte complement synthesis in normals or Type I HAE patients. Therefore the mechanism of action of anabolic steroids in correcting C1-inhibitor deficiency, could not be investigated.

Monocytes isolated from patients with Type II HAE synthesised C1-inhibitor with a similar molecular weight to normals, but one of the two patients investigated synthesised monocyte C1-inhibitor with a lower specific functional activity than normals or Type I HAE patients.

The results from these investigations suggest that the same gene encodes for C1-inhibitor synthesis in the monocyte and the hepatocyte, which maintains levels of plasma C1-inhibitor. However, since monocytes from Type I HAE patients did not synthesise the predicted reduced levels of C1-inhibitor, it is suggestive that the defect occurs in the regulatory gene, rather than the structural gene. Since the defect was not manifest in monocytes of these patients, it would suggest that Type I HAE is the result of a tissue-specific regulatory defect. Unfortunately, only two patients with Type II HAE were studied, and the results obtained were conflicting. Thus, I was unable to draw any conclusions about the underlying genetic defect in Type II HAE.

I: INTRODUCTION.

1:1 The Complement System

The complement system comprises a group of twenty self-assembling plasma proteins, which are involved in the humoral immune defence mechanisms of the body. The name "complement" was applied after the early observations that serum proteins complemented the ability of antibody to inactivate bacteria and foreign materials. The three main functions of complement are now recognised as cytolysis of invading micro-organisms; opsonization of foreign material and immune complexes to enhance phagocytosis; and thereby, activation of cells by the cleavage products generated during the cascade reactions.

The complement system is composed of two inter-related enzyme cascade systems, classified as the classical and alternative pathways, which converge into the final common route of the terminal pathway, which forms the membrane attack complex of complement. The system will be discussed under the following headings: the classical, alternative and terminal pathways, regulation of complement activation, the biological activities of the complement system, inherited deficiencies of complement, and synthesis of complement.

1:2 The Classical Pathway: C1q, C1r, C1s, C4 and C2

1:2:1 C1q

The molecule which recognises antigen-antibody complexes, and is responsible for the activation of the classical pathway of complement is C1q (Fig. 1). It possesses an unusual chemical composition in that it contains a higher than normal proportion of the hydroxylated amino acids, hydroxylysine and

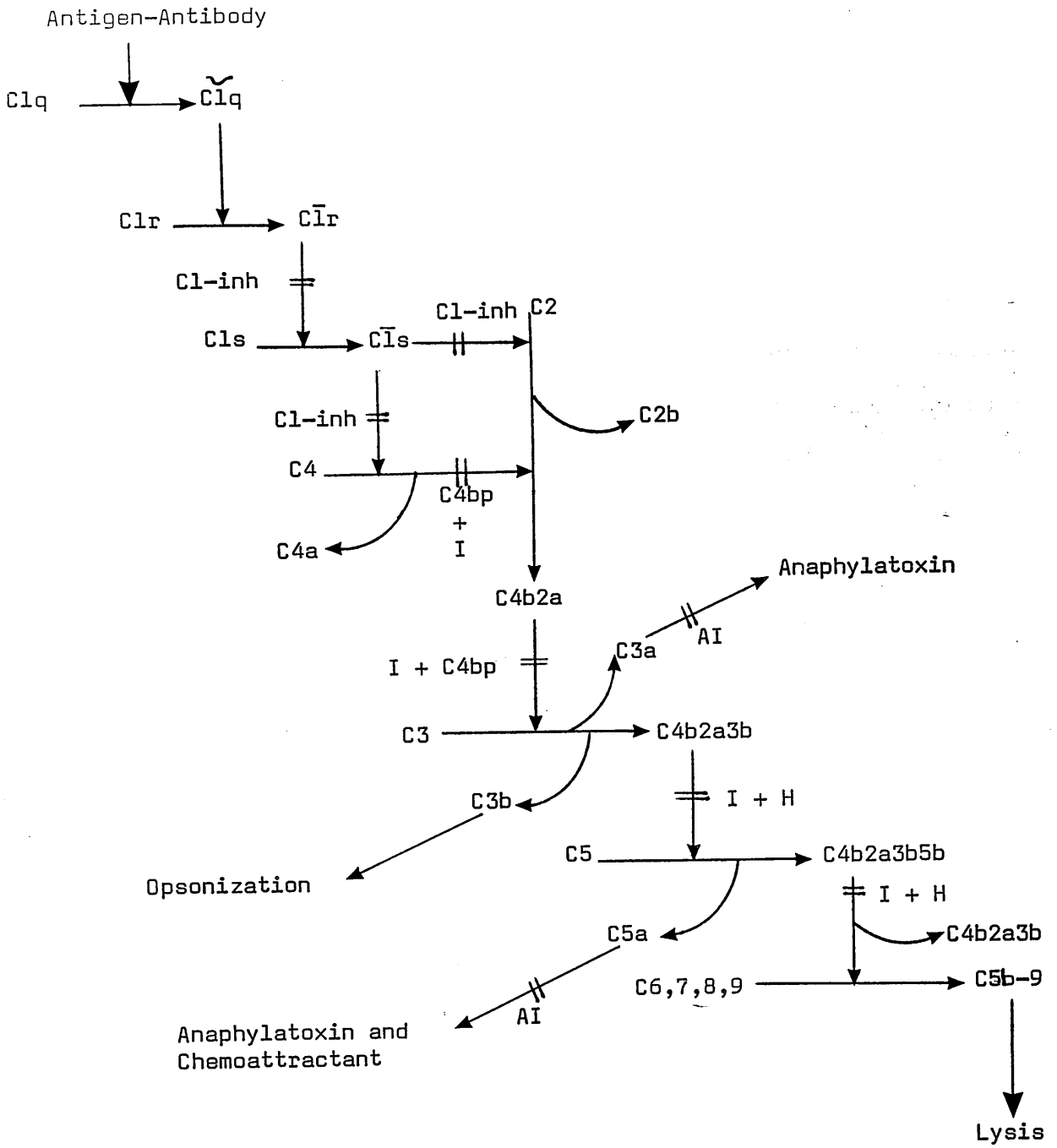


Fig. 1: The Classical and Terminal Pathway of Complement

Enzymatically active components are denoted by a bar above the component, e.g. $\bar{C1r}$. Conformational changes are denoted e.g. $\tilde{C1q}$. Control of the activity of the pathway, by the controlling enzymes, occurs at various stages denoted ⟶‖
AI denotes anaphylatoxin inactivator.

hydroxyproline; a large proportion of glycine; repeating sequences of X-Y-glycine, where X is often proline, and Y hydroxylysine or hydroxyproline (Reid and Porter, 1976). This suggested to investigators that the structure of C1q was very similar to that of collagen.

Close to the N-terminus there is a region of collagen-like helical structure of about 80 residues, whereas the C-terminal 110 residues form a globular region. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) studies of the C1q molecule have demonstrated that it is composed of equimolar amounts of three non-identical chains, A, B and C (Reid, Lowe and Porter, 1972). The six A and six B chains are linked by disulphide bonds to form six AB dimers, as are the six C chains, to form three CC dimers. The structural arrangement of the dimers is such that one AB dimer associates non-covalently with a C chain, whilst the other C chain of the dimer associates non-covalently with an adjacent AB dimer (Reid and Thompson, 1978).

From the data available on C1q, Reid and Porter (1976) proposed a model for the structure of C1q, whose profile is best represented by a bunch of six tulips. The stems represent the collagen-like connecting strands, whilst the flower heads are represented by the C-terminal globular heads, which appear to have a cleft at the end.

The collagen-like regions, although similar in each chain, are not identical. An important area where divergence from the X-Y-Glycine repeating structure occurs is in the middle of the collagen-like portions, around positions 35 to 40.

Pathway	Component	Molecular weight (Daltons)	Serum Conc. µg/ml	Polypeptide chain structures	Cleavage Products
Classical	C1q	410 000	250	6A + 6B + 6C	-
	C1r	95 000	100	1	H & L chains
	C1s	87 000	80	1	H & L chains
	C4	204 000	430	3(α,β,γ)	C4a & C4b
	C2	100 000	20	1	C2a & C2b
Terminal	C3	190 000	1300	2(α,β)	C3a, C3b, iC3b, C3c, C3d-g, C3e
	C5	185 000	75	2(α,β)	C5a & C5b
	C6	128 000	60	1	-
	C7	121 000	60	1	-
	C8	150 000	80	3(α,β,γ)	-
	C9	79 000	50	1	-
Alternative	B	93 000	150	1	Ba & Bb
	D	24 000	2	1	-
	P	220 000	30	3 or 4	-

TABLE 1: Human Complement Components of the Classical, Alternative and Terminal Pathways.

Substitutions in the A, B and C chains, breaking the X-Y-glycine sequence interferes with the triple helical structure, causing a bend or "kink" in the molecule (Porter and Reid, 1978). This region of divergence from the central stalk, is thought to possess some flexibility enabling it to act as a type of hinge.

The ability of Clq to bind, via its globular heads, to immunoglobulin is vital in its role as the recognition unit for activating the classical pathway of complement. Specificity of binding varies between immunoglobulin classes: Ig G and M bind, whereas Ig A, D, and E do not. Within the IgG class, IgG_3 binds more strongly than IgG_1 or IgG_2, whereas IgG_4 does not bind at all. It is thought that each globular head possesses an immunoglobulin binding site, thus Clq may have a valency of six, or multiples of six, for IgG (Schumaker, Calcott, Spiegelberg, Müller-Eberhard, 1976).

1.2.2: Cl Macromolecule

The Cl macromolecule is assembled in plasma and the enzymatic activity of this molecule resides in the Cls subunit, which is activated by the Clr subunit (Fig. 1). Clr and Cls have almost identical amino acid compositions and molecular weights, as shown in Table 1, and are thought to have arisen as a result of gene duplication.

Clr exists as a non-covalently, linked dimer of a single polypeptide chain of approximately 95 000 Daltons (95 kD) (Sim, Porter, Reid and Gigli,1977). Upon activation the

polypeptide chain is cleaved into a 60 kD heavy chain, and a 35 kD light chain, the two chains being linked by disulphide bonds, with the 35 kD light chain possessing the active site of the enzyme. C1s in the absence of calcium exists as a single chain of molecular weight 87 kD, however in the presence of calcium dimerization occurs (Valet and Cooper, 1974). If C1r is introduced into this system, tetramers consisting of two C1r and two C1s molecules are formed; this occurs naturally in serum.

Upon activation by $C\bar{1}r$, C1s is cleaved to a 59 kD heavy chain, and a 28 kD light chain which contains the enzyme's active site. The heavy and light chains are linked by disulphide bridges, as in the C1r molecule (Sim et al., 1977).

As stated previously, in the presence of calcium ions, C1r and C1s can interact to form complexes. Electron microscopic studies of these $C1r_2$ $C1s_2$ complexes has revealed a linear chain of six to eight globular domains, most of which were present in the configuration of a reversed "S" shape (Strang, Seigel, Phillips, Poon and Schumaker, 1982). On the basis of these observations, it was suggested that the more stable C1r dimer formed a core, onto which the C1s monomers attach at each end (Tschopp et al., 1980). Each C1r and C1s was visualised as possessing two globular domains: a catalytic domain, represented by Cs or Cr, and an interaction domain, represented by Is or Ir. Thus the proposed linkage of the $C1r_2$ $C1s_2$ complex would be Cs-IsIr-CrCr-IrIs-Cs, hence portraying the idea of globular domains connected by interdomain

links. This model structure has also been supported by electron microscopy studies (Tschopp et al., 1980).

Assembly of C1r and C1s components into a complex alters the properties of the individual molecules as follows. Firstly, the spontaneous activation of C1r is stabilised and does not occur as readily in the $C1r_2$ $C1s_2$ complex (Arlaud et al., 1980b). Secondly, the complex has the ability to interact with C1q, whilst neither individual component on its own possesses this property (Valet and Cooper, 1974).

The next stage in the assembly of macromolecular C1 involves the calcium-dependent interaction between C1q and the $C1r_2$ $C1s_2$ complex. Due to the differences in association constants between macromolecular C1 and the free C1q and $C1r_2$ $C1s_2$ complexes, the resultant interaction reaches an equilibrium in serum where approximately 70% to 90% of the C1 subcomponents are present in the macromolecular C1 complex (Seigel and Schumaker, 1983). However when C1 is activated, the association constant between $\tilde{C1q}$ and $C1r_2$ $C1s_2$ is ten fold lower, thus activated C1 tends to be present in serum as the dissociated forms: $\tilde{C1q}$ and activated $C1r_2$ $C1s_2$ complexes (Seigel and Schumaker, 1983).

Investigation of the concentrations of the three subcomponents in the C1 macromolecule, have revealed a molar ratio of 1:2:2, C1q:C1r:C1s. This has been substantiated by the finding that the molecular weight of the C1 macromolecule (739 kD) approximates to that calculated for the sum of the constituent proteins (774 kD) (Seigel, Schumaker and Poon, 1981).

As discussed previously, electron micrographic studies have presented good evidence to enable the construction of models for C1q and the $C1r_2$ $C1s_2$ complex. Many hypothetical models have been constructed for the C1 macromolecule, to account for its structural and physicochemical properties, however only the most recent model proposed by Colomb and his colleagues will be discussed (Colomb, Arlaud and Villiers, 1984). Their model for the assembly of macromolecular C1 has been constructed using the C1q model of Reid and Porter (1976), and the $C1r_2$ $C1s_2$ model of Tschopp et al. (1980).

In the C1 macromolecule model, it was proposed that the linear C1s-C1r-C1r-C1s complex distorts and interweaves around two opposite arms of the C1q molecule, bringing the two C1s subunits into close proximity with each other and the C1r subunits. Thus the tetrameric complex is said to "assume the shape of a distorted figure eight in which a clear structural grouping of the domains appears" (Colomb et al., 1984). On the areas outwith the C1q arms the interaction domains cluster, controlling the interactions of C1r, C1s and C1q, whilst the catalytic domains are clustered within the funnel-shaped cone created by the C1q arms and globular heads. It is within this area, that the proteolytic cleavages of C1r, C1s, C4 and C2 are thought to occur, in addition to the controlling inhibitory activity of the C1-inhibitor protein (Colomb et al., 1984).

In order to activate the classical pathway of complement, C1 must bind to and be activated by some type of trigger. The most commonly described trigger for C1 activation is an immune complex or aggregates of immunoblogulins of the IgG (IgG_1, IgG_2 and IgG_3) or IgM classes (Cooper, 1985). In

addition to immunoglobulins, other non-immune substances can bind to, and activate C1, to a greater or lesser degree. The range of substances includes certain bacteria, viruses, parasites, mycoplasma, transformed cells, subcellular membranes, lipids, proteins, carbohydrates and polyions (Reviewed by Cooper, 1985).

For activation of the classical pathway to occur, the globular heads of the C1q molecule must interact with the CH_2 domain on at least two correctly orientated IgG molecules, within the complex or aggregate. In the case of IgM, only one molecule would be required since the spatial orientation of the pentamer is such that C1q can easily bind to two of the CH_4 domains in the IgM pentamer.

The reaction kinetics for C1 activation are thought to involve three phases (Tschopp, 1982). The first step involves the rapid binding of the C1q heads to the activator; this is followed by the slower autocatalytic activation of C1r; thirdly, the $C\bar{1}r$ rapidly activates C1s.

1.2.3: C4

The second component involved in the classical pathway of complement is C4, a substrate for $C\bar{1}$ (Fig. 1 and Table 1). It is a 204 kD protein consisting of three polypeptide chains (Table 1) (Schreiber and Müller-Eberhard, 1974).

The 95 kD α chain of C4 is cleaved by $C\bar{1}$ or $C\bar{1}s$, to release C4a, a 6 kD polypeptide, from the remaining C4b molecule (Schreiber and Müller-Eberhard, 1974). During the activation, an internal thiol ester group is exposed which

enables the C4b to react with hydroxyl or amino groups on the activator, to form ester or amide bonds, thus the C4b becomes surface or antibody bound. However, if this does not occur within 50 milliseconds of cleavage, the labile binding site becomes inactive, the C4b becomes fluid phase and has little further role in the complement cascade (Cooper and Müller-Eberhard, 1968).

The degradation of C4b is controlled by I and C4bp, both of which will be discussed in the section on regulation of complement activation (Section 1.5).

1.2.4: C2

C2, a 100 kD single chain glycoprotein, is cleaved by $C\bar{1}$ or $C\bar{1}s$ into two non-covalently bound polypeptides. The larger C2a (70 kD) possesses the enzymatic site, and the smaller C2b (30 kD) is responsible for binding to the magnesium-dependent binding site on the C4b molecule (Nagasawa and Stroud, 1977). Although C2 can be cleaved by $C\bar{1}$ and $C\bar{1}s$ in the absence of C4b, Gigli and Austen (1969) showed that the reaction was more efficient in the presence of the latter molecule.

The resultant magnesium-dependent C4b2a enzyme is a very unstable complex since C2a decays rapidly from the enzyme, thus losing its C3 cleaving capacity (Nagasawa and Stroud, 1977). Consequently the study of the C3 convertase under laboratory conditions has been somewhat difficult. This has been overcome by oxidising the C2 with iodine, which results in a more stable enzyme because the rate of decay of C2a is reduced. This leads to a ten to twenty fold increase in

haemolytic activity (Polley, and Müller-Eberhard, 1967).

The assembly of the C3 convertase, C4b2a, brings to an end the activity of the classical pathway of complement. This C3 convertase can then control the activation of the terminal components of the complement pathway: C3, C5, C6, C7, C8 and C9 (Fig. 1).

1.3: The Terminal Pathway: C3, C5, C6, C7, C8 and C9.

1.3.1: C3

C3, the most abundant complement component in serum, is the central protein of the two complement activation pathways. It is a 190 kD protein consisting of two polypeptide chains, α (110 kD) and β (70 kD), held together by both disulphide bridges and non-covalent bonding (Tack and Prahl, 1976).

The C2a component of the C3 convertase, cleaves a susceptible arginyl-serine bond at position 77 in the α chain of C3 (Tack and Prahl, 1976), resulting in the release of C3a (9 kD). The 171 kD C3b molecule consists of an unaltered β chain and the cleaved α chain (Fig. 5). During cleavage a buried thiolester group is exposed providing a site for covalent attachment of C3b to surfaces, in a similar manner to C4b. The labile binding site of C3b has a very short half-life, approximately 50 milliseconds, during which a hydroxyl or amide group on the activating surface forms a covalent ester or amide linkage between the C3b and the surface. (Pangburn and Müller-Eberhard, 1980). If C3b fails to bind to a surface,

the thiolester site combines with water to form fluid-phase iC3b, which is subject to further degradation (Fig. 5) and plays little further role in the complement cascade (Budzko, Bokisch and Müller-Eberhard, 1971).

Binding of the C3b molecule to the surface in close proximity to the C3 convertase, alters its specificity rendering it a C5 convertase (Daha, Fearon and Austen, 1976). The catalytic site still resides in the C2a moiety of the C5 convertase, whilst C3b offers a binding site for C5, allowing activation of the terminal sequence.

In addition to the ability of C3b to bind covalently to surfaces via its labile binding site, it can bind via a distinct stable binding site to a specific receptor on cell surfaces called complement receptor 1 (CR1). These have been shown to be present on the membranes of several cell types, which will be discussed later in a section on the biological activities of complement (Section 1.6.5).

Activity of C3b is closely regulated by its control protein I, and its cofactors H and CR1, the activities of which will be discussed under regulation of complement activation (Section 1.5).

1.3.2: C5

Assembly of the C5 convertase, C4b2a3b, allows the last of the enzymatic steps of the terminal sequence of complement activation to proceed: cleavage of C5 by C2a (Fig. 1). C5 is very similar to C3 in many respects (Fig. 1);

it is a 185 kD protein composed of two polypeptide chains, α (115 kD) and β (75 kD), which are linked by disulphide and non-covalent bonds (Nilsson, Tomar and Taylor, 1972). Activity of the C5 convertase on the N-terminus of the α chain of C5 results in the cleavage and release of a 12 kD polypeptide, C5a (Fernandez and Hugli, 1978) which is chemotactic for mononuclear phagocytes and polymorphonuclear leukocytes, as well as possessing anaphylatoxin activity. The role of C5a is discussed further under the biological activities of complement (Section 1.6.3).

C5b, the larger molecule which remains after C5 cleavage, lacks enzymatic activity, but functions to initiate the assembly and binding of the membrane attack complex of complement. The half-life of the binding site generated on C5b during cleavage is short (50 milliseconds) and enables it to bind to cell membranes via non-covalent forces, such as electrostatic and hydrophobic interactions (Ogle and Ogle, 1983). Failure to bind, which occurs in over 90% of cleavage reactions, results in decay of the binding site and conformational changes to form unreactive fluid-phase C5b.

1.3.3: C6 and C7

C6 and C7 are similar proteins: they both consist of single chain polypeptides which possess partial α helical structure and have molecular weights of 128 kD and 121 kD respectively (Podack, Kolb and Müller-Eberhard, 1976).

C5, C6 and C7 can form reversible complexes free in solution, however, a much more stable complex is formed as a result of C5 cleavage to C5b. One molecule of C6 binds to the metastable binding site on C5b, to form a C5b6 complex, which allows the binding of one molecule of C7, to form the trimolecular complex C5b67. Binding of C7 stabilises C5b6, which otherwise is unstable and can decay from the membrane (Podack et al., 1978). The C5b67 binds avidly to phospholipid bilayers, thus allowing attachment of the initiating complex, for the assembly of the complete membrane attack complex of complement.

Activity of C5b67 is regulated by the S protein, which will be discussed further under regulation of complement activity (Section 1.5.5).

1.3.4: C8

C8 consists of three polypeptide chains: α (77 kD), β (63 kD) and γ (14 kD) linked by disulphide bridges between the α and γ chains, with the β chain being attached by non-covalent forces (Kolb and Müller-Eberhárd, 1976).

1.3.5: C9

The terminal component in the complement sequence, C9, is a single polypeptide chain of molecular weight 79 kD (Hadding and Müller-Eberhard, 1969).

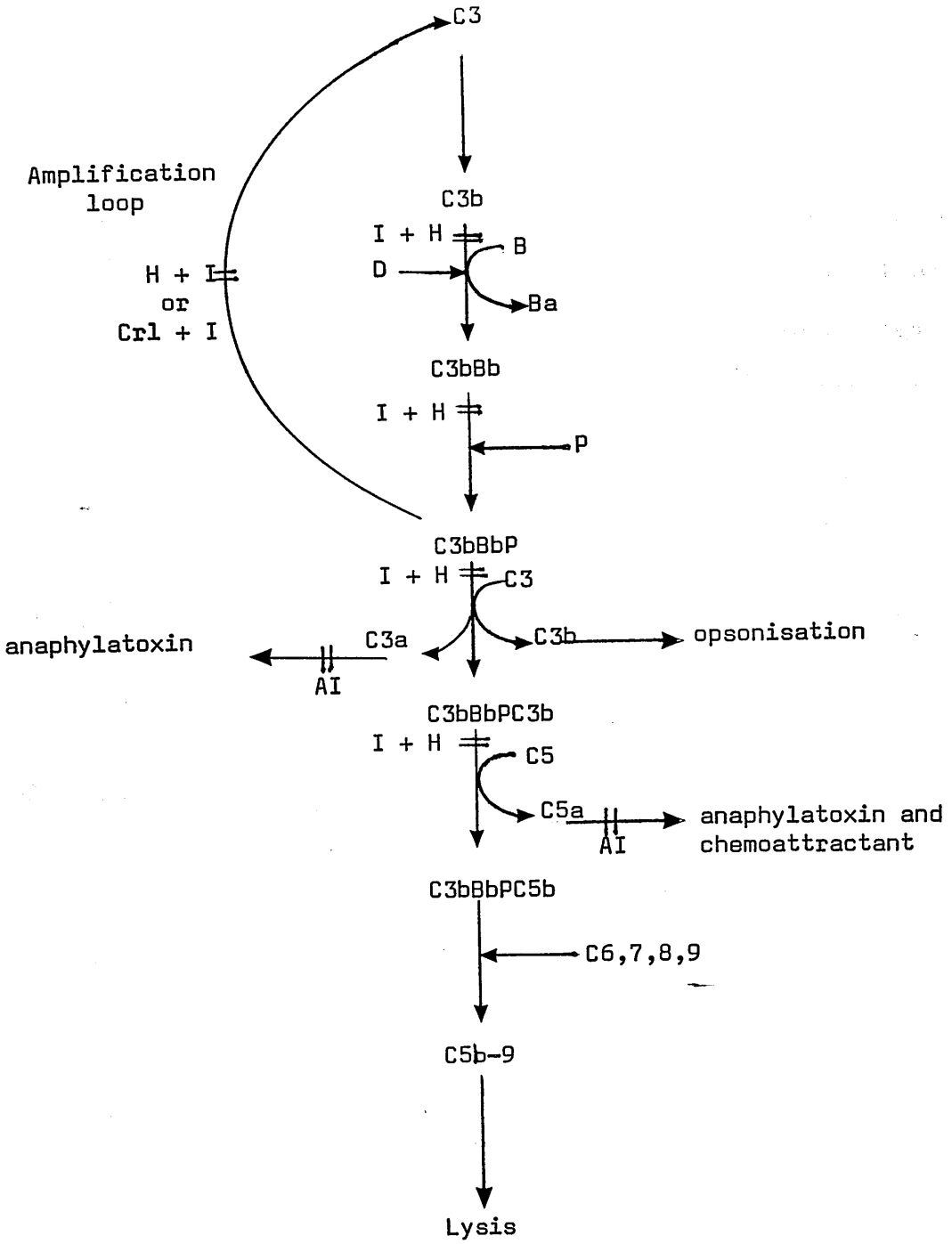


Fig. 2: The Alternative and Terminal Pathway of Complement.

Control of the activity of the pathway, by the controlling enzymes occurs at various stages, denoted by ⟶‖⟶
AI denotes Anaphylatoxin Inactivator.

1.3.6: The Membrane Attack Complex of Complement

The membrane attack complex of complement, first described by Mayer (1972), is a self-assembling group of proteins which forms channels across cell membranes, resulting in lysis of the target cells.

As described C5b67 binds avidly to phospholipid bilayers, however membrane destruction only occurs upon binding of C8 and 9 or C8 alone (Stolfi, 1968). One molecule of C8 binds to the C5b67 complex via the C8 β chain, whilst the hydrophobic α chain is involved in insertion of C8 into the lipid bilayer, and it also carries a binding site for C9. C9 has a tendency to polymerize spontaneously, however C5b678 accelerates the polymerization of C9 and enables its binding to the target cell membrane (Tschopp, 1984).

Insertion of the C5b-9 complex, which can contain between 12 and 18 C9 molecules, into the membrane results in holes with diameter ranging from 9 to 12 nm (Tschopp, 1984). The complex can be viewed as a cylindrical structure with a hollow central hydrophilic channel. The outer surface is hydrophobic and interacts with the lipid bilayer.

1.4: The Alternative Pathway: C3b, Factors B, D and Properdin

The alternative pathway of complement activation (Fig. 2, and Table 1), originally named the properdin system, is an antibody-independent complement pathway which is involved in the destruction of bacteria, the neutralization of viruses and the lysis of certain red cells. The proteins involved

in the assembly of the alternative pathway C5 convertase are C3b, factors B and D and properdin (P), whilst the control of turnover is regulated by H and I, the two control proteins.

There are certain similarities between the two complement sequences: the roles of C1s and D are similar; the role of classical pathway C4b is similar to alternative pathway C3b; the roles of C2 and factor B are analogous, both of which in their cleaved form possess the enzymatic activity for cleaving C3.

1.4.1: C3b

Preformed C3b, as described earlier in the terminal sequence (Section 1.3.1) is an essential component involved in the amplification of the alternative pathway.

1.4.2: D

D is a 24 kD polypeptide serine protease, present in serum in its activated form. In the presence of C3b and magnesium ions, D cleaves factor B to form the alternative pathway C3 convertase, C3bBb (Lesavre and Müller-Eberhard, 1978).

1.4.3: Factor B

Factor B is a 93 kD glycoprotein, which in the presence of magnesium ions binds to C3b, and is subsequently cleaved by D. This releases the 30 kD Ba fragment leaving Bb, which contains the enzymatic activity, bound to C3b (Fearon, Austen and Ruddy, 1973).

1.4.4: Properdin

Properdin is a 220 kD glycoprotein consisting of three or four identical chains held together by non-covalent forces. Properdin stabilizes the C3 and C5 convertases of the alternative pathway (Fig. 2) by binding to C3b, and reducing the rate of decay of Bb from the convertases (Fearon and Austen, 1975).

1.4.5: Alternative Pathway C3 and C5 Convertase Assembly.

The C3 convertase, C3bBb, is assembled as a result of D-catalysed cleavage of B bound to C3b, and is further stabilized by the binding of properdin, producing the properdin-stabilized C3 convertase, C3bBbP (Fearon and Austen, 1975). This C3 convertase has the ability to cleave more C3, thus forming the positive feedback amplification loop, (Fig. 2). The resulting C3b generated can act as an opsonin, binding to bacterial cell walls, leading to their enhanced phagocytosis and killing.

The lytic phase of the alternative pathway requires an alteration in the specificity from a C3 convertase, to a C5 convertase: this occurs when a subsequent C3b molecule binds to the C3 convertase. The lytic sequence from the C5 convertase onwards is identical to that described for the classical pathway: C5 is cleaved by the Bb moiety of the C5 convertase, which is followed by assembly of the membrane attack complex.

1.4.6: Activation of the Alternative Pathway

In blood, the alternative pathway is thought to be constantly turning over. However, the rate is extremely slow, and held in check by the control proteins H and I. Thus, it would appear that for activation of the alternative pathway to occur, the control mechanisms must be overcome.

The alternative pathway is activated by yeasts and bacteria, due to the presence of complex carbohydrate structures on their surface membranes. The most influential component appears to be the sialic acid content: the lower the sialic acid content of the membrane, the better the activator of the alternative pathway (Fearon, 1978).

Low grade fluid-phase turnover of C3 generates C3b, which binds to the activating surface, rendering the C3b resistant to inactivation by the control proteins H and I. This enables the binding of B and P to the C3b bound to the surface, resulting in the formation of C3 convertases which lead to further amplification. Thus, the low grade fluid-phase turnover is converted to assembly of the C3 and C5 convertases on the activating surface.

There are two other known substances which have a profound effect on the turnover of the alternative pathway: cobra venom factor (CoVF) and nephritic factor. CoVF is cobra C3b, a 150 kD glycoprotein, which acts like human C3b, binding to B and producing a C3 convertase (CoVFBb). However, cobra C3b is resistant to the activities of human I and H, thus, the result is uncontrolled activation of the alternative pathway (Alper and Balavitch, 1976; Nagaki et al., 1978).

Component	Molecular weight (Daltons)	Serum conc. µg/ml	Polypeptide Chain structures
C1-inhibitor	105 000	180	1
C4 Binding protein (C4bp)	540 000	100	8
I	93 000	50	2
H	150 000	300	1
S protein	88 000	600	1
Anaphylatoxin inactivator	310 000	35	8

Table 2: Human Complement Control Proteins.

Nephritic factor is an IgG antibody directed against both components of the alternative pathway C3 convertase, C3b and Bb, and is found in the serum of some patients with mesangiocapillary glomerulonephritis (Daha, Austen and Fearon, 1978). The binding of nephritic factor stabilizes the C3 convertase activity, and renders it resistant to inactivation by H. As a result, increased uncontrolled activation of the alternative pathway occurs.

1.5: Regulation of Complement Activation

In a system as complex as the two interlinked complement pathways described, it is obvious that for an efficient system, its activation must be controlled by extremely sensitive regulatory mechanisms. Regulation is important to conserve "normal concentrations" of components in serum and body fluids, and to regulate the generation of biologically active by-products, produced as a result of activation and cleavage of complement components.

Figures 1 and 2 demonstrate the site of action of the major complement control proteins which will be discussed: C1-inhibitor (C1-Inh), C4 binding protein (C4 bp), H, I, S protein and anaphylatoxin inactivator (AI) (Table 2).

1.5.1: C1-inhibitor

C1-inhibitor is a single chain 105 kD glycoprotein first described by Ratnoff and Lepow (1957), which acts as the only known physiological inhibitor of the first component of complement (Sim *et al.*, 1979). In addition to its major role

in the complement cascade, C1-inhibitor is a multi-specific inhibitor of the enzymes of other plasma mediator systems. These include the coagulation system (factors XIa, XIIa and XIIf), fibrinolytic system (plasmin) and the kinin system (kallikrein) (Harpel and Cooper, 1975; Ratnoff *et al.*, 1969; Gigli *et al.*, 1970).

C1-inhibitor is one of the most heavily glycosylated serum proteins, containing approximately 33% carbohydrate by weight (Harrison, 1983) most of which is located on the N-terminal 120 residues (Bock *et al.*, 1986). Upon deglycosylation of purified C1-inhibitor, the apparent molecular weight decreased from 105 kD to 78 kD. This was thought to represent the polypeptide portion of the glycoprotein, which was PAS-negative on staining (Harrison, 1983).

The carbohydrate composition is unusual since it possesses a high galactose and N-acetyl galactosamine content, as well as elevated levels of aliphatic hydroxyl-containing amino acids. This appears to suggest to protein biochemists that O-glycosidic linkages may be involved in linkage between the carbohydrate and protein moieties (Harrison, 1983). The purpose of the high degree of glycosylation of C1-inhibitor is unknown. However, Harrison (1983) has suggested that all of the carbohydrate does not appear to be necessary for C1-inhibitor functional activity. Minta (1981) has reported that removal of 60% of the sialic acid and 20% of the galactose residues does not alter the functional capacity of C1-inhibitor, however it does lead to its increased hepatic clearance.

The results of studies investigating the 3-dimensional structure of C1-inhibitor have revealed it to be a highly elongated molecule, consisting of two domains. A globular head with a diameter of 4 nm, and the second domain a rod-like tail structure of diameter 2 nm and length 33 to 36 nm (Odermatt, Berger and Sano, 1981).

C1-inhibitor inhibits activated C1, thus limiting the turnover of the classical pathway of complement. It does this by binding irreversibly, at or close to the catalytic site of activated C1r and C1s, thus disassembling the C1 macromolecule, releasing a complex of C1r: C1s: C1-inhibitor (Ziccardi and Cooper, 1979). The C1q remains attached to the activating surface, or becomes fluid-phase: it is thought then to interact with C1q receptors which are found on various cell types (Sobel and Bokisch, 1975; Tenner and Cooper, 1979).

The dissociated complex contains two 105 kD C1-inhibitor molecules: one in association with one 95 kD $C\bar{1}r$, and one in association with one 87 kD $C\bar{1}s$. The molecular weight of the complex was estimated by SDS-PAGE to be 330 kD, which was in close agreement with the calculated sum of the polypeptide chains of 382 kD (Ziccardi and Cooper, 1979). Thus the molecular composition of the complex released from the $C\bar{1}$ was 1:1:2, $C\bar{1}r$: $C\bar{1}s$: C1-inhibitor, and two such complexes were shown to be released from each activated C1 molecule (Ziccardi and Cooper, 1979). The complex was shown to be of the same composition whether isolated from classical-pathway activated serum, or reconstituted purified components (Ziccardi and Cooper, 1979). The $C\bar{1}r$:$C\bar{1}s$: C1-inhibitor$_2$

complex formed is extremely stable and is not dissociated by EDTA (Ziccardi and Cooper, 1979), by SDS under reducing conditions, by other dissociating agents such as guanidine and 4M urea (Reboul, Arlaud and Colomb, 1976), or by high ionic strength, 1.2M sodium chloride (Chesne et al., 1982). Stability of the $C\bar{1}r$: $C\bar{1}s$: C1-inhibitor$_2$ complex under these various conditions suggests that the bonding between the $C\bar{1}$ subcomponents, $C\bar{1}r$ and $C\bar{1}s$, and the C1-inhibitor is likely to be covalent. Hydroxylamine can dissociate the complex (Sim et al., 1979) which suggests that an ester bond is involved, possibly with the reactive serine in the active sites of $C\bar{1}r$ and $C\bar{1}s$.

Chesne et al. (1982) studied the interactions of C1-inhibitor with $C\bar{1}r$, $C\bar{1}s$, and the $C\bar{1}r_2$ - $C\bar{1}s_2$ complex using sucrose density gradient centrifugation techniques which allowed investigation into the type of inter-proteinase bonds formed in the complex with C1-inhibitor. The bonding within the $C\bar{1}r$ dimer, the $C\bar{1}s$ dimer and the $C\bar{1}r_2$ - $C\bar{1}s_2$ tetramer differs in that $C\bar{1}r$ dimers have calcium-independent bonding, $C\bar{1}s$ dimers have calcium dependent bonding (Arlaud et al., 1980) and the $C\bar{1}r_2$ - $C\bar{1}s_2$ complex has calcium-independent bonding (Chesne et al., 1982). However, one common property in construction of the dimers and tetramers is that the interacting chains in all cases are the heavy chains (Arlaud et al., 1980), leaving the light chains with the active enzymatic sites accessible to C1-inhibitor. Binding of C1-inhibitor to the active sites of $C\bar{1}r_2$ - $C\bar{1}s_2$, induces

conformational changes in the heavy chains. Consequently there are decreased $C\bar{1}r$ - $C\bar{1}r$ interactions as a result of decreased intermonomer non-covalent bonding (Chesne et al., 1982). Concurrently, there is a reinforcement of bonding between the $C\bar{1}r$ and $C\bar{1}s$, leading to stronger noncovalent bonding between $C\bar{1}r$ and $C\bar{1}s$ within the released $C\bar{1}r$:$C\bar{1}s$: Cl-inhibitor$_2$ complex (Chesne et al., 1982).

Salvesen and his colleagues (1985) have produced evidence that a single reactive/inhibitory site is present on the Cl-inhibitor molecule. Preformed complexes of plasma-kallikrein and Cl-inhibitor are unable to inhibit $C\bar{1}s$, and vice-versa, even after prolonged incubation at 37°C. The complex formed upon interaction of $C\bar{1}s$ with Cl-inhibitor have a molecular weight of 120 kD, confirming the complex is formed between Cl-inhibitor and the light chain of $C\bar{1}s$, which contains the active enzymatic site.

Various groups have performed sequencing studies on Cl-inhibitor (Salvesen et al., 1985; Harrison, 1983; Davis et al., 1986; Bock et al., 1986). Salveson et al. (1985) studied preformed $C\bar{1}s$-Cl-inhibitor complexes, and demonstrated an alteration in the amino acid sequence of the complexed Cl-inhibitor. They proposed that the new sequence arose from cleavage of an arginine-threonine bond in Cl-inhibitor, releasing a 5 kD polypeptide from the C-terminal region, following the formation of a complex between $C\bar{1}s$ and Cl-inhibitor.

Ziccardi (1982b) has proposed that C1-inhibitor, as well as inhibiting the activity of $C\bar{1}$, may play an important role in the control of auto-activation of unactivated C1, thus suggesting it functions earlier in the complement sequence than previously proposed.

The concentrations of C1-inhibitor investigated ranged from 0.15 to 2 times physiological levels, with normal physiological levels of C1. The findings illustrated that concentrations of C1-inhibitor as low as 0.35 times physiological, were effective at blocking spontaneous auto-activation of C1. However, concentrations of C1-inhibitor below 0.25 times physiological, were ineffective at inhibiting spontaneous auto-activation of physiological levels of C1. This observation has important clinical implications in hereditary angio-oedema (HAE) patients whose sera contain reduced levels of functional C1-inhibitor, often 0.05 to 0.30 times that of the normal physiological levels. Consequently, the spontaneous auto-activation of C1 observed in the sera of these patients will proceed, unaffected by the residual low levels of C1-inhibitor.

In addition to the inhibition of auto-activation of C1 by C1-inhibitor, its effect on the inhibition of $C\bar{1}$ activated by different types of stimuli were investigated (Ziccardi, 1982b). The two types of stimuli investigated were immune complex stimulation, such as tetanus anti-tetanus complexes and antibody coated erythrocytes (EA), as well as the non-immune stimuli, DNA and heparin. The results demonstrated that at 37°C, C1-inhibitor could inhibit C1 activation induced by the non-immune stimuli, whereas, it had no effect

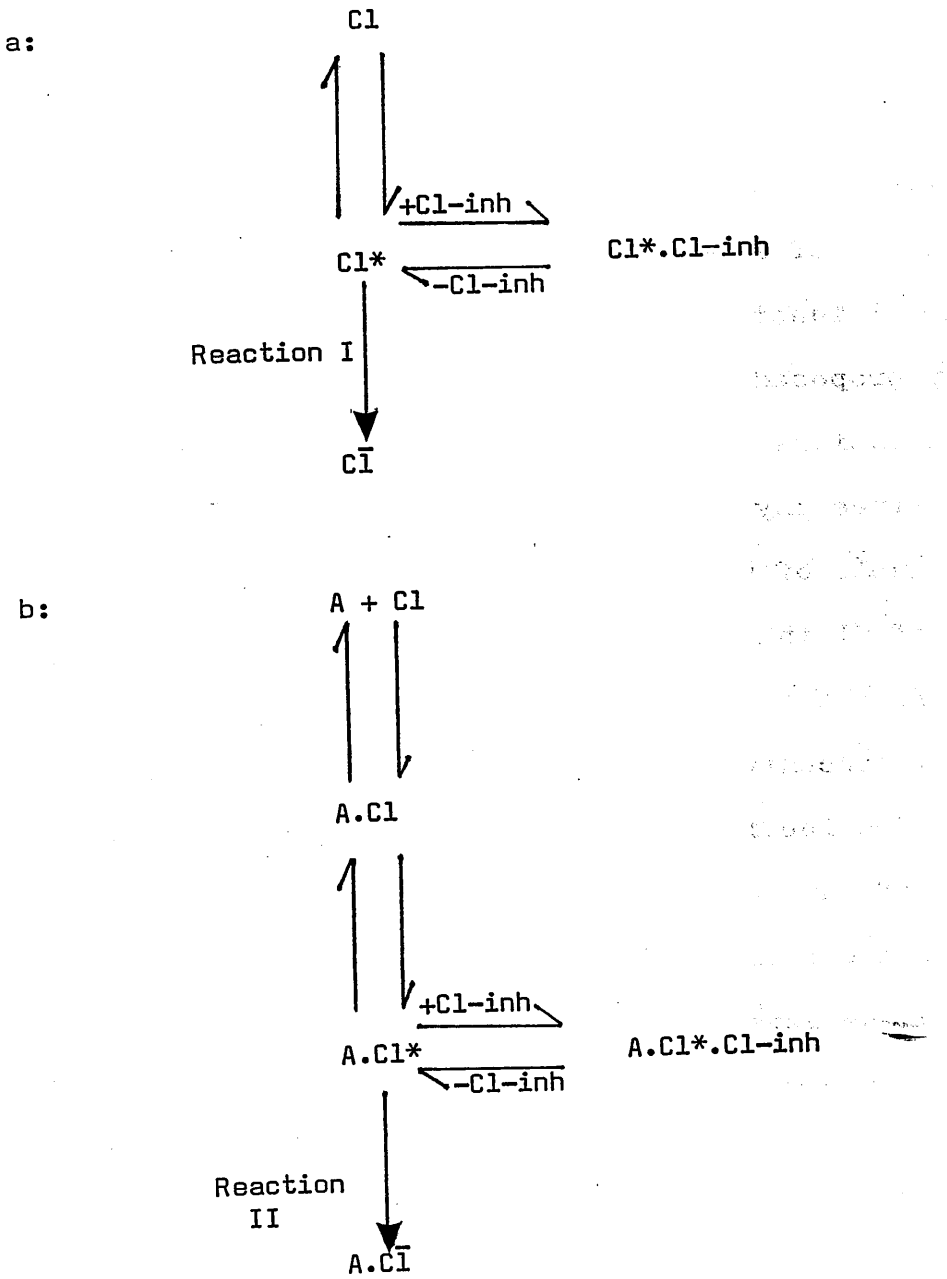


Fig. 3: The Role of C1-inhibitor in the activation of C1, by Non-immune (a) and Immune (b) Stimuli.

C1* denotes a transitional state of C1, to which C1-inhibitor can bind; A denotes an activating surface. See text for full explanation of reaction mechanisms.

on C1 activation by immune stimuli. When the incubation temperature was reduced to 20°C, Ziccardi showed that C1-inhibitor could block almost all of the EA induced C1 activation. Upon further investigation of the activation state of the C1, it was demonstrated that 90% of the C1-inhibitor was bound specifically to unactivated C1 present on the EA surface, and only 10% was bound to $C\bar{1}r$ and $C\bar{1}s$.

Incubation of reconstituted C1 and purified C1-inhibitor in free solution, does not lead to the formation of stable complexes which can be isolated by sucrose-density gradient centrifugation. With the results obtained, Ziccardi has proposed a model for the interactions between C1-inhibitor and C1 (Fig. 3).

In order to explain the interaction of C1-inhibitor with unactivated C1, an intermediate or transitional state of C1 was postulated, denoted as C1* in Figure 3. This is conformationally distinct from native C1, but is not yet cleaved to activated C1. Panel a Figure 3, demonstrates that C1-inhibitor binds to the transitional state C1*, thus blocking spontaneous activation of C1 which is known to occur (Ziccardi, 1982a). Secondly, Panel b, Figure 3, demonstrates the proposed interaction of C1 with an activating surface (A), producing C1 bound to the activating surface (A.C1). The transitional state A.C1* again occurs: this is the point at which C1-inhibitor can interact, leading to the inhibition of activator-induced C1 activation. In an attempt to explain the differing results observed between immune and non-immune stimuli, Ziccardi has

proposed that the conversion of A.C1* to A.C$\bar{1}$ (reaction II, panel b, Fig. 3) in immune complex stimulated C1, is too rapid at 37°C to be inhibited by C1-inhibitor. However, the ability of C1-inhibitor to block this reaction at 20°C would be explained by the decrease in reaction rate of reaction II (panel b, Fig. 3) at the lower temperature. This reaction I, in non-immune induced C1 activation is thought to be a slower conversion process at 37°C, and thus allows the intervention of C1-inhibitor to block the non-immune induced C1 activation (Ziccardi, 1982b). Thus, Ziccardi has suggested that C1-inhibitor blocks the auto-activation of C1 and also has an important physiological role in the blocking of undesired, non-specific C1-activation by non-immune activators. This demonstrates the selectivity of C1-inhibitor under physiological conditions, based on the faster rate of C1 activation induced by immune activators, compared to non-immune activators.

Several groups of workers have independently performed amino acid sequencing studies on C1-inhibitor, and cDNA probes spanning part of, or the whole gene sequence have been prepared (Davis et al., 1986; Bock et al., 1986). The consensus of opinion is that the C1-inhibitor gene maps to chromosome 11, and they have finally proven that it is valid to include C1-inhibitor in the serpin superfamily. This is a group of homologous protease inhibitors which includes α_1 antitrypsin, antithrombin III, as well as ovalbumin, angiotensinogen, and α_2 antiplasmin as some of its members (Bock et al., 1986).

The general reaction mechanism which is accepted to represent the interaction between protease enzymes (E) and their inhibitors (I), is illustrated in equation 1 below:

$$E + I \underset{}{\overset{A}{\rightleftharpoons}} EI \xrightarrow{B} EI \qquad \text{Equation 1}$$

The reaction is proposed as a two step mechanism consisting of a rapid second order reaction (A) resulting in the formation of a reversible unstable intermediary complex, EI. The second step is a slower first order rearrangement (Equation 1, reaction B), to form a stable and irreversible enzyme-inhibitor complex. This general reaction is also proposed as the mode of inactivation of $C\bar{1}r$ and $C\bar{1}s$ by C1-inhibitor, although only the stable end product has been observed.

At 37°C and in the presence of calcium ions, the overall second order reaction rate for $C\bar{1}s$ with C1-inhibitor was $12.5 \times 10^3\ M^{-1}s^{-1}$, and for $C\bar{1}r$ with C1-inhibitor the reaction rate was slower, at $1.53 \times 10^3\ M^{-1}s^{-1}$ (Sim, Arlaud and Colomb, 1980). Although there is a difference in reaction rates, the affinities of $C\bar{1}r$ and $C\bar{1}s$ for C1-inhibitor are more similar (12×10^{-8}M and 9.6×10^{-8}M respectively), and are unaltered by the presence or absence of calcium ions (Sim, Arlaud and Colomb, 1980). The activation energy for $C\bar{1}r$-C1-inhibitor binding was 44.3 kcal mol^{-1} and for $C\bar{1}s$-C1-inhibitor was 11.7 kcal mol^{-1}. EDTA does not affect the reaction rate of C1-inhibitor with $C\bar{1}s$, however it increases the reaction rate of C1-inhibitor with $C\bar{1}r$ two to three fold, up to $2.84 \times 10^3\ M^{-1}\ s^{-1}$ (Sim, Arlaud and Colomb, 1980).

This kinetic data demonstrates that $C\bar{1}r$ and $C\bar{1}s$ have a similar affinity towards C1-inhibitor, however $C\bar{1}r$ reacts much more slowly with C1-inhibitor than does $C\bar{1}s$. The slower reaction rate of C1-inhibitor with $C\bar{1}r$ supports previous observations (Reboul et al., 1977) that $C\bar{1}r$ free in solution, does not compete with $C\bar{1}s$ effectively for C1-inhibitor binding. The converse has been shown to occur when $C\bar{1}r$ is present in macromolecular $C\bar{1}$ bound to immune complexes (Sim, Arlaud and Colomb, 1980). In this situation a four-fold increase in reactivity of $C\bar{1}r$ towards C1-inhibitor occurs. There is no change in the reactivity of $C\bar{1}s$ towards C1-inhibitor when present as $C\bar{1}$ bound to immune complexes. This may have physiological significance since most of the activated C1r and C1s in plasma will be present together in a $C\bar{1}$ complex bound to immune aggregates, rather than being present as free proteases. In addition, the closer reaction rates of $C\bar{1}r$ and $C\bar{1}s$ within the $C\bar{1}$ complex is most important in that C1-inhibitor must firstly bind to $C\bar{1}r$, before it can induce disassembly of the $C\bar{1}$ macromolecule, thus limiting turnover of the classical complement pathway. (Sim, Arlaud and Colomb, 1979).

In addition to its role in the control of the first component of complement, C1-inhibitor is known to inhibit plasma kallikrein, plasmin and the coagulation factors XIa, XIIa and XIIf, (an enzymatically active fragment derived from factor XIIa) (Gigli et al., 1970; Harpel and Cooper,

1975; Ratnoff et al., 1969; Schreiber, Kaplan and Austen, 1973). C1-inhibitor appears to have a similar reaction mechanism, regardless of its substrate: SDS-PAGE studies have shown that C1-inhibitor forms a stable 1:1 complex with its substrate, as was described for C1r and C1s (Reboul et al., 1977; Salvesen et al., 1985; Ziccardi and Cooper, 1979).

Plasma kallikrein circulates as an inactive precursor zymogen called prekallikrein, whose cleavage is catalysed by high molecular weight kininogen (HMWK) (Fig. 4). The HMWK then acts as a substrate for kallikrein which cleaves the HMWK to kininogen, with subsequent reactions resulting in the generation of bradykinin, a vasoactive peptide which is involved in vascular permeability and the generation of the inflammatory response.

The intrinsic pathway of blood clotting is initiated when factor XII is cleaved to factor XIIa, in the presence of a negatively charged surface such as kaolin or glass and HMWK, which functions as a non-enzymatic cofactor to stimulate the reaction (Fig. 4) (Davis, 1981). This initial enzymatic conversion stimulates a cascade of reactions where inactive precursors are converted to active enzymes: factor XII is cleaved to factor XIIa; factor XIIa then converts factor XI to factor XIa; through several other reactions with the terminal reaction being the conversion of fibrinogen to fibrin, catalysed by thrombin. The fibrin monomers then undergo aggregation leading to the formation of a fibrin gel, with factor XIIIa cross-linking the fibrin gel with covalent bonds (reviewed by Davie, 1981).

Factor XIIa, also known as activated Hageman factor, and kallikrein are involved in the initiation of the fibrinolytic system, as demonstrated in Figure 4. Factor XIIa, generated in the coagulation system, can convert plasminogen to plasmin. This conversion can also be catalysed by kallikrein which is generated in the kinin generating system. Plasmin is a proteolytic enzyme which digests fibrin to fibrinogen as well as being able to activate C1, to $C\bar{1}$.

The multiplicity of the inhibitory capacity of C1-inhibitor is demonstrated when one considers the wide range of its substrates, all of which are involved in the plasma mediator systems. However, the observation that C1-inhibitor is active against these serine proteases *in vitro*, does not necessarily demonstrate that physiologically, in the plasma, it will perform the same functional role. *In vitro*, plasmin and C1-inhibitor react rapidly, however, in the plasma the predominant inhibitor of plasmin is α_2 antiplasmin, the secondary inhibitor is α_2 macroglobulin, and C1-inhibitor does not appear to play an important inhibitory role (Aoki *et al.*, 1977). Thus, C1-inhibitor may not be important in the control of the fibrinolytic system in plasma, unless there is a deficiency of α_2 antiplasmin or α_2 macroglobulin.

In contrast, $C\bar{1}$-inhibitor does appear to have a physiologically important role in the control of the plasma kinin forming system and the coagulation system. In plasma, 90% of the inactivation of factor XIIa and factor XIIf is performed by C1-inhibitor. This was demonstrated by the isolation of XIIa-C1-inhibitor and XIIf-C1-inhibitor as the

major complexes on SDS-PAGE, after incubation of I^{125}-labelled factors XIIa and XIIf with the purified plasma protease inhibitors: C1-inhibitor, antithrombin III, α_2 antiplasmin, α_1 antitrypsin, α_2 macroglobulin, in addition to various normal human sera and sera depleted of the aforementioned enzymes (Agostini et al., 1984).

Kinetic analysis has shown that in normal plasma, C1-inhibitor is the major inhibitor of plasma kallikrein, to a level of 58%, with a secondary inhibitory role played by α_2 macroglobulin to a level of 38%, the remaining 4% of activity being accounted for by all the remaining plasma inhibitors (Schapira, Scott and Colman, 1982). In support of these data it has been demonstrated in normal human plasma that $C\bar{1}$-inhibitor binds 57% of exogenously activated kallikrein, using kaolin, and 84% of endogenously activated kallikrein, using factor XIIf (Harpel, Lewin and Kaplan, 1985). Therefore, although C1-inhibitor has the capacity to inactivate $C\bar{1}$ and its subcomponents, kallikrein, factors Xia, XIIa, XIIf, and plasmin in vitro, in vivo its major functional role is thought to be the inactivation of $C\bar{1}$ and its subcomponents. Less major functions of C1-inhibitor in vivo, are the control of kallikrein, factors Xia, XIIa and XIIf, since these enzymes also possess other inhibitors which can be used in their inactivation; the role of C1-inhibitor in the control of plasmin in vivo is thought to be unimportant.

From the discussion of the biochemical interactions and the physiological role of C1-inhibitor in the plasma, it is obviously of widespread importance in the control of several of the plasma mediator systems. Hereditary deficiency of this protein, displayed in the clinical syndrome of hereditary angio-oedema, therefore leads to complications in the control of these systems, which will be discussed under C1-inhibitor deficiency (Section 1.7.1).

1.5.2: C4 Binding Protein (C4 bp)

C4 bp is a serum glycoprotein composed of several disulphide-linked 70 kD subunits, which are assembled into a polypeptide chain of 540 kD (Nussenzweig and Melton, 1981).

C4bp regulates formation and activity of the classical pathway C3 convertase by binding specifically to C4b, although it cannot cleave C4b on its own. It has been suggested that it performs this role by competing with C2a for the binding site on C4b, and it can displace C2a from the C4b2a complex (Nussenzweig and Melton, 1981).

In order to control C3 convertase activity, both C4bp and factor I are required to act together: C4bp binds to C4b in the C3 convertase, and renders it susceptible to cleavage by factor I. Cleavage occurs at two sites in the C4b α chain releasing an α_2 fragment of 47 kD, denoted C4d, from the remaining molecule, denoted C4c. C4c is composed of the intact β and γ chains and the two α chain cleavage products, α_3 (25 kD) and α_4 (17 kD) (Fujita, Gigli and

Nussenzweig, 1978). Inactivation of C4b to C4c and C4d renders it unable to bind C2, thus reducing the formation of the classical pathway C3 convertase, and prevents its binding to the C3b/C4b receptor (CR1), which is involved in immune adherence.

1.5.3: H

H is a single polypeptide chain glycoprotein of 150 kD whose two main roles are as cofactors: firstly in the inactivation of C3b by factor I (Whaley and Ruddy, 1976; Harrison and Lachman, 1980) (see Section 1.5.4) and secondly in accelerating the decay of the alternative pathway C3 and C5 convertases, by binding stoichiometrically to C3b. When C3bBb or C3bBbP are formed, I cannot gain access to C3b to cleave it, due to Bb binding. However, H can bind to C3b in the convertase, accelerating decay and releasing fluid phase C3b which can then be inactivated by I (Whaley and Ruddy, 1976; Weiler et al., 1976).

1.5.4: I

I is a 93 kD glycoprotein consisting of two polypeptide chains of 55 kD and 42 kD, held together by disulphide bridges and non-covalent bonding. I is important in the regulation of the alternative pathway feedback amplification loop, along with its cofactor activity of H or CR1, in a purified form (Medof et al., 1982) or on the surface of human erythrocytes (Medicus, Melamed and Arnaout, 1983).

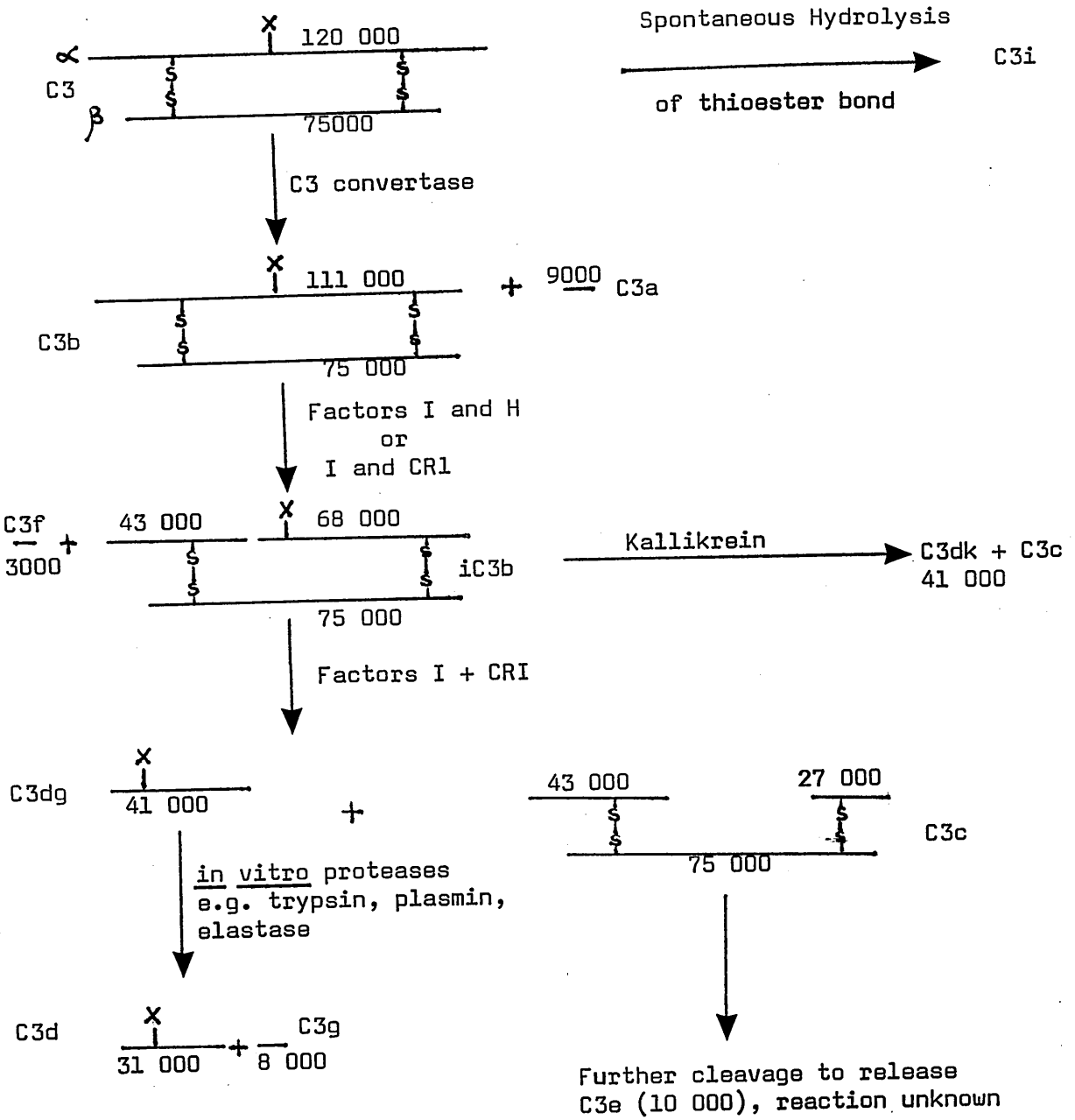


Fig. 5: Cleavage Patterns of C3.

X Denotes binding site.

The cleavage patterns of C3b to its degradation products is illustrated in Figure 5. I, in the presence of CR1 or H, cleaves the α chain of C3b at two sites to form iC3b, and the fluid phase C3f (3 kD). The haemolytically inactive iC3b consists of two α chain fragments (68 kD and 43 kD) covalently bound to the intact β chain. Further cleavage of iC3b is carried out again by I, with its CR1 cofactor, resulting in the generation of a fluid phase 140 kD C3c fragment, and a 41 kD C3d-g fragment which remains bound and possesses the binding site, denoted as X in Figure 5 (Medicus, Melamed and Arnaout, 1983). In serum, this is the end of C3b degradation, however C3d-g can be further cleaved *in vitro*, by proteases such as trypsin, plasmin and elastase to the fragments C3d (31 kD) and C3g (8 kD) (Medicus, Melamed and Arnaout, 1983). Cleavage of fluid-phase C3c is thought to release C3e, a 10 kD polypeptide with leukocytosis-inducing activity, however the mechanism of cleavage is unknown (Gherbrehiwet and Müller-Eberhard, 1979).

Thus, the activity of I along with its cofactors H, C4bp and CR1, results in the control of fluid and solid phase C3b activity; control of the alternative pathway C3 and C5 convertases; and control of the classical pathway C3 convertase, by its action on C4b (Section 1.5.2). The important role played by I in regulation is displayed in its hereditary deficiency. Unopposed alternative pathway turnover occurs due to failure to degrade C3b, and to prevent inactivation of C3bBbP. This C3-convertase enzyme cleaves more C3 and the

alternative pathway continues to turn over until the supply of B is exhausted (Thompson and Lachman, 1977).

1.5.5: S Protein

S protein, an 88 kD glycoprotein, is involved in the regulation of the activity of the membrane attack complex of complement. It is proposed that the S protein binds to the hydrophobic regions on C5b, at the C5b67 stage of MAC formation, thus inhibiting its insertion and interaction with lipid bilayers (Kolb and Müller-Eberhard, 1975).

1.5.6: Anaphylatoxin Inactivator (AI, Figs. 1 and 2)

During activation of C3 and C5, cleavage products C3a and C5a which have anaphylatoxic properties, are released. In addition C5a is a powerful chemoattractant for neutrophils, eosinophils and macrophages. In order to control the activities of these peptides, anaphylatoxin inactivator (also known as carboxypeptidase N) is brought into play. It is a 310 kD protease, which cleaves the C-terminal arginine residue from both peptides. The resulting C3a-des-Arg and C5a-des-arg do not possess anaphylatoxic activity, although the latter remains a powerful chemoattractant (Bokish and Müller-Eberhard, 1970).

1.6: The Biological Activities of the Complement System

1.6.1: Cytolysis

It is due to this function of complement that the system was recognised and discovered. As discussed previously, the formation of the membrane attack complex and its insertion into the membrane, leads to development of hydrophilic channels. Through these water and electrolytes flow, eventually leading to equilibration with the surrounding media, resulting in lysis of the cell, that is cytolysis.

1.6.2: Increased Vascular Permeability

The cleavage products from C3 and C5, C3a and C5a, are known to be potent anaphylatoxins which bind to receptors on mast cells and basophils. The result is degranulation, releasing vasoactive amines such as histamine, which is associated with increased vascular permeability. C3a and C5a can act directly on smooth muscle to cause contractions, which would affect the endothelial cells of the post-capillary venules, increasing the intracellular gaps, leading to the exudation of fluid. C3a and C5a are inactivated by anaphylatoxin inactivator, which cleaves the C-terminal arginine residue, thus dramatically reducing their anaphylotoxic activity (Bokish and Müller-Eberhard, 1970).

1.6.3: Chemotaxis

C5a and C5a-des-arg are powerful chemotactic agents for eosinophils, neutrophils and mononuclear phagocytes,

with the latter two groups possessing specific receptors for these C5 cleavage products. This is thought to be important in host defences, because the attraction of neutrophils and mononuclear phagocytes to an area of complement turnover due to bacterial infection, results in the phagocytosis and killing of the opsonised bacteria (Damerau et al., 1978; Fernandez et al., 1978).

1.6.4: Leukocytosis

Another breakdown product of C3, C3e, causes an initial leukopenia, followed by leukocytosis: this is probably due to it mobilising neutrophils from the bone marrow (McCall et al., 1974).

1.6.5: Phagocytosis

C3b-coated immune complexes or any C3b-coated particulate material, can interact with C3b receptors (CR1) on the surface of numerous cell types including polymorphonuclear leukocytes (PMN), monocytes, macrophages, B lymphocytes, erythrocytes and granulocytes. When C3b is present on the activating surface on its own, adherence but not ingestion occurs. However, when C3b and IgG are both present on the activating surface, ingestion occurs by phagocytic cells such as PMNs, monocytes and macrophages. Thioglycollate elicited macrophages are one of the groups of cells which are capable of ingesting C3b-coated particles in the absence of IgG (Bianco et al., 1975).

Pathway/ Component	Associated Disease	
	Frequent	Infrequent
Classical		
C1q	SLE	Recurrent infections
C1r	SLE	Recurrent infections
C1s	SLE	Recurrent infections
C4	SLE	Recurrent infections
C2	SLE; glomerulonephritis	Recurrent infections, vasculitis, inflammatory bowel disease
C1-inhibitor	HAE, SLE	Glomerulonephritis
Alternative		
P	Neisserial infections	
D	Recurrent upper respiratory infections	
I	Neisserial infections	Immune complex disease
H	Neisserial infections	
Terminal		
C3	Neisserial infections, glomerulonephritis	Fever, rash, arthralgias
C5	Neisserial infections	SLE
C6	Neisserial infections	SLE
C7	Neisserial infections	SLE, scleroderma, rheumatoid arthritis, purpura, fever and ankylosing spondylitis
C8	Neisserial infections, SLE	Xeroderma pigmentosa, fever, hypergammaglobulinaemia, eosinophilia
C9	None	

Table 3: Diseases associated with hereditary complement component deficiencies

Receptors for other C3 breakdown products are present on the surfaces of cells: CR2, whose ligands include iC3b, C3dg and C3d, are present on B lymphocytes, monocytes and polymorphs; CR3, whose ligands include iC3b and C3dg, are present on granulocytes, monocytes, macrophages and null cells (Arnaout and Colten, 1984).

1.7: Inherited Complement Component Deficiencies

Hereditary deficiencies of the complement components and their control proteins has been reported for all of the components, with the exception of factor B and C4 binding protein. There is frequently a disease association (Table 3), which varies depending on the component which is deficient. Generally, deficiency of the classical pathway components is associated with immune complex disease and recurrent bacterial infections. Deficiency of P, C5, C6, C7 or C8 is associated with a high incidence of recurrent systemic Neisserial infections; C3 deficiency is associated with immune complex disease and severe recurrent pyogenic infections. Deficiency of H and I result in recurrent infections as a result of C3 depletion, because of uncontrolled alternative pathway turnover. The only deficiency which does not regularly appear to be associated with a diseased state, is C9 deficiency, however, one case has been associated with Neisserial infections (Rynes and Pickering, 1985). Deficiency of C1-inhibitor results in the clinical condition of hereditary angio-oedema (HAE), which will be discussed in further details later.

The study of inheritance patterns of complement deficiencies has demonstrated autosomal recessive modes of inheritance, with the exceptions being properdin, which is sex-linked, and C1-inhibitor deficiency which is transmitted in an autosomal dominant fashion with heterozygotes developing the disease.

1.7.1: C1-inhibitor Deficiency

Hereditary angio-oedema (HAE) is the result of C1-inhibitor deficiency, and is one of the most commonly occurring inherited complement deficiencies, its incidence estimated to be one in 150,000. It was first reported by Osler in 1888, and was identified later as a deficiency of C1-inhibitor by Donaldson and Evans (1963).

Clinically HAE is characterised by recurrent bouts of acute, circumscribed, non-inflammatory oedema, involving the subcutaneous tissues and mucous membranes. The oedema is usually painless, non-pitting and non-puritic and can affect any area of the body, including the skin, gastrointestinal tract, respiratory system and genitalia. The most endangered system to be involved is the upper respiratory tract, where if the airways and larynx experience oedema would result in respiratory obstruction and death due to asphyxiation. This is the only known fatal symptom of HAE and accounts for as many as 25% of early deaths, if the disease is left untreated (Donaldson, 1979).

The attack commences usually by a sensation of tightness or tingling, which develops over several hours into an oedematous area ranging from a few centimetres, to the involvement of an entire limb. The oedema increases for 24 to 48 hours, and then recedes over the next 2 to 3 days. Most patients experience their first attack around puberty, although evidence has accumulated that 50% of cases present before their sixth birthday (Ballogh and Whaley, 1980). However some cases may present as late as their sixtieth year. The frequency of attacks can vary from as often as one per week, to experiencing only one in a lifetime; the frequency of attacks does not usually display any regularity, however, some women appear to have symptoms related to their menstrual cycle (Kerr and Yeung Laiwah, 1987).

The precipitating factor for an attack often can be identified in about half of the cases: such triggering factors have been identified as local tissue trauma, as observed during dental extractions, and tonsillectomy. These have been associated with laryngeal oedema; prolonged pressure or vibrations, infections, over-exposure to extremes of heat, cold and sunlight, emotional stress and anxiety have all been associated with triggering attacks. The severity of the attack is not always a reflection of the severity of the triggering stimulus, and the major trauma of surgery to areas other than the neck and head, often does not precipitate an attack (Ballogh and Whaley, 1980; Al-Abdullah and Greally, 1986).

The deficiency of serum C1-inhibitor is inherited in the unusual manner of an autosomal dominant trait, with heterozygotes being affected by the disease (Donaldson, 1979). The disease can normally be traced back through several generations displaying autosomal dominant inheritance, without any sex preference. Occasionally, the disease may present late on in life often without a positive family history, this is classified as acquired C1-inhibitor deficiency and has been associated, in more than 90% of cases, with B cell malignancies. Geha and his colleagues (1985) demonstrated that three patients had circulating anti-idiotypic antibodies to the monclonal immunoglobulin expressed on the surface of their B cells, whilst in one patient, the antibodies were directed against the immunoglobulins in the cytoplasm of their bone marrow cells. The anti-idiotypic antibodies reacted with the myeloma (M) component circulating in their autologous serum, but did not cross react with heterologous M proteins. These results suggested to the authors that interaction between the M component and the anti-idiotypic antibody, resulted in formation of immune complexes which increased consumption of C1 and C1-inhibitor. This led to activation and cleavage of C4 and C2, but not C3 since the C3 convertase formed in the fluid phase is ineffective at cleaving C3. This accounts for the observed clinical profile in these patients: decreased serum C2, C4 and C1-inhibitor, increased C1q consumption and normal C3 levels (Geha *et al.*, 1985).

The clinical profiles observed in the hereditary deficiency of C1-inhibitor, has led to the identification of two types

of C1-inhibitor deficiency. In approximately 85% of families, the C1-inhibitor protein in serum is present in very low antigenic levels and functional activity, however the specific activity of the protein is similar to that observed in normals. This is designated as Type I HAE and is characterised by a low C2, C4, CH_{50} and C1-inhibitor level (Al-Abdullah and Greally, 1986). The remaining 15% of cases present with antigenically normal or elevated amounts of plasma C1-inhibitor, but a complete absence or very low level of functional activity. This is designated as Type II HAE and is characterised by low C2, C4 and CH_{50}, but normal or increased antigenic levels of C1-inhibitor.

Currently there are three approaches to the treatment and management of HAE patients (Ballogh and Whaley, 1980; Al-Abdullah and Greally, 1986). Firstly, the use of anti-fibrinolytic drugs, such as epsilon-aminocaproic acid (EACA) and tranexamic acid (cyclokapron), which are thought to act by conserving the C1-inhibitor, due to blocking the enzymes which are normally inhibited by C1-inhibitor. The second method of treatment is the administration of fresh frozen plasma, which provides a rapid and effective treatment in acute attacks. However, it has been argued that this also provides a further source of C1, C4 and C2 which may be consumed to release more "C2 kinin", a small kinin-like peptide, if this theory is the view accepted, although there is some debate about the theory. The purification of C1-inhibitor has led to infusions of concentrates of purified C1-inhibitor in patients who require rapid treatment, especially during

the life-threatening laryngeal/bronchial attacks. These approaches do not treat the underlying biochemical defect, thus C4, C2, C1-inhibitor and CH_{50} all remain subnormal during these types of therapy. The third method of treatment involves the administration of anabolic steroids or attenuated androgens such as methyl testosterone, danazol or stanozolol, which appear to stimulate hepatic synthesis of C1-inhibitor (Gelfand et al., 1976). It was observed that only the 17α alkylated compounds were effective, those non 17α alkylated compounds tested had no effect on the angio-oedema. After a period of treatment, with attenuated androgens, ranging from as little as 5 days up to 2 weeks, the clinical profile had dramatically changed: CH_{50}, C4, C2 and C1-inhibitor levels had increased almost to within the normal limits, and the episodes of angio-oedema had ceased to occur. In Type II HAE, during androgen therapy, there was a decrease in the non-functional C1-inhibitor, and an increase in the functionally normal C1-inhibitor.

These observations demonstrate that C4 and C2 levels are controlled by C1 which in the absence of sufficient C1-inhibitor, autoactivates and subsequently cleaves C4 and C2. Ziccardi (1982b) demonstrated that in vitro C1-inhibitor at levels ranging from 0.35 to 2 times physiological levels were effective at inhibiting the spontaneous auto-activation of physiological concentrations of C1. However, C1-inhibitor concentrations as low as 0.25 times physiological levels were ineffective at controlling spontaneous C1 auto-activation. The levels of

C1-inhibitor in the sera of untreated HAE patients is usually 0.05 to 0.30 times that of physiological concentration, and therefore would probably be somewhat ineffective at inhibiting spontaneous C1 auto-activation. During androgen therapy, serum C1-inhibitor levels may only require to be raised to half that of normal in order to become effective at blocking complement turnover (Ziccardi, 1982b).

The underlying biochemical agent causing the angiooedema is unknown, but the pathogenesis of HAE appears to be related to the inability of C1-inhibitor to inhibit activated C1, and subsequently cleavage of C4 and C2, its substrates. In the plasma, C1 can be activated by three distinct means: firstly by autoactivation, secondly by immune and non-immune stimuli, and thirdly by the action of plasmin, which can cleave C1 to $C\bar{1}$. Normally in plasma, plasmin activation is regulated by its inhibitors α_2 antiplasmin and α_2 macroglobulin; thus plasmin cleavage of C1 should not be a major source of $C\bar{1}$. As discussed earlier under regulation (Section 1.5.1), C1-inhibitor present in normal physiological levels can regulate C1 auto-activation and activation of C1 by non-immune stimuli. However in the serum of HAE patients, C1-inhibitor levels are very low and cannot effectively block cleavage and hence activation of C1. This activation in the serum of HAE patients results in the uninhibited cleavage of C4 and C2, releasing C4a and C2b, and formation of the classical pathway C3 convertase, in the fluid phase. Since fluid-phase C3 convertases are ineffective at cleaving C3, the complement cascade does not proceed any further.

A theory has been postulated that as a result of C2 activation, a small polypeptide fragment with kinin-like (C2 kinin) activity is released, probably from C2a (Donaldson, Rosen and Bing, 1977). This C2 kinin, which is claimed to be distinct from bradykinin, has been shown to increase vascular permeability, which could be responsible for the angio-oedema attacks observed in patients with low C1-inhibitor levels. However, there are also criticisms against this theory: these include the puzzling observation that C2-kinin can be detected in the serum of patients even during remission phases (Donaldson, Rosen and Bing, 1977). Other workers in this field (Fields, Ghebrehiwet and Kaplan, 1983; Smith and Kerr, 1983, 1985), have failed to demonstrate the generation of C2 kinin from C2 during cleavage by $C\bar{1}$ or plasmin. Highly purified C2, at up to twenty fold greater than plasma levels, incubated with plasma deficient in C1-inhibitor or with kallikrein, plasmin, or $C\bar{1}s$ failed to generate kinin activity (Smith and Kerr, 1985). It has been demonstrated that the kinin-like activity generated from C1-inhibitor depleted plasma, may derive from kallikrein and is most probably bradykinin. The supportive evidence for this is that soya bean trypsin inhibitor, a known potent inhibitor of the kallikrein/kininogen system, has been shown to abolish C2 kinin generation in HAE plasma, and has been shown to have no effect on the rate of cleavage of C2 or the products formed (Smith and Kerr, 1985). Experiments involving plasma depleted of kallikrein, or kininogen deficient plasmas

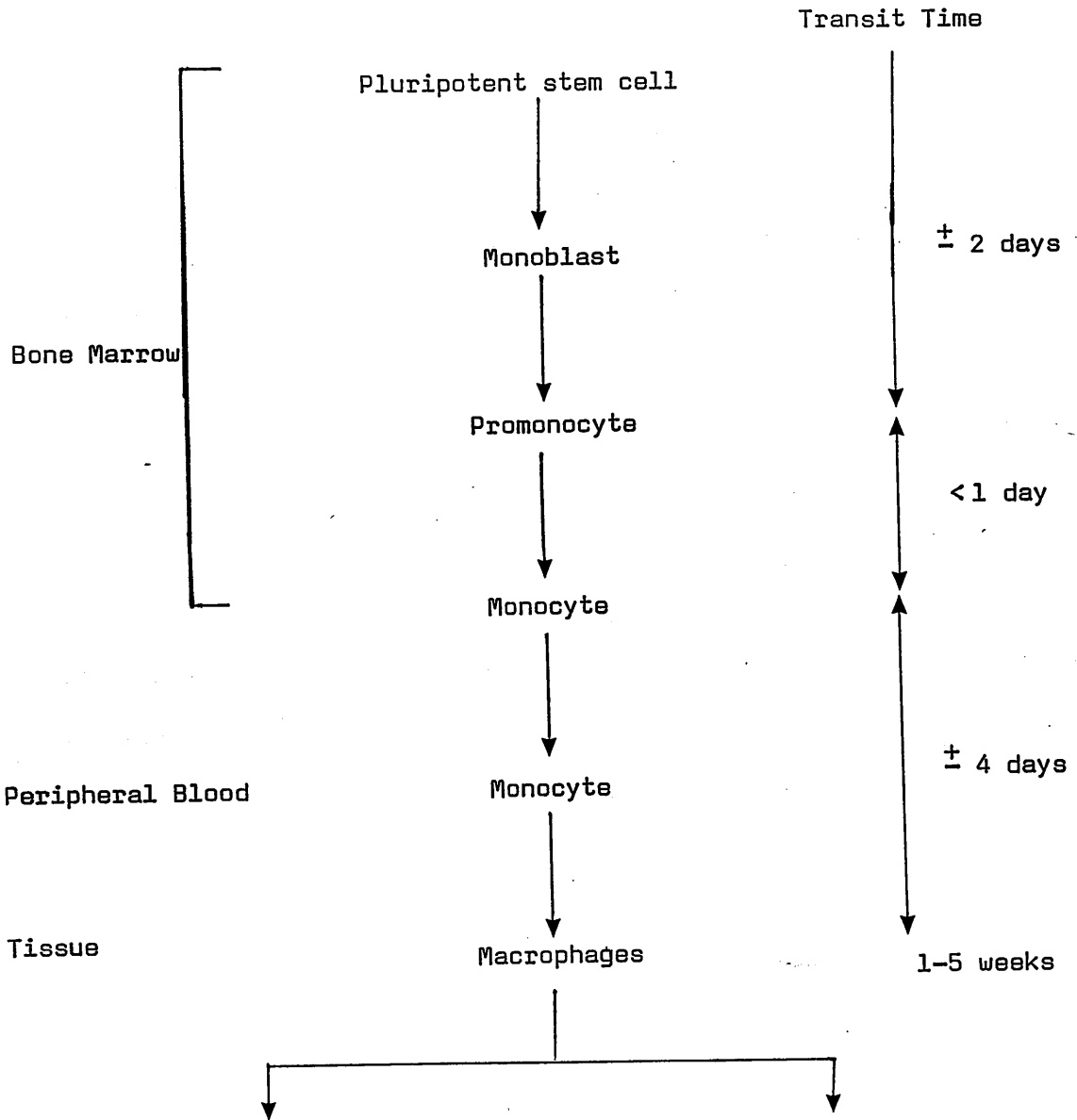
depleted of C1-inhibitor, demonstrated that no kinin activity was generated. This therefore supports the theory that kinin generation involves the kallikrein/kininogen system, and this may be what is responsible for the observed angio-oedema in HAE patients.

The precise nature of the underlying biochemical basis of HAE remains so far unresolved, and warrants further attention.

1.8: Synthesis of Complement

As a result of *in vivo* and *in vitro* studies, the synthesis of many of the complement components has now been localised to specific cell types within certain tissues. The liver has been demonstrated to be the major source of synthesis of C3, C4, C5, C6, C8, C9, factor B and C1-inhibitor, the hepatocyte being the main cell type involved. This was first demonstrated following orthotopic liver transplantation in humans, which resulted in the conversion of the allotypes of C3 (Alper *et al.*, 1969), C6 (Hobart, Lachmann and Calne, 1977), C8 and B from that of the recipient to those of the donor.

C1 and its subcomponents have been demonstrated to be synthesised in the intestine and genito-urinary tract, the major cells involved being the epithelial cell (columnar/transitional) and the macrophage (Colten *et al.*, 1966). Extrahepatic synthesis of C4 (Colten, 1976,; Whaley, 1980), C2 (Einstein, Schneeberger and Colten, 1976), C3 (Lai A Fat and van Furth, 1975) C5 (Colten, 1973), B, D, P (Bentley, Fries and Brade, 1978), H and I (Whaley, 1980; De Ceulaer, Papazoglou and Whaley, 1980) has been demonstrated; the



Connective Tissue: Histiocyte
Liver: Kupffer cell
Lung: alveolar macrophage
Lymph Nodes: free and fixed macrophages
Bone Marrow: fixed macrophages
Serous Cavities: pleural and peritoneal macrophages
Bone: Osteoclasts
Central Nervous System: CSF macrophages and brain macrophages
Skin: Histiocyte
Synovia: Type A cell
Other organs: Tissue macrophages

Exudate macrophage
Exudate-resident macrophage
Epithelial cell
Multinucleated giant cell.

Fig. 6: Development of Cells of the Mononuclear Phagocyte Lineage.

cells of the mononuclear phagocyte lineage are probably responsible due to their wide tissue distribution. This is the area which is of greatest relevance to this thesis, and will therefore be discussed in greater detail (Section 1.9.3).

1.9: Mononuclear Phagocytes

1.9.1: General Discussion of Mononuclear Phagocytes

Mononuclear phagocytes are a family of mobile, long-lived cells which are derived from precursors in the bone marrow, circulate in peripheral blood, and take up specific locations in tissues. These cells are specialised in the uptake and degradation of particulate and soluble macromolecules, and in the release of many regulatory products. They play central roles in wound healing, host defences against microbes and tumours, and in the inflammatory process, especially in chronic inflammatory lesions, where they mediate host tissue injury.

The mononuclear phagocyte cell line originates from the division of a bone marrow precursor cell, called the pluripotent stem cell (Fig. 6). The resultant initiating cell of this line, and the most immature is the monoblast, which in turn divides giving rise to two daughter promonocytes (Fig. 6). At this stage of development the first "macrophage-like" characteristics are observed, for example the presence of peroxidase positive granules, some adhesive properties and the presence of Fc receptors (Tomida, 1985). Further division and differentiation of each promonocyte gives rise to two monocytes which remain in the bone marrow for a brief period, of up to one day. Monocytes express more characteristics of

the mononuclear phagocyte cell line, in that they have developed the expression of more Fc and C3 receptors; they have a greater phagocytic capacity, and they possess many more lysosomes (Tomida, 1985). Monocytes then leave the bone marrow and are transported via the blood to the tissues, where they perform their main functional role as tissue macrophages (Fig. 6).

Upon migration of blood monocytes into tissues, there is a further sequential development to resident macrophages, which display similar characteristics to monocytes. The development of monocytes to resident macrophages is characterised by staining and examining the patterns of peroxidase activity.

Thus, the life span of the mononuclear phagocyte exhibits three distinct phases: firstly, the bone marrow phase where the stem cell proliferates to form monocytes; secondly, the transportation phase, where the monocytes are transported to the body tissues and cavities via the circulation; and thirdly, the development into the functional cell, the tissue macrophage. The control of proliferation of stem cells *in vivo* to form the mononuclear phagocyte clone, is somewhat unclear. *In vitro*, colony stimulating factors are important in the proliferation of bone marrow precursor cells to macrophages, and in the maintenance of differentiated phenotypes of macrophages (Tomida, 1985).

Macrophages are known to be present in all tissues, although they do not all possess the same functional, morphological and antigenic characteristics. This would be

expected due to the differing micro-environments within the different tissues. Depending on the tissue in which the macrophage is located, and upon its activation state, it is named differently (Fig. 6) (van Furth, 1980).

The fate of macrophages after they serve their function in the tissues is unknown. It is known that there is little recirculation of macrophages via the peripheral blood, and it is postulated that they may migrate to local lymph nodes, where they die and are cleared from the body (van Furth, 1980). In the tissues they are replaced by monocytes infiltrating from the blood, since macrophages do not possess the ability to proliferate, as demonstrated by low ^{3}H-thymidine incorporation into tissue macrophages (van Furth, 1980).

In the normal state the numbers of tissue macrophages are maintained at a steady level; however during acute or chronic inflammation, there is a vast increase in the numbers of mononuclear phagocytes present in tissues and exudates. They are recruited from the peripheral blood as monocytes, and have various paths of development open to them, leading to the formation of the four types of mononuclear phagocytes present in inflammatory exudates. Firstly, they can become exudate or exudate-resident macrophages; secondly they can transform into epitheliod cells of two types, activated or secretory; finally they can fuse with other mononuclear phagocytes to become multinucleated giant cells (van Furth, 1980).

When the inflammation subsides, the stimulus for recruitment of blood monocytes ceases, and there is a decrease in the number of macrophages in the exudate, either due to cell death, or by migration of macrophages away from the site of inflammation.

1.9.2: Characterization of Mononuclear Phagocytes

The initial identification of cell types primarily involves the morphological characteristics; however, this alone is often insufficient to classify mononuclear phagocytes at different stages of development. Since mononuclear phagocytes are not known to possess any property unique to themselves, it has become necessary to develop a battery of characteristics which can be used in the identification of the cells of this lineage: these are summarised in Table 4.

For a cell to be classified a mononuclear phagocyte, it must possess a number of characteristics including morphological appearances, the presence of non-specific esterase, peroxidase positive granules, lysozymes, Fc and C3 receptors. In addition they must be able to phagocytose IgG coated red cells or opsonized bacteria, and exhibit pinocytosis (van Furth, 1981). Generally, one would not find all of the cells in a homogenous population positive for all of the above criteria, thus a generally acceptable level of 90% positive cells would be sufficient, so long as they display a minimum of three different characteristics.

1.9.2a: Membrane Receptors of Mononuclear Phagocytes

Mononuclear phagocytes are known to possess receptors on their surface membranes for such components as the Fc portion of IgG (Bodmer, 1985; Unkeless, 1980, 1986), IgA, IgE and IgM (Bodmer, 1985). (Table 5); for complement components and their cleavage products; for fibronectin (Bodmer, 1985); for mannose-N-acetyl glucosamine (Bodmer, 1985); for low-density lipoproteins (Kaplan and Buys, 1985) and for hormones including polypeptide hormones, growth factors, steroid hormones and neurohormones (Oropeza and Werb, 1985).

The binding of immunoglobulin, in the form of immune complexes, to Fc receptors for IgG on the cell surface of mononuclear phagocytes, results in the triggering of phagocytosis and the release of mediators of inflammation from the cell. Thus, Fc receptors are important in the clearance of immune complexes from the circulation by mononuclear phagocytes.

Mononuclear phagocytes from a variety of sources, including rabbit, mouse and human all express surface receptors for the complement components C1q, C5a, C4b and C3 breakdown products (Table 5). The types of complement receptor and their numbers present on the surface can vary, and hence can be used as an indicator of the state of activation of a cell. Another factor which influences the levels of complement receptors expressed includes *in vitro* culture conditions (Bodmer, 1985).

1.9.2b: Surface Antigens of Mononuclear Phagocytes

Cells of the mononuclear phagocyte system are known to express several identifiable surface antigens, which can be used to distinguish them from other cells of similar haemopoetic origin. With the advent and perfection of raising monoclonal antibodies (Mabs), there has become available a wide range of Mabs which react with surface antigens. For a full comprehensive list of Mabs against mononuclear phagocyte antigens, see Springer and Unkeless (1984), Hogg et al. (1986) and Springer and Anderson (1986).

A family of three inter-related human monocyte/macrophage surface antigens has been characterised, using monoclonal antibodies, and includes Mac-1, LFA-1 and p150, 95 (Springer and Anderson, 1986). The Mac-1 antigen is composed of two subunits of 170 kD (α) and 95 kD (β), which are associated non-covalently in a tight α,β complex (Springer and Unkeless, 1984). Mac-1 is a universal macrophage marker, and blocking studies strongly suggest identity between Mac-1 and complement receptor type 3 (Springer and Unkeless, 1984).

The second surface antigen, LFA-1, appears to have the same β subunit structure as Mac-1, but different cell distribution, function, and α subunit structure. LFA-1 has a 180 kD α subunit and an identical 95 kD β subunit to Mac-1, and has been shown to be involved in mononuclear phagocyte adhesion reactions (Springer and Anderson, 1986).

The third associated antigen, p150, 95, is composed of a 150 kD α chain and a β chain of 95 kD, identical to that recognised by Mac-1 and LFA-1 (Springer and Anderson, 1986). The distribution of these three surface antigens varies depending on the developmental stage of the mononuclear phagocyte and its state of activation: for example Mac-1 and p150, 95 can increase their expression when stimulated by f-Met-Leu-Phe and C5a anaphylatoxin.

Mac-1 antigen's cellular distribution encompasses monocytes, macrophages, granulocytes, and large granular lymphocytes; the LFA-1 antigen's cellular distribution encompasses lymphocytes, monocytes, granulocytes and large granular lymphocytes; the p150, 95 is expressed on monocytes, macrophages and granulocytes. Therefore, it can be seen that although these antigens are expressed by cells of the mononuclear phagocyte lineage, they are also expressed by granulocytes and lymphocytes (Springer and Anderson, 1986).

Recently, a monoclonal antibody, 27E10, has been raised which reacts with a subset of human monocyte/macrophages isolated from peripheral blood and present in inflammatory tissue, but absent in normal tissue (Zwaldo, Schlegel and Sorg, 1986). The Mab recognises a 17 kD surface antigen which is present on approximately 20% of freshly isolated peripheral blood monocytes; expression increased reaching a maximum at day 2 or 3, then decreased with further culture. Expression of the antigen was enhanced with LPS, TPA and γ interferon, which suggests that the 27E10 antigen is expressed by an activated-type of monocyte/macrophage (Zwaldo, Schlegel and Sorg, 1986).

Hydrolytic Enzymes	Lysosomal hydrolases e.g. cathepsin B, hyaluronidase, acid phosphatase, β glucuronidase. Neutral proteases e.g. collagenase, elastase, plasminogen activator. Lysozyme Other hydrolases e.g. angiotensin-converting enzyme, alkaline alpha-glucosidase, arginase, lipoprotein lipase.
Cell Stimulatory Agents	Colony stimulating factor Interferon Interleukin I
Arachidonic Acid Metabolites	Prostaglandins E_2, I_2, $F_{2\alpha}$ Thromboxane A_2 Leukotriene C_4 and B_4
Reactive Oxygen Intermediates	Superoxide anion Hydrogen peroxide Hydroxyl radical
Coagulation Components	Thromboplastin, Factors V11, 1X, X/Xa, V, Prothrombinase, Prothrombin.
Complement	C1, 2, 3, 4, 5, B, D, P, H, I, C1-inhibitor.

Table 6: Biologically Active Secretory Products of Mononuclear Phagocytes

The antigen is not expressed by platelets, lymphocytes and many cell lines, but is expressed by 15% of granulocytes. The antigen is not expressed by monocytes and resident macrophages on fresh tissue sections of skin, lung and colon; however in inflammatory tissue, monocytes and macrophages express the antigen. Thus, it is thought that the Mab 27E10 detects a differentiation antigen present on the surface of a subset of monocytes/macrophages, which appear in the primary stages of an inflammatory reaction (Zwaldo, Schlegel and Sorg, 1986).

1.9.3: Secretory Products of Mononuclear Phagocytes

One of the major functions of mononuclear phagocytes in addition to the clearance of foreign materials and debris from the body, is synthesis and secretion. The macrophage is primarily a secretory cell, and the array of biologically active products which it secretes (Table 6) exhibits the multipotency of this cell. The secreted products fall into one of two categories: firstly, constitutive products which are secreted continuously at the same rate irrespective of the state of activation of the cell, for example, lysozyme. Secondly, inducible products, such as complement components, which are not secreted or are only secreted in small amounts by resting cells, but following activation secretion increases dramatically. The discussion of the secretory products of mononuclear phagocytes will be restricted to lysozyme and complement components, which are the most pertinent to the work carried out in this thesis.

As described previously, outwith the liver the cells which have been established as the principal source of complement components are those of the mononuclear phagocyte system. Their contribution to the levels of serum complement is negligible; however it is at the local tissue level that their biosynthetic capacity is of considerable importance. Under normal physiological conditions, the extravascular spaces will experience a relatively low level of complement components, thus resident tissue macrophages will play an important role in the maintenance of local complement levels. Mononuclear phagocytes are often situated at areas where micro-organisms enter, thus they can synthesise the components required in the opsonization and phagocytosis of the invading micro-organisms (McPhaden et al., 1985). At sites of inflammation there will be increased complement activation and the initial levels will soon be depleted, as demonstrated in the inflammatory exudates from the joints of rheumatoid arthritis patients (Ruddy, Fearon and Austen, 1975).

It has been demonstrated that macrophages isolated from the synovium of rheumatoid arthritis patients, synthesise C2, C3, C4 and C5, whereas the macrophages in the synovium of patients with degenerative or traumatic arthritis, synthesise very little complement (Ruddy and Colten, 1974). Monocytes, synovial fluid macrophages and synovial membrane macrophages were cultured from patients with rheumatoid arthritis. Functionally active C2, B, D, P, H and I were synthesised by all three types of cells studied, whereas C4, C3 and C5 could

only be detected by antibody-specific precipitation of components which had incorporated ^{14}C-amino acids, since these components were not detected in functional haemolytic assays. Synovial fluid macrophages synthesised the greatest quantities of all the components measured; the three types of cells isolated from rheumatoid arthritis patients synthesised greater quantities of components than the cells isolated from patients with degenerative joint disease (De Ceular, Papazoglou and Whaley, 1980).

Thus, the presence of macrophages in the synovial tissue and fluid, in joints of patients with rheumatoid arthritis, supply a constant source of selected complement components allowing continued activation of the complement pathways. This generates the cleavage products C3a, C5a and Bb which are thought to play a major role in the inflammatory process, degeneration and destruction observed in the joints of patients suffering from rheumatoid arthritis (Ruddy and Colten, 1974; De Ceulaer, Papazoglou and Whaley, 1980).

The presence of complement components in monocyte culture supernatant can be detected by various types of functional haemolytic assay; immunochemical assays such as radioimmunoassay and ELISA; or the incorporation of ^{35}S-methionine into immunoprecipitable protein, which can subsequently be analysed by SDS-PAGE and fluorography.

The development of these techniques has enabled investigators to demonstrate that cells of the mononuclear phagocyte lineage from mouse, guinea pig, and human, between them synthesise the following array of complement components:

Table 7: Synthesis of Complement Components by Mononuclear Phagocytes

Complement component	Mouse peritoneal macrophages	Guinea-pig macrophage				Human monocytes	Human macrophages				
		Peritoneal	Broncho-alveolar	Splenic	Bone Marrow		Peritoneal	Broncho-alveolar	Breast milk	Synovial fluid	Colostral
C1(C1q,C1r, C1s)	C1q only	+				±	+				
4	+	+	+	+	+	−					
2		+	+	+		+		+	+	+	+
3	+	+				+	+	+	+	+	+
5	+									+	
6											
7											
8											
9											
B	+	+				+		+	+	+	+
D		+				+				+	
P		+				+				+	
C1-inhibitor						+				+	
I						+				+	
H						+				+	

C1, C2, C3, C4, C5, B, D, P, H, I and C1-inhibitor, as demonstrated in Table 7. There is accumulating evidence that they do not synthesise C7 and C9; however, it is not certain whether they produce C8 and C6 (McPhaden et al., 1985).

As shown in Table 7, there are important differences in the components synthesised by various species: for example C4 and C5 are synthesised by guinea pig and mouse macrophages, but are not easily detected in the supernatants of human monocytes and macrophages, with the possible exception of synovial fluid macrophages.

In addition to the species difference, the profile of complement components synthesised varies as mononuclear phagocytes differentiate from monocyte to macrophage. At any one time in culture, not all mononuclear phagocytes in a given population will be actively synthesising the complement components being assayed for. It is not known if this represents the existence of subpopulations of cells, or whether all cells have the ability to synthesise complement, but do so at different times. Also, whether the basis for the differing synthetic capacities resides in the precursor population, or in the individual tissue environment is somewhat of a mystery (Alpert et al., 1983).

Cole et al. (1985) studied the production of C2, factor B, C3 and lysozyme by freshly isolated monocytes, monocytes maintained in culture for 14 days, breast milk macrophages and bronchoalveolar macrophages. They measured the proportion of C2-producing cells, the average single-cell production rate of C2, the post-translational glycosylation and kinetics of secretion of C2 and B, and the amount of C2 and B mRNA in each group of cells. Their results demonstrated that there

were comparable rates of synthesis of C3 and lysozyme, and similar post-translational glycosylation and kinetics of secretion of C2 and B. However, the freshly isolated tissue macrophages differed from *in vitro* monocyte-derived macrophages in the proportion of C2-producing cells, in the average single-cell production rates of complement synthesised and in the amounts of specific C2 and B mRNA. The results have demonstrated that the differences are tissue specific because the C2-specific mRNA of broncho-alveolar macrophages is considerably greater than that of breast milk macrophages, although the amounts of B mRNA are comparable. The authors suggest that there is tissue-specific regulation of complement production in human monocytes, which occurs at a pre-translational level, that is at mRNA transcription, processing or degradation (Cole *et al.*, 1985).

The levels of lysozyme, C2, C3, B and C1-inhibitor synthesised by peripheral blood monocytes, and macrophages isolated from the synovial fluid of patients with rheumatoid arthritis, has been compared as representing two different stages in mononuclear phagocyte development (Lappin *et al.*, 1986). The study has demonstrated that synthesis of lysozyme from monocytes and macrophages is very similar, 4700 and 4300 x 10^3 molecules/cell/hr respectively. However synthesis of the complement components C2, C3, B and C1-inhibitor differed by factors of 2.7, 3.7, 5.0 and 6.2 fold greater respectively in macrophages compared to monocytes. Another point which was highlighted by this study was that the lag observed over the first three days in culture in the synthesis of C2, C1-inhibitor and B by monocytes, was not manifested in the synthesis of these components by macrophages (Lappin *et al.*, 1986).

The synthesis patterns of individual complement components by human monocytes in culture will now be discussed.

1.9.3a: C1 subcomponents and C1-inhibitor

Monocytes have been shown to synthesise C1q and C1s after 5 days in culture: C1q was produced at a constant linear rate over two weeks, whilst C1s production plateaued almost immediately. Upon stimulation of monocytes with conditioned supernatants from mitogen-, antigen- or allogenic-stimulated lymphocyte cultures, C1q synthesis was increased between 2 and 7 fold; C1s production was reactivated; and C1r and C1-inhibitor became detectable in the culture supernatants (Bensa, Reboul and Colomb, 1983).

1.9.3b: C4

There is some dispute as to whether monocytes synthesise C4 in culture: incorporation of ^{14}C-amino acids into protein which was tested for C4 by double immunodiffusion and the plates exposed to X-ray film, as well as the immunofluorescent localisation of C4, suggests that human monocytes in culture synthesise C4 (Whaley, 1980). However, the unsuccessful attempts to demonstrate haemolytic activity (Colten, 1976), C4 mRNA (Cole et al., 1985) immunogenic protein by RIA and ELISA, and the inability to precipitate C4 from ^{35}S-methionine pulse-chased cultures, tends to suggest that human monocytes do not synthesise C4 which is homologous with its serum component.

1.9.3c: C2

The synthesis of C2 by human monocytes in culture has been demonstrated by several groups and is one of the most widely studied secreted complement components (Einstein, Schneeberger and Colten, 1976; Whaley, 1980). The synthesis of C2 over short periods of culture, up to 12 days, demonstrated that there was a slight lag in production over the first 2 or 3 days. The production was then linear up to 5 or 6 days, after which a plateau was observed (Whaley, 1980). Einstein, Schneeberger and Colten (1976) found the kinetics to differ in long term primary monocyte cultures: after a 6 day lag, C2 was synthesised at a linear rate in culture, and did not plateau. The average rates of synthesis and secretion of C2 per cell, were 2 to 3 times the initial rate in culture which had been maintained for 2 to 4 and 6 to 8 weeks. When Whaley (1980) investigated cumulative C2 synthesis it was shown to be linear over 10 days, whilst continuous synthesis demonstrated linear production only over the first 5 to 6 days (Whaley, 1980).

C2 precipitated from monocyte lysates during pulse-chase experiments has demonstrated an intracellular molecular weight of around 90 kD, with the extracellular secreted form being of a slightly higher molecular weight. The addition of 0.3 µg tunicamycin/ml before pulse-chasing resulted in the intracellular C2 having a molecular weight reduced by about 12 kD (Cole et al., 1985).

1.9.3d: C3

Synthesis of C3 has been demonstrated in monocyte culture supernatants using functional assay RIA, ELISA and specific precipitation of ^{35}S-methionine labelled C3.

Intracellularly C3 is precipitated as a 185 kD precursor called pro-C3; extracellularly it is secreted as a 116 kD α chain and 75 kD β chain. The addition of tunicamycin did not appear to alter the molecular weight of pro-C3 (Cole et al., 1985).

Initial attempts to demonstrate functionally active C3 in monocyte culture supernatants failed (Whaley, 1980); however, recently a modified method for culturing monocytes, under serum free conditions and processing of the supernatant, has led to the demonstration of its presence (Strunk, Kunke and Giclas, 1983), with the same range of specific activity as serum C3.

The use of an ELISA has enabled the kinetics of secretion of C3 by human monocytes to be investigated (Lappin et al., 1986): unlike C2, C3 is secreted from the onset of culture, and appeared to plateau after about 5 days synthesis.

1.9.3e: B

Precipitation and analysis of ^{35}S-methionine labelled B has demonstrated a 100 kD protein of similar molecular weight intracellularly and extracellularly; tunicamycin appeared to reduce the molecular weight by about 8 kD (Cole et al., 1985).

Factor B activity in monocyte culture supernatants has been estimated using a haemolytic assay and ELISA (Whaley, 1980; Lappin et al., 1986). This analysis has demonstrated that B synthesis has a similar pattern of continuous synthesis as C2: delayed onset, linear production between days 3 and 5 or 6, followed by a plateauing (Whaley, 1980; Lappin et al., 1986). Investigation of cumulative B synthesis demonstrated similar results to C2: synthetic rates remained constant and no plateau was observed up to day 10 (Whaley, 1980).

1.9.3f: D

Using a haemolytic assay, D synthesis by monocytes in culture does not have a lag phase: it is secreted at a constant rate up to day 5, after which time synthesis plateaus. Cumulative synthesis was linear over 10 days (Whaley, 1980).

1.9.3g: Properdin and H

Synthesis of properdin was demonstrated by a haemolytic assay and the synthesis of H by a RIA (Whaley, 1980). The kinetics of secretion under continuous and cumulative culture were of a similar pattern to that observed for C2 and B (Whaley, 1980).

1.9.3h: I

Synthesis of I by monocytes in culture has been demonstrated by a haemolytic assay; the patterns of continuous and cumulative synthesis were similar to these observed with D. (Whaley, 1980).

Thus the synthesis of complement by monocytes in culture has been demonstrated, and represents one of the vast array of inducible products of mononuclear phagocytes.

1.9.3j: Lysozyme

Lysozyme is an example of a constitutive secretory product of mononuclear phagocytes. Secretion of lysozyme by mononuclear phagocytes *in vitro* has been shown to occur continuously, and at a remarkably constant rate, regardless of the origin of the macrophage population. Unstimulated mouse macrophages synthesise 0.6 to 0.8 µg lysozyme/day/10^6 cells after the first day in culture, whilst human monocytes synthesise 0.3 µg lysozyme/day/10^6 cells from day 0 in culture, these values quoted being the secreted lysozyme and represent approximately ninety percent of the total lysozyme synthesised (Gordon, Todd and Cohn, 1974). Thus, in culture mononuclear phagocytes synthesise and secrete lysozyme at a relatively constant rate over a period of up to seventeen days. The relatively constant secretory rate observed in human monocytes from the beginning of culture is in contrast to the dramatic morphological changes which occur, as the monocytes mature to macrophages after the first few days in culture.

Under a wide variety of culture conditions such as different seeding densities, presence or absence of serum, during active phagocytosis, stimulation by lymphokines and other biological agents, lysozyme synthesis appears to remain very stable. One way in which lysozyme synthesis could be

modified was by completely or partially blocking protein synthesis using cyclohexamide or colchicine. Thus, it was concluded that lysozyme was a constitutive, rather than an inducible, secretory product and a specific cell marker of mononuclear phagocytes (Gordon, Todd and Cohn, 1974).

It was noted that polymorphonuclear leukocytes contained approximately 0.6 μg lysozyme/10^6 cells; however, under similar culture conditions these cells released their lysozyme into the supernatant, and died several hours later. Therefore, it was concluded that PMNs only possessed preformed enzyme, and unlike the mononuclear phagocyte, did not have the capacity to synthesise lysozyme (Gordon, Todd and Cohn, 1974).

Until recently it has been accepted that lysozyme was a constitutive secretory product of mononuclear phagocytes in culture, its synthetic rate being unaffected by most stimuli with the exception of protein synthesis inhibitors. However work on the effects of lipopolysaccharide (LPS) and mouse fibroblast-derived interferon (containing 80% β interferon and 20% α-interferon) on lysozyme synthesis and secretion by mouse resident peritoneal macrophages, thioglycolate elicited and protease-peptone elicited macrophages has suggested that production of lysozyme can be down-regulated (Warfel and Zucker-Franklin, 1986).

There are a few reports documenting significant stimulatory effects on lysozyme synthesis and secretion; however, Prydz and Lyberg have reported an increased release of lysozyme when human monocytes were challenged with immune complexes (>800%

of the unstimulated control) (Prydz and Lyberg, 1980) TPA (>600%) PDD (>600%), PDA (about 500%) and anthralin (>250%) (Lyberg and Prydz, 1981). The only other report to be encountered which documented a reduction in lysozyme secretion, was upon the addition of Fc fragments to human monocytes (Passwell et al., 1980).

Thus, the traditional theory of lysozyme being a constitutive secretory product of mononuclear phagocytes appears to require modification to take these findings into account.

1.9.4: Regulation of Monocyte Complement Biosynthesis

The synthesis of complement by monocytes can be influenced by many environmental factors, drugs and agents which can cause stimulation or inhibition of complement synthesis: also, a specific agent does not always have the same effect on all of the components synthesised. The synthesis of the most widely studied component C2, is stimulated and inhibited by many agents, thus its control of production is possibly the best understood.

The inhibition of C2 production is thought to be mediated by increasing the intracellular levels of cAMP, which can occur as a result of adenyl cyclase activation, or the inhibition of phosphodiesterase activity. Examples of these are histamine, (Lappin and Whaley, 1980), PGE_2, PGD_2, 6-keto $PGF_{2\alpha}$, TXB_2 (Lappin and Whaley, 1982b), adenosine (Lappin and Whaley, 1984), and serum treated immune complexes (Hamilton et al., 1984)

all of which are known to directly or indirectly stimulate adenyl cyclase activity; whereas IBMX and theophylline (Lappin et al., 1984) are examples of phosphodiesterase inhibitors. Addition of all of these agents to monocytes in culture results in an increase in cAMP levels and a decrease in C2 synthesis.

Enhancement of C2 synthesis is not such a simple picture as was originally proposed, and is now thought to involve calcium and sodium ions and channel formation, in addition to the decrease in cAMP levels. Agents which directly or indirectly stimulate C2 production can be classified into three groups: firstly, those which stimulate phosphodiesterase activity for example imidazole (Lappin and Whaley, 1983); secondly, those which inhibit adenyl cyclase activity for example acetylcholine, carbamylcholine (Whaley, Lappin and Barkas, 1981), adrenaline, noradrenaline and phenylephrine (Lappin and Whaley, 1982a). The last three are also thought to cause calcium channel formation, which is the proposed mechanism for stimulating C2 synthesis when immune complexes and poly IgG1 and IgG3 are added to monocytes (McPhaden, Lappin and Whaley, 1981). Thirdly, prostaglandin synthetase inhibitors act to reduce prostaglandin synthesis, which are known to be activators of adenyl cyclase. Examples of these synthetase inhibitors are ETYA, indomethacin and benoxaprophen (Lappin et al., 1984).

The role of calcium and sodium ions and channels was elucidated using channel blockers and calmodulin antagonists (Lappin and Whaley, 1985). Calcium and sodium channel

blockers could abrogate the stimulatory effects on C2 synthesis produced by phenylephrine, carbamylcholine and immune complexes, as did the calmodulin agonists upon simultaneous addition to culture (Lappin and Whaley, 1985).

1.10: Aims of Project

Monocytes in culture, have been shown to synthesise some of the components of the classical, alternative and terminal pathways of complement, in addition to some of the regulatory proteins. Synthesis of C1-inhibitor by monocytes in culture at the commence of this project, in 1982, had not been investigated in any great detail. Thus, it was proposed to use the *in vitro* culture of monocytes as a model system to study C1-inhibitor synthesis by normal controls, and patients with the clinical syndrome of hereditary angio-oedema, which is characterised by a deficiency of plasma C1-inhibitor functional activity.

It was also proposed to use this method to study the effect of anabolic steroids and other steroids on monocyte complement synthesis, in particular, to attempt to elucidate the mechanism by which anabolic steroids correct C1-inhibitor deficiency. It was also hoped that the investigations would help elucidate the underlying genetic defect in HAE.

2: MATERIALS AND METHODS.

2.1: General Materials and Methods

All general reagents used were of analytical grade or the highest quality available as supplied by British Drug Houses (BDH) Limited, other than those stated in each method.

2.1.1: Preparation of General Reagents

Phosphate Buffered Saline (PBS) pH 7.2 to 7.4.

Isotonic PBS (0.15M) was prepared by dissolving 8g NaCl, 0.34g K H_2PO_4 and 1.21g K_2HPO_4 in deionised water, to a final volume of 1 litre.

0.12M EDTA

0.12M disodium ethylenediaminotetra acetic acid (EDTA) was prepared by dissolving 44.79g EDTA in deionised water, to a final volume of 1 litre.

0.086M EDTA pH 7.2 to 7.6

Isotonic EDTA (0.086M) was prepared by mixing 500 ml 0.12M EDTA and 197.7 ml 0.3M sodium hydroxide (12g NaOH/litre).

Isotonic Saline (0.9% NaCl)

Isotonic saline, 0.15M or 0.9%, was prepared by dissolving 9g sodium chloride (NaCl) in deionised water to a final volume of 1 litre.

5 x Veronal-buffered Saline (5 x VBS) pH 7.4 to 7.6.

5 times isotonic strength veronal buffered saline was prepared by dissolving 85g NaCl, 3.75g sodium barbitol ($NaC_8H_{11}Na_2O_3$) and 5.75g barbituric acid ($C_8H_{12}N_2O_3$) in hot deionised water to a final volume of 2 litres.

0.01M EDTA/PBS

0.01M EDTA in PBS was prepared by mixing 500 ml PBS and 65 ml 0.086M EDTA.

0.5M Sodium Hydroxide (NaOH)

0.5M Sodium hydroxide was prepared by dissolving 20.0g NaOH in deionised water to a final volume of 1 litre.

0.5M Hydrochloric Acid (HCl)

0.5M HCl was prepared by diluting 42.9 ml stock 36% HCl to a final volume of 1 litre with deionised water.

Coomassie Brilliant Blue Solution (0.1%)

1g Coomassie brilliant blue was dissolved in 1 litre of methanol/acetic acid/water in the proportions of 5/1/4 (v/v/v), and filtered before use.

Destain Solution

Prepared by mixing methanol/acetic acid/water in the proportions of 1/1/8 (v/v/v).

2.1.2: Preparation of Serum from Plasma

2.1.2a: Materials Used in the Preparation of Serum

Blood Transfusion Service St. Vincent Street, Glasgow and Law Hospital, Carluke.	:	Expired frozen plasma packs
Millipore Harrow, Middlesex	:	Sterile 0.22 µm millex GS filters

Preparation of Materials

1M Calcium Chloride ($CaCl_2$)

1M calcium chloride was prepared by dissolving 14.7g $CaCl_2.2H_2O$ in deionised water, to a final volume of 100 ml.

2.1.2b: Preparation of Serum from Plasma

Serum was required for the culture of human monocytes and as a source of C1 and C4 for use in the preparation of haemolytic intermediates.

Outdated frozen plasma packs, obtained from the Blood Transfusion Service, were thawed and coagulation was produced by adding 4 ml 1M calcium chloride to each unit. The clot was retracted onto a stirrer by incubating overnight at $4^{o}C$ and separated from the serum by filtration.

2.1.2c: Preparation of AB Serum for Monocyte Cultures

AB serum, prepared as described above, was heat inactivated at $56^{o}C$ for 2 hr; dialysed against PBS and ultracentrifuged at 80 000g for 30 min at $4^{o}C$ using an SW27 head in a Beckman Model L2 65B ultracentrifuge, to remove aggregates and lipid.

The serum was sterilized by filtration through sterile 0.22 μm Millex GS filters, pooled and stored at $-20^{o}C$ or $4^{o}C$.

2.1.3a: Preparation of Chromatography Gels

Biorex 70 and DEAE-cellulose gels were prepared for chromatography according to the manufacturers' recommendations prior to use. After equilibration in their respective starting

buffers, the gels were degassed and cooled to 4°C, after which the pH and conductivity were measured and adjusted as required.

After preparation, the gel was poured into the appropriate column, allowed to settle under gravity, and buffer pumped through to ensure there was no further packing of the gel. The buffer being pumped over the column and that being collected off the column, were monitored for pH and conductivity to ensure complete equilibration before sample application.

All columns were run in the coldroom between 0° and 4°C; all gels and buffers were degassed before use, to allow better flow rates to be attained.

2.1.3b: Measurement of pH and Conductivity

The pH of solutions used were measured on a Pye Model 292 pH meter; the conductivity was measured on a DM3 conductivity meter (Radiometer-Copenhagen).

The tubes containing fractions from chromatography columns were immersed in ice, in order that pH and conductivity measurements were performed at a constant temperature (0°C).

2.1.4: Measurement of Protein Concentration

2.1.4a: Optical Density (OD) 280 nm

In order to determine the amount of protein present in the fractions collected from the columns, the absorbance at 280 nm was estimated. Most proteins absorb light in the ultraviolet (260-280 nm) region of the spectrum, due to the

presence of the aromatic ring-containing amino acids tyrosine and tryptophan . The absorbance of proteins containing different proportions of tyrosine and tryptophan will vary considerably, however this can be corrected for by using the Beer-Lambert law and the specific extinction coefficient.

2.1.4b: Folin Assay (Lowry et al., 1951)

The presence of certain inhibitors added to column samples and buffers during purification, such as benzamidine interfered with the $OD_{280}{}^{nm}$ measurement since the compound itself contains aromatic rings. To overcome this problem and enable the measurement of low protein concentrations, the colorimetric Folin assay was used. This is based on the amount of tyrosine present in a protein: the tyrosine reacts with the yellow Folin and Ciocalteau reagent, to produce a blue coloured product, the intensity of which may be read spectrophotometrically at 600, 700 or 750 nm.

Materials Used in Folin Assay

Sigma Chemical Company, Poole, Dorset. : Folin and Ciocalteau Reagent
Bovine Serum Albumin, fraction V.

Preparation of Solutions used in Folin Assay

Folin A Solution

100 ml Folin A was prepared by dissolving 2g Na_2CO_3 and 20 mg sodium potassium tartrate in 0.1M NaOH solution.

Folin B Solution

0.15g $CuSO_4 \cdot 5H_2O$ was dissolved in deionised water to a final volume of 100 ml.

Folin C Solution

Folin C was made just before use; by mixing Folin A and Folin B in the proportions of 5 ml Folin A to 0.1 ml Folin B.

Folin and Ciocalteau Solution

For use in the assay, the stock solution was diluted in an equal volume of deionised water.

Protein Standard: Bovine Serum Albumin (BSA) 1 mg/ml.

The standard was prepared by dissolving 1 mg BSA in 1 ml deionised water.

Folin Assay Method

A standard curve was constructed using BSA at 2.5, 5, 10, 25, 50, 75, 100 and 125 µg and added to a set of tubes; samples to be measured were added to a set of tubes, the volume of sample depending on the expected protein concentration. A blank was prepared by adding a volume of buffer, equal to that of the samples, to a tube.

2 ml Folin C solution and 0.2 ml Folin and Ciocalteau reagent were added to all samples and standards, mixed and incubated at room temperature for 30 minutes. The $OD_{700}{}^{nm}$ was determined and plotted (y axis) against the BSA concentration (x axis), producing a straight line which passed through the origin. The line, which was linear up to about 100 µg BSA, was used as a standard curve to calculate the protein concentration of the samples.

2.1.5: Measurement of Concentrations of Specific Proteins using Radial Immunodiffusion Plates (RID) (Mancini et al, 1965)

Materials used for R.D Plates

Gibco, Paisley, Renfrewshire. : 53 mm Nunclon Plastic Petri Dishes

Behring Diagnostics Hounslow. : Antiserum to Human C1-inhibitor and Factor B.

Preparation of Solutions for RID Plates

1.5% Agarose Solution

100 ml 1.5% agarose solution was prepared by heating the following in a glass bottle in a pressure cooker for 5 min; 20 ml 5 x VBS, 11.6 ml 0.086M EDTA, and 1.5g agarose, made up to 100 ml with deionised water.

Preparation of RID Plates

1.5% agarose was melted and maintained at 56°C, 3.5 ml agarose per plate to be poured. Antiserum was heated to 56°C for a few minutes and the appropriate dilution prepared in the agarose; the antibody containing agarose was poured with a swirling motion into plates; allowed to cool on a levelling table and stood inverted in a humidified atmosphere at 2°C. Nineteen evenly spaced 2 mm diameter holes were punched in the agrose using a 2mm punch.

The wells were filled with column fractions or a series of samples containing known concentrations of the protein in question. A 48 hr incubation at room temperature in a moist box, allowed diffusion of the antigen and the formation of precipitin rings of antigen-antibody complexes. The square of the diameter of these rings was proportional to the antigen concentration. The square of the diameter (D^2) was plotted

(y axis) against the concentration of standard (x axis); the line constructed was used to calculate the concentration of component in the samples.

2.1.6: Testing the Purity of Complement Protein Preparations

Complement components were purified and their purity tested by immunoelectrophoresis (IEP) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), before being used as antigens for the production of monospecific antisera.

2.1.6a: Immunoelectrophoresis (IEP) (Scheidigger, 1955)

This procedure involved a combination of electrophoretic separation of proteins in agarose gel, with their immunological detection using antisera.

Materials Used in IEP

Sigma Chemical Company, Poole, Dorset.	:	Bromophenol Blue Coomassie Brilliant Blue
Scottish Antibody Production Unit, Law Hospital, Carluke.	:	Antiserum to whole normal human serum, antiserum to human albumin.

Solutions used in IEP

1.5% Agarose solution

1.5% agarose was prepared in veronal buffer pH 8.4 containing 2 mM EDTA. The veronal buffer was prepared by heating 9g sodium barbitone, 65 ml 100 mM sodium hydroxide and 23 ml 0.086M EDTA in deionised water, to a final volume of 1 litre.

Veronal Tank Buffer pH 8.4

1 litre veronal tank buffer was prepared by mixing 17g sodium barbitone and 23.5 ml 1.0M HCl in deionised water, to a final volume of 1 litre. The solution was diluted in an equal volume of water before use.

0.5% Bromophenol Blue Solution

0.5% Bromophenol blue was prepared by dissolving 0.5g Bromophenol blue in 100 ml deionised water.

Method for IEP

The 1.5% agarose was melted and 9.6 ml used to coat a clean 80 mm x 80 mm glass plate placed on a horizontal levelling table, ensuring the complete surface was evenly coated. After the plate had solidified, the template was placed over, and 1.2 mm diameter wells punched and the troughs cut using a scalpel blade. The plugs of agarose were removed, but the troughs were left in place until after the electrophoretic separation was completed.

Column fractions to be screened and a normal human serum standard were loaded into separate wells, each being filled to the rim. Prior to loading a small quantity of Bromophenol Blue was added to the samples, to enable the distance migrated by albumin to be visualised. The loaded plate was placed in the electrophoretic tank, in direct contact with the wicks which had been soaked in running buffer. The samples were electrophoresed under a constant current of 1.5 mA/cm^2, until the free Bromophenol Blue had migrated to within 1 cm of the anode.

The plate was then placed on a horizontal table, the troughs carefully removed, and refilled to the rim with the appropriate antisera to be tested. For example in the C1-inhibitor preparation, neat antisera to C1-inhibitor, normal human serum and albumin were tested against normal human serum and C1-inhibitor purification pools.

The diffusion was allowed to develop at room temperature for 24 hrs in a humidified box, the precipitin arcs being observed with incident light against a dark background. The non-precipitated proteins were washed out of the gel by soaking in 75 mM NaCl, then deionised water. The gel was dried to the plate by covering with several layers of Whatman filter paper, on top of which a heavy weight was placed.

Once the gel was dried, it was stained for about 10 minutes with stain solution containing 0.1% Coomassie brilliant blue, and destained until the precipitin arcs were observed.

The material being tested was assumed to be homogeneous when a single line was observed against antiserum to normal human serum. The identity of the protein was ascertained by formation of a precipitin arc with a monospecific antiserum of determined specificity.

2.1.6b: SDS-PAGE (Weber and Osborn , 1975)

The addition of the ionic detergent SDS to protein, results in the formation of SDS-protein complexes which possess a strong negative charge. Thus, SDS-PAGE separates proteins by molecular sieving on the basis of their molecular weight,

as they migrate towards the anode. By incorporating standard proteins of known molecular weight, the molecular weight of the protein in question can be determined. Furthermore, if the disulphide bonds are reduced prior to electrophoresis, the polypeptide chain composition of the protein is revealed.

Materials Used in SDS-PAGE

Sigma Chemical Company Poole, Dorset.	:	Bromophenol Blue Coomassie Brilliant Blue Lauryl Sulphate (SDS) Ammonium persulphate N,N,N',N'-tetramethylenediamine (TEMED) Mercaptoethanol Molecular Weight Standards (30,000 to 200,000)
Evans Medical Greenford, Middlesex.	:	Glycerol

Preparation of Solutions used in SDS-PAGE

10% SDS solution

10% SDS was prepared by dissolving 10g SDS in deionised water, to a final volume of 100 ml.

0.75M Tris

0.75M Tris was prepared by dissolving 90.8g Tris in deionised water, to a final volume of 1 litre.

0.75M Tris-HCl pH 6.8

Prepared as for 0.75M Tris but pH to 6.8 using HCl solution, before making up to the final volume.

0.75M Tris-HCl pH 8.8

Prepared as for 0.75M Tris, but pH to 8.8 using HCl solution, before making up to the final volume.

0.86M Glycine Solution

0.86M glycine was prepared by dissolving 32.2g glycine in deionised water, to a final volume of 500 ml.

30% Acrylamide/0.8% bisacrylamide Solution

Acrylamide/bisacrylamide solution was prepared by dissolving 30g acrylamide and 0.8g bisacrylamide in deionised water, to a final volume of 100 ml.

Resolving and Stacking Gels

The gels were prepared as shown in Table 8.

Running Buffer

Running buffer was prepared by mixing 10 ml 10% SDS, 33 ml 0.75M Tris and 220 ml 0.86M glycine, diluted in deionised water, to a final volume of 1 litre.

Sample Application Buffer: Reducing

Reducing sample application buffer was prepared by mixing 3 ml 10% SDS, 0.85 ml 0.75M Tris pH 6.8, 1 ml glycerol, and 0.5 ml mercaptoethanol in deionised water, to a final volume of 10 ml. Bromophenol blue solution was added to colour the buffer.

Coomassie Brilliant Blue Stain and Destain Solution

These were prepared as described previously (Section 2.1.1).

Method for SDS-PAGE

The method used was that described by Laemmli (1970) which used polyacrylamide gel in Tris-HCl buffer pH 8.8 for the resolving gel, pH 6.8 for the stacking gel and a buffer system of Tris-glycine at pH 8.3.

% Gel Solution	Acrylamide/ bisacrylamide (ml)	Water (ml)	Tris/HCl pH 8.8 (ml)	Tris/HCl pH 6.8 (ml)	10% SDS (µl)	TEMED (µl)	Ammonium Persulphate (mg)	Total Volume (ml)
3% Stacking gel	1.8	13	0	3	180	9	18	18
7.5	10	9.6	20	-	400	20	40	40
10	10	4.7	15	-	300	15	30	30
15	10	0	10	-	200	10	20	20

Table 8: SDS-PAGE Slab Gel Recipes.

Clean glass plates were swabbed with ethanol, allowed to air dry and clamped together with 1.5 mm perspex spacers inserted along three sides. These 3 sides were sealed with resolving gel containing excess N,N,N',N'-tetramethylenediamine (TEMED). The appropriate percentage of resolving gel was prepared by mixing the volumes of the components as listed in Table 8, with the omission of TEMED. The TEMED was added and gently mixed and the solution poured between the two sealed glass plates, to within about 4 cm of the top of the shaped glass plate. The gel was overlaid with isobutanol, levelled and allowed to polymerise at room temperature for about 60 min. The 3% stacking gel was prepared as described in Table 8, with the omission of TEMED. The isobutanol was washed from the resolving gel surface, 9 µl TEMED was added to the stacking gel and the solution gently poured over the resolving gel. A comb with the appropriate size or number of wells was gently inserted between the plates, and the stacking gel allowed to polymerise.

Molecular weight standards and samples (if using column fractions containing approximately 20 µg protein) were added to microcap tubes, diluted with at least an equal volume of sample application buffer and boiled for 2 min at 100°C.

The gel within the glass plates was loaded into the lower reservoir filled with running buffer and sealed; the comb was removed and the upper reservoir filled with running buffer. The standards and samples were applied to the wells and electrophoresed with 25 to 40m Amps constant current,

until the dye front reached within 1 cm of the end of the resolving gel.

The gel was removed from the glass plates, fixed and stained in 0.1% Coomassie brilliant blue for 1 hr at 37°C. The gel was destained in several changes of destain solution until the protein bands were clearly visible.

The migration of the samples relative to the known molecular weight standards enabled an estimate of their molecular weight to be performed. The distance (mm) from the buffer front to the middle/or front of each of the proteins in the mixture of standards was measured; the same procedure was carried out for the samples. A linear plot of distance migrated (y axis) against $\log_{10}$ molecular weight (x axis) enabled estimates of polypeptide chain molecular weights, based on their migration distances under identical conditions in the same gel.

The SDS-PAGE system along with IEP was used to check that the C1-inhibitor preparations were pure before being used to immunise rabbits.

2.1.7: Immunisation of Rabbits with C1-inhibitor

Each of three New Zealand White rabbits were injected at three sites with a total of 38 µg C1-inhibitor emulsified in 0.5 ml Freund's complete adjuvant (Difco). One third of the antigen-adjuvant mixture was injected subcutaneously into the back, and one third intramuscularly into each thigh.

Approximately two weeks later, a booster dose was given to all three rabbits: 40µg C1-inhibitor in Freund's incomplete adjuvant (Difco), injected subcutaneously in each rabbits' back.

Seven, nine and fourteen days after the booster injection, each rabbit was bled of 30 to 40 ml from the marginal ear vein, and the serum separated. The specificity and monospecificity of each of the antisera was tested on double-diffusion plates.

2.1.8: Double Diffusion in Agarose Gel (Ouchterlony, 1958)

Antiserum and antigen placed in adjacent wells of an agarose gel, were allowed to diffuse and form precipitin lines. The patterns of cross-reactivity of the arcs were used to determine the specificity and monospecificity of the test antiserum.

Materials used in Double Diffusion Plates

Scottish Antibody Production Unit (SAPU), Blood Transfusion Service, Law Hospital, Carluke.	: Antiserum to whole normal human serum.
Behring Diagnostics, Hounslow.	Antiserum to human C1-inhibitor.

Preparation of Reagents

1.5% Agarose Solution

This was prepared as described for IEP procedures (Section 2.1.6a).

Method of Double Diffusion

Clean 76 mm x 26 mm microscope slides were swabbed in ethanol and allowed to air dry. 1.5% agarose in EDTA/VBS buffer was melted and 3 ml poured onto the slides situated on a horizontal table. After the slides had solidified, 3 mm diameter wells were punched in the agarose in a pattern of one central well surrounded by six wells: the distance between the centres of the middle and the peripheral wells was 10 mm.

The wells were loaded with the test samples which included purified C1-inhibitor, NHS, antiserum to NHS, commercially purchased antiserum to C1-inhibitor as well as the raised rabbit antiserum to C1-inhibitor. The gel was placed in a humid container at room temperature and allowed to diffuse for 48 to 72 hr. If the precipitin arcs produced were faint, the slides were stained as described for IEP slides (2.1.6a).

Double diffusion plates were also used to test the titre of the antiserum to C1-inhibitor. This was done by placing NHS in the centre well and increasing dilutions of antiserum in the peripheral wells. Thus, the higher the titre of antiserum, the higher the dilution that a precipitin line was observed to occur.

2.2: Purification of Human C1-inhibitor

C1-inhibitor purified from human citrated plasma by Dr A.C. Yeung Laiwah and Professor K. Whaley using the method of Reboul *et al.* (1977), was further purified by gel filtration over a Biogel column. A summary of the initial purification

procedures performed by Dr A.C. Yeung Laiwah and Professor K. Whaley will be given, followed by the details of the final purification over Biogel.

2.2.1: Materials Used in C1-inhibitor Purification

Biorad : Biogel A 0.5M (200-400 mesh)
Watford,
Hertfordshire.

2.2.2: Buffer Used in C1-inhibitor Purification

Biogel A 0.5M Buffer

10 mM Tris-HCl pH 8.0 + 100 mM sodium chloride + 50 mM benzamidine + 3 mM sodium azide.

2.2.3: Initial Purification Procedure

During the purification procedure inhibitors of proteolytic enzymes and antibacterial agents were added to plasma and buffers as follows:- 4 mM phenylmethyl-sulphonyl fluoride (PMSF) in dimethylformamide, 5 mM benzamidine, 1.5 mM sodium azide and 20 mM EDTA. The first three stages of purification consisted of polyethylene glycol (PEG) precipitation, ion exchange chromatography using the anion exchanger DEAE cellulose, followed by affinity chromatography using Con A sepharose.

PEG 600 was added to 1 litre of fresh citrated plasma, to a final concentration of 6% (w:v), and stirred for 30 min at 4°C. After centrifugation the supernatant was collected and dialysed against the starting buffer for DEAE cellulose. The dialysed supernatant was applied to a 2.5 cm x 100 cm

column of DEAE cellulose, and the bound protein eluted with a linear salt gradient from 50 mM sodium chloride up to 200 mM sodium chloride. The fractions containing C1-inhibitor (conductivity at 0°C of around 8 mS) were pooled, concentrated and dialysed against the starting buffer for the Con-A sepharose column.

The dialysed sample was applied to a 2.6 x 40 cm column of ConA sepharose, and the bound protein eluted with starting buffer containing 2% methyl α-D-mannopyranoside. The fractions containing C1-inhibitor were pooled, concentrated and dialysed against the starting buffer for the Biogel column.

2.2.4: Biogel A 0.5M Chromatography

8 mg of C1-inhibitor was applied at 20 ml/hr to a 2.6 cm x 90 cm column of Biogel A 0.5M equilibrated in starting buffer. 2 ml fractions were collected and screened for protein content by Folin assay (Section 2.1.4) and C1-inhibitor by RID (Section 2.1.5).

The C1-inhibitor containing fractions were pooled into six pools and tested for homogeneity and purity by IEP (Section 2.1.6a) and SDS-PAGE (Section 2.1.6b). The pools were divided into aliquots and stored at -70°C.

2.3: Purification of Human C2

2.3.1: Materials used in Purification of C2

Biorad, Watford, Hertfordshire.	:	Biorex 70
Whatman	:	DEAE Cellulose

Aldrich, Gillingham, Dorset.	:	Benzamidine
Sigma Chemical Company, Poole, Dorset.	:	Dimethylformamide Phenylmethylsulphonyl fluoride
Blood Transfusion Service St. Vincent Street, Glasgow and Law Hospital, Carluke	:	Expired frozen plasma.
Scientific Instrument Centre Ltd., Eastleigh, Hants.	:	Dialysis Tubing.

2.3.2: Buffers used in Purification of C2

Biorex 70 Chromatography Starting buffer:

10 mM sodium phosphate pH 7.0 + 2 mM EDTA + 6 mM sodium azide.

Biorex 70 Chromatography Gradient:

Starting buffer and starting buffer containing 1M sodium chloride.

DEAE-cellulose chromatography starting buffer:

10 mM Tris-HCl pH 7.5 + 25 mM Sodium Chloride + 2 mM EDTA + 5 mM benzamidine + 0.5 mM PMSF (in dimethylformamide).

2.3.3: Purification of C2 (Whaley, 1985)

C2 was purified for use in haemolytic assays by the method of Whaley (1985) which involves three major purification stages: sodium sulphate precipitation followed by 2 ion exchange chromatography steps involving Biorex-70 and DEAE-cellulose.

2.3.3a: Sodium Sulphate Precipitation

Five expired frozen citrated plasma units, from the Blood Transfusion Service, were thawed at 37°C, pooled (1280 ml) and inhibitors added as follows: sodium azide (6 mM) and EDTA (2 mM). Whilst stirring continuously, 254g solid sodium sulphate was added slowly to the plasma at room temperature (final concentration of 20% sodium sulphate). After stirring for a further 1 hour at room temperature, the suspension was centrifuged at 5000g for 15 min at room temperature in a MSE High Speed 25 centrifuge. The pellet was discarded and the supernatant (965 ml) incubated overnight at 4°C to allow crystallisation of the sodium sulphate. The supernatant (700 ml) was dialysed three times against 8 litres of 10 mM sodium phosphate pH 7.0 with 2 mM EDTA and 6 mM sodium azide (starting buffer for Biorex 70 column).

2.3.3b: Biorex 70 chromatography

The dialysed supernatant (860 ml) was applied at 50 ml/hour to a 5 cm x 90 cm column of Biorex 70 equilibrated in starting buffer. 25 ml fractions were collected and the column washed with starting buffer until an OD_{280}nm of less than 0.04 was attained, and no further protein was eluted from the column. A linear gradient consisting of 3 litres starting buffer, and 3 litres starting buffer containing 1M sodium chloride was applied to the column. 25 ml fractions were collected and assayed for protein content by OD_{280}, conductivity, C2 by haemolytic assay (Section 2.6.6), and

factor B by radial immunodiffusion (Section 2.1.5). The fractions containing C2 were pooled and dialysed three times against 8 litres of 10 mM Tris-HCl pH 7.5 containing 2 mM EDTA, 5 mM benzamidine, 0.5 mM PMSF, 25 mM sodium chloride (starting buffer for DEAE-cellulose chromatography).

2.3.3c: DEAE-cellulose Chromatography

The equilibrated pool (375 ml) was applied at 70 ml/hour to a 2.6 cm by 40 cm column of DEAE-cellulose equilibrated in starting buffer. The sample was washed through with starting buffer, 10 ml fractions were collected and assayed for protein content by OD_{280}^{nm}, C2 by haemolytic assay, and factor B by radial immunodiffusion. The fractions containing C2 were pooled, adjusted to pH 6.9 with 1M HCl, concentrated on an Amicon PM10 membrane, divided into aliquots and stored at -70°C. Since this purified C2 was used only as a standard in haemolytic titrations, it was unnecessary to determine its purity.

2.4: Purification of C1

2.4.1: Materials used in purification of C1

Blood Transfusion Centre Law Hospital, Carluke.	: Expired frozen plasma
Animal House, Department of Pathology, Western Infirmary, Glasgow.	: Guinea pig serum

2.4.2: Buffers for Purification of C1

5 mM sodium phosphate pH 7.5 with 15 mM sodium chloride.
5 mM Sodium phosphate pH 7.5 with 300 mM sodium chloride.
1M orthophosphoric acid.

2.4.3: Method of Purification of C1

Purified C1 was prepared by the method of Lachman and Hobart, (1978) from guinea pig serum and human serum.

1 volume of serum, prepared from citrated plasma as described (Section 2.1.2), was cooled to 0°C and diluted 1 in 4 with deionised water at 0°C. The solution was adjusted to pH 7.5 with 1M HCl or 1M NaOH (0.1M HCl and 0.1M NaOH were used in the small quantities of guinea pig serum) and the euglobulin allowed to precipitate for 1 hr at 0°C, whilst constantly monitoring the pH. The euglobulin precipitate was harvested by centrifugation at 1000g for 10 minutes at 0°C in a MSE Coolspin centrifuge. The supernatant was discarded and the precipitate resuspended in 1 volume of 5 mM sodium phosphate buffer pH 7.5 containing 15 mM sodium chloride, at 0°C. The pH of the resulting solution was carefully adjusted to 5.4 using 1M orthophosphoric acid. Incubation for 5 to 10 min at 0°C resulted in flocculation of the precipitate, which was harvested by centrifugation at 1000g for 10 min at 0°C. The supernatant was discarded and the pellet dissolved in 5 mM sodium phosphate buffer pH 7.5 containing 300 mM sodium chloride, to a volume of about 1/10 that of the initial serum volume. The C1 was divided into aliquots and stored at -70°C.

The purified C1 was used in the preparation of haemolytic intermediates and in haemolytic assays, therefore its purity was not determined.

2.5: Preparation of Human Monocyte Cultures

2.5.1: Materials Used in Preparation of Monocyte Culture

Flow Laboratories, Rickmansworth, Herts.	:	1 x RPMI 1640 (with HEPES, without sodium bicarbonate and glutamine) 24 well Linbro multiwell tissue culture plates Ham's F12 Medium, 1M HEPES.
Gibco, Paisley, Renfrewshire.	:	100 x Antibiotic/antimycotic solution, 200 mM L-glutamine solution, 7.5% Sodium bicarbonate solution BHK 21 Medium-Glasgow Modified (10 x concentrated) 100 x Penicillin/Streptomycin solution. 75 cm^2 Nunclon Tissue culture flasks Powdered Hanks balanced salt solution. Foetal Calf Serum.
Department of Virology University of Glasgow.	:	BHK 21 clone 13 cells Tryptose phosphate broth solution Amphotericin B solution.
Blood Transfusion Service: St. Vincent Street, Glasgow, and Law Hospital, Carluke.		Buffy Coat Preparations from blood donations, anticoagulated with citrate phosphate dextrose solution. AB plasma which was used to prepare serum.
Pharmacia Ltd., Milton Keynes, Bucks.	:	Ficoll 400.
Sterling Research Laboratories, Surbiton Upon Thames, Surrey.	:	45% Sodium diatrazoate (Hypaque).
Evans Medical, Greenford, Middlesex.	:	Mucous heparin, without preservative. 1000 units/ml.

2.5.2: Preparation of Reagents used in Monocyte Culture

Ficoll-Hypaque

9% Ficoll was prepared by dissolving 90g Ficoll 400 in deionised water, to a final volume of 1 litre.

45% Hypaque was diluted to 33.9% by mixing 20 ml hypaque and 6.5 ml deionised water.

9% Ficoll and 33.9% hypaque were mixed in proportions of 24:10 respectively, to produce Ficoll-hypaque with a specific gravity of 1.077 g/l.

BHK 21 Cell Culture Medium

This medium was prepared from stock 10x concentrated BHK 21 medium - Glasgow modified and the additives listed. To a bottle containing 300 ml sterile distilled water the following were added: 36 ml 10 x concentrated medium, 14 ml 7.5% sodium bicarbonate, 8 ml 200 mM L-glutamine, 4 ml 100 x penicillin/streptomycin, 0.4 ml amphotericin B, 36 ml Tryptose phosphate broth and 36 ml foetal calf serum (FCS).

RPMI 1640

RPMI 1640 was purchased with HEPES, and without bicarbonate and glutamine which were added as follows: 5 ml 200 mM L-glutamine (2 mM final concentration) and 5 ml 7.5% sodium bicarbonate (0.075% final concentration) per 500 ml bottle. Antibiotic antimycotic solution 100x, was also added (5 ml/ 500 ml bottle) (Penicillin 100 u/ml, Streptomycin 100 mcg/ml and Fungizone 0.25 mcg/ml, final concentrations).

Foetal Calf Serum (FCS)

FCS was purchased and the complement activity destroyed by heat inactivation at 56°C for 2 hr.

RPMI/10% FCS

Prepared by mixing 5 ml FCS with 45 ml RPMI.

RPMI/20% FCS

Prepared by mixing 10 ml FCS with 40 ml RPMI.

Human AB Serum

This was prepared as previously described (Section 2.1.2c).

RPMI/10% AB

Prepared by mixing 5 ml AB serum with 45 ml RPMI.

EDTA/PBS and RPMI/FCS Mixture

Prepared by mixing equal volumes of 0.01M EDTA/PBS and RPMI/10% FCS.

Hanks Balanced Salts Solution (HBSS)

1 pack of powdered HBSS was added to 500 ml sterile water containing 2.5 ml 7.5% sodium bicarbonate.

Ham's F-12 Medium

To a 500 ml bottle of medium the following were added 10 ml 1M Hepes (20 mM final), 5 ml 100 x Glutamine (2 mM final) and 5 ml antibiotic-antimycotic solution (Penicillin 100 u/ml, streptomycin 100 mcg/ml and fungizone 0.25 mcg/ml, final concentrations).

Preparation of Monocyte Culture

Monocyte monolayers were prepared under sterile conditions from 60 ml heparinised human venous blood donations, or from unwanted buffy coat fractions prepared from citrated venous blood donations taken for transfusion purposes by the Blood Transfusion Centre. Two methods were employed for their preparation:

Method 1, the BHK method, which involved purification of monocytes by adherence to microexudate-coated flasks, removal of the adherent monocytes using EDTA, followed by readherence to tissue-culture treated plates. Method 2 involved the direct adherence of monocytes to tissue-culture treated plates.

2.5.3: Method 1: The BHK Method: Modification of the method by Douglas, Zuckerman and Ackerman, 1981.

2.5.3a: Preparation of BHK-microexudate coated flasks (BHK flasks)

The microexudate coated flasks were prepared by growing Baby Hamster Kidney fibroblasts - 21 (BHK-21), clone 13, donated by Dr G. Clements, Virology Department, University of Glasgow, on 75 cm^2 Nunclontissue culture flasks. The cells were seeded at a density of 2 x 10^6 cells per flask in BHK-21 medium - Glasgow modified containing sodium bicarbonate, supplemented with 10% FCS, 10% tryptose phosphate broth, antibiotics, antimycotics, and allowed to grow to confluence at 37°C in a humidified atmosphere (3 or 4 days). The medium was decanted and fresh medium was added on two consecutive days, allowing the cells to reach "superconfluence". The medium was decanted and about 10 mls 0.01M EDTA in PBS pH 7.4 (EDTA/PBS) added to the flask and incubated at room temperature for 5 min, to allow detachment of the fibroblasts. The solution with fibroblasts was discarded, replaced with 10 ml fresh EDTA/PBS and the flask gently shaken to remove any remaining fragments of fibroblasts, leaving the microexudate intact; this was repeated once. If the flask was

to be used immediately it was rinsed twice with Hanks balanced salts solution (HBSS), or if it was to be stored at 4°C for later use, 5 ml EDTA/PBS were added. The flasks have been stored and satisfactorily used after periods of up to ten weeks.

2.5.3b: Preparation of Monocyte Cultures using Heparinised Venous Blood

60 ml peripheral venous blood was withdrawn from human volunteers, and mixed with 600 units preservative free mucous heparin. Each 20 ml of blood was mixed with 15 ml RPMI, gently layered over 15 ml Ficoll-Hypaque (specific gravity: 1.077 g/ml) and separated by centrifugation at 400g for 45 minutes at room temperature. The plasma/RPMI layer was discarded, the interfacial mononuclear cell layer was collected from the three gradients, pooled and diluted to 72 ml with RPMI/10% FCS.

The cell suspension was equally divided between 6 BHK flasks, and incubated at 37°C in a humidified atmosphere for 45 min, to allow adherence of the monocytes. The medium containing non-adherent cells was discarded and the adherent cells washed with gentle agitation, using about 5 ml HBSS which had been prewarmed to 37°C. This was routinely performed three times, or until no loose cells were observed floating in the medium.

Platelets which were distributed around the adherent monocytes, were detached by incubating the contents of each flask with about 5 ml of a 1:1 mixture of 0.01M EDTA/PBS and RPMI/10% FCS, for 20 to 30 seconds at room temperature. The medium was decanted and a fresh 5 ml of the 1:1 EDTA/FCS mixture was added to each flask, and incubated at 37°C for 15 min, to allow detachment of the monocytes. Each flask was gently shaken to ensure complete detachment of the monocytes, the contents of the six BHK flasks were pooled in a 50 ml Falcon centrifuge tube, and the cells harvested by centrifugation at 200g for 15 min at room temperature. The supernatant was discarded and the pellet resuspended by tapping the tube prior to the addition of 4 ml RPMI/10% AB serum. An aliquot of the cell suspension was removed for counting in a Neubauer Haemocytometer; the cells were resuspended to a concentration of 0.7 to 0.9 x 10^6 cells/ml RPMI/10% AB. 1 ml of the cell suspension was plated into each well of the tissue-culture treated Linbro multi-well plates, and the cells allowed to adhere overnight at 37°C in a humidified atmosphere.

The supernatant was gently removed from each well and replaced by 1 ml RPMI/20% FCS, in which the cells were cultured for seven days. After this incubation period which allowed maturation and better attachment of the monocytes, the supernatant was removed, the monolayers washed twice with prewarmed RPMI and replaced with 1 ml fresh RPMI/20% FCS, along with any agents which were to be studied. This was designated as day 0 in all experiments.

2.5.3c: Preparation of Monocyte Cultures using Citrated Buffy Coats

Freshly prepared citrated buffy coats were obtained from the Blood Transfusion Service, and used in the preparation of monocyte cultures, as described for venous blood, with the following exceptions. The buffy coats were diluted in PBS, since the Ca^{++} and Mg^{++} present in RPMI was of a high enough concentration to reverse the clotting process inhibited by the citrate. The mixture was layered over 15 ml Ficoll-Hypaque and separated by centrifugation, the resulting cell suspension being incubated in 6 to 9 BHK flasks, with the appropriate scaling up of all other parameters.

2.5.4: Method 2

This method involved Ficoll-Hypaque separation of blood followed by direct adherence of monocytes to tissue culture treated Linbro multi-well plates, and did not involve any prior purification of monocytes. The method was carried out using citrated buffy coats and heparinized venous blood donations, the latter of which will be described first using a 60 ml blood volume.

Each 20 ml of heparinised venous blood was mixed with 15 ml RPMI, gently layered over 15 ml Ficoll-Hypaque and separated by centrifugation at 500g for 30 min at room temperature. The plasma/RPMI mixture was discarded and the interfacial mononuclear cell layer was removed from the three gradients, pooled and transferred to a 50 ml sterile Falcon tube. The volume was made up to 50 ml with HBSS in order to

wash the cells and remove most of the unattached, contaminating platelets and Ficoll-Hypaque. The cells were harvested by centrifugation at 400g for 10 min at room temperature; following resuspension in HBSS the cells were recentrifuged at 200g for 10 min at room temperature. The resuspended cells were washed twice with about 15 ml HBSS, harvesting the cells by centrifugation at 200g for 5 min at room temperature. The supernatant was decanted, the cells resuspended by tapping the tube and 5 ml RPMI/20% FCS was added. An aliquot of the cell suspension was removed for counting in a Neubauer haemocytometer and the cells resuspended to a density of 6 to 9 x 10^6 cells/ml RPMI/20% FCS. The cell suspension was plated out at 1 ml per well in Linbro multi-well plates, and the monocytes allowed to adhere for 1 hr at 37°C in a humidified atmosphere.

The supernatants containing the non-adherent cells were removed, and the monolayers washed three times with about 0.4 ml warm HBSS. The monocyte monolayers were cultured in 1 ml RPMI/10% AB serum per well at 37°C in a humidified atmosphere for three days to allow firm attachment and maturation of the monocytes. On day 3 the supernatants were removed, each monolayer was washed twice with about 0.4 ml warm RPMI and the culture continued in 1 ml RPMI/20% FCS per well. Any agents to be studied were added to the monocytes at this stage, which was designated as day 0.

The modifications of this method when using citrated buffy coats were as follows: the buffy coat was diluted with PBS instead of RPMI; the interfacial mononuclear cell layer was removed, pooled and transferred to two 50 ml sterile Falcon tubes, PBS replacing the HBSS used in the first one or two washes; after completing the washes, the cells were resuspended in 20 ml RPMI/20% FCS for counting instead of 5 ml. Otherwise, all stages are similar to that described for the venous blood method.

2.5.5: Monocyte Culture under Serum-Free Conditions

Monocyte monolayers set up as described with buffy coats using the BHK method (Section 2.5.3) were maintained in RPMI/20% FCS for about 10 days, after which the supernatant was removed, the monolayers washed with prewarmed RPMI, and further culture continued in Ham's F-12 medium in the absence of serum. After three days the culture supernatants were harvested, pooled, centrifuged to remove any loose cells, dialysed against PBS, concentrated approximately 20 fold by sucrose dialysis, and stored at -70^{o}C until use.

This modified culture procedure was adopted to allow investigation of functional C1-inhibitor activity synthesised by monocytes in culture, since serum was inhibitory in this functional assay. (Methods in Section 2.6.7).

2.5.6: Characterisation of Mononuclear Phagocytes

The characteristics used to determine that the separated, cultured cells were mononuclear phagocytes included microscopic examination; Giemsa and non-specific esterase staining (both of which were kindly performed by Mr John Stewart, Department of Pathology, Western Infirmary, Glasgow); phagocytosis of serum treated zymosan; testing for C3b and Fc receptors using EAC 43b and IgG-coated sheep E cells respectively (kindly donated by Dr E.R. Holme, Department of Pathology, Western Infirmary, Glasgow).

The cells for staining were prepared by the BHK method (Section 2.5.3b) and readhered to glass coverslips in Linbro wells to allow easier handling during the staining procedure. Once stained with the Giemsa technique, the proportion of monocytes in the total cell population was counted on the basis of morphology: monocytes having a kidney-shaped nucleus and a large volume of cytoplasm, compared to the very rounded nuclei and small volume of cytoplasm of lymphocytes, and multi-lobed nuclei of PMNs. The proportion of non-specific esterase positive cells was also counted on the basis of staining patterns: monocytes showed diffuse granular reddish-brown staining distributed evenly throughout their cytoplasm.

Monocytes were washed, the various particles added and incubated at 37°C for 30 minutes. The non-phagocytosed and unbound particles were removed and the cells washed to allow easier counting of cells which were positive or negative for phagocytosis and rosette formation.

2.5.7: Materials added to Monocyte Cultures

Sterling Windthrop Surbiton-Upon-Thames, Surrey.	:	Danazol Stanozolol.
Organon Laboratories Ltd., Cambridge.	:	Dexamethasone as Oradexon-Organon 8 mg/2 ml.
Dr A. Campbell, Organon Laboratories Ltd., Newhouse.	:	Organon 6632
Syntex Corporation Palo Alto, California.	:	Oxymetholone
Aldrich Chemical Co. Ltd., Gillingham, Kent.	:	Desoxycorticosterone
Sigma Chemical Company, Poole, Dorset.	:	Hydrocortisone hemisuccinate Prednisolone Corticosterone Testosterone β-D-glucuronide Testosterone Benzoate 17α methylestosterone Testosterone 17β hemisuccinate Estrone-3-sulphate Estriol 17β glucuronide 17α estradiol Diethylstilbestrol Δ^4 androstene 3,17 dione 11α hydroxy progesterone 17α hydroxy progesterone 17α hydroxy pregnenolone Fluoxymesterone Aldosterone Cycloheximide Indomethacin
Dr John Toy, Biogen SA, Geneva, Switzerland.	:	Interferon-gamma (recombinant, *E. coli*-derived. Activity of 1.4 antiviral units/ng).

2.5.8: Preparation of Materials Added to Monocyte Culture

The materials added to monocytes in culture were most frequently prepared as a concentrated stock solution in RPMI/20% FCS, which was sterilised by filtration through 0.22 µm Millex GS filters. When the effect of a dose response curve was to be investigated, the solutions were prepared as ten times concentrated stock solutions, and 100 µl added per 1 ml

culture medium to each well. For example, if a dose response curve of hydrocortisone was to be investigated between the doses of 10^{-4}M and 10^{-8}M, a stock solution of 10^{-3}M would be prepared. 1/10 dilutions in RPMI would be serially performed to 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}M, and 100 µl of each stock added per well containing 1 ml, giving a final dilution range of 10^{-4}M to 10^{-8}M.

Some reagents to be added were obtained in solution (dexamethasone) or were able to be dissolved in RPMI/20% FCS (hydrocortisone hemisuccinate, cyclohexiмide, interferon-gamma); however others were not soluble in RPMI/20% FCS (androgen, derivatives, oestrogen derivatives, anabolic steroids, testosterone derivatives, mineralocorticoids, progesterone derivatives, corticosterone, prednisolone, Org 6632, and indomethacin). These were dissolved to the highest concentration possible in absolute alcohol, usually 10^{-2} or 10^{-3}M; the solution was then diluted out in RPMI/20% FCS until the appropriate dose range was reached. A corresponding control dilution containing only absolute alcohol in RPMI/20% FCS was prepared, and added to alcohol control wells in the culture. This was to ensure that any effect observed was due to the drug added, and not to the alcohol alone.

2.5.9: Sampling of Monocyte Cultures

Samples were removed throughout the culture period which usually consisted of 14 days in total: as described previously, the cells were cultured initially for 7 days without any

manipulations to allow maturation. After this the medium was changed, the cells washed with RPMI, and 1 ml fresh RPMI/20% FCS replaced along with any drugs to be investigated.

Samples were removed (200-500 µl) on specified days, usually 1, 3, 5 and 7, and the medium replaced with an equal volume of RPMI/20% FCS. In the experiments involving monocytes isolated from patients with hereditary angio-oedema, and their normal controls, culture was usually continued for longer periods of up to 17 to 21 days.

To investigate cumulative and continuous synthesis of complement components by monocytes, the total 1 ml of medium was removed and replaced with a fresh 1 ml RPMI/20% FCS on days 1, 3, 5 and 7, which represents cumulative synthesis; continuous synthesis was sampled as described previously with a fixed volume.

2.6: Haemolytic Assays

2.6.1: Materials used in Haemolytic Assays

Evans Medical, Greenford, Middlesex.	:	Glycerol.
Travenol Laboratories, Thetford, Norfolk.	:	20% Mannitol solution.
Gibco, Paisley, Renfrewshire.	:	Sheep whole blood in Alsever's Solution (50%).
Nordic	:	Rabbit antiserum to sheep erythrocytes.

2.6.2: Preparation of Buffers Used in Haemolytic Assays

10% Gelatin

10g gelatin was dissolved in deionised water, by heating in a pressure cooker for 5 min, and diluted to a final volume of 100 ml.

30 mM Calcium Chloride

3.3g $CaCl_2.6H_2O$ was dissolved in deionised water, to a final volume of 500 ml.

100 mM Magnesium Chloride

10.2g $MgCl_2.6H_2O$ was dissolved in deionised water, to a final volume of 500 ml.

GVB^{++}

GVB^{++} was prepared by mixing 200 ml 5 x VBS, 10 ml 100 mM $MgCl_2$, 5 ml 30 mM $CaCl_2$ and 10 ml 10% gelatin in deionised water, to a final volume of 1 l.

$D5W^{++}$

$D5W^{++}$ was prepared by mixing 50g D-glucose, 5 ml 30 mM $CaCl_2$ and 10 ml 100 mM $MgCl_2$ in deionised water to a final volume of 1 litre.

$DGVB^{++}$

$DGVB^{++}$ was prepared freshly when required by mixing equal volumes of GVB^{++} and $D5W^{++}$.

GVB^{--}

GVB^{--} was prepared by mixing 200 ml 5 x VBS and 10 ml 10% gelatin in deionised water to a final volume of 1 litre.

0.04M EDTA/GVB^{--}

Prepared by mixing 232.5 ml 0.086M EDTA with 267.5 ml GVB^{--}.

0.01M EDTA/GVB^{--}

Prepared by mixing 58.1 ml 0.086M EDTA with 441.9 ml GVB^{--}.

C-Rat Solution

C-rat was prepared by diluting rat serum 1/15 in 0.04M EDTA/GVB^{--}.

Glycerol Buffer for freezing of EAC4 cells. pH 6.8

Glycerol buffer was prepared by mixing 57.1g glycerol, 1.34 ml sodium lactate solution, 12.75 ml 20% mannitol, 1 ml 500 mM NaH_2PO_4 and 1 ml 500 mM KCl, pH to 6.8 and adjusted to 100 ml with deionised water.

D50S

Prepared by dissolving 50g glucose, and 5.95g NaCl in deionised water, to a final volume of 100 ml.

Mannitol/GVB^{++}

Prepared by mixing 9 ml 20% mannitol and 3 ml GVB^{++}.

2.6.3: The Use of Sheep Erythrocytes in Haemolytic Assays

Functional haemolytic assays for the various complement components utilizes sheep erythrocytes (E) as the target cell since they are readily available, they lyse easily when coated with antibody (EA) and complement, and are easily quantitated. Suspensions of sheep erythrocytes are easily standardised on the basis that haemoglobin has three major light absorbance peaks at 576, 541 and 414 nm. For standardisation of sheep erythrocytes used in complement work the optical density of 100 µl of cell suspension lysed in 2.9 ml deionised water is determined at either 541 nm (OD_{541} 0.192 = 5×10^8 erythrocytes/ml or .385 = 1×10^9/ml) or at 414 nm (OD_{414} .327 = 1×10^8 erythrocytes/ml or 0.654 = 2×10^8 erythrocytes/ml).

2.6.4: Preparation, Freezing and Reconstitution of EAC4 cells

The three haemolytic assays performed in the process of this work were for the components C1, C2 and C1-inhibitor, all of which use the same starting haemolytic intermediate, EAC4 cells. These cells were prepared in bulk quantities and stored frozen in glycerol buffer at -70°C until required, thus the preparation of EAC4 cells will be the first method described, followed by the assays utilizing these cells: C1, C2 and C1-inhibitor haemolytic assays.

2.6.4a: Preparation of EA (Rabbit Antibody Coated Sheep Erythrocytes)

50 mls sheep erythrocytes (E) in Alsevers solution were washed twice in saline and twice in $DGVB^{++}$, harvesting the cells by centrifugation at 1000 g for 10 minutes at 4°C using an MSE Coolspin centrifuge. The cells were standardised to a concentration of 1 x 10^9/ml in 0.01M EDTA/GVB^{--} as described previously (OD 541_0.385) and warmed to 37°C. A subagglutinating dose of antibody to sheep erythrocytes (rabbit anti-sheep haemolysin, at 1/1000 dilution) was diluted, to give a volume equal to that of the sheep E, and warmed to 37°C. The two solutions were mixed whilst being constantly shaken at 37°C in a shaking water bath, and incubated for a further 30 min. The resulting EA were washed twice in $DGVB^{++}$, harvesting the cells by centrifugation at 1000g for 10 min at 4°C, and resuspended to 5 x 10^8/ml (OD 541_0.192) in $DGVB^{++}$.

2.6.4b: Preparation of EAC1

Pretitrated human C1 (prepared as described in Section 2.4) was added neat to EA (5×10^8/ml) at a C1 concentration of 400 units C1/5×10^8 cells and incubated with shaking at 37°C for 15 min. The EAC1 cells were harvested by centrifugation at 1000 g for 10 min and 4°C and resuspended in $DGVB^{++}$ to 5×10^8/ml (OD 541.192).

2.6.4c: Preparation of EAC4 (Borsos and Rapp, 1967)

Preparation of EAC4 from EAC1 required a source of fresh normal human serum to supply the C4, this was either obtained as a clotted blood donation from a laboratory volunteer, or was prepared as described (Section 2.1.1) from fresh frozen plasma packs obtained from the Blood Transfusion Service.

Normal human serum was diluted 1 in 10 in 0.01M $EDTA/GVB^{--}$ to a volume 2.5 times the volume of erythrocytes at 5×10^8/ml. Both solutions were separately cooled, with mixing to prevent freezing, on salted ice to 0°C. The serum and cells were then mixed and incubated for 15 min at 0°C, shaking constantly. Following centrifugation at 1000 g for 10 min at 0°C, the EAC1 were washed twice in ice cold 0.01M $EDTA/GVB^{--}$, then resuspended to 5×10^8/ml in 0.01M $EDTA/GVB^{--}$. The EAC14 were warmed to 37°C and incubated for 30 min with shaking to decay off any residual C1 and C2. The cells were harvested by centrifugation at 1000 g for 10 min at 4°C, washed once in 0.01M $EDTA/GVB^{--}$, twice in $DGVB^{++}$ and resuspended in $DGVB^{++}$.

At this stage EAC4 could be stored successfully for up to 2 weeks at 4°C, or for prolonged periods of storage at -70°C by freezing in glycerol buffer.

2.6.4d: Freezing of EAC4 Cells (Whaley, 1985)

EAC4 to be frozen were standardised to a 50% suspension in GVB^{++}. Each aliquot of EAC4 contained 4×10^9 EAC4 in 0.8 ml glycerol buffer. The cell suspension and glycerol buffer were warmed to 37°C in a shaking water bath, and 1.8 times the cell volume of glycerol buffer was added dropwise with constant shaking as follows: 0.15 times cell volume over 10 min, 0.45 times cell volume over 10 min, 1.2 times cell volume over 10 min. Aliquots of 0.8 ml glycerolised cells were dispensed into screw-capped tubes, snap frozen in a dry ice and methanol mixture, and stored at -70°C.

2.6.4e: Reconstitution of EAC4

All buffers used were prewarmed to 37°C and added dropwise with shaking.

An aliquot of EAC4 was thawed at 37°C and reconstituted by the addition of D50S: 0.23 ml over 5 min; mannitol/GVB^{++}: 1 ml over 2 min, 2 ml over 2 min, 8 ml over 5 min; $DGVB^{++}$: 5 ml over 2 min, 10 ml over 2 min, 10 ml over 2 min, and 10 ml over 5 min. The cell suspension was centrifuged and the EAC4 washed with $DGVB^{++}$ until no further lysis occurred (usually 3 or 4 times).

The EAC4 were suspended to a concentration of 1 x 10^8/ml (OD_{414} .327) in $DGVB^{++}$, and stored at 4°C. The cell recovery varied from about 25 ml to 30 ml of cells at 1 x 10^8/ml, per batch.

2.6.5: Haemolytic Assay for Titration of C1

Purified human or guinea pig C1 (prepared as described, Section 2.4) was double diluted in $DGVB^{++}$ from about 1 in 2000 to 1 in 1024 000 dilution. 100 µl of diluted C1 was transferred to assay tubes and prewarmed to 37°C before the addition of 100 µl prewarmed EAC4 at 1 x 10^8/ml $DGVB^{++}$. The mixture was incubated with shaking at 37°C for 15 min, to allow formation of EAC14. The tubes and contents were transferred to a 30°C shaking water bath, and 100 µl human C2 at 50 units/ml, prewarmed to 30°C, was added to the appropriate tubes and incubated for the T_{max} time (see Section 2.6.6b). The haemolytic sites were developed by the addition of 300 µl C-RAT to the appropriate tubes, followed by incubation at 37°C for 1 hour with shaking. The controls included the reagent blank (RB) which accounts for any lysis which occurs not due to C1; the cell blank (CB) which accounts for spontaneous lysis of the EAC4 cells; the complement colour (CC) which accounts for any absorbance due to haemoglobin present in the C-RAT; and the 100% lysis, which was lysed with 2 ml of deionised water, and represents the 100% standard against which the lysis in other tubes were compared. The reagents added to these control tubes are tabulated in Table 9.

Control	DGVB^{++} µl	C2 µl	EAC4 µl	C-RAT µl	Saline (s) or Water 2 ml (w)
RB	200	-	100	300	S
CB	500	-	100	-	S
CC	300	-	-	300	S
100 %	100	100	100	300	W

Table 9: Controls for C1 Haemolytic Titration.

The EAC4 were suspended to a concentration of 1 x 10^8/ml (OD_{414}•327) in $DGVB^{++}$, and stored at 4°C. The cell recovery varied from about 25 ml to 30 ml of cells at 1 x 10^8/ml, per batch.

2.6.5: Haemolytic Assay for Titration of C1

Purified human or guinea pig C1 (prepared as described, Section 2.4) was double diluted in $DGVB^{++}$ from about 1 in 2000 to 1 in 1024 000 dilution. 100 µl of diluted C1 was transferred to assay tubes and prewarmed to 37°C before the addition of 100 µl prewarmed EAC4 at 1 x 10^8/ml $DGVB^{++}$. The mixture was incubated with shaking at 37°C for 15 min, to allow formation of EAC14. The tubes and contents were transferred to a 30°C shaking water bath, and 100 µl human C2 at 50 units/ml, prewarmed to 30°C, was added to the appropriate tubes and incubated for the T_{max} time (see Section 2.6.6b). The haemolytic sites were developed by the addition of 300 µl C-RAT to the appropriate tubes, followed by incubation at 37°C for 1 hour with shaking. The controls included the reagent blank (RB) which accounts for any lysis which occurs not due to C1; the cell blank (CB) which accounts for spontaneous lysis of the EAC4 cells; the complement colour (CC) which accounts for any absorbance due to haemoglobin present in the C-RAT; and the 100% lysis, which was lysed with 2 ml of deionised water, and represents the 100% standard against which the lysis in other tubes were compared. The reagents added to these control tubes are tabulated in Table 9.

2 ml saline (0.9% NaCl) was added to all tubes except the 100% lysis, to which 2 ml deionised water was added. The OD_{414} was determined on the supernatant after unlysed cells had been pelleted by centrifugation at 1000 g for 5 min at $4^{o}C$.

2.6.5a: Calculation of Results

The lysis (y) was calculated for each sample using the following formula:-

$$y = \frac{OD_{sample} - OD_{RB}}{OD_{100\%} - OD_{RB}} \qquad \text{equation 1}$$

The percentage lysis was represented by y multiplied by 100. Mayer and his colleagues (Borsos et al., 1961) demonstrated that the plot of y against the dilution of the component (x axis) was concave to the x axis, which led them to postulate the one hit theory of complement action. This was based on the hypothesis that complement mediated lysis was due to a single effective hit by a complement molecule, rather than to cumulative successive hits.

With mathematical manipulations, the number of effective molecules per cell (z) can be calculated on the basis of the above equation transformed to

$$z = -\ln(1-y) \qquad \text{equation 2}$$

When z was plotted against dilution of complement component (x axis, log scale) a straight line passing through the origin was obtained, as shown in Figure 7.

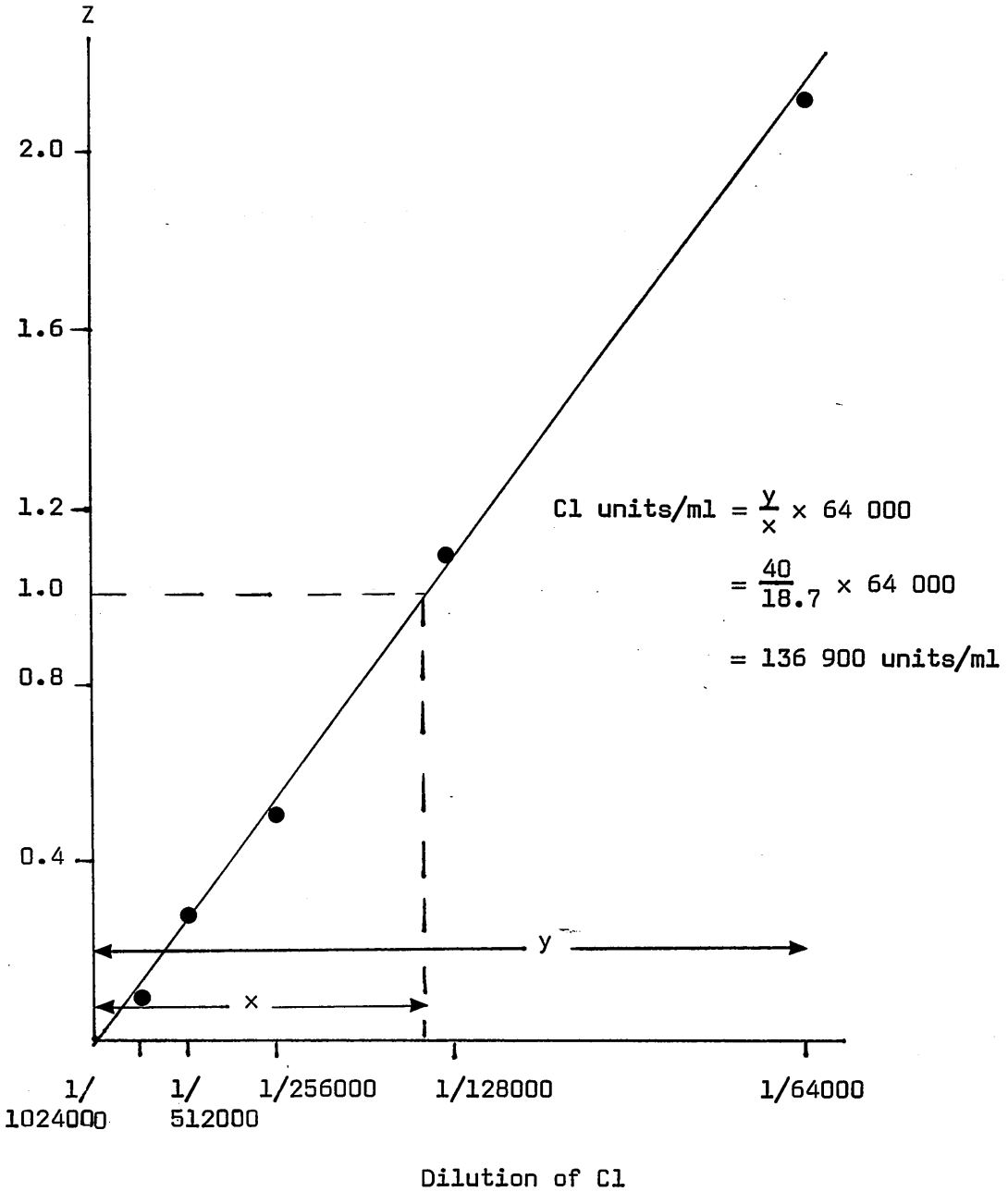


Fig. 7: Plot and calculation of haemolytic titration of Cl.

When z, the number of effective molecules per cell was equal to 1, then $-\ln(1-y) = 1$, therefore y must equal 0.632, that is 63.2% of the cells added were lysed. To standardise titrations, the effective molecules were calculated as follows: a vertical line was drawn from the point z = 1 to the corresponding dilution on the x axis. The units/ml were calculated as shown in Figure 7 as follows:

$$\frac{\text{Distance along x axis to initial dilution (y)}}{\text{Distance along x axis to z = 1 (x)}} \times \text{initial dilution}$$

equation 3

In the haemolytic assays 1×10^7 E/tube were used, therefore the concentration of effective molecules is represented as $\varepsilon m \times 10^7$/ml; in a titration of components the results are reported as units/ml.

2.6.6: Haemolytic Assay for C2

Stoichiometric haemolytic assays for C2 were performed on culture supernatants using purified C2 as a standard (Section 2.3), using a modified version of the method developed by Rapp and Borsos, 1970. The sample or C2 standard was incubated with freshly formed EAC1$\bar{4}$ cells, resulting in the production of C3 convertases on the E surface, which were developed by the addition of C-RAT in EDTA buffer which supplied the terminal lytic components, C3 to C9.

2.6.6a: Preparation of EAC1$\bar{4}$

Equal volumes of freshly washed EAC4s standardised to 1×10^8/ml in $DGVB^{++}$, and human C1 at 200 units/ml in $DGVB^{++}$ (prepared as in Section 2.4) were prewarmed to 37°C,

Controls	$DGVB^{++}$ µl	EAC14 µl	C2 µl	C-RAT µl	Saline (S) or Water (W) 2 ml
RB	100	100	-	300	S
CB	300	100	100	-	S
CC	200	-	-	300	S
100%	-	100	100	300	W

Table 10: Controls for T_{max} Assay.

mixed and incubated at 37°C with shaking for 15 min. The EAC14 cells were harvested by centrifugation at 1000 g for 5 min at 4°C and resuspended to their original volume in $DGVB^{++}$ i.e. at 1 x 10^8/ml.

2.6.6b: T_{max} Assay

The titration of C2 haemolytic activity is complicated by the natural decay of C2a from the C3 convertase (C4b2a), thus the kinetics of formation and decay of the convertase must be determined for each new batch of EAC4 prepared. The T_{max} is defined as the time taken for EAC142 formation to reach its maximum, and is a function of the concentration of C4b present on the EAC4 cells, and is independent of the concentration of C1 or C2. Thus, the more functionally active C4b present on the EAC4 cells the shorter the T_{max} time: the T_{max} for most of the batches of cells prepared varied from one to four minutes.

2.6.6c: Procedure for T_{max} Assay

3 ml EAC4 at 1 x 10^8/ml in $DGVB^{++}$ were converted to EAC14 as described previously (Section 2.6.6a) and warmed to 30°C. 3 ml purified C2 (Section 2.3) at 1 unit/ml was warmed to 30°C. The two solutions were mixed and incubated with shaking at 30°C; at timed intervals from 0.5 to 15 min, duplicate 200 µl of cell suspension were removed, transferred to tubes containing 300 µl C-RAT, incubated at 37°C for 1 hr to allow completion of the lytic sequence. The controls consisted of RB, CB, CC and 100%, the contents of which are

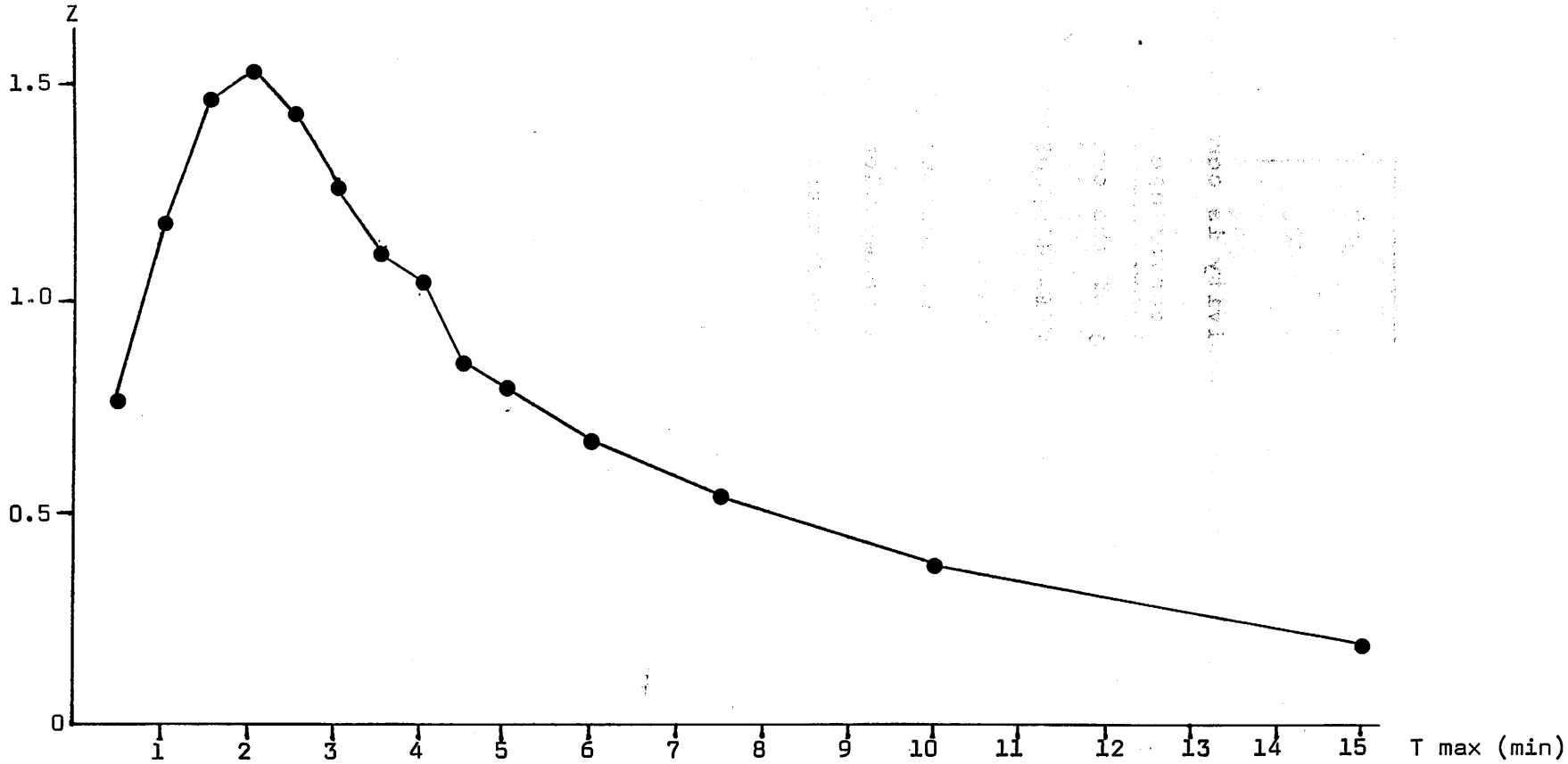


Fig. 8: Plot of T_{max} Assay.

detailed in Table 10. After incubation the samples were diluted with 2 ml saline, or water for the 100% lysis, the amount of lysis being determined spectrophotometrically as described previously (Section 2.6.5).

The number of effective molecules/cell (z) was calculated as described previously (Section 2.6.5a) and plotted against incubation time, as shown in Figure 8. The T_{max} was determined as the time when maximum lysis occurred: 2 minutes in Figure 8.

2.6.6d: C2 Assay Protocol

EAC14 prepared as described (Section 2.6.6a) were standardised to 1 x 10^8/ml in $DGVB^{++}$ and warmed to 30°C. Doubling dilutions of human purified C2 standard were titrated from about 1/2000 to 1/256000 and 100 µl of each added to tubes; samples of monocyte culture supernatants ranging from 5 µl to 50 µl were added to tubes containing 100 µl $DGVB^{++}$; all tubes were warmed to 30°C. At timed intervals 100 µl EAC14 were added to each tube, incubated at 30°C with shaking for the T_{max} time, after which 300 µl warm C-RAT was added and incubated for 1 hr at 37°C with shaking, to develop the lytic sequence. The controls included RB, RB', CB, CC and 100% containing the reagents as shown in Table 11. The reactions were terminated by the addition of 2 ml saline and the amount of lysis determined as described previously (Section 2.6.5). The number of effective molecules (z) were calculated as described previously (Section 2.6.5a, equations 1 & 2) for each sample, and the titration of the C2 standard plotted as described for

Controls	DGVB^{++} µl	EAC14 µl	C-RAT µl	RPMI-FCS µl	Saline (s) or Water (w) 2 ml
RB	100	100	300	-	S
RB'	100	100	300	As sample volume	S
CB	400	100	-	-	S
CC	200	-	300	-	S
100%	100	100	300	-	W

Table 11: Controls for C2 Assay.

C1 (Fig. 7). The z was corrected to 1 ml for the culture supernatants taking into account the sample volume used in the assay; for example for a 5 µl sample, the z value obtained would be multiplied by 200, to give z/ml for the sample.

2.6.7: Haemolytic Assay for C1-inhibitor

The haemolytic assay for C1-inhibitor is based on its ability to bind to fluid-phase C1, and therefore inhibit the formation of EAC14 and thus reduce haemolysis. The procedure used was a modification of the method developed by Gigli, Ruddy and Austen (1968). The assay involves four steps consisting of the fluid-phase reaction of a limiting amount of C1 with C1-inhibitor; incubation of the resulting mixture with EAC4, allowing reaction of any unreacted C1 to form EAC14; formation of the C3 convertase (EAC142) by incubation with purified C2; and development of the lytic sequence by the addition of C-RAT.

2.6.7a: Procedure

Purified guinea pig C1 ($C1^{gp}$) (Section 2.4) was titrated as described (Section 2.6.5) and standardised to give a limiting amount of C1 of about 1 to 1.5 u/ml. The culture samples to be assayed were titrated by performing 100 µl doubling dilutions in $DGVB^{++}$, starting at about 1/50 dilution. Similar doubling dilution titrations were performed in $DGVB^{++}$ with purified C1-inhibitor to serve as a standard, and culture medium (concentrated Hams F12 or RPMI/20% FCS) to assess background inhibition of the assay due to culture medium and not to C1-inhibitor activity.

Controls	$DGVB^{++}$ µl	EAC4 µl	C1 µl	C2 µl	C-RAT µl	Saline (s) or Water (w) 2 ml
RB	200	100	-	100	300	S
CB	600	100	-	-	-	S
CC	400	-	-	-	300	S
100%	100	100	100	100	300	W
C1 Solo	100	100	100	100	300	S

Table 12: Controls for C1-inhibitor Assay.

All samples and solutions were prewarmed to 30°C. $C1^{gp}$ (100 µl) was added to all tubes containing the samples, purified C1-inhibitor or controls, and incubated for 30 min at 30°C to allow C1-inhibitor to interact with C1. EAC4, standardised to 1 x 10^8/ml, were prewarmed to 30°C and 100 µl of the suspension added to each tube and the incubation continued for a further 30 min at 30°C. This step allowed formation of EAC14 to proceed. Human C2, at 50 units/ml, was prewarmed to 30°C and 100 µl added to each tube for the T_{max} time, to allow formation of the C3 convertase. The lytic sequence was developed by the addition of 300 µl C-RAT to each tube, followed by incubation at 37°C for 1 hr with shaking. Addition of saline and the measurement of lysis were carried out as described previously (Section 2.6.5). The controls included RB, CB, CC, 100% and a C1 solo which contained the components as detailed in Table 12.

The haemolytic assay for C1-inhibitor depends upon a limiting amount of C1, thus it would be very difficult to perform this assay in serum. Gigli, Ruddy, and Austen (1968) developed a method by which serum can be depleted of most of its C1, and thus allow measurement of C1-inhibitor functional activity.

Serum was diluted 1 in 20 in 5 mM sodium phosphate buffer pH 7.5, adjusted to pH 7.5, and stirred slowly overnight at 4°C. The resulting C1 euglobulin precipitate was removed by centrifugation at 6000 g for 10 minutes at room temperature, the supernatant was then used to perform a C1-inhibitor haemolytic assay, as described above.

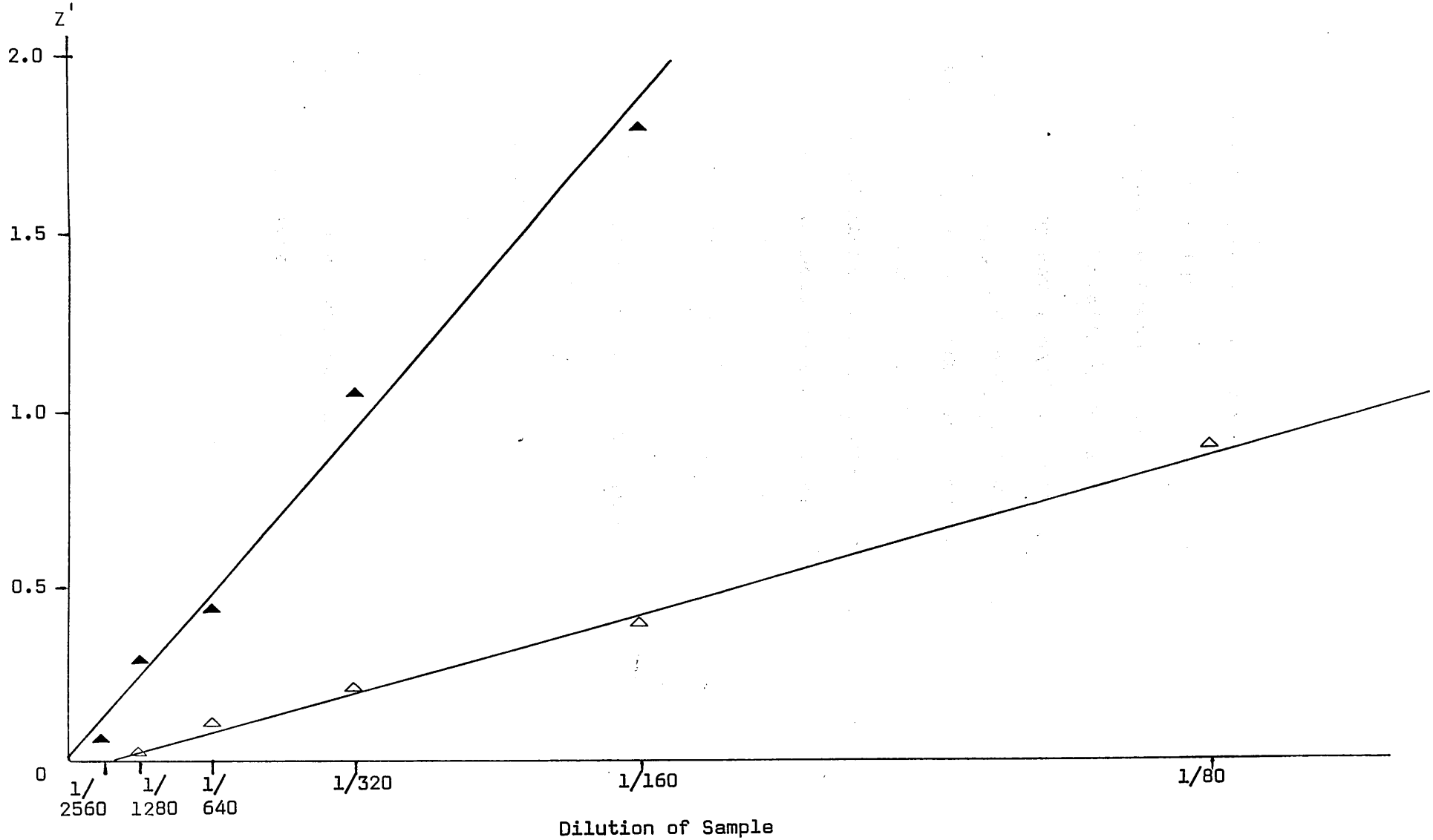


Fig. 9: Haemolytic Titration of C1-inhibitor activity in Monocyte Culture supernatant (▲, 298 u /ml) and RPMI/20% FCS (△, 69 units/ml), with a C1 solo value of 1.3 units/ml: thus corrected titrations were 387 units/ml for culture supernatant and 90 units/ml for RPMI/20% FCS.

2.6.7b: Calculation of Results

C1-inhibitor activity is expressed in inhibitory units (z' units), calculated as follows:-

$$z' = -\ln\left(\frac{OD\ sample - RB}{OD\ solo - RB}\right)$$

The z' values were plotted (y axis) against the sample dilution (x axis log dilution) as shown in Figure 9, and the graph used to calculate inhibitory units/ml, as described previously for C1 (Section 2.6.5a).

The titre of C1-inhibitor activity has been defined by Gigli, Ruddy and Austen (1968) as the product of the reciprocal of the dilution producing z' = 1.0 and the number of haemolytic units (z) present in the uninhibited C1 solo reaction. Thus if the C1 solo generates for example 1.32u/ml, then the z' titration for the sample (equal to 298 u/ml, Fig. 9) must be multiplied by 1.3, thus the titration for this sample would be estimated at 387 units/ml. This enables standardisation of all C1-inhibitor assays to a C1 solo of z = 1, and therefore assays performed at different times with different C1 solo values, may be directly compared.

In C1-inhibitor functional assays performed on monocyte culture supernatants containing 20% FCS, there was an inhibitory effect due to the 20% FCS alone. Thus, when RPMI/20% FCS culture supernatants were titrated, fresh RPMI/20% FCS was titrated in the same assay, at the same dilutions. In the calculation of results, the titrations were estimated for the monocyte culture supernatant and for the RPMI/20% FCS alone, which was subtracted from the supernatant value, in order to

correct for the background. As shown in Figure 9, the titre for the monocyte culture supernatant was estimated at 387 u/ml and for the RPMI/20% FCS 90 u/ml, therefore the actual titre due to C1-inhibitor activity synthesised by the monocyte and secreted into the culture supernatant, was 297 u/ml.

2.7: Enzyme-linked Immunosorbent Assay (ELISA) for Complement Components

The ELISA procedure used was a double antibody sandwich ELISA; the conjugates were prepared using the periodate method which linked horse radish peroxidase (HRP) to the purified IgG fractions of specific antisera. Incorporation of dilutions of a standard serum containing a known concentration of component being measured, enabled the construction of a standard curve and allowed estimation of concentrations present in unknown samples.

2.7.1: Materials used in ELISA

Sigma Chemical Company, Poole, Dorset.	:	Horse Radish Peroxidase (HRP) Tween 20 Type VI Bovine Serum Albumin Fraction V O-phenyldiamine (OPD)
Dr Michael Kerr, Ninewells Hospital, Dundee.	:	Antiserum to human C_2
Atlantic Antibodies (ATAB) American Hospital Supply (U.K.) Ltd., Didcot, Oxfordshire.	:	Antiserum to human C3 (IgG fraction) factor B (IgG fraction) properdin (IgG fraction)
Dynatech Laboratories Ltd., Billingshurst, Sussex.	:	96 well flat-bottom micro-elisa plates.

2.7.2: Preparation of Reagents for ELISA

Wash buffer: 0.05% Tween 20 in PBS (PBS-Tween).

PBS was prepared as described (Section 2.1.1) and 0.5 ml Tween 20 added to each 1 litre PBS.

Coating Buffer: 0.05M Carbonate-bicarbonate buffer pH 9.6

Coating buffer was prepared by dissolving 0.79g sodium carbonate and 1.46g sodium hydrogen carbonate in deionised water, to a final volume of 500 ml.

Blocking Buffer: 0.1% BSA in PBS

Blocking buffer was prepared by dissolving 0.5g BSA in 500 ml PBS, pH 7.4.

0.1M Citric Acid

Prepared by dissolving 10.5g citric acid in deionised water, to a final volume of 500 ml.

0.2M $Na_2H\ PO_4$

Prepared by dissolving 14.2g Na_2HPO_4 in deionised water, to a final volume of 500 ml.

Substrate Solution: citrate-phosphate buffer containing ODP and H_2O_2.

Substrate solution pH 5.6, was prepared by mixing approximately 14 ml 0.2M Na_2HPO_4 and approximately 11 ml 0.1M citric acid, the pH checked and the solution diluted to a final volume of 50 ml with deionised water. To this was added 17 mg O-phenyldiamine (OPD) and, just before use, 20 µl 30% hydrogen peroxide. This solution was light-sensitive and was protected from light.

2.7.3: Periodate Method of Conjugating IgG and HRP (Wilson and Nakane, 1978)

The method will be described for the conjugation of 12 mg IgG to Horse radish peroxidase (HRP), and can be scaled up or down, depending on the amount of IgG to be conjugated.

6 mg HRP was dissolved in 1 ml deionised water and 0.2 ml, freshly prepared 0.1M sodium periodate solution was added; the resulting solution was stirred at room temperature for 20 min. The solution was transferred to 1/4" diameter dialysis tubing and dialysed against four, one hourly changes of 2 litres 1 mM sodium acetate buffer pH 4.4, followed by an overnight dialysis at 4°C against the same buffer.

The dialysate was transferred to a glass universal, 1 ml carbonate-bicarbonate buffer pH 9.6 was added along with 12 mg IgG (i.e. twice the weight of IgG to HRP used). The solution was stirred for 2 hr at room temperature. 0.1 ml freshly prepared 0.1M sodium borohydride solution was added, and the resulting solution allowed to stand at 4°C for at least 2 hr. It was then transferred to dialysis tubing and dialysed twice against 1 litre of saline for 30 minutes, and once against 1 litre PBS for 30 min.

The conjugate was ready for testing, and was stored aliquoted at 4°C with 0.01% thiomersal.

In order to determine the optimum conditions for each ELISA assay, checkerboard experiments were performed where various concentrations of IgG were used to coat the plate, for example 1, 2.5 and 5 µg IgG/ml; this was cross-reacted with various dilutions of HRP-IgG conjugate, for example,

Complement Component	Antibody (IgG) Source	Coating IgG µg/ml	Conjugate Dilution	S6STD µg/ml
C2	M. Kerr	10	1/500	Est. 20
C3	ATAB	5	1/5000	1194
B	ATAB	5	1/5000	207
P	ATAB	5	1/2000	Est. 30

Table 13: ELISA assay conditions and serum standard 6 values.
C2 and properdin values were estimated as no standardised serum was available.

1/250, 1/500 and 1/1000 dilutions. Three suitable dilutions of a serum standard covering a wide concentration range were used along with a PBS-Tween blank, to determine which conditions gave the best standard curve with the lowest backgrounds. These conditions were then used in subsequent assays using the same conjugate and coating antibody. A chequerboard assay was performed for each of the ELISA procedures established, and the resulting conditions for each assay, and source of antiserum are summarised in Table 13. Most of the assay conditions were determined by Dr Lindsay Morrison, who also kindly supplied most of the reagents used in the ELISA assays performed.

2.7.4: ELISA Protocol

This method will be described for the C2 ELISA assay; however all other assays were performed in a similar manner using the IgG coating concentrations and conjugate dilutions stated in Table 13. All incubations unless stated were carried out at room temperature in a humidified container, in the light.

ELISA plates (96 well) were coated with IgG anti-C2 at 10 µg/ml in carbonate-bicarbonate buffer pH 9.6, by adding 100 µl/well using a Titertek multichannel pipette. The plates were incubated overnight at $4^{o}C$ in a humidified atmosphere to allow IgG binding.

The plate was washed with PBS containing 0.05% Tween-20 (PBS Tween) using a Dynatech autowash 2000, the programme

consisting of four wash cycles, followed by an aspiration cycle. All subsequent washes were carried out in a similar manner.

Unreacted sites on the wells were blocked by the addition of 250 µl PBS + 0.1% BSA (blocking buffer) per well, and incubated for 1 hour. After washing the plate, samples of monocyte culture supernatant were added, the volumes used ranging from 25 µl to 100 µl, with a final volume of 100 µl/well being made up by the addition of PBS-Tween. Serum standard (S6 kindly donated by Mrs Jean Veitch) was diluted in PBS-Tween from 1/50 to 1/2500, and 100 µl added to the plate in duplicate; PBS-Tween blanks of 100 µl were added to two wells, as was RPMI/20% FCS, the volume used being the same as that of the culture supernatant sample.

The samples were incubated for 2 hr, a wash procedure executed and the conjugate added: HRP anti-C2 at a dilution of 1/500 in PBS-Tween was used, and 100 µl added to all wells. After incubation for 1 hr, the conjugate was washed out and 100 µl substrate added per well. The next 30 min incubation was carried out in the dark at room temperature as OPD is light sensitive. Normally 30 min was the incubation time used, however, in some assays the conjugate appeared to react very rapidly with the substrate, and hence required very short incubation periods, for example C3 only required 5 min incubation, and properdin 20 minutes incubation.

The reaction was stopped by the addition of 25 µl/well 4N H_2SO_4. The absorbance of the solutions in each of the wells was read at OD 492 nm using a Dynatech MR700 microplate

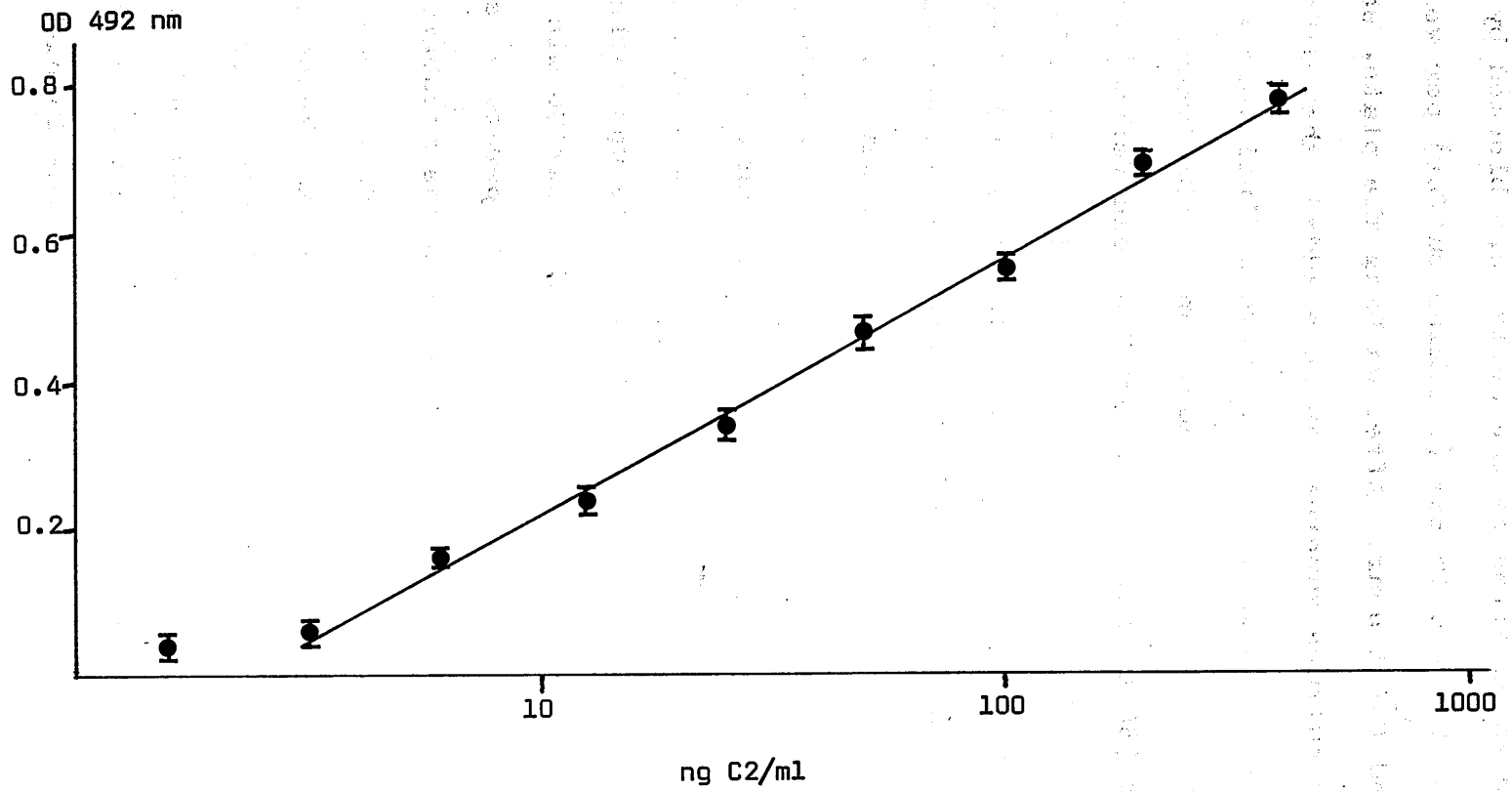


Fig. 10: ELISA standard curve for C2.

reader, zeroing the machine on the PBS-Tween blank.

2.7.5: Calculation of Results

A plot of the OD 492 nm (y axis) against the $\log_{10}$ of the concentration of C2 standard (x axis) was constructed as shown in Figure 10. The linear portion of the graph (3.13 ng/ml to 400 ng/ml) was used to perform a linear regression analysis, and the corresponding concentrations of complement components calculated for each sample. If a culture supernatant sample of less than 100 µl was used in the assay, the concentration was corrected for this, for example if a 25 µl sample gave a result of 3 ng/ml, then the actual concentration corrected for sample volume would be 12 ng/ml ($3 \times \frac{100}{25}$).

2.8: Radioimmunoassay for C1-inhibitor

A competetive double-antibody radioimmunoassay (RIA) was developed for measuring C1-inhibitor in monocyte cultures, using a modification of the procedure as first described by Morgan and Lazarow (1963). Purified human C1-inhibitor (Section 2.2) was radiolabelled with I^{125} using the Chloramine T method (McConahey and Dixon, 1966). However the radiolabelled protein failed to react with antibody to C1-inhibitor, probably because it had been denatured during the radiolabelling procedure. Successful iodination was achieved using the Bolton and Hunter reagent, which is a radiolabelled ester to which the protein in question is conjugated.

The RIA developed was a competitive double-antibody assay which involved two stages:- the reaction of C1-inhibitor antigen with rabbit antiserum to C1-inhibitor, followed by the precipitation of the antigen-antibody complexes by the addition of a second antibody. The second antibody used was a donkey anti-rabbit IgG, with normal rabbit serum (NRS) as the carrier protein, both used at predetermined dilutions which produced maximum precipitation. A carrier protein was used since precipitation only occurs at high concentrations of antigen and antibody, thus the NRS acted as a source of rabbit IgG to assist in the precipitation of the primary complexes formed between C1-inhibitor and its antiserum.

2.8.1: Materials Used in Radiolabelling of C1-inhibitor and RIA

Sigma Chemical Company, Poole, Dorset.	:	Bovine serum albumin, Fraction V.
Sarsted, Leicester	:	4.5 ml conical plastic tubes.
Amersham International	:	Bolton and Hunter Reagent in benzene solution.
Scottish Antibody Production Unit, Blood Transfusion Centre, Law Hospital, Carluke.	:	Normal Rabbit Serum Donkey anti-rabbit IgG.
Pharmacia, Milton Keynes, Buckinghamshire.	:	Sephadex G50 (medium grade).

2.8.2: Preparation of Solutions used in Radiolabelling of C1-inhibitor and RIA

0.1M borate buffer pH 8.5

0.1M borate buffer pH 8.5 with 0.2M glycine (0.75g glycine/ 50 ml buffer)

0.1M sodium phosphate buffer pH 7.6 with 0.25% gelatin.
0.01M sodium phosphate buffer pH 7.6 with 1% BSA.

0.01M EDTA/PBS with 1% BSA

Prepared by mixing 13 ml 0.086M EDTA with 100 ml PBS and dissolving 1.13g BSA in the solution.

2.8.3: Radiolabelling of C1-inhibitor with Bolton and Hunter Reagent

Purified human C1-inhibitor was dialysed against 0.1M borate buffer pH 8.5 prior to iodination. The Bolton and Hunter reagent was supplied as a solution in benzene; prior to use the benzene was evaporated by directing a fine stream of dried nitrogen gas onto the surface. The nitrogen was dried by passing through a column of calcium chloride chips, which had been previously dried by heating in an oven at 160°C for two to three hours. The nitrogen/benzene vapour leaving the reaction vial was bubbled through a trap containing sodium hydroxide solution to absorb any I^{125} evaporated during the process.

The vial containing the dry Bolton and Hunter reagent (1.7 mCi for the experiment reported) was opened carefully, placed on ice and 5 µg of the dialysed C1-inhibitor were added (a volume of 20 µl) and incubated on ice for 30 min, with occasional gentle shaking.

The reaction was stopped by the addition of 0.5 ml of 0.1M borate buffer pH 8.5 containing 0.2M glycine, with further incubation at 0°C for 10 min. Two 10 µl samples were removed from the vial and added to two glass tubes containing

250 µl 0.01M phosphate buffer pH 7.6 with 1% BSA. These were used for counting to determine the radioactivity present in the starting material.

The remaining solution was then separated into free iodine and I^{125} bound to C1-inhibitor by applying to a 10 ml column of Sephadex G50 (medium grade) which had previously been equilibrated in 0.05M phosphate buffer pH 7.6 containing 0.25% gelatin. The vial was washed out with 0.5 ml of the same buffer, which was also applied to the column. 20 to 30 fractions consisting of 10 drops were collected in glass tubes containing 250 µl 0.01M phosphate buffer pH 7.6 with 1% BSA (w/v), whilst the column was being washed with 0.05M phosphate buffer pH 7.6 containing 0.25% gelatin.

2.8.4: Counting of Radioactivity and Calculation of Labelling Efficiency

Counting of radioactivity in the fractions, starting material, Sephadex and vial were kindly performed by Mrs Sylvia Johnstone in the Gardiner Institute, using an external counter. Counts per 10 seconds were taken with the samples in their containers balanced in a glass beaker on top of the counter. The results obtained were used to calculate the specific activity of the label, the % efficiency of the conjugation reaction, the protein recovery, and hence the dilution to be performed to standardise the I^{125}-C1-inhibitor to 50 ng/ml. The calculations were performed as described below.

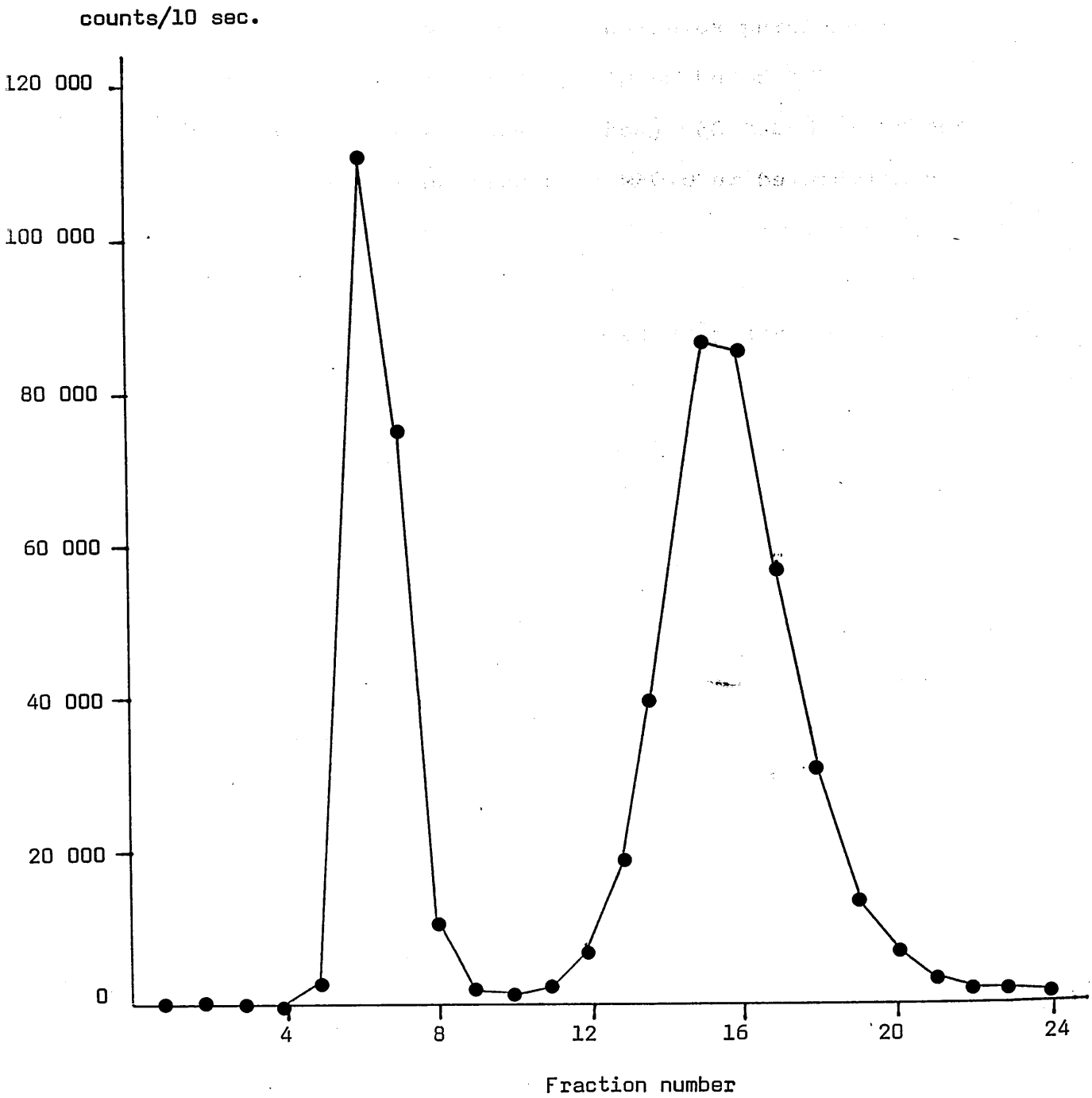


Fig. 11: Sephadex G50 Profile for C1-inhibitor Bolton and Hunter Labelling.

Table 14 shows the counts per 10 seconds obtained in the starting material and the fractions from the Sephadex G50 column (Fig. 11). These counts were converted to µCi on the basis of a fresh 1m Ci standard of I^{125} giving 622 525 counts in 10 seconds.

10 µl of starting material which represent 1/52 of the total volume (500 µl borate buffer with glycine and 20 µl C1-inhibitor) gave 20 638 c/10s, thus the total counts applied to the column was 1 073 176, representing 1724 µCi. The total radioactivity (RA) accountable in the vial was calculated as follows:-

RA over column + RA remaining in vial + RA remaining in Sephadex = 1724 + 32.9 + 6.8 = 1763.7 µCi.

The percentage of total counts recovered in the protein pool from the column was calculated:-

$$\frac{201\ 182}{569\ 785} \times 100 = 35\%$$

The radioactivity in the protein pool was calculated:-

$$\frac{201\ 182}{622\ 525} = 323.2\ \mu Ci$$

The assumption was made that the 5 µg C1-inhibitor was all labelled, and that the resulting I^{125}-C1-inhibitor was evenly distributed throughout the solution initially contained in the vial. Since there was radioactivity remaining in the original vial and on the Sephadex, both must contain some I^{125}-C1-inhibitor. Therefore to account for the total radioactivity in the 5 µg C1-inhibitor used, the following calculation must be performed:-

Fraction Number	Counts per 10 Sec.
1	5
2	3
3	3
4	6
5	3048
6	111 737
7	75 974
8	10 423
9	2 378
10	1 826
11	2 518
12	7 032
13	19 727
14	48 503
15	87 192
16	85 696
17	56 075
18	30 847
19	13 009
20	6 496
21	3 405
22	1 802
23	1 227
24	853
Starting Material (10 µl)	20 638
Total Counts (F1-24)	569 785
Protein Pool (F5-8)	201 182
Protein Peak (F6.7)	187 711
1m Ci standard	622 525

Table 14: Radioactivity in Column fractions, starting material and 1m Ci standard.

RA in vial + RA in Sephadex + RA in Protein Pool from Column = 32.9 + 6.8 + 323.2 = 362.9 μCi.

Thus the specific activity of the I^{125}-C1-inhibitor was calculated as:-

$$\frac{362.9}{5} = 72.6 \text{ μCi/μg C1-inhibitor.}$$

The % yield of conjugation was:-

$$\frac{362.9 \times 100}{1000} = 36.3\%$$

The % C1-inhibitor recovered from the column was calculated:-

$$\frac{323.2}{362.9} \times 100 = 89.1\%$$

Therefore the mass of C1-inhibitor recovered was calculated:-

$$\frac{89.1}{100} \times 5 = 4.45 \text{ μg.}$$

Not all of the I^{125}-C1-inhibitor protein pool was used in the RIA, only the two or three fractions containing the highest counts were pooled, in this case fractions 6 and 7 (Table 14).

C1-inhibitor recovered in fractions 6 and 7 was calculated:-

$$\frac{187711}{201182} \times 4.45 = 4.15 \text{ μg.}$$

C1-inhibitor for use in the RIA was stored at a concentration of 50 ng/ml; since fractions 6 and 7 contained 4150 ng, they were diluted in 0.05M phosphate buffer pH 7.6 containing 1% BSA to a final volume of 83 ml. This was aliquoted into 2 ml fractions and stored at -20°C until use.

2.8.5: <u>TCA Precipitation of I^{125} C1-inhibitor</u>

A TCA precipitation assay was performed to determine the amount of TCA-precipitable I^{125}-labelled C1-inhibitor present.

200 µl 10% BSA (w/v) in PBS was added to each of 6 tubes; 10 µl I^{125}-C1-inhibitor at 50 ng/ml was added to three of the tubes and mixed, the other three tubes acted as backgrounds. 1ml cold 20% TCA was added to all six tubes, vortexed and the resulting precipitate pelleted by centrifugation at 6000g for 10 min at room temperature.

The supernatants were decanted and placed in glass carrier tubes, as were the pellets. All samples were counted in a Packard γ counter, and the % TCA precipitable I^{125}-C1-inhibitor calculated thus:-

$$\frac{\text{(Pellet - background pellet) x 100}}{\text{(Pellet-background pellet) + (Supernatant-background supernatant)}}\%$$

2.8.6: Precipitin Curve of Normal Rabbit Serum (NRS) and Donkey anti-rabbit IgG (D α R IgG)

After the initial reaction of C1-inhibitor with rabbit anti-C1-inhibitor, the complexes formed must be precipitated by the addition of donkey anti-rabbit IgG (D α R IgG), using NRS as a carrier for a source of rabbit IgG. However, before this second step could be undertaken a precipitin curve of NRS and D α R IgG had to be performed to determine the optimum conditions for precipitation by the second antibody (D α R IgG).

NRS was serially diluted in 1% BSA in PBS (w/v) from 1/25 down to 1/1600 and 100 µl of each dilution added in triplicate to a set of tubes containing 200 µl 1% BSA in PBS. D α R IgG was added (100 µl) to each set of tubes at three dilutions: 1/2.5, 1/5 and 1/20, and incubated for 1 hr at 37°C with shaking. After an overnight incubation at 4°C, the insoluble complexes were precipitated by centrifugation

Dilution of NRS	µg Protein precipitated with D & R IgG at 1/2.5	1/5.0	1/20.0
1/25	120	100	32
1/50	140	140*	20
1/100	160	103	18
1/200	65	50	30
1/400	10	12	0
1/800	0	0	0
1/1600	0	0	0

Table 15: Results of Precipitin Curve between NRS and Donkey anti-rabbit IgG (D ∝ R IgG).

* indicates the conditions chosen for the second antibody:donkey anti-rabbit IgG at 1/5 and NRS at 1/50.

at 6000g for 10 min. at room temperature, washed twice in cold PBS, and the final precipitate dissolved in 0.2 ml 1M sodium hydroxide. A Folin assay was performed on the precipitates using BSA as a standard curve (Section 2.1.4b).

The results, shown in Table 15, resulted in the use of D ∝ R IgG at a dilution of 1/5, with NRS at a dilution of 1/50.

2.8.7: Determination of Antiserum Dilution for use in C1-inhibitor RIA

The second antibody precipitation conditions were determined by performing a precipitin reaction; however such small quantities of C1-inhibitor and antiserum were used in the formation of the initial complexes that they would be undetectable by this method. Therefore, a chequerboard titration was set up where a purified C1-inhibitor standard was titrated from about 3 µg/ml down to about 1.5 pg/ml and various batches of rabbit anti-human C1-inhibitor antiserum (Section 2.1.7) were diluted 1/2000, 1/4000, 1/8000 and 1/16 000 in assay buffer (0.01M EDTA/ PBS with 1% BSA v/w). Each set of standard curves were cross-reacted with each dilution of antiserum (100 µl of each) in the presence of 400 µl assay buffer and 100 µl I^{125} C1-inhibitor at 5 ng/ml. After an overnight incubation at $4^{o}C$, 100 µl NRS (diluted 1/50) and 100 µl D ∝ R IgG (diluted 1/5) were added to all tubes, vortexed and incubated overnight at $4^{o}C$. The insoluble complexes were precipitated by centrifugation at 1000 g for 30 min at $4^{o}C$, the supernatants aspirated and the radioactivity

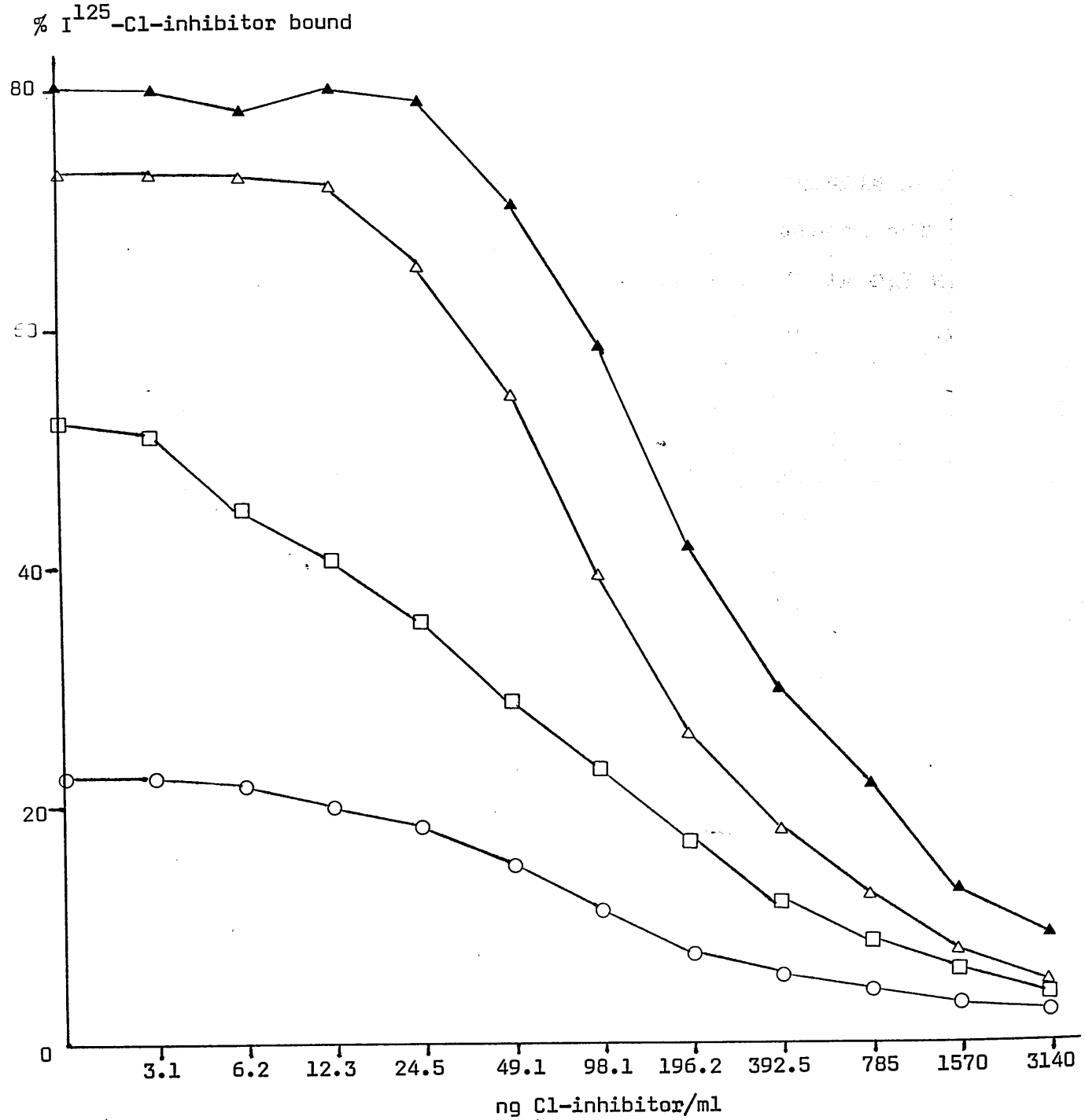


Fig. 12: <u>Standard Curves for C1-inhibitor RIA:</u> antiserum dilutions used were 1/2000 (▲), 1/4000 (△), 1/8000 (□) and 1/16000 (○).

in the pellets counted in a γ -counter.

The controls included a total input, where 100 µl I^{125}-C1-inhibitor was added and the supernatant was not removed, and a set of backgrounds which did not receive any I^{125}-C1-inhibitor. To determine the amount of I^{125}-C1-inhibitor which was immunoprecipitable, a set of three tubes containing no cold C1-inhibitor and 100 µl I^{125} C1-inhibitor, and 100 µl anti-C1-antiserum at a low dilution (1/250); the samples were then processed as described above.

The results obtained were used to calculate the percentage I^{125}-C1-inhibitor bound by the antibody as follows:-

$$\frac{\text{CPS sample - CPS background}}{\text{CPS total - CPS background}} \times 100\%$$

The % I^{125}-C1-inhibitor bound was plotted (y axis) against a log scale of C1-inhibitor concentration (x axis). The results obtained for rabbit antiserum $R2_3$ at 1/2000, 1/4000, 1/8000 and 1/16 000 dilutions are shown in Figure 12. The antibody dilution which should be chosen is that which binds about 50% of the input I^{125}-C1-inhibitor and gives a slope which has a sufficiently steep gradient, and is sufficiently long that small differences can be determined accurately and small amounts of C1-inhibitor can be measured with precision. The dilution chosen for the antiserum under study in Figure 12 was 1/8000; however the other antisera were used at dilutions ranging between 1/4000 and 1/12 000.

Sample	Assay Buffer (µl)	I^{125}-C1-inh. at 5 ng/ml (µl)	Purified C1-inh. standard (µl)	S5 or S6 at 1/10000 (µl)	Culture Supern./ medium (µl)	αC1-inh. (µl)	NRS (1/50) (µl)	D ≪ R IgG (1/5) (µl)
Total Input	500	100	0	0	0	100	100	100
Background	600	0	0	0	0	100	100	100
Standard curve	400	100	100	0	0	100	100	100
Standard serum	400	100	0	100	0	100	100	100
Supernatant Samples	450 400 300	100	0	0	50 100 200	100	100	100
RPMI/20% FCS	450 400 300	100	0	0	50 100 200	100	100	100

Table 16: <u>C1-inhibitor RIA Protocol</u>.

2.8.8: Cl-inhibitor RIA Protocol

All dilutions were performed in assay buffer which consisted of 1% BSA (w/v) in 0.01M EDTA/PBS; the assays were performed in plastic 4.5 ml conical centrifuge tubes. Purified Cl-inhibitor of a known concentration was used as a standard and serially diluted in triplicate from about 3 µg/ml to about 1.5 ng/ml. Serum standard S6 was diluted 1/10 000 and included in each assay to monitor the inter-assay variation. A tissue-culture medium blank consisting of RPMI/20% FCS was included to ensure no background Cl-inhibitor was present in the foetal calf serum.

The assay was set up, with controls, as described in Table 16: 400 µl assay buffer, 100 µl I^{125} Cl-inhibitor 100 µl purified Cl-inhibitor standard or monocyte culture supernatant (50 µl to 200 µl) and 100 µl anti-Cl-inhibitor antiserum (predetermined dilution). After an overnight incubation at 4°C, 100 µl NRS (diluted 1/50) and 100 µl D∝R IgG (diluted 1/5) were added to all tubes, vortexed and incubated overnight at 4°C.

All tubes except the total input and the background tubes were centrifuged at 1000 g for 30 min at 4°C to pellet the insoluble complexes. The supernatants were aspirated and the radioactivity in the pellets and control tubes counted in the γ counter.

The % bound I^{125} Cl-inhibitor was calculated as described previously and used to construct a standard curve such as that shown in Figure 13 and described previously (Section 2.8.7). The linear area of the graph was used to perform a linear

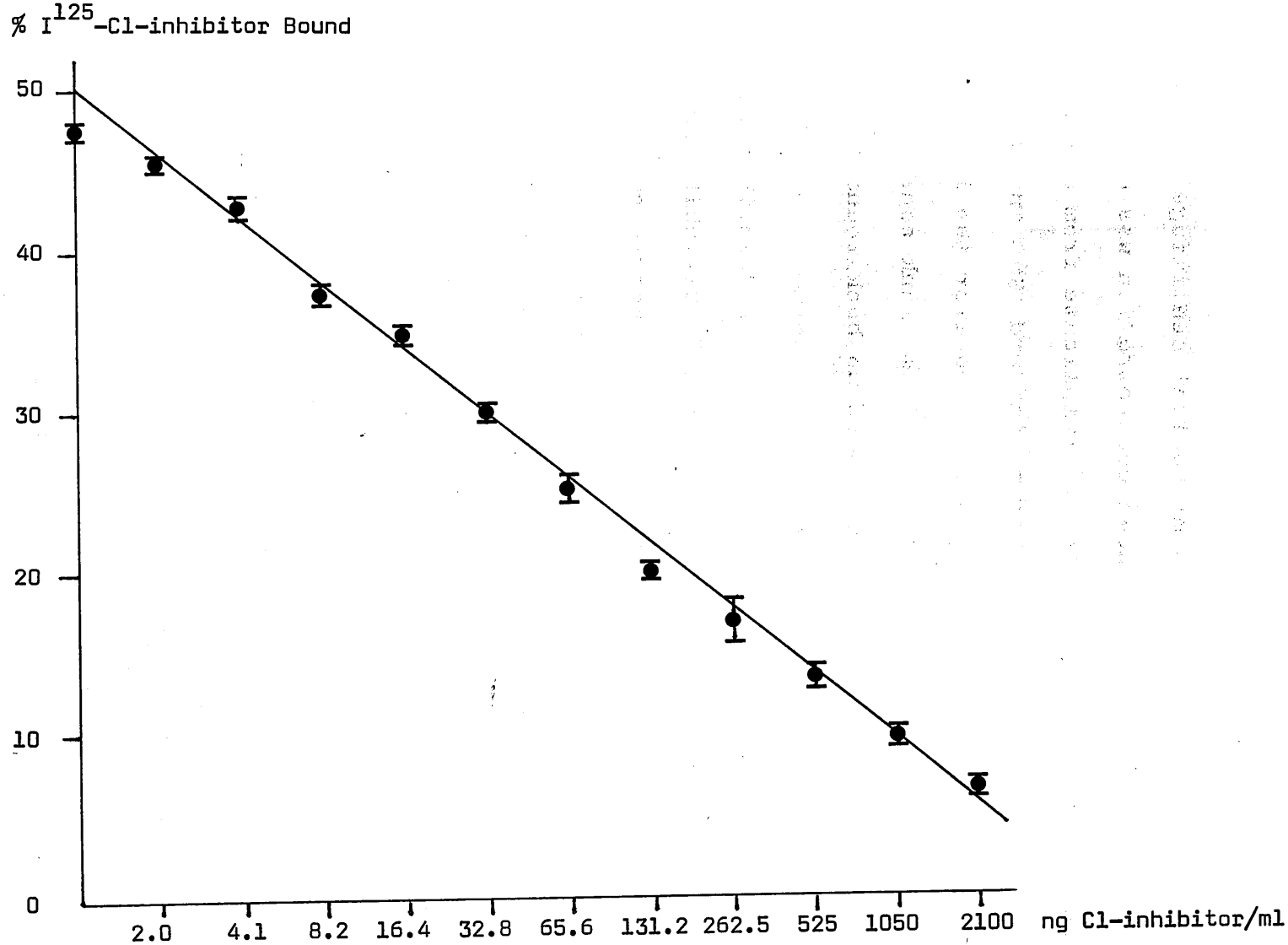


Fig. 13: Cl-inhibitor RIA Standard Curve.

regression analysis, and the amount of C1-inhibitor in the monocyte culture supernatant samples was calculated.

2.9: Estimation of DNA Content of Monocyte Cultures

The DNA content of the monocyte cultures was an important parameter to measure since it allowed standardisation between different monolayers, and enabled direct comparison between cultures containing different numbers of cells, to be made. Thus, after haemolytic assays, ELISA, and RIA were performed, the value of complement component obtained for each well was divided by the DNA for the same well, to express all results as for example pg C1-inhibitor/µg DNA.

The DNA assay used was a modified version of the method described by Cesarone, Bolognesi and Santi (1979), which was based on the increase in fluorescence at excitation-emission wavelengths of 350/490 nm or at 360/450 nm, when Hoechst 33258, a benzimidazole derivative, reacted with double stranded DNA.

2.9.1: Materials used in DNA Assay

Sigma Chemical Company, Poole, Dorset.	:	Lauryl sulphate (SDS) Calf thymus DNA. Hoechst 33258.

2.9.2: Preparation of Materials used in DNA Assay

DNA Stock Solution: 100 µg DNA/ml.

DNA was dissolved by prolonged stirring at 100 µg DNA per 1 ml deionised water.

Hoechst 33258: Stock solution 7.5×10^{-3}M

A stock solution of 100 times concentrated dye was prepared by dissolving 0.4g in 100 ml deionised water. This was stored frozen at -20°C, thawed and diluted 1/100 in PBS before use, to give a 7.5×10^{-5}M solution.

2.9.3: Protocol for DNA Assay

At the end of the culture period, supernatants were harvested, transferred to microcap tubes and any non-adherent cells harvested by centrifugation at 300 g for 5 min at room temperature. This was repeated for three 1 ml aliquots of RPMI/20% FCS, which acted as backgrounds for the supernatants, and were treated similarly throughout. The supernatants were transferred to a new set of microcap tubes and stored at -70°C until further use. The DNA assay on adherent and pelleted non-adherent cells, could be performed immediately, or the samples stored at -20°C and assayed at a later date.

The adherent and non-adherent cells were treated similarly: the monocytes were lysed by the addition of 200 µl 0.05% SDS to each sample, mixed well, and diluted by the addition of 1 ml PBS to each sample. The contents of each adherent and non-adherent cell lysate (1.2 ml) was transferred separately to a set of tubes containing 4 ml PBS.

A standard curve was constructed, consisting of 0, 0.5, 1, 2, 3, 4 and 5 µg DNA using the stock DNA solution (100 µg/ml). To each standard 200 µl 0.05% SDS was added, and the final volume made up to 5.2 ml with PBS. 20 µl Hoechst dye at

7.5×10^{-5} M was added to all tubes, vortexed and incubated in the dark for a minimum of 15 min at room temperature. The fluorescence at excitation 364 nm and emission 498 nm was measured in each sample, using the 0 and 5 µg DNA standards to calibrate the fluorimeter, which then gave fluorescence values directly proportional to the DNA content of the sample.

If it was observed that there was a rather high density of adherent monocytes at the end of the culture period, the cells were lysed as normal and 1 ml PBS added to each sample. However, only 0.6 ml (i.e. 50%) of the cell lysate was used in the DNA assay, with the corresponding addition of only 100 µl 0.05% SDS in the standard curve, which had a final volume of 4.6 ml. The fluorescence value obtained for this half of the lysate was then multiplied by two, to give the total DNA content for the culture.

During the harvesting of the non-adherent cells, the FCS used in the culture supernatant appeared to interfere with the assay, producing a background fluorescence representing between 0.2 and 1 µg DNA. Thus the background fluorescence obtained in the RPMI/20% FCS blanks, was subtracted from that obtained for each of the non-adherent cell samples.

2.10: Estimation of Lysozyme Content of Monocyte Culture Supernatants

The measurement of lysozyme secretion by monocytes in culture was used as an indication of the viability of the monocytes, and it can indicate cell loss from a monolayer due to toxicity of the agents being added to the culture.

The method used for measuring lysozyme was a modification of the method described by Strunk, Kunke and Musson (1980) using the lysis of Micrococcus lysodeikticus, which was measured spectrophotometrically.

2.10.1: Materials Used in Lysozyme Assay

Sigma Chemical Company, Poole, Dorset.	:	Human Milk Lysozyme (100 000 units/mg)
		Micrococcus lysodeikticus.

2.10.2: Preparation of Reagents used in Lysozyme Assay

0.1M PBS pH 6.3 (PBS with 0.1M sodium chloride)

Prepared by dissolving 0.34g K H_2PO_4, 1.21 g K_2HPO_4 and 5.48 g NaCl in deionised water, pH to 6.3 with orthophosphoric acid, made up to a final volume of 1 litre with deionised water.

2.10.3: Method for Lysozyme Assay

Monocyte culture supernatants (50 µl to 200 µl) were added to a set of assay tubes. Purified human lysozyme standard (12 000 units/ml) was serially diluted in 0.1M phosphate buffer pH 6.3 from about 600 units/ml down to about 2 units/ml, and 100 µl of each dilution transferred in triplicate to a set of assay tubes. M. lysodeikticus was dissolved to about 0.4 mg/ml in 0.1M PBS pH 6.3 (an OD_{450}^{nm} of about 1.0); 2.5 ml of the solution was added to all of the tubes and incubated at 37°C in a shaking water bath for 30 mins. The controls included a set of triplicate tubes with only M. lysodeikticus present, this acted as the starting OD; a set of triplicate tubes containing a volume of RPMI/20% FCS equal to that of the

culture supernatant added, with 2.5 ml M.lysodeikticus, to ensure there was no background lysozyme activity due to FCS.

The OD_{450}nm of all standards, controls and samples was measured and plotted (y axis) against a log scale of the concentration of lysozyme standard (x axis). The linear area of the curve was used to perform a linear regression analysis, upon which the amounts of lysozyme in the monocyte culture supernatant samples was calculated. A correction was performed if a sample of supernatant other than 100 µl was used.

2.11: ^{35}S-methionine-labelling of Monocyte Proteins: Pulse-chase studies

This method of investigating the synthetic products of monocytes, involves internally labelling the proteins by the addition of radiolabelled amino acids into the culture medium. The structure and patterns of secretion of the proteins can then be studied by immunoprecipitation, SDS-PAGE and fluorography.

2.11.1: Materials Used in ^{35}S-Methionine Labelling of Monocyte Proteins

Gibco, Paisley, Renfrewshire.	:	Dulbecco's Minimal Essential Medium without Methionine.
New England Nuclear, Boston.	:	^{35}S-methionine (approx. 1000 Ci/mmol)
Sigma Chemical Company, Poole, Dorset.	:	Bovine Serum Albumin Fraction V. Lauryl Sulphate (SDS). Insoluble Protein A (Staph A)cell suspension. Phenylmethylsulphonyl fluoride (PMSF).
Whatman	:	GF/C Filters.

Aldrich, Gillingham, Dorset.	:	Salicyclic Acid (Sodium salt)
Kodak, Manchester.	:	8" x 10" X-Omat AR5 X-ray films FX40 Developing Solution LX24 Fixing Solution.
Behring Diagnostics, Hounslow.	:	Antiserum to C1-inhibitor.

2.11.2: Preparation of Buffers Used in Pulse Chase Experiments

Lysis Buffer

100 ml lysis buffer was prepared by mixing the following in deionised water, and pH to 7.5 with 1M hydrochloric acid:- 0.292g NaCl (50 mM), 0.745g KCl (100 mM), 0.606g Tris (50 mM), 5.8 ml 0.086M EDTA (5 mM), 0.5g BSA (0.5%), 0.5g Deoxycholate (0.5%), 1 ml Triton X-100 (1%), 0.5 ml 1M PMSF (5 mM), 0.02g Sodium azide (0.02%) and 0.156g benzamidine (10 mM).

2.11.3: Pulse-Chase Method

Monocyte cultures (prepared by method 2, Section 2.5.4) were maintained in RPMI/10% AB for the first three days, changed to RPMI/20% FCS and culture continued until the pulse chase was performed, sometime between days 4 and 10. Triplicate sets of wells were used throughout, and pooled after pulse-chasing.

The monocyte cultures were washed gently with warm RPMI twice, and once with Dulbecco's minimal essential medium (DMEM) without methionine. 330 µl D-MEM containing ^{35}S-methionine at 500 µCi/ml was added to each well and incubated at 37°C in a humidified 5% CO_2/air atmosphere for the pulse period which ranged from 1 to 3 hr. The supernatants were removed and

transferred to microcap centrifuge tubes and made 5 mM PMSF, 5 mM EDTA and 5 mg BSA/ml by the addition of concentrated stock solutions. After gentle washing of the monocyte cultures with warm RPMI, 330 µl RPMI was added to each well and incubated at 37°C in a humidified atmosphere for the chase periods, which included 0, 0.25, 0.5, 1, 2, 4 and 8 hr. The supernatants were removed, pooled in microcap centrifuge tubes, and treated with PMSF, EDTA and BSA as described for the pulse supernatants. All supernatant samples were centrifuged at 300 g for 2 min at room temperature to remove any loose cells, and the supernatants transferred to new microcap centrifuge tubes.

The monocytes were lysed by the addition of 330 µl lysis buffer to each well; the lysates were pooled in microcap centrifuge tubes and centrifuged at 6000 g for 10 min at room temperature to remove any debris; the lysates were transferred to new tubes. All lysates and supernatants had SDS added to a final concentration of 0.5% in an attempt to reduce non-specific background binding to the Staph A used later.

In addition to the time points listed above, a triplicate control was carried out in a similar manner, at a selected time point (either 0 or 1 hr). This was used as a non-specific control to determine which radio-labelled proteins were binding non-specifically to the Staph A.

All samples were stored at -20°C until used for immuno-precipitations.

2.11.4: Measurement of Acid Precipitable Protein Incorporated into Monocyte Proteins

A measurement of the acid (Trichloroacetic Acid, TCA) precipitable protein was carried out in order to determine the level of incorporation of ^{35}S-methionine into intracellular and extracellular secreted monocyte protein.

This was performed by spotting 5 µl of each lysate and supernatant, in triplicate, on to GF/C Whatman filters, previously presoaked in 2 mM methionine. After drying, the precipitation was performed by soaking each filter in the following sequence of solution for 1 minute, at room temperature: 10% TCA, 5% TCA, 100% acetone, 50% acetone 50% ethanol, and 100% ethanol.

After drying of each filter, they were counted, in vials containing 2.5 ml Unisolve, for 1 minute in a Packard β counter. A total input was counted, which consisted of 5 µl of pulse medium containing ^{35}S-methionine. In order to correct for non-specific binding of ^{35}S-methionine to tissue culture plates, 330 µl of pulse medium was incubated in an empty well and treated as for the samples: lysed with 330 µl lysis buffer, and a TCA precipitation performed on the lysate and supernatant samples.

2.11.5: Calculation of Incorporation and Secretion of ^{35}S-methionine into Acid Precipitable Protein

The % incorporation of ^{35}S-methionine into acid-precipitable protein was calculated, using the samples corrected for background non-specific absorbance.

The CPM of the empty well lysate or supernatant was subtracted from the CPM for monocyte supernatants or lysates:-

$$\% \text{ incorporation} = \frac{\text{CPM lysate + CPM supernatant}}{\text{CPM Total Input}} \times 100$$

The % incorporated radioactivity to be secreted over X hrs was calculated thus:-

$$\frac{\text{CPM supernatant at x hr}}{\text{CPM supernatant (X hr) + CPM lysate (X hr)}} \times 100\%$$

2.11.6: Immunoprecipitation of ^{35}S-methionine-labelled Monocyte Proteins

The method involved the addition of specific antisera to the complement component under study, the immune complexes formed being isolated by their property of binding to Staph A, which was added to the supernatant. After sequential precipitation of the specific proteins to be studied, they were analysed by separation on SDS-PAGE and subject to fluorography, in order to visualise the radiolabelled proteins.

Protocol for Immunoprecipitations

10% Staphylococcus aureus (Staph A) Cowan Type I strain was washed twice in PBS, and resuspended in PBS to a 10% solution. 100 µl Staph A was added to all supernatants and lysates, incubated with end over end mixing for 1 hr at room temperature, in order to preabsorb any materials which bound non-specifically to Staph A. The Staph A was pelleted by centrifugation at 6000 g for 2 min at room temperature; the supernatants were transferred to new tubes containing 5 µl 1/20 dilution of the appropriate antiserum. The control (0 or 1 hr) supernatant and lysate did not receive any antiserum; the contents of all tubes were mixed and incubated overnight at 4°C.

The immune complexes which had formed were isolated by the addition of 20 µl freshly washed 10% Staph A to each tube including the controls; end over end mixing was carried out at room temperature for 30 min to allow binding. The immune complexes bound to the Staph A were harvested by centrifugation at 6000 g for 2 min at room temperature, and the supernatants transferred to a new set of tubes to enable further immunoprecipitations.

The Staph A/immune complex pellets were washed six times with 1 ml PBS containing 1% triton and 0.5% SDS, to remove any non-specifically bound radiolabelled material. The samples were centrifuged at 6000 g for 2 min to precipitate the pellets between each wash, and then prepared for SDS-PAGE analysis as described previously (Section 2.1.6b). After addition of 30 µl reducing sample buffer and boiling, the dissociated IgG and radiolabelled proteins were separated from the Staph A by centrifugation at 6000 g for 2 min at room temperature. The supernatants were then applied to an SDS-PAGE gel of the appropriate percentage for separation, usually 7.5%. An aliquot of ^{14}C-labelled molecular weight markers were reduced and run on the same gel to enable estimation of protein molecular weights.

2.11.7: Fluorography of Gels

After electrophoresis, the gel was soaked in destain solution for 30 min to fix the proteins; the destain solution was washed out by soaking in water for 30 min, and the gel

was impregnated with the scintillant, salicyclic acid (1M) for 30 min, all incubations being carried out at room temperature. The gel was placed on a sheet of Whatman filter paper, covered with clingfilm and dried under vacuum with heat for about 2 hr on a Pharmacia Slab Gel dryer (GSD 4).

The clingfilm was removed and the dried gel secured in an x-ray casette containing fast tungstate intensifying screens. In the dark room, a sheet of X-omat AR5 photographic film was placed in direct contact with the gel, and allowed to develop at -70°C for a period of 2 days up to 6 weeks.

The bands on the film were visualised by incubation in a 1/5 dilution of Kodak A40 developer for 5 minutes at room temperature, followed by fixation in a 1/5 dilution of Kodak LX24 fixer for 5 min at room temperature. The fluorograph was washed in cold running water, and dried in a warm air oven.

The tracks containing specific antisera to the component being investigated were observed for bands which did not occur in the control precipitation tracks. The migration of the molecular weight standards were used to construct a standard curve, and calculation of the molecular weight of the specifically precipitated proteins was carried out as described previously (Section 2.1.6b).

2.11.8: Immunoprecipitation of C1-inhibitor from Human Serum

In the pulse-chase experiments involving monocytes isolated from HAE patients and their normal controls, C1-inhibitor was

precipitated from autologous serum samples obtained during venesection. The precipitated C1-inhibitor was run on the same gel as the ^{35}S-methionine labelled C1-inhibitor precipitated from monocyte culture supernatants, stained with Coomassie Brilliant Blue to visualise (see Section 2.1.6b), and the molecular weights of the two C1-inhibitors compared.

200 µl serum from normals and patients was added to 3 sets of microcap tubes. Antiserum to C1-inhibitor (as used in pulse-chase immunoprecipitations) was double diluted in PBS, and 200 µl added to the tubes above at neat, 1/2 and 1/4 dilutions of antiserum. The samples were mixed and incubated at 37°C for 1 hour, and then transferred to 4°C where they were incubated overnight to allow precipitation. The precipitates were harvested by centrifugation at 6000 g for 5 min at room temperature and washed four times in 1 ml cold PBS. The precipitates were treated in the same way as the precipitates from the pulse chase experiment: reduced, run on SDS-PAGE, stained with Coomassie blue and destained (Section 2.1.6b).

3: RESULTS.

Fig. 14: Elution profile of C1-inhibitor from Biogel A 0.5M column.

C1-inhibitor was measured by Radialimmunodiffusion.

Samples were pooled as described in the text.

Vo: Void volume

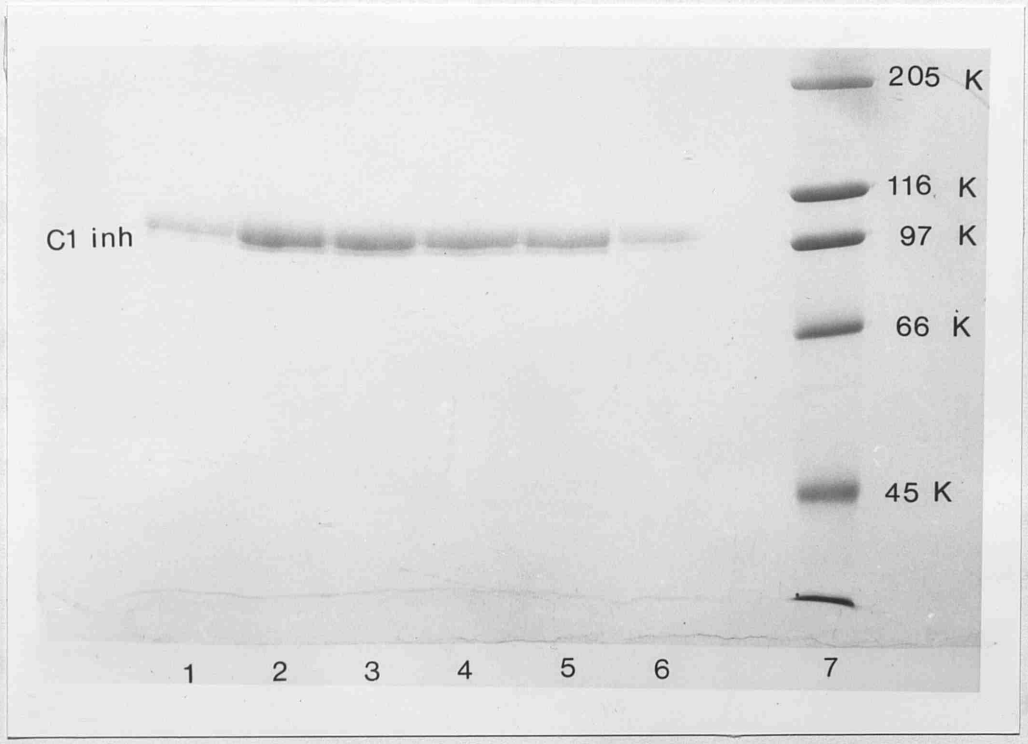


Fig. 15: SDS-PAGE of C1-inhibitor Pools from Biogel Column. Tracks 1 to 6 represent the pools of purified human C1-inhibitor; track 7 shows the molecular weight standards which included myosin (205 kD), β galactosidase (116 kD), phosphorylase B (97 kD), bovine serum albumin (66 kD) and ovalbumin (45 kD). The purified C1-inhibitor had an estimated molecular weight of 94 kD.

3.1: Purification of Complement Components and Specificity of Antisera

3.1.1: Purification of C1-inhibitor

Figure 14 shows the elution profile obtained when C1-inhibitor was chromatographed over a Biogel A0.5M gel filtration column, as described in Section 2.2. Six pools of C1-inhibitor were prepared from the column by pooling the various fractions as follows: pool 1, fractions 118 and 119; pool 2, fractions 120, 121 and 122; pool 3, fractions 123 and 124; pool 4, fractions 125 and 126; pool 5, fractions 127 and 128; pool 6, fractions 129 and 130. The purity of the six pools was assessed by SDS-PAGE and IEP.

The gel obtained, shown in Figure 15, demonstrated that loading the samples at 20 µg C1-inhibitor per track, pools 3 to 6 contained pure C1-inhibitor which was free from contaminating proteins. The purified C1-inhibitor migrated as a doublet, with a molecular weight of 94 kD.

Pools 1 and 2 appeared to contain minor contaminants of molecular weight about 65 kD and 35 kD, however upon IEP the 65 kD contaminant was proven not to be albumin, which was one of the contaminating proteins observed in pre-gel filtration samples. Pools 3 and 4 were used to immunise rabbits, to raise monospecific polyclonal antisera.

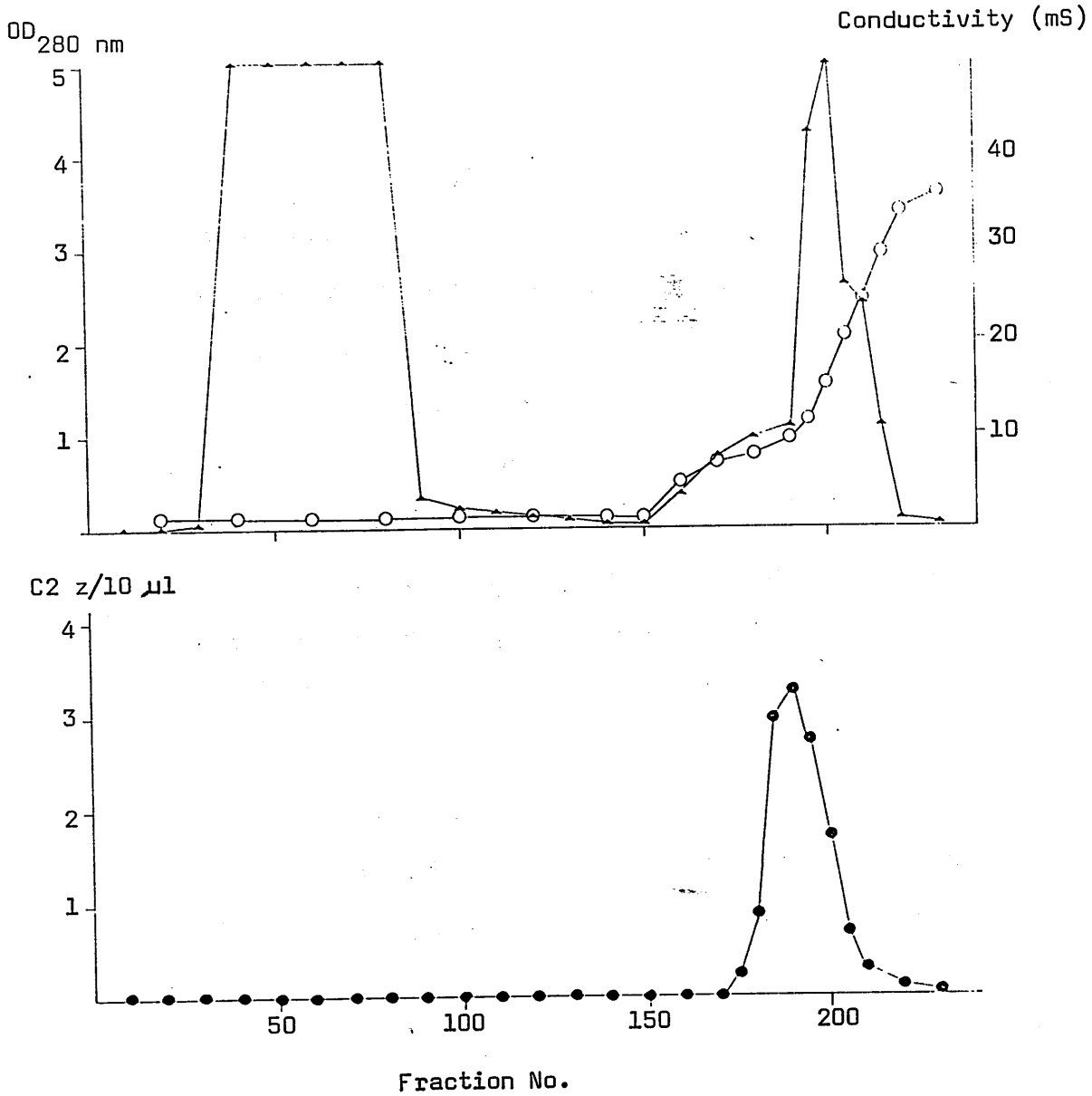


Fig. 16: Elution Profile from Biorex 70 column of C2 Purification showing OD_{280nm} (▲), conductivity mS (○) and C2 z/10 µl sample (●).

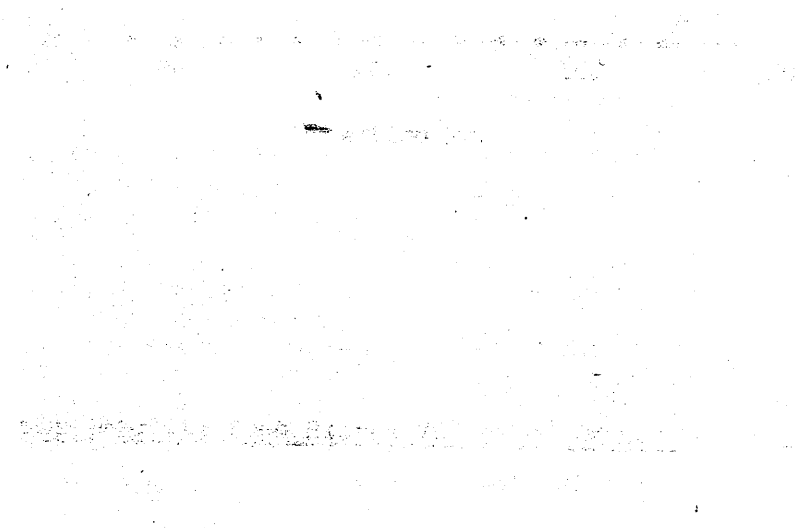
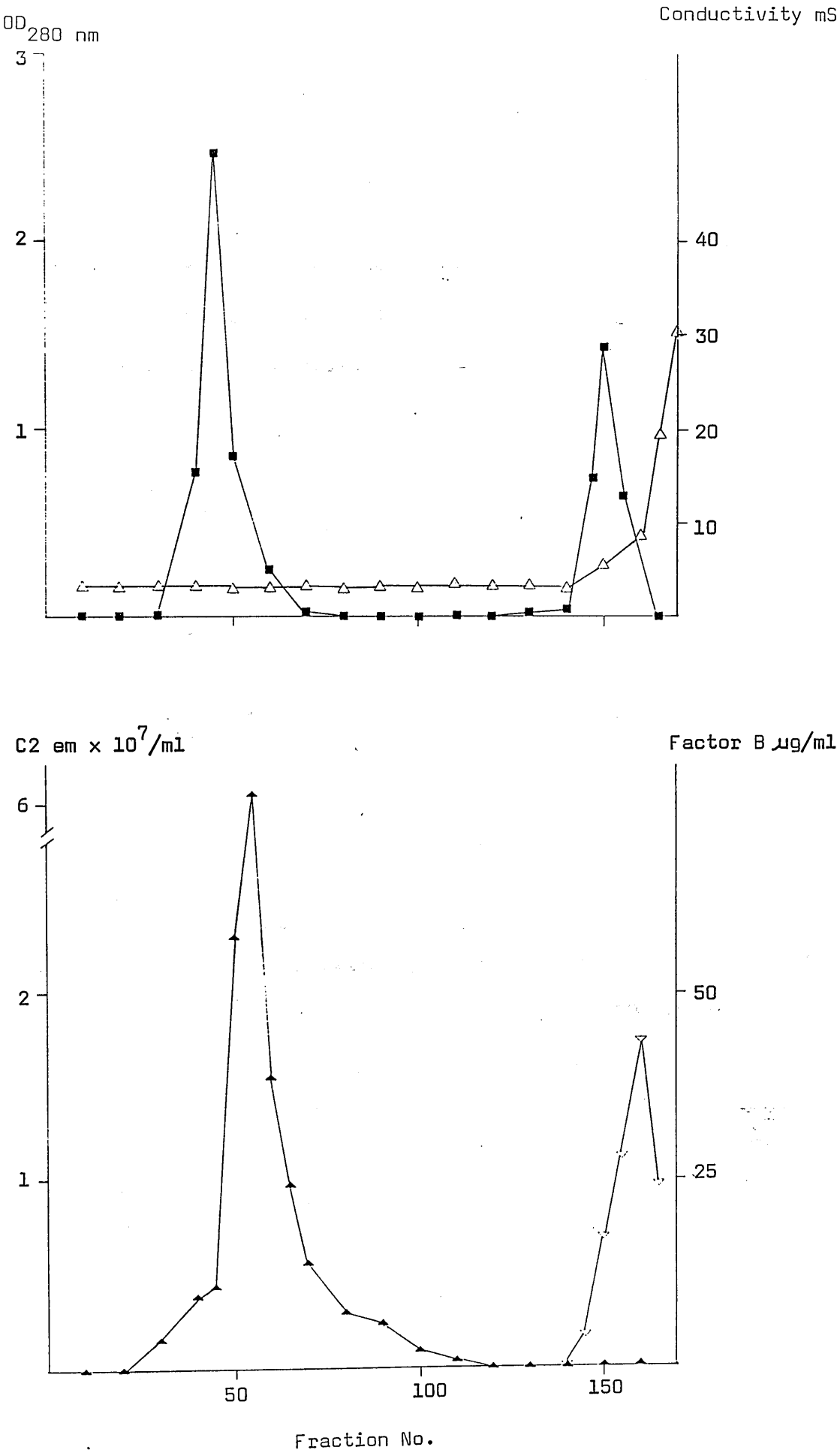


Fig. 17: Elution profile from DEAE Cellulose Column of C2 Purification showing OD_{280nm} (■), conductivity mS (△), C2 z/10 µl of sample diluted 1/100 (▲) and Factor B as µg/ml (▽).


OD 280 nm
Conductivity mS
3
2
1
40
30
20
10
C2 em x 10^7/ml
Factor B µg/ml
6
2
1
50
25
50
100
150
Fraction No.

3.1.2: Specificity of Antisera

The specificity of the antisera was tested on double diffusion plates, as described in Section 2.1.8, against purified C1-inhibitor and NHS, using commercially purchased antiserum to C1-inhibitor and NHS as the standards. The results of the double diffusion showed a single precipitin line for the reaction between raised antiserum to C1-inhibitor and purified C1-inhibitor or NHS. Both the commercial and locally produced antisera to C1-inhibitor showed a reaction of identity when tested against purified C1-inhibitor or NHS.

From this it was assumed that the antisera raised against purified C1-inhibitor were specific. The various batches of antisera raised against C1-inhibitor were tested in double diffusion plates using NHS at 1/2 dilution, in order to determine the precipitation titre. The results varied for each batch of antiserum ranging from 1/2 to 1/32.

3.1.3: Purification of C2

The elution profile obtained for the Biorex 70 column in the initial purification of C2 is shown in Figure 16. The bound C2 protein, which eluted at a conductivity of around 8 mS, was pooled (fractions 183 to 199), dialysed against starting buffer for the next column, and chromatographed over a DEAE cellulose column, as described in Section 2.3.

The elution profile of the DEAE cellulose column is shown in Figure 17 and demonstrates that the C2 protein was eluted in the exclusion peak (fractions 40 to 80). A linear salt gradient was applied to the column to elute the bound factor B, which was further purified by a colleague.

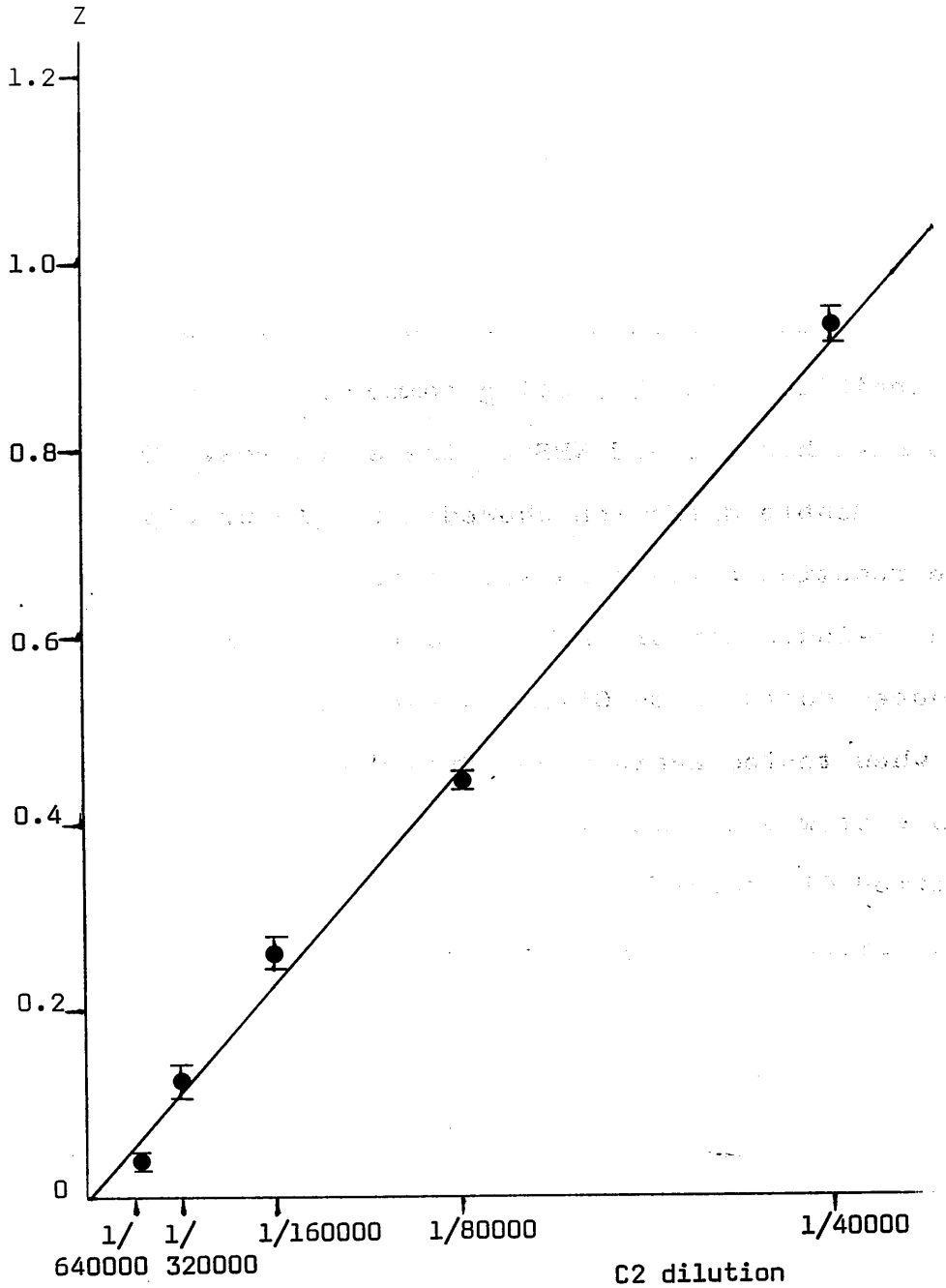


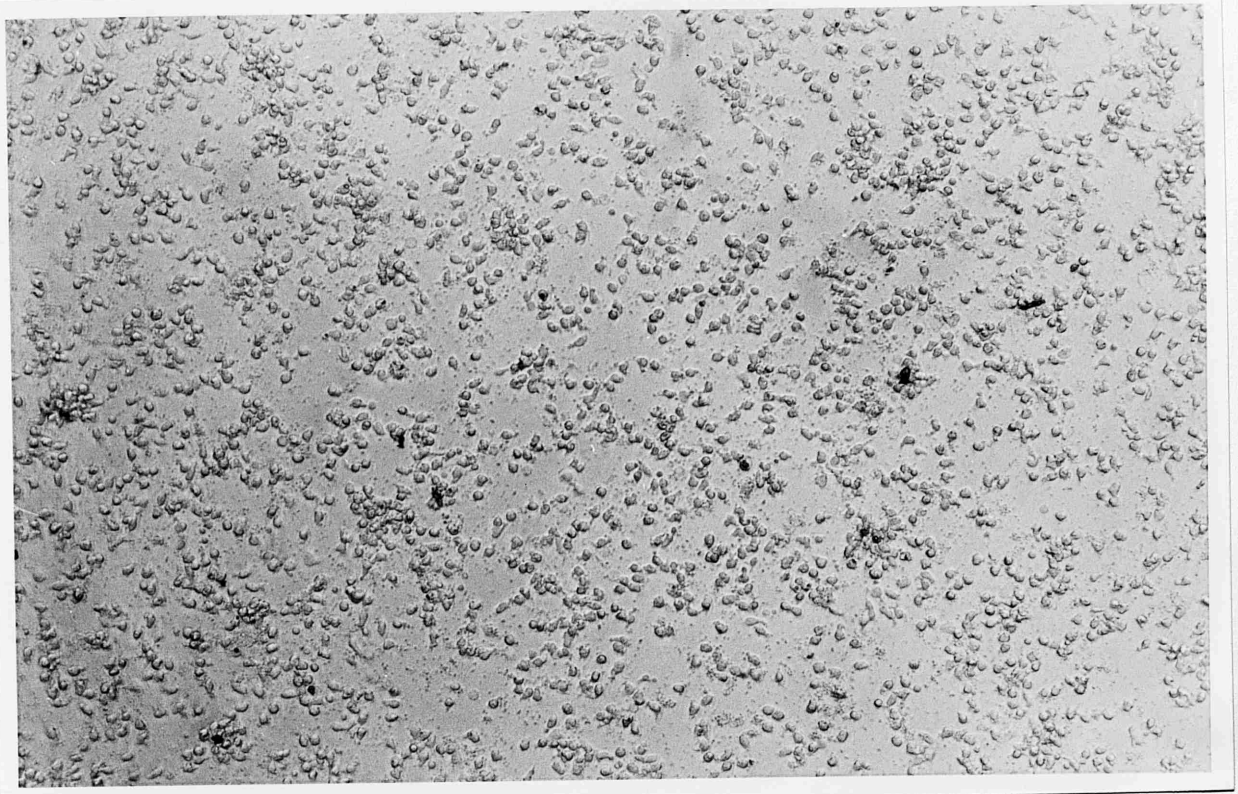
Fig. 18: Titration of Purified C2: 36 700 units/ml

Fig. 19: Photographs of monocytes on day 1 (A) and 12 (B) of culture.

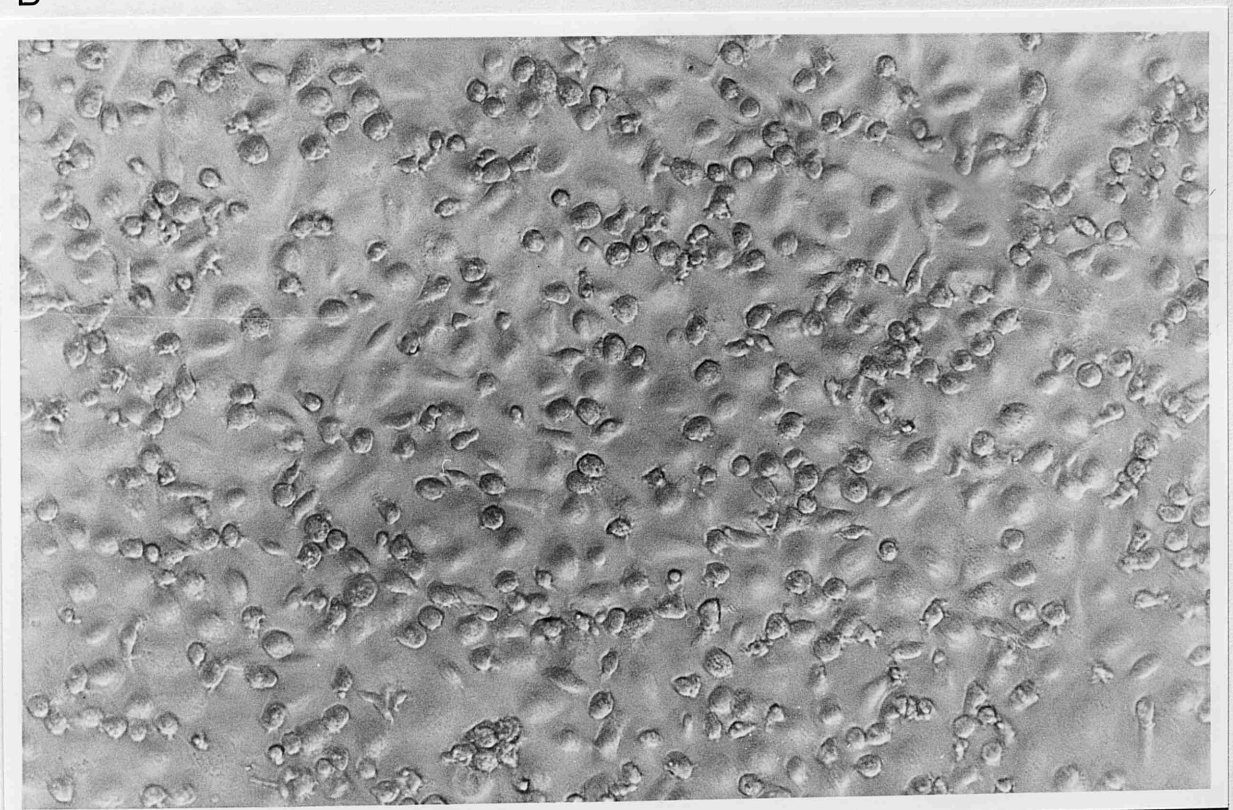
Microscope magnification A x 40

B x 100

A



B



The final pool of C2 (fractions 49 to 63, Fig. 17) was concentrated by pressure ultrafiltration (from 150 ml to approximately 32 ml) and the final solution divided into aliquots and stored at -70°C. The haemolytic activity of this batch of C2 was titrated (Section 2.6.6) and found to be 36,700 units/ml, as shown in Figure 18.

3.2: The Study of Human Monocytes under Control Conditions

3.2.1: Characteristics of Human Monocytes

Morphological examination of monocytes in culture was one of the major methods used throughout the study in order to characterise the cell content of the monolayers. Using phase contrast microscopy, lymphocytes appeared smaller than monocytes and were very rounded in comparison. At the beginning of culture, it was sometimes difficult to distinguish monocytes from lymphocytes, since the monocytes were still very small and rounded, as shown in Figure 19A. As the culture was maintained, the few lymphocytes or PMNs which were present detached and floated in the culture supernatant, which was always removed and discarded before any experimental procedures were carried out. This initial phase of culture (3 or 7 days) allowed maturation of the monocytes which spread and became well attached to their substratum, as seen in Figure 19B. At this stage some showed well defined granular nuclei, and had developed various irregular shapes.

Characteristics	% Monocytes in Culture	
	1	2
Morphology 1) Giemsa Stain	98	96
2) Non-specific Esterase	98	95
Phagocytosis of serum treated zymosan	87	91
Rosetting with 1) EAC 43b	88	90
2) IgG coated E	92	90

Table 17: Characterisation of Cell Populations

Performed using morphology (Giemsa and non-specific esterase stains), phagocytosis (serum treated zymosan) and rosetting (EAC43b and IgG coated E).

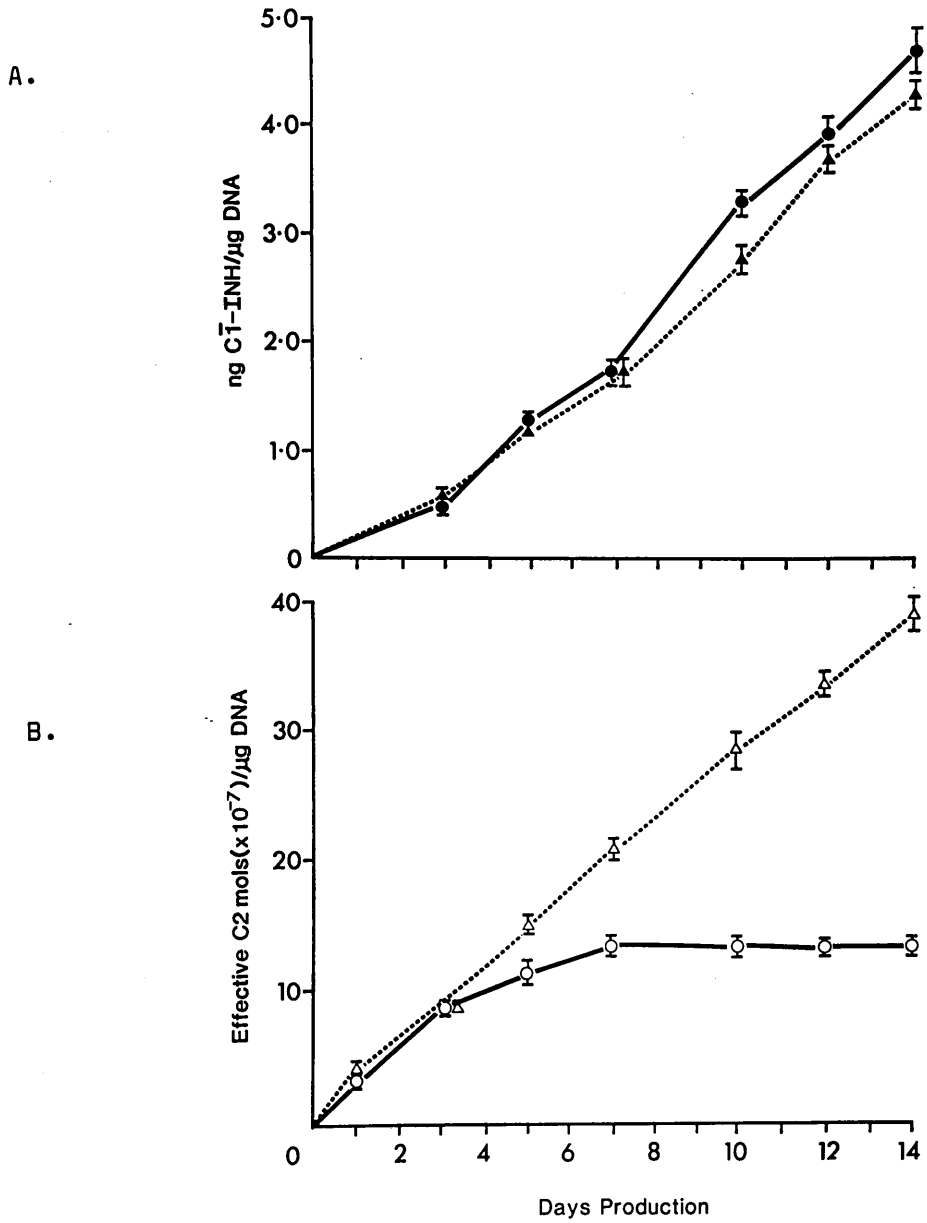


Fig. 20: Continuous and Cumulative production of C1-inhibitor as ng/µg DNA (A) and C2 as em x 10^7/µg DNA (B) by monocytes in culture.

○ : continuous C2 production.

△ : cumulative C2 production.

● : continuous C1-inhibitor production.

▲ : cumulative C1-inhibitor production.

The results of the Giemsa, and non-specific esterase staining, phagocytosis and receptor-binding experiments for two separate sets of cultures are shown in Table 17. Using these criteria, 87% to 98% of the cell population being used in the cultures satisfy the characteristics fulfilled by cells of the mononuclear phagocyte lineage.

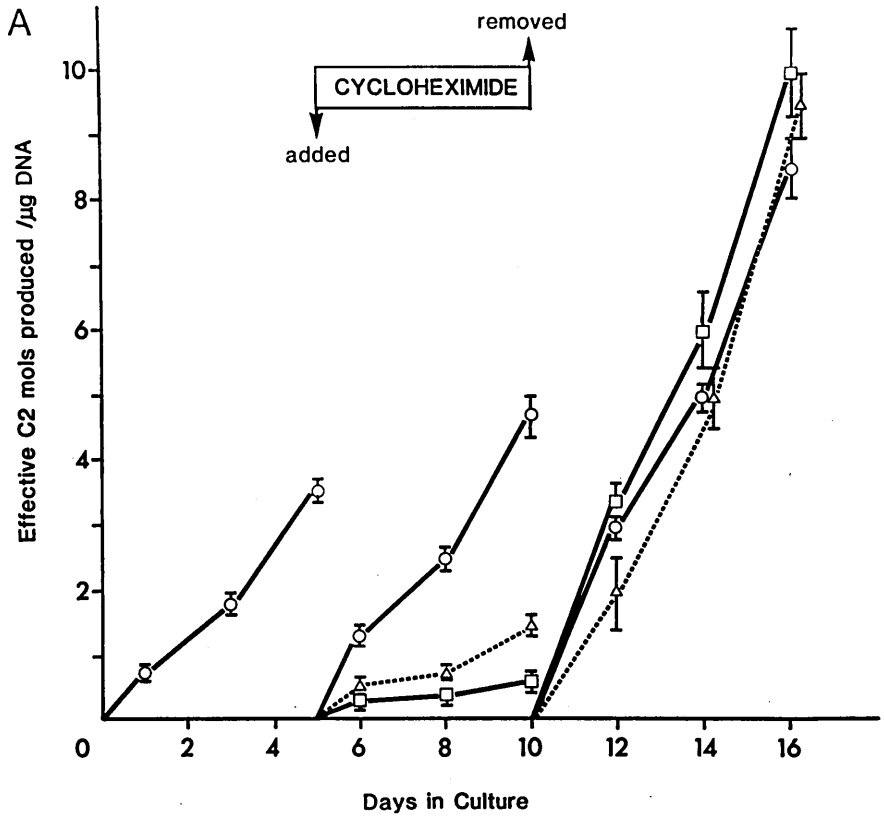
3.2.2: Continuous and Cumulative Complement Synthesis by Monocytes

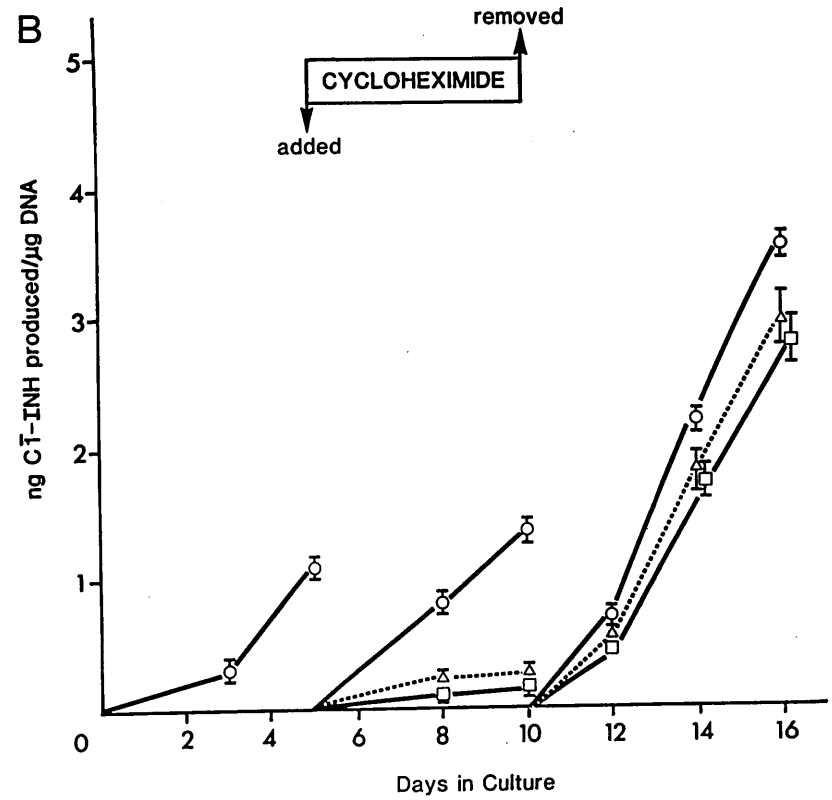
Continuous and cumulative synthesis of the complement components C2 and C1-inhibitor by human monocytes in culture were investigated (as described in Section 2.5.9), for total periods of up to 21 days. When the continuous synthesis of C2 was measured, in two out of three cultures, the rate of synthesis was not linear over the entire culture period of 14 days. One such example, shown in Figure 20B, demonstrated that concentrations of C2 reached their maximum level by day 7, and remained constant for the remainder of the culture period. However, when cumulative synthesis was measured (Fig. 20B), C2 appeared to be synthesised at a linear rate over the 14 day culture period.

Upon investigation of C1-inhibitor synthesis (Fig. 20A) both continuous and cumulative synthesis closely mirrored each other, and were linear over the 14 days measured. This occurred in all of the three monocyte cultures tested, a representative one of which is shown in Figure 20A. Thus, there did not appear to be a switching off of C1-inhibitor synthesis, as was demonstrated with C2 synthesis.

Fig. 21: <u>The effect of cycloheximide at 0.5 µg/ml (△) and 1.0 µg/ml (□) on the synthesis of C2 (A) and C1-inhibitor (B) by monocytes in culture.</u>

Cycloheximide was added on day 5 and removed on day 10, synthesis of C2 and C1-inhibitor being assayed before, during and after cycloheximide treatment. Control cultures were denoted by ⧖ and treated cultures by ⧗ for 0.5 µg cycloheximide/ml, and ⧖ for 1 µg cycloheximide/ml.


A
removed
CYCLOHEXIMIDE
added
Effective C2 mols produced /µg DNA
0
2
4
6
8
10
12
14
16
Days in Culture


B
removed
CYCLOHEXIMIDE
added
ng C1-INH produced/µg DNA
0
1
2
3
4
5
6
8
10
12
14
16
Days in Culture

3.2.3 Inhibition of Monocyte Complement Synthesis by Cycloheximide

Cycloheximide, at 0.5, 1.0 and 2.5 µg/ml, was added to 5 day monocyte cultures which were synthesising C2 and C1-inhibitor. After 5 days incubation with the monocytes, the cycloheximide was washed out and culture continued; during all periods samples were removed and assayed for C2 and C1-inhibitor.

The results of one such representative experiment are shown in Figure 21, where cycloheximide at 0.5 µg/ml and 1.0 µg/ml was added to monocyte cultures on day 5. Before addition of the cycloheximide all wells were shown to be synthesising C2 and C1-inhibitor as shown in Figure 21A and B, days 1, 3 and 5. Cycloheximide was added on day 5 and culture continued for 5 days. Both C2 and C1-inhibitor synthesis were inhibited (Fig. 21A and B, days 5 to 10) by cycloheximide at 0.5 µg/ml, to a level of 72 and 76% respectively. With cycloheximide at 1 µg/ml, the levels of inhibition were 82% and 84% respectively for C2 and C1-inhibitor. The range of inhibition over three cultures due to the addition of cycloheximide at 1 µg/ml for C2 was 75 to 86%; for C1-inhibitor the inhibition levels were 60% to 84%. The various doses of cycloheximide used, all appeared to be equally effective at inhibiting monocyte complement synthesis to about the same degree, although in one culture using cycloheximide at 0.5 µg/ml, was much less effective at inhibiting C1-inhibitor synthesis.

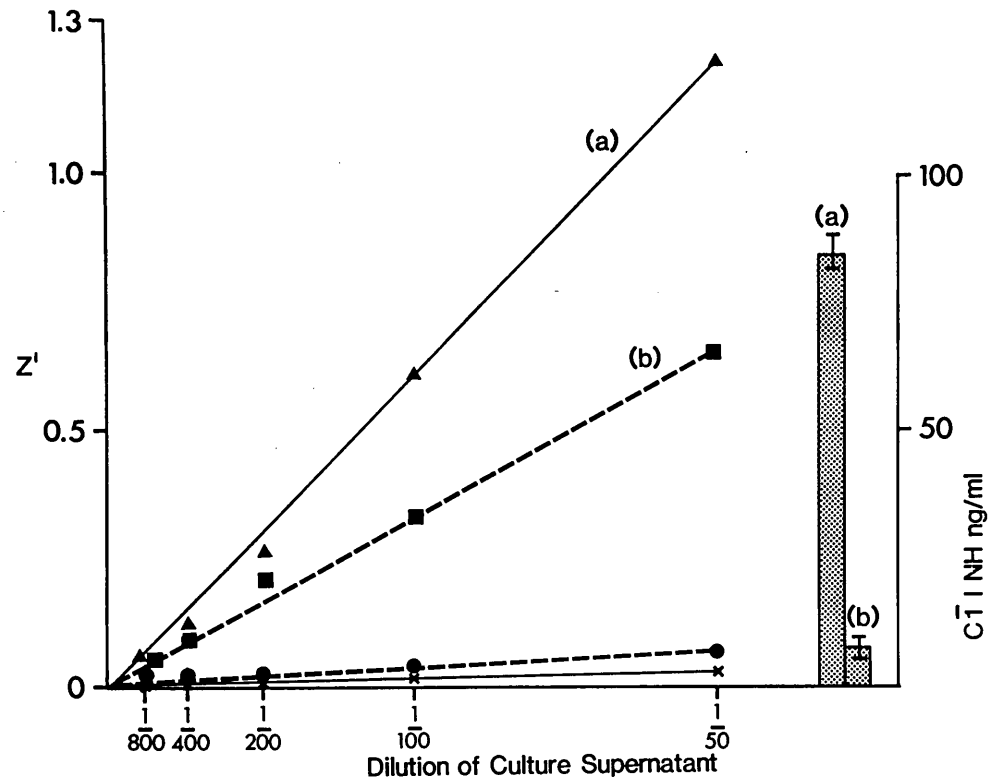


Fig. 22: Measurements of monocyte C1-inhibitor activity by haemolytic assay (titrations) and RIA (bars), in control supernatants (a) and cycloheximide treated (1µg/ml) supernatants (b).
Concentrated culture medium with (●) or without (X) cycloheximide had negligible effects on the assay.
This culture is designated as Supernatant I in Table 10.

Conditions	Approximate concentration factor	ng C1-inh/ml RIA	% inhib-ition	units C1-inh/ml by haemolytic assay	% inhib-ition	Specific activity as units C1-inh/ng C1-inh
Control Supernatant 1	9	85	93	103	44	0.57
Cycloheximide Supernatant 1	9	6		58		
Control Supernatant 2	10	102	95	245	60	1.53
Cycloheximide Supernatant 2	9	5		97		
Control Supernatant 3	8	79	94	91	67	0.82
Cycloheximide Supernatant 3	9	5		30		
Medium	10	0		0		0
Cycloheximide Medium	9	0		0		

Table 18: Functional Activities of Monocyte C1-inhibitor.

Upon removal and washing of the cycloheximide out of the culture, synthesis of C2 and C1-inhibitor resumed at comparable rates in cycloheximide and non-cycloheximide treated cultures (Fig. 21A and B, days 10 to 16).

3.2.4: Functional Activity of Monocyte C1-inhibitor

Established monocyte cultures (10 days) were cultured for short periods under serum free conditions (as described in Section 2.5.5), in the presence and absence of cycloheximide at 1 μg/ml. The supernatant was harvested, pooled, concentrated, and assayed for C1-inhibitor activity by RIA and haemolytic assay. The results of three sets of experiments are summarised in Table 18 and Figure 22.

When cycloheximide at 1 μg/ml was added to cultures, the C1-inhibitor measured by RIA in all three cases was inhibited by more than 90%; however the functional activity of C1-inhibitor was only inhibited by 44, 60 and 67% in the three cultures tested (Table 18 and Fig. 22). Concentrated Hams F12 medium, with or without cycloheximide, did not appear to contain any C1 inhibitory activity (Fig. 22). Thus, in monocyte culture supernatant there appeared to be a second C1-inhibitory activity, which was assumed to be insensitive to cycloheximide. By using simultaneous equations, the specific functional activity of C1-inhibitor protein synthesised by monocytes as units C1-inh/ng C1-inh was calculated and for the three sets of cultures tested was 0.57, 1.53 and 0.82 units/ng (Table 18).

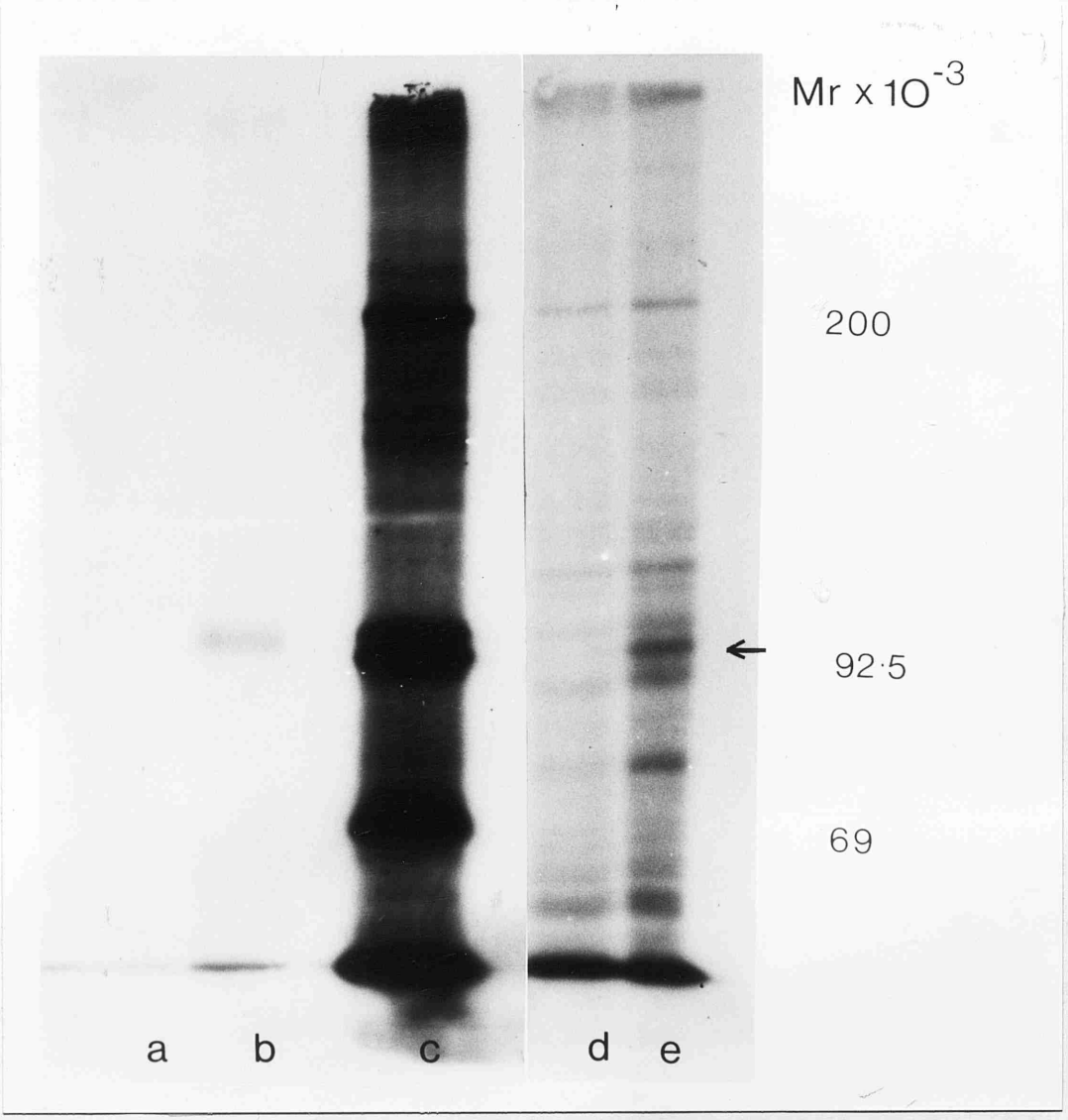


Fig. 23: Immunoprecipitation of C2 from Monocyte Supernatant and Lysate.

Tracks A and D: Non-specific immunoprecipitation.

Track B : Specific precipitation from monocyte supernatant (97 kD).

Track E : Specific precipitation from monocyte lysate (94 kD).

Track C : ^{14}C-methylated molecular weight markers:
200 kD (myosin), 92.5 kD (phosphorylase b),
69 kD (BSA), 4b kD (ovalbumin),
30 kD (Carbonic anhydrase).

3.2.5: Pulse Chase Studies under Control Conditions

Incorporation of ^{35}S-methionine into immunoprecipitable protein during pulse chase studies of monocytes in culture was performed for C1-inhibitor and C2.

3.2.5a: C2

Precipitation of C2 from the supernatant and lysate of a monocyte culture which was pulsed for 2 hr is shown in Figure 23. A specific band of molecular weight 97 kD was precipitated from the supernatant (track b) and represents the extracellular secreted form of C2; a lower molecular weight of 94 kD represented the intracellular form of C2 (Fig. 23, track e). In two other cultures precipitated for C2, the secreted forms displayed molecular weights of 100 and 102 kD, whilst the intracellular forms were 93 and 95 kD respectively.

An initial problem which was encountered in pulse-chase experiments was the high non-specific background binding of intracellular components to the protein A, as demonstrated in tracks d and e, Figure 23. This method was later modified, for C1-inhibitor immunoprecipitations, by adding SDS to a final concentration of 0.5% to the lysates before immunoprecipitations were commenced, and including 0.5% SDS along with the 1% triton in the PBS which was used for washing the Protein A-antigen-antibody complexes. This, in addition to an overnight incubation with the specific antibody, improved the immunoprecipitation technique by decreasing the background non-specific binding to protein A.

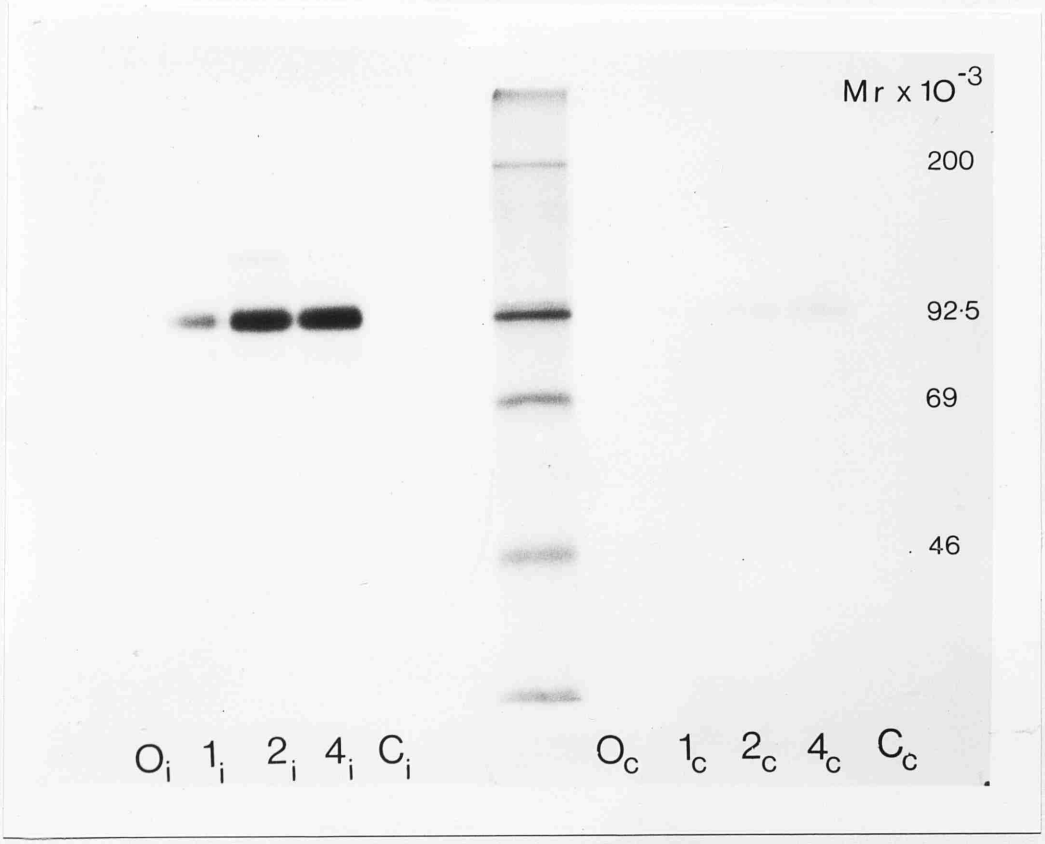


Fig. 24: Cl-inhibitor Immunoprecipitations of Supernatants from a pulse-chase study of control and γ interferon treated monocytes.

Control monocytes are denoted c; γ interferon (1 μg/ml) treated monocytes are denoted i. C denotes non-specific immunoprecipitations.

Cultures were pulsed and chased for time periods of 0, 1, 2 and 4 hr. Cl-inhibitor in both control and γ interferon-treated monocyte supernatants displayed a molecular weight of 96 kD. Molecular weight markers are as described in legend to Figure 23.

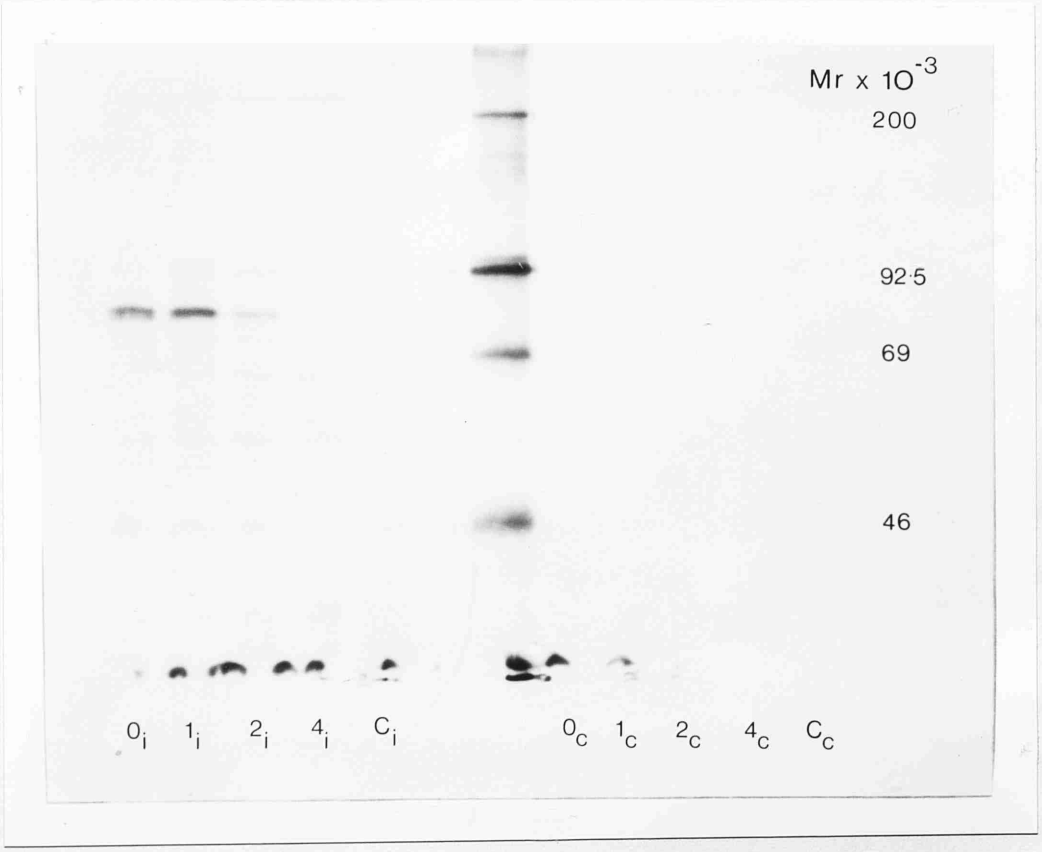


Fig. 25: Cl-inhibitor Immunoprecipitates of Lysates from a Pulse-Chase Study of Control Monocytes and Monocytes Stimulated with ɣ interferon.

Control monocytes are denoted c; ɣ interferon (1 µg/ml) treated monocytes are denoted i. C denotes non-specific immunoprecipitations.

Cultures were pulsed and chased for 0, 1, 2 and 4 hr. Cl-inhibitor in both control and ɣ interferon-treated monocyte lysates displayed a molecular weight of 83 kD. Molecular weight markers are as described in legend to Figure 23.

3.2.5b: Cl-inhibitor

The results of a pulse chase study demonstrating intracellular and extracellular forms of Cl-inhibitor precipitated from a monocyte culture are shown in Figure 24 and 25, on the right sides of the molecular weight markers, labelled 0c, 1c, 2c, 4c and Cc. The culture was pulsed for 1 hr with 500 µCi ^{35}S-methionine/ml, chased and time points taken at 0, 1, 2 and 4 hours.

The molecular weight of the secreted form of Cl-inhibitor was calculated as 96 kD (Fig. 24). A range of molecular weights obtained for four sets of immunoprecipitations of Cl-inhibitor from supernatants showed a mean of 100 kD, with a range from 93 kD to 103 kD. The lysates again showed lower molecular weight bands with a mean of 82 kD, ranging from 80 kD to 85 kD.

The patterns of secretion from the lysate and appearance in the supernatant showed that at 0 time all of the Cl-inhibitor was present intracellularly, but by 1 hour secretion had started, as demonstrated by a faint band in the supernatant, and a decrease in the intensity of the intracellular precursor in the lysate. This secretion continued and by 4 hours virtually all of the Cl-inhibitor had been secreted.

Culture Number	interferon ng/ml	C1-inhibitor			C2		
		Day	ng C1-inh/µg DNA	Relative amount	Day	C2-em x 10^7/ µg DNA	Relative amount
1	0	1	0.3 ± 0.1		7	151.4±10.4	
	10 000	1	114.9 ± 17.7	383 ± 6 1	7	345.1±29.7	2.3± 0.2
	1 000	1	16.3 ± 2.0	54 ± 7	7	412.2±49.5	2.7± 0.3
	100	1	7.4 ± 0.6	25 ± 2	7	548.8±30.5	3.6 ± 0.3
	10	1	3.9 ± 0.2	13 ± 0.5	7	276.2±22.6	1.8 ± 0.2
	1	1	2.9 ± 0.2	10 ± 0.7	7	278.3±2.0	1.8 ± 0.1
	0.1	1	1.6 ± 0.4	5 ± 1.3	7	297.1±16.4	2.0 ± 0.1
	0.01	1	1.6 ± 0.2	5 ± 0.6	7	213.1±53.9	1.4 ± 0.4
2	0	1	0.2 ± 0		7	148.0±3.3	
	10 000	1	142.8 ± 34.5	893 ± 216	7	294.1±8.9	2.0 ± 0.1
	1 000	1	35.3 ± 4.5	221 ± 28	7	311.4±11.1	2.1 ± 0.1
	100	1	6.5 ± 0.5	41 ± 3	7	319.0±47.1	2.2 ± 0.3
	10	1	7.5 ± 0.5	47 ± 3	7	330.1±8.4	2.2 ± 0.1
	1	1	4.8 ± 1.3	30 ± 1.6	7	274.6±20.8	1.9 ± 0.2
	0.1	1	2.6 ± 0.9	16 ± 12.6	7	203.4±12.4	1.4 ± 0.1
	0.01	1	1.4 ± 0.7	9 ± 6.4	7	198.6±8.4	1.3 ± 0.1
3	0	1	0.1 ± 0		1	10.1±1.0	
	10 000	1	59.1 ± 4.6	603 ± 47	1	33.7±4.6	3.3 ± 0.5
	1 000	1	20.3 ± 2.2	206 ± 23	1	32.9±3.6	3.3 ± 0.4
	100	1	2.3 ± 0.5	23 ± 5	1	18.1±1.9	1.8 ± 0.2

Table 19: Stimulation of monocyte C1-inhibitor and C2 synthesis by X interferon at 0.01 ng/ml up to 10 000 ng/ml.

Values reported as mean ± SEM for triplicate samples in each culture. C1-inhibitor was measured by RIA and C2 by functional assay.

3.3: The Effect of ɣ Interferon on Monocyte Complement Synthesis

3.3.1: Dose Response Curves

Various doses of ɣ interferon, ranging from 0.01 ng/ml to 10,000 ng/ml, were added to monocytes in culture and the effect on complement synthesis over a period of seven days examined. The results for C1-inhibitor, C2 and C3 synthesis are summarised in Tables 19 and 20 and Figures 26, 27 and 28.

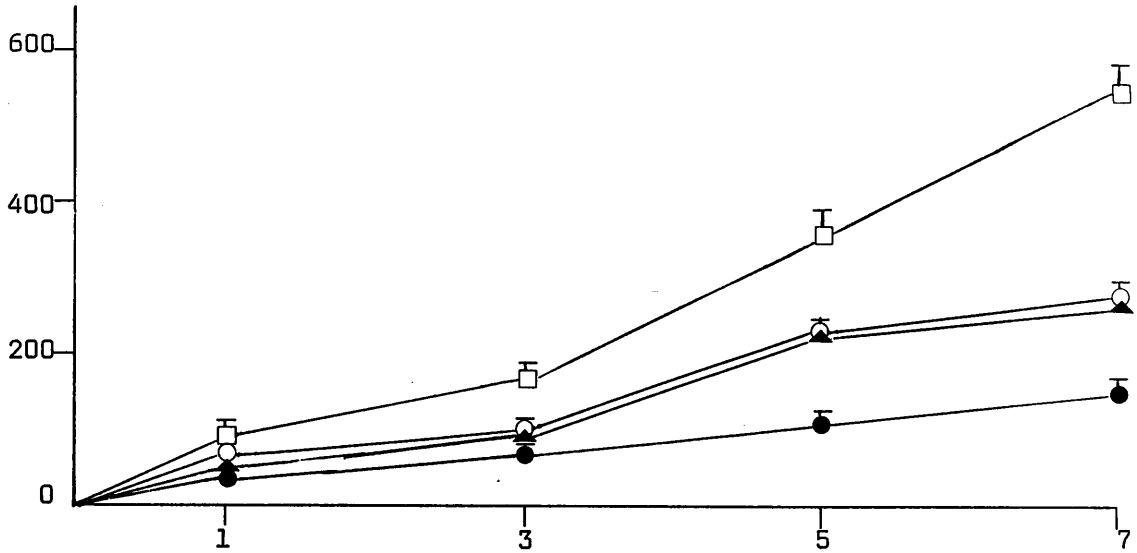
When monocytes were stimulated with ɣ interferon they showed a dramatic stimulation of C1-inhibitor synthesis over the whole dose range tested (0.01 ng/ml to 10 000 ng/ml) and stimulation showed no signs of reaching a plateau (Figs. 26 and 27). Maximum stimulation of C1-inhibitor synthesis in the three cultures reported in Table 19 was shown to occur on day 1, on which the synthesis with ɣ interferon (10 000 ng/ml) was increased by between 383 to 893 fold over that found in control cultures. Even at the lowest dose of ɣ interferon tested (0.01 ng/ml) there was stimulation over control synthesis: five and nine-fold for cultures 1 and 2 respectively (Table 19). Thus stimulation of C1-inhibitor synthesis by ɣ interferon was shown to occur over a wide dose range (Fig. 27), with doses as low as 0.01 ng/ml causing enhancement of C1-inhibitor synthesis up to 9 fold over control (Table 19).

C2 synthesis was stimulated by ɣ interferon, however the dose response curve (Fig. 27) demonstrated lower levels of enhancement compared to that observed with C1-inhibitor. Enhancement of C2 synthesis appeared to reach a plateau at

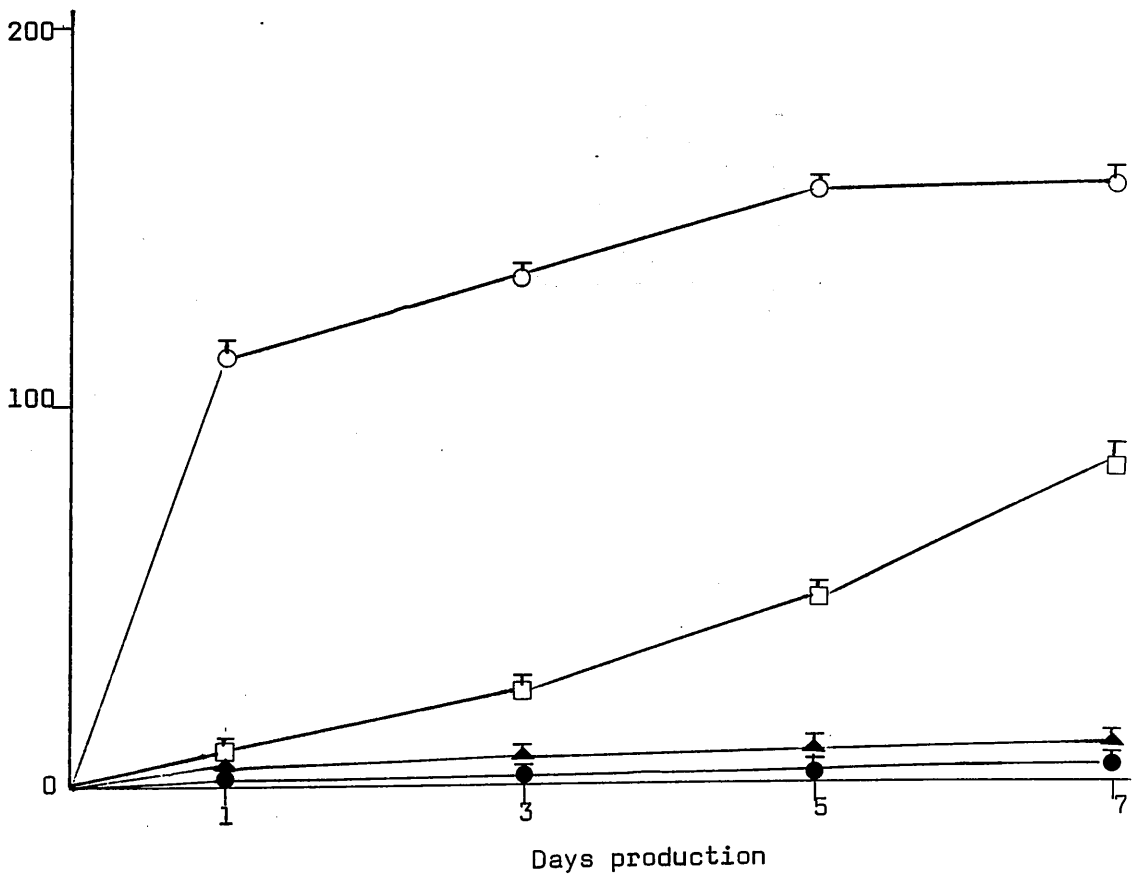
Fig. 26: Effect of γ interferon on Monocyte C2 and C1-inhibitor Synthesis.

Time course of C2 synthesis (A as C2 em x 10^7/μg DNA) and C1-inhibitor synthesis (B as ng C1-inh/μg DNA) by monocytes in culture, on days 1, 3, 5 and 7 (x axis) with γ interferon at 10,000 ng/ml (○), 100 ng/ml (□) and 0.1 ng/ml (▲) and control production (●). These results correspond to culture number I in Table 19.

A. C2 em x 10^7/µg DNA



B. ng C1-inhibitor/µg DNA



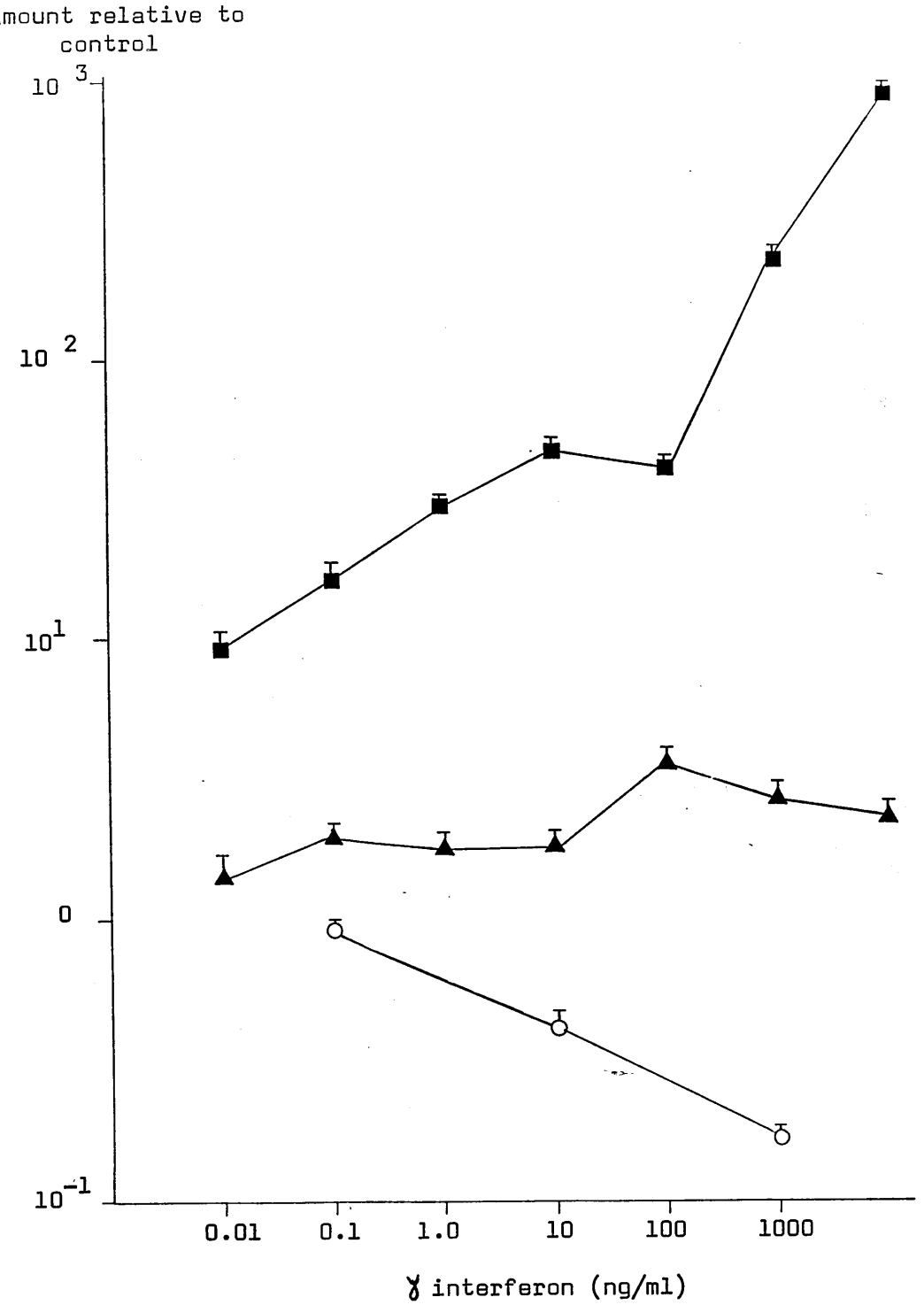


Fig. 27: Dose response curve for the effect of γ interferon on the synthesis of monocyte C1-inhibitor (■), C2 (▲) and C3 (○).

The values are expressed as relative amounts of those present in the control cultures, which were designated an arbitrary value of one. This is a representative set of results from one of the three experiments performed. ▮ denotes the mean ± SEM for triplicate samples.

Culture Number	γ interferon ng/ml	C1-inhibitor			C2			C3		
		Day	ng/μg DNA	Relative amount	Day	ng/μg DNA	Relative amount	Day	ng/μg DNA	% inhibition
4	0	1	0.23 ± 0		3	5.2 ± 0.2		1	6.3 ± 0.2	
	1000	1	17.4 ± 1.0	76 ± 4.6	3	9.8 ± 1.7	1.9 ± 0.5	1	3.6 ± 0.8	43 ± 8
	10	1	4.1 ± 0.1	18 ± 0.2	3	6.7 ± 0.1	1.3 ± 0.3	1	3.4 ± 0.3	46 ± 5
	0.1	1	1.5 ± 0.1	7 ± 0.3	3	5.3 ± 0.1	1.0 ± 0.1	1	3.1 ± 0.2	51 ± 2
5	0	1	1.6 ± 0.3		7	16.3 ± 2.6		7	250.6 ± 15.0	
	1000	1	107.4	67	7	29.9	1.8	7	67.8	65
	10	1	21.9 ± 1.1	14 ± 0.7	7	36.3 ± 2.7	2.2 ± 0.2	7	94.0 ± 13.1	63 ± 5
	0.1	1	10.5 ± 3.7	7 ± 2.3	7	27.1 ± 1.7	1.7 ± 0.1	7	157.2 ± 0.6	37 ± 1
6	0	5	2.4 ± 0.1		7	13.6 ± 0.3		3	177.2 ± 13.4	
	1000	5	72.5 ± 4.6	30 ± 1.9	7	26.1 ± 3.4	1.9 ± 0.3	3	28.3 ± 2.1	84 ± 1
	10	5	31.2 ± 7.3	13 ± 1.8	7	22.8 ± 0.9	1.7 ± 0.1	3	70.0 ± 19.2	60 ± 11
	0.1	5	8.2 ± 0.1	3 ± 0.1	7	22.8 ± 1.8	1.7 ± 0.1	3	159.0 ± 2.5	10 ± 1

Table 20: Effect of γ interferon at 0.1, 10 and 1000 ng/ml on monocyte C1-inhibitor, C2 and C3 synthesis.

Values are reported as mean ± SEM, and were selected to show maximum enhancement and inhibition levels observed over the 7 day culture period.

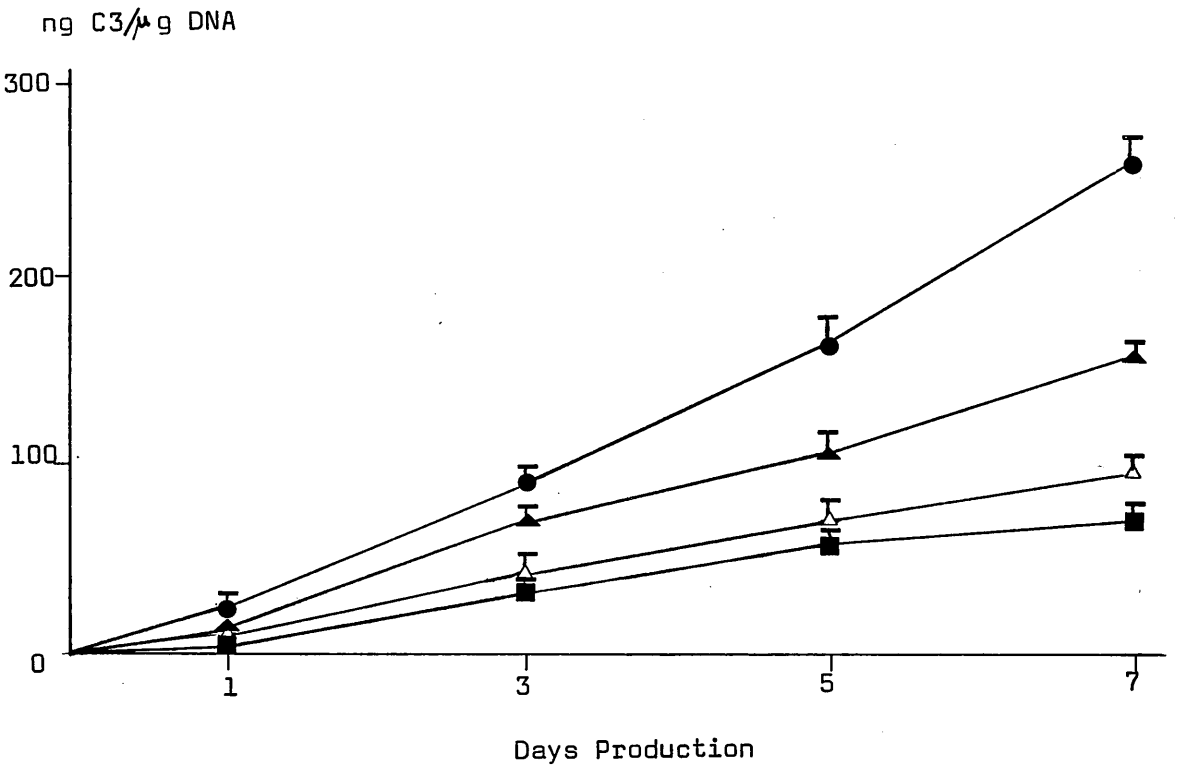


Fig. 28: Effect of ɣ interferon on monocyte C3 synthesis.

Time course of C3 synthesis (ng C3/µg DNA) by monocytes in culture on days 1, 3, 5 and 7 (x axis) with ɣ interferon at 1000 ng/ml (■), 10 ng/ml (△) and 0.1 ng/ml (▲) and control synthesis (●). This corresponds to culture 5 in Table 20.

doses above about 10 ng/ml (Fig. 27), showing maximum enhancement over control of about 2 to 3.6 fold (Table 19). At the lowest dose of γ interferon used (0.01 ng/ml) a low level of enhancement was observed of 1.3 and 1.4 fold over controls.

The results for the effects of γ interferon on the synthesis of C3 by monocytes are shown in Table 20 and Figures 27 and 28. The dose response curve to γ interferon over the dose range tested (0.1 ng/ml to 1000 ng/ml), demonstrated that there was a reduction in C3 synthesis at all doses tested (Fig. 27). The peak inhibitory dose for 2 of the 3 cultures tested, was 1000 ng γ interferon/ml which was shown to inhibit C3 synthesis by 65 and 84% (Table 20, cultures 5 and 6 respectively). However, in culture 4 the maximum degree of inhibition of C3 synthesis of 51% occurred at a dose of 0.1 ng/ml. Thus, C3 synthesis was reduced by γ interferon over the whole dose range tested, even at the lowest dose investigated, 0.1 ng/ml.

An experiment was performed to investigate the effect of γ interferon (1 μg/ml) on the synthesis of several monocyte secretory products including C2, C1-inhibitor, C3, B, properdin and lysozyme. A triplicate set each of control and γ interferon-treated cultures were harvested on days 3, 5 and 7, and assayed for the aforementioned components. The results, shown in Table 21, demonstrated that γ interferon

Component	Day 3		Day 5		Day 7	
	Control	ɣ interferon	Control	ɣ interferon	Control	ɣ interferon
C2 em x 10^7/ µg DNA	2.5 ± 0.1	5.2 ± 0.7	4.9 ± 0.9	14.0 ± 2.1	9.3 ± 0.6	13.1 ± 1.9
C1-inh ng/ µg DNA	2.2 ± 0.2	34.9 ± 2.1	2.5 ± 0.3	62.1 ± 4.7	4.8 ± 0.6	106.8 ± 2.6
B ng/µg DNA	0.9 ± 0.1	1.3 ± 0.1	1.4 ± 0.2	3.2 ± 0.1	1.0 ± 0.1	2.8 ± 0.1
P ng/µg DNA	3.6 ± 0.1	2.1 ± 0.1	5.2 ± 0.2	2.7 ± 0.2	8.5 ± 0.6	2.9 ± 0.2
C3 ng/µg DNA	68.7 ± 7.9	36.3 ± 3.3	42.3 ± 4.5	48.4 ± 12.9	58.4 ± 12.8	56.9 ± 3.8
Lysozyme u/µg DNA	8.6 ± 0.2	7.2 ± 0.4	16.8 ± 1.7	20.7 ± 1.0	40.5 ± 1.5	39.2 ± 1.7

Table 21: Effect of ɣ interferon (1µg/ml) on monocyte C2, C1-inhibitor, B, P, C3 and Lysozyme Synthesis on Days 3, 5 and 7.

Values reported as mean ± SEM for triplicate samples.

stimulated monocyte C2 and B synthesis to similar levels; it stimulated C1-inhibitor synthesis to a much greater level; it inhibited the synthesis of C3 and properdin to similar levels; it had no effect on the synthesis of lysozyme.

Thus, from this study it is apparent that γ interferon has its greatest effect on the synthesis of monocyte C1-inhibitor (maximum effect of greater than 890 fold increase over control), with a steep dose response curve which showed no signs of levelling off, even when high doses of γ interferon were used (Fig. 27). Interferon stimulated C2 synthesis to a much lower level (maximum effect of 3.6 fold increase) and it did not demonstrate such a well defined dose-response curve as C1-inhibitor (Fig. 27). C3 synthesis was inhibited by γ interferon to a maximum of 84% at 1 000 ng/ml, although the maximum inhibition did not always occur at the highest dose tested.

3.3.2: Pulse-Chase Experiments

The effect of γ interferon (1 µg/ml) on the synthesis of monocyte C1-inhibitor was investigated and the results of one such pulse-chase experiment are shown in Figs. 24 and 25. The results demonstrated that when the supernatant and lysate of γ interferon-treated monocytes were precipitated for C1-inhibitor, there was a dramatic enhancement of C1-inhibitor synthesis. Figure 24 demonstrates the specific bands obtained from the supernatant of γ interferon treated (denoted by suffix i, Fig. 24) and control (denoted by suffix c, Fig. 24) monocytes after a one hour pulse with ^{35}S-methionine, followed by chase periods of 0, 1, 2 and 4 hr. Although the

amounts of ^{35}S-methionine labelled C1-inhibitor differed markedly, as denoted by the intensity of the bands, similar secretion rates were observed. Rates of disappearance of 50% of the intracellular precursors, and the appearance of 50% of the secreted form of C1-inhibitor was similar in control and γ interferon-treated cultures, as assessed by densitometry and by radioactivity measured in the bands cut from the gels. Between 1 hr and 2 hr the intensity of the bands observed in the supernatant increased markedly, whereas between 2 hr and 4 hr, there was little increase in band intensity. The molecular weight of secreted C1-inhibitor from stimulated and unstimulated monocytes was calculated to be 96 kD (Fig. 24).

Figure 25 demonstrates pro-C1-inhibitor in the lysates of γ interferon-treated (denoted by suffix i in Fig. 25) and control (denoted by suffix c in Fig. 25) monocytes. There appeared to be very little pro-C1-inhibitor in control monocytes as the specific immunoprecipitation band was of low intensity and difficult to visualise. However, the pattern of secretion was similar to that found in the γ-interferon-treated cells. The band at 1 hr was slightly less dense than the 0 hr; by 2 hr chase time the band intensity had decreased dramatically and by 3 hr virtually no intracellular pro-C1-inhibitor remained. This pattern coincided with the appearance of increasing amounts of labelled C1-inhibitor in the culture supernatant: a small amount had appeared extracellularly by 1 hr, but by 2 hr virtually all of the C1-inhibitor had been secreted (Fig. 24).

Glucocorticoid	Component	N	Peak effect dose (M)	% Change
Hydrocrotisone	Cl-inh	3	10^{-5}	229, 55, 112
	C2	3	10^{-5}	94, 61, 55
	C3	1	10^{-4}	-73
	Lysozyme	2	10^{-4}	-46, -81
Prednisolone	Cl-inh	4	10^{-5},10^{-6},10^{-7},10^{-5}	273, 196, 115, 54
	C2	4	10^{-6},10^{-5},10^{-6},10^{-6}	225, 106, 85, 95
	C3	1	10^{-6}	-48
	Lysozyme	3	10^{-5},10^{-6},10^{-5}	-35, -26, -68
Dexamethasone	Cl-inh	3	10^{-5},10^{-6},10^{-7}	513, 83, 232
	C2	3	10^{-5},10^{-6},10^{-5}	245, 99, 161
	C3	1	10^{-4}	-53
	Lysozyme	2	10^{-5},10^{-4}	-36, -78
Org 6632	Cl-inh	3	10^{-8}	303, 320, 160
	C2	3	10^{-8},10^{-9},10^{-10}	250, 75, 96
	C3	2	10^{-7},10^{-8}	-70, -46
	Lysozyme	2	10^{-7},10^{-8}	-77, -67

Table 22: Peak effective dose and effect of glucocorticoids on Cl-inhibitor, C2, C3 and lysozyme synthesis by monocytes.

The values are quoted as % change, with positive values denoting enhanced synthesis and negative values denoting inhibition of synthesis, compared to control. N represents the number of cultures in which each glucocorticoid has been tested.

3.4 The Effect of Steroid Hormones on Monocyte Complement Synthesis

3.4.1 The Effect of Glucocorticoids on Monocyte Complement Synthesis

The effect of the following glucocorticoids on monocyte C2, C1-inhibitor, C3 and lysozyme synthesis were tested over the dose range 10^{-10} to 10^{-4}M: corticosterone, hydrocortisone, prednisolone, dexamethasone, and Org 6632.

With the exception of corticosterone, all glucocorticoids tested produced stimulation of monocyte C2 and C1-inhibitor synthesis, whereas C3 and lysozyme synthesis were inhibited. The data summarised in Table 22 and Figures 29-32 show the mean $\pm$ SEM expressed as % change with positive values demonstrating enhanced synthesis, and negative values demonstrating inhibition of synthesis. The values reported for each set of cultures were chosen to demonstrate the maximum effect observed throughout the entire culture.

3.4.1a Corticosterone

Corticosterone, over a dose range of 10^{-9} to 10^{-4}M did not produce any significant effect on monocyte C2 or C1-inhibitor synthesis, in the three cultures tested.

3.4.1b Hydrocortisone

Hydrocortisone stimulated monocyte C1-inhibitor synthesis over the complete dose range tested, 10^{-9} to 10^{-4}M, at some time during the culture.

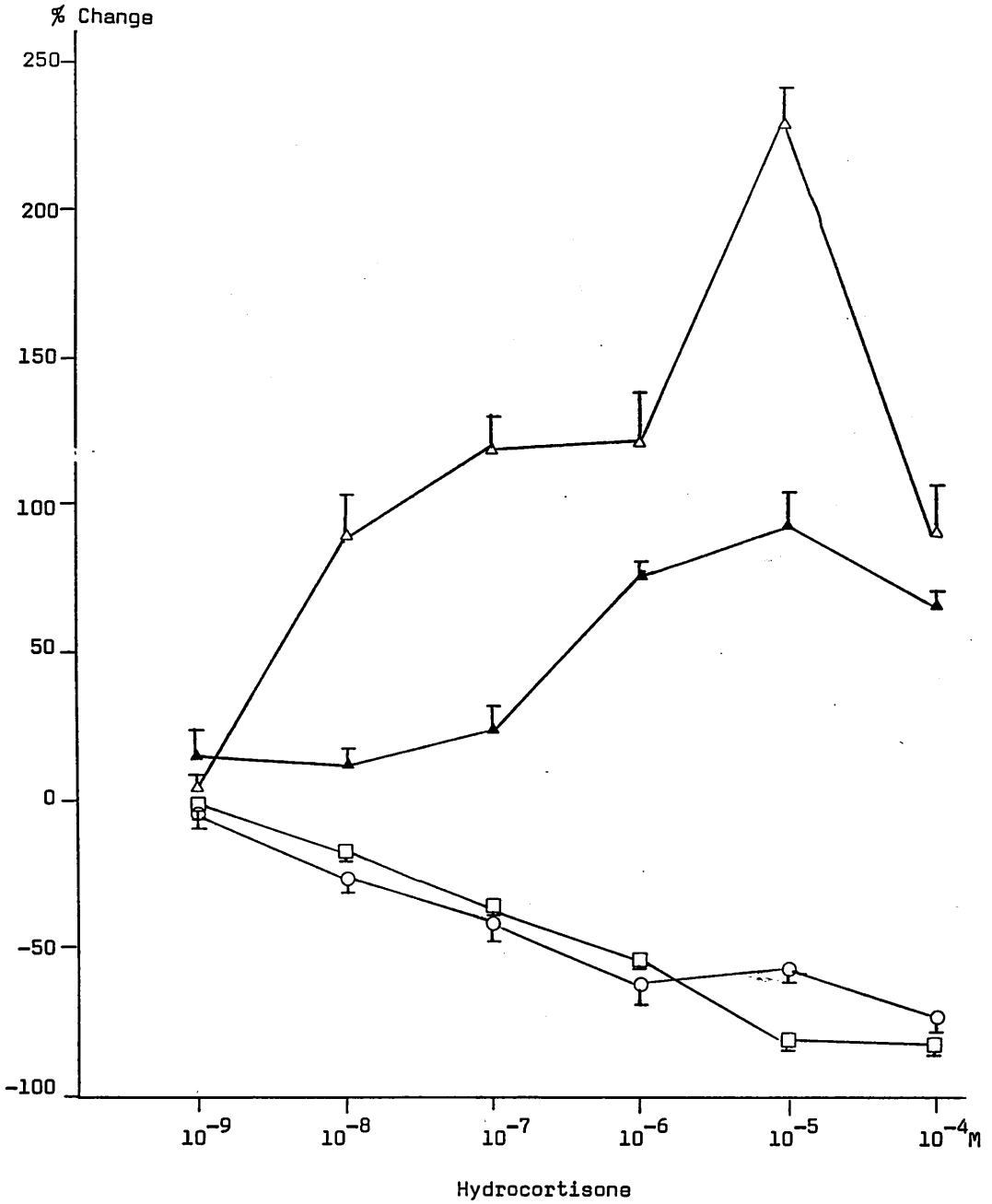


Fig. 29: Dose Response curves of monocyte C1-inhibitor (△), C2 (▲), C3 (○) and lysozyme (□) synthesis to hydrocortisone (10^{-9} to 10^{-4}M).

The values reported represent the mean ± SEM for a triplicate set of samples from one representative experiment. The peak effective dose and % change values obtained for this and the remaining two cultures tested with hydrocortisone are shown in Table 22.

Table 22 shows that out of the three cultures tested the peak enhancing dose for C1-inhibitor in all three cultures was 10^{-5}M, with the levels of enhancement ranging from 55% up to 229%.

C2 synthesis by monocytes was also stimulated by hydrocortisone over the same dose range, 10^{-9} to 10^{-4}M (Fig. 29). The peak enhancing dose for C2 in all three cultures tested was again 10^{-5}M, with the levels of enhancement ranging from 55% to 94% (Table 22).

Monocyte C3 and lysozyme synthesis were inhibited by hydrocortisone (Fig. 29 and Table 22). Lysozyme synthesis was inhibited over the dose range 10^{-8} to 10^{-4}M, with the maximum inhibition occurring at 10^{-4}M, to a level of 46 and 81%. The maximum inhibitory effect of hydrocortisone on lysozyme synthesis was usually achieved by concentrations of 10^{-6}M.

C3 synthesis was inhibited by hydrocortisone over the complete dose range tested, 10^{-9} to 10^{-4}M, to a maximum of 73% at 10^{-4}M. As shown in the dose response curve there was a plateau area observed above 10^{-6}M hydrocortisone.

3.4.1c Prednisolone

Prednisolone stimulated C1-inhibitor synthesis over the dose range 10^{-7} to 10^{-4}M (Fig. 30) in all four cultures tested (Table 22), one of which was also stimulated at 10^{-8}M. The peak enhancing dose in two of the cultures was 10^{-5}M with C1-inhibitor synthesis being stimulated by 273% and 54% over control (Table 22). In the other two cultures the peak enhancing doses occurred at 10^{-6}M and 10^{-7}M, with levels

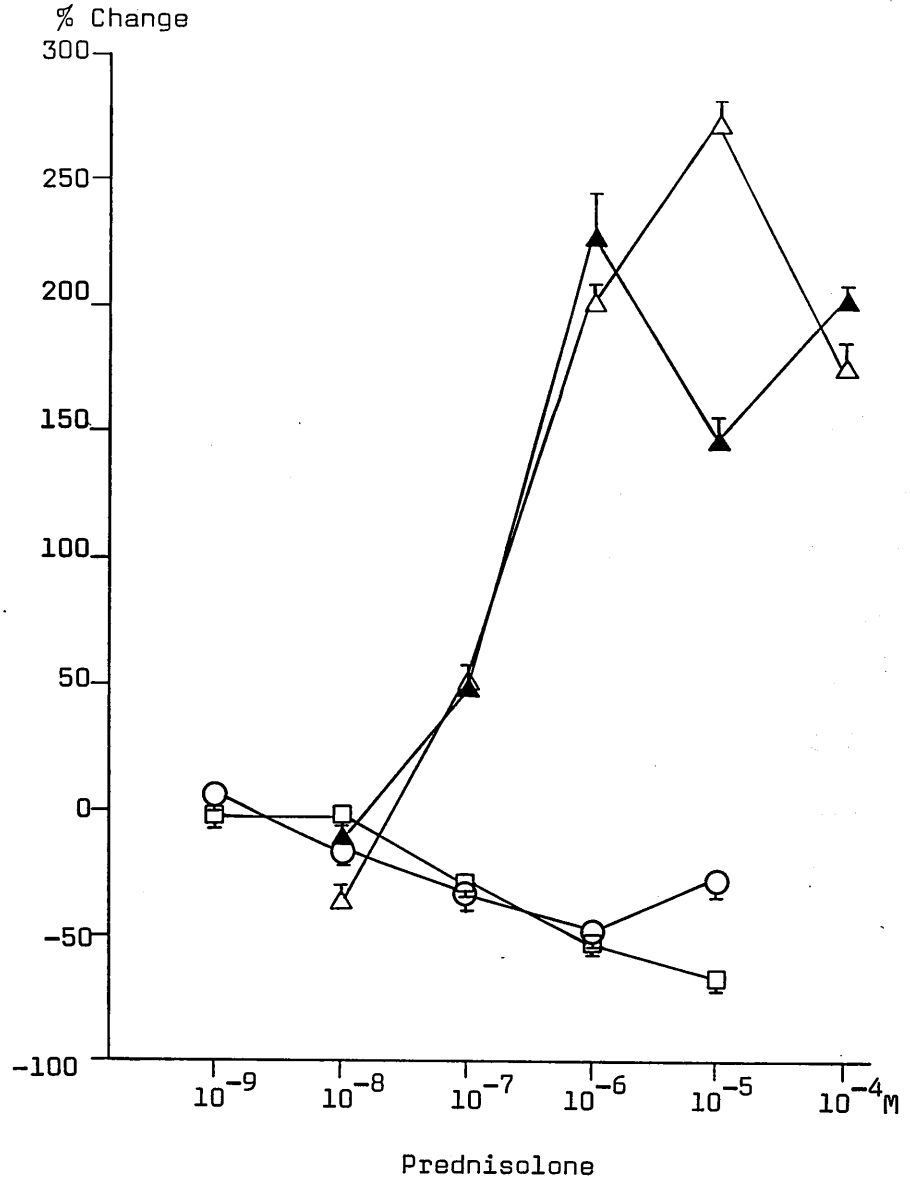


Fig. 30: Dose Response Curves of monocyte C1-inhibitor (△), C2 (▲), C3 (○) and lysozyme (□) synthesis to prednisolone (10^{-9} to 10^{-4}M).

The values reported represent the mean ± SEM for a a triplicate set of samples from one representative experiment. The peak effective dose and % change values obtained for this and the remaining three cultures tested with prednisolone are shown in Table 22.

of enhancement of 196% and 115% respectively over control (Table 22).

Prednisolone stimulated C2 synthesis over the dose range 10^{-7} to 10^{-4}M (Fig. 30), with one of the four cultures also being stimulated at 10^{-8}M. The peak enhancing dose for three out of the four cultures was 10^{-6}M, with the levels of enhancement ranging from 85% to 225% over controls (Table 22). The fourth culture showed a peak enhancing dose of 10^{-5}M, with a level of enhancement of 106% (Table 22).

C3 synthesis by monocytes was inhibited by prednisolone over the dose range 10^{-8} to 10^{-5}M as demonstrated (Fig. 30), where the greatest level of inhibition attained was 48% at 10^{-6}M.

Lysozyme synthesis was inhibited by prednisolone: two of the three cultures were inhibited over the dose range 10^{-6} to 10^{-4}M, the third culture was also inhibited at 10^{-7}M (Fig. 30). Once again, a plateau of inhibition was observed in each culture at concentrations of prednisolone above 10^{-6}M.

3.4.1d Dexamethasone

Dexamethasone was shown to cause an enhancement of C1-inhibitor synthesis over the complete dose range from 10^{-9} to 10^{-4}M (Fig. 31). However, it did not stimulate C1-inhibitor synthesis at 10^{-10}M in the one culture in which it was tested. The peak enhancing dose of dexamethasone for stimulation of C1-inhibitor synthesis appeared to vary between 10^{-5}M, 10^{-6}M and 10^{-7}M, showing levels of enhancement of 513%, 83% and 232% respectively (Table 22).

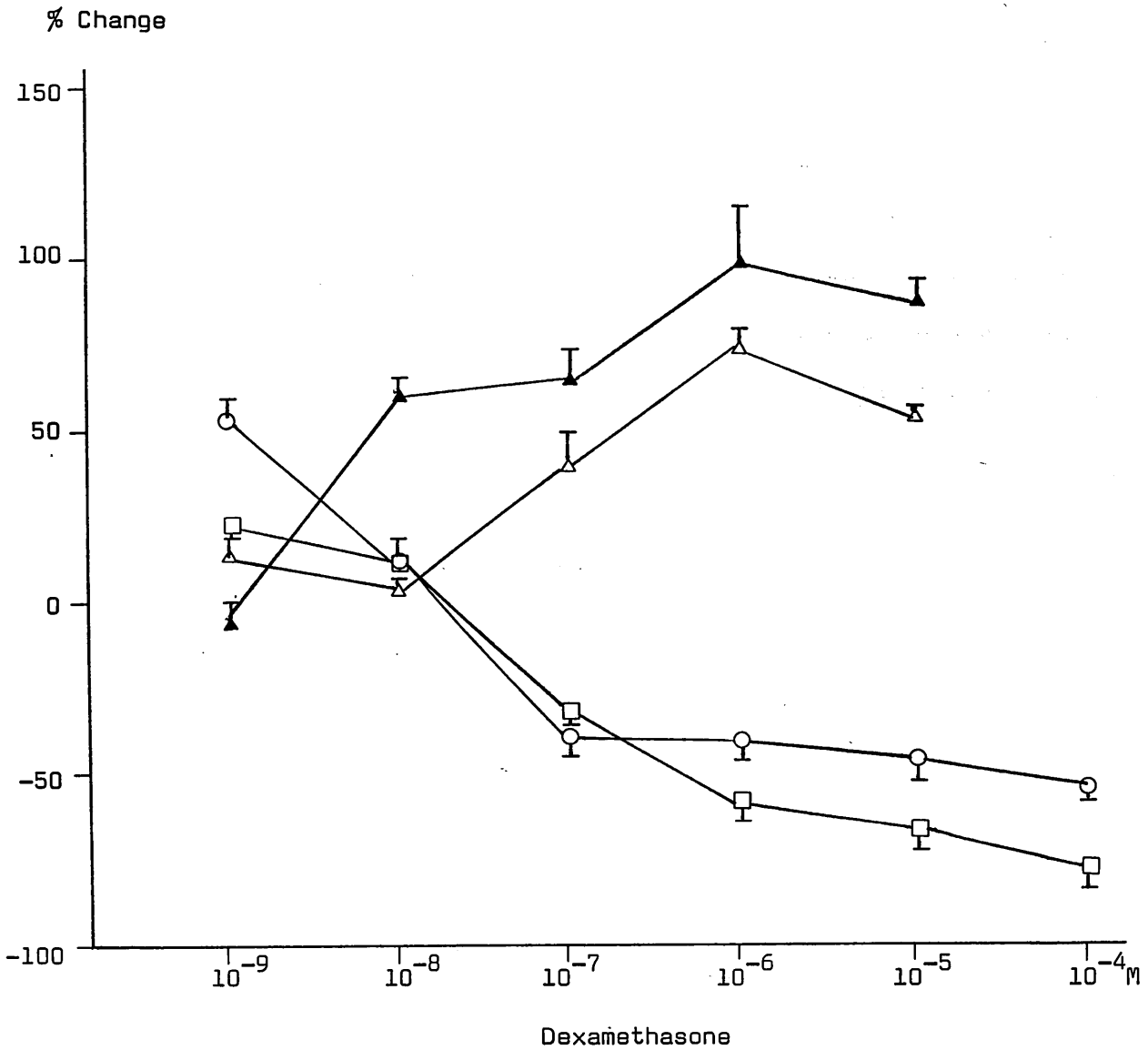


Fig. 31: Dose Response Curve of Monocyte C1-inhibitor (△), C2 (▲), C3 (○) and lysozyme (□) synthesis to Dexamethasone (10^{-9} to 10^{-4}M). The values reported represent the mean ± SEM for a triplicate set of samples from one representative experiment. The peak effective dose and % change values obtained for this and the remaining two cultures tested with dexamethasone are shown in Table 22.

C2 synthesis was stimulated by dexamethasone over the dose range of 10^{-8}M to 10^{-4}M in two cultures (Fig. 31 shows one culture), whilst the third culture was also stimulated at 10^{-9}M. The peak enhancing dose for C2 when stimulated with dexamethasone varied from 10^{-5}M (showing 245% enhancement) down to 10^{-7}M (showing 232% enhancement).

C3 synthesis was inhibited by dexamethasone between 10^{-7}M and 10^{-4}M, but was not inhibited at lower doses, to the contrary, at 10^{-9}M C3 synthesis appeared to be slightly stimulated (Fig. 31). The maximum level of inhibition of C3 synthesis occurred at 10^{-4}M showing 53% inhibition (Table 22). However, this was on a plateau area which occurred at doses of 10^{-7}M and above (Fig. 31).

Lysozyme synthesis in one of the two cultures was inhibited across the complete dose range tested (10^{-9} to 10^{-4}M), whereas it was only inhibited between 10^{-7} and 10^{-4}M in the other culture (Fig. 31). The peak levels of inhibition observed varied from 36% at 10^{-5}M to 78% at 10^{-4}M for the two cultures tested.

3.4.1e Org 6632

Org 6632, a glucocorticoid supplied by Organon Laboratories, produced a similar profile of stimulation and inhibition of monocyte complement and lysozyme synthesis to the other glucocorticoids studied, but it was effective at lower doses (Table 22 and Fig. 32).

C1-inhibitor synthesis by monocytes was stimulated over the complete dose range tested, 10^{-10}M to 10^{-5}M, (Fig. 32) with the peak enhancing dose in all three cultures

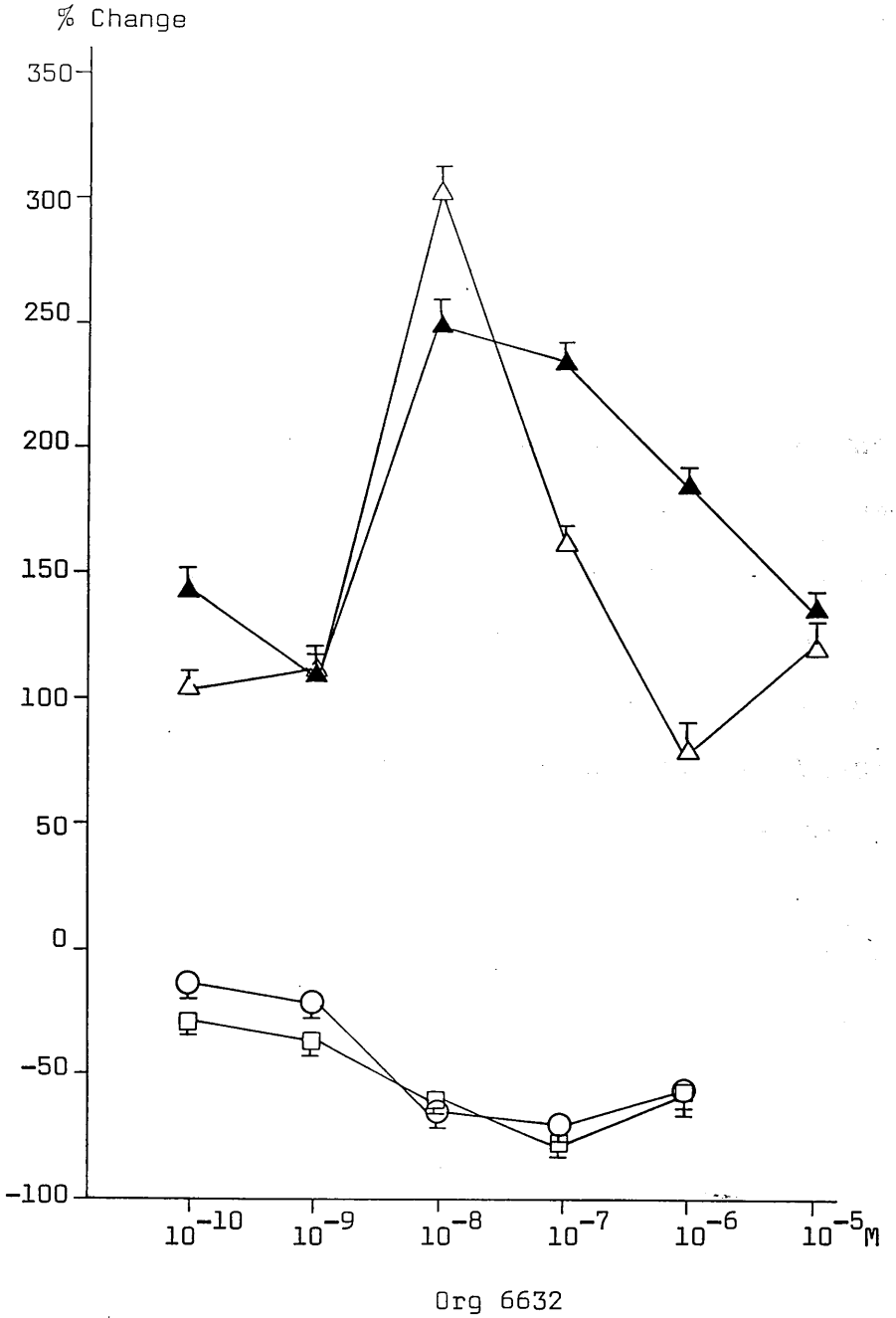


Fig. 32: Dose Response Curve of Monocyte C1-inhibitor (△), C2 (▲), C3 (○) and lysozyme (□) synthesis to Org 6632 (10^{-10} to 10^{-5}M).

The values reported represent the mean ± SEM for a triplicate set of samples from one representative experiment. The peak effective dose and % change values obtained for this and the remaining two cultures tested with Org 6632 are shown in Table 22.

being 10^{-8}M, and the level of stimulation ranging from 160% up to 320% (Table 22). The dose response for this glucocorticoid was very wide, and was the only one which was stimulatory at a dose as low as 10^{-10}M (Fig. 32).

C2 synthesis was stimulated over the complete dose range tested, 10^{-10}M to 10^{-5}M, with the peak enhancing dose varying in all three cultures tested (Table 22). Peak enhancement occurred in different cultures at 10^{-8}M (250%), 10^{-9}M (75%) and 10^{-10}M (96%) (Table 22).

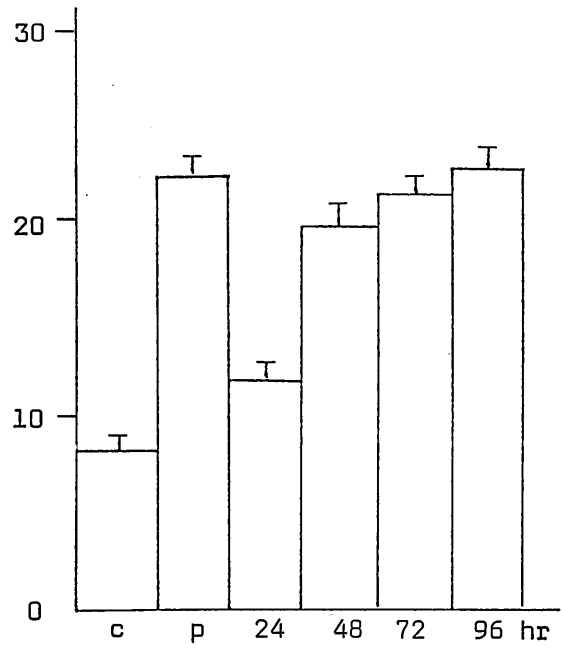
C3 synthesis was inhibited over the whole dose range tested, 10^{-10}M to 10^{-6}M, (Fig. 32) with the maximum inhibition occurring at 10^{-7}M (70%) and 10^{-8}M (46%) (Table 22). These two cultures displayed the familiar plateauing effect of inhibition of C3 synthesis; however in this case the plateau started at a lower dose of about 10^{-8}M.

Lysozyme synthesis was inhibited over the complete dose range tested, 10^{-10}M to 10^{-6}M (Fig. 32) with maximum inhibitory levels occurring at 10^{-7}M (77%) and 10^{-8}M (67%) on the plateau area of inhibition.

Thus the glucocorticoids hydrocortisone, prednisolone, dexamethasone and Org 6632 all appear to have similar effects on monocyte complement and lysozyme synthesis. They showed enhancement of monocyte C1-inhibitor and C2 synthesis and inhibition of lysozyme and C3 synthesis. Hydrocortisone stimulated C1-inhibitor and C2 maximally at 10^{-5}M; dexamethasone and prednisolone stimulated C2 and C1-inhibitor synthesis maximally over the range of 10^{-7}M to 10^{-5}M. However, Org 6632 appeared to be the most potent, stimulating C1-inhibitor

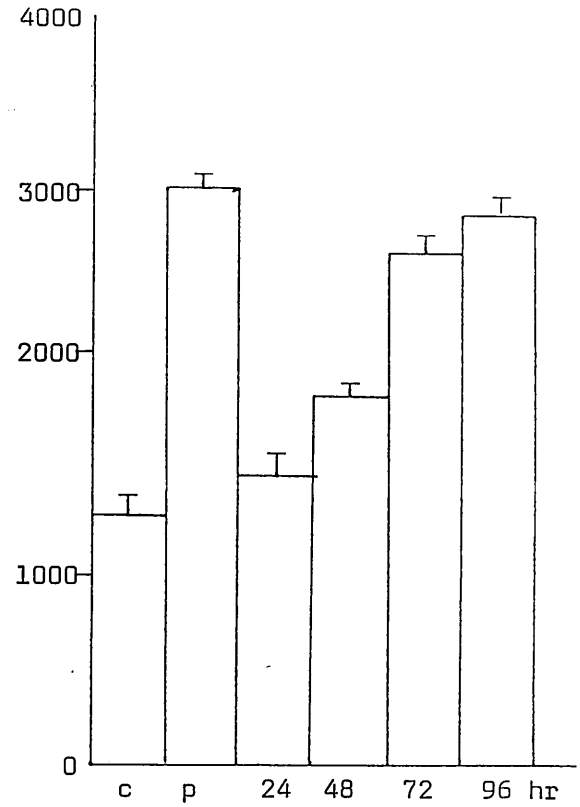
Fig. 33: Glucocorticoid Time Exposure Experiment.
Monocytes were pre-exposed to 10^{-6}M dexamethasone (A and B) or 10^{-5}M Prednisolone (C and D) permanently (P) or for 24, 48, 72 or 96 hr, and the levels of C2 (A and C) and C1-inhibitor (Band D) measured at the end of a 7 day culture period. C denotes unstimulated control monocytes.

A C2 em x 10^7/µg DNA



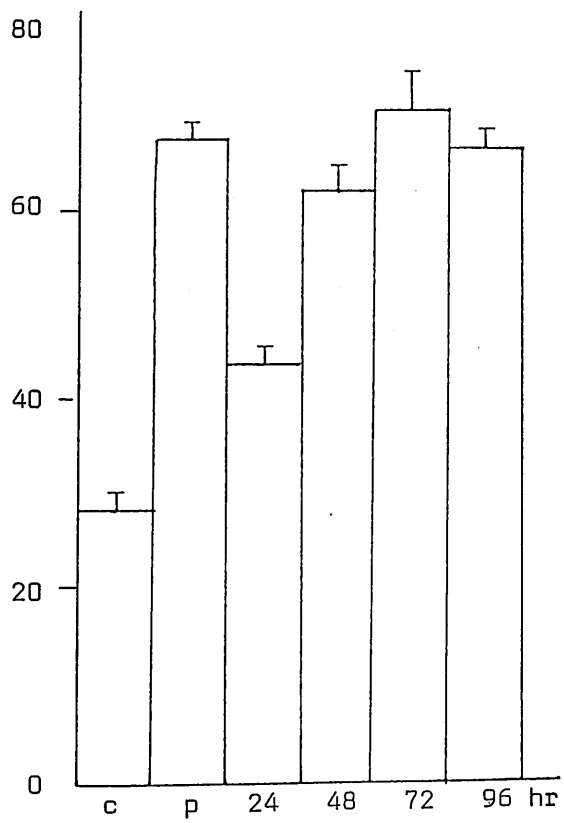
Dexamethasone 10^{-6}M

B pg C1-inh/µg DNA



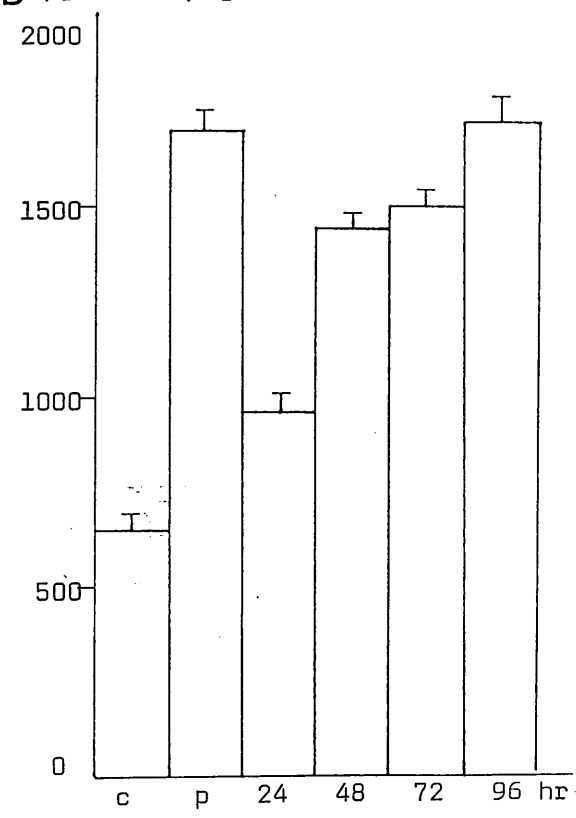
Dexamethasone 10^{-6}M

C C2 em x 10^7/µg DNA



Prednisolone 10^{-5}M

D pg C1-inh/µg DNA



Prednisolone 10^{-5}M

and C2 synthesis maximally between 10^{-7}M and 10^{-8}M.

Inhibition of lysozyme and C3 synthesis by the glucocorticoids, hydrocortisone, prednisolone and dexamethasone, was usually maximal at concentrations of 10^{-6}M. In comparison, Org 6632, produced maximal inhibition of C3 and lysozyme synthesis at 10^{-8}M.

3.4.1f Glucocorticoid Time Exposure Experiments

Experiments were performed to determine the length of time glucocorticoids had to be present in the monocyte cultures in order to produce their enhancing effects on C2 and C1-inhibitor synthesis. The first set of experiments involved exposing monocytes to 10^{-6}M dexamethasone or 10^{-5}M prednisolone for 5, 15, 30 min, 1, 2 or 4 hrs; washing the steroid out of the culture, adding fresh medium and culturing for a further seven days. One set of wells had the glucocorticoid added back to determine the maximum stimulation which would have been observed when the glucocorticoid was present all of the time (denoted P in Fig. 33). These exposure times did not result in any enhancement of C2 or C1-inhibitor synthesis, therefore the times of exposure were lengthened to 24, 48, 72 and 96 hr, before the drug was removed. Culture was again continued in fresh medium for 7 days, and the results obtained for C2 and C1-inhibitor are reported in Figure 33, when the cells were pretreated with dexamethasone (10^{-6}M) (Fig. 33A and B) or prednisolone (10^{-5}M) (Fig. 33C and D).

The results for enhancement of C2 were very similar for dexamethasone and prednisolone. It demonstrated that to get enhancement of C2 synthesis, to a similar level to that observed when the glucocorticoids were present all of the time (Fig. 33 denoted P), the drug had to be present for a minimum preincubation period of 48 hrs. When it was only preincubated for 24 hr, the levels of C2 measured were less than 40% enhanced over control, compared to 162 and 120% for dexamethasone and prednisolone respectively, when present all of the time in culture (Fig. 33A and C).

When measuring enhancement of C1-inhibitor synthesis by dexamethasone and prednisolone over the same time course, the results were not so clear (Fig. 33, B and D). The time of preincubation appeared to be longer at 72 hr for dexamethasone before enhancement became equal to that when the drug was present permanently. For prednisolone, 48 hr exposure appeared to be long enough, although the level of enhancement was slightly lower (120%) than that when the drug was present permanently (160%); however these differences were not significant.

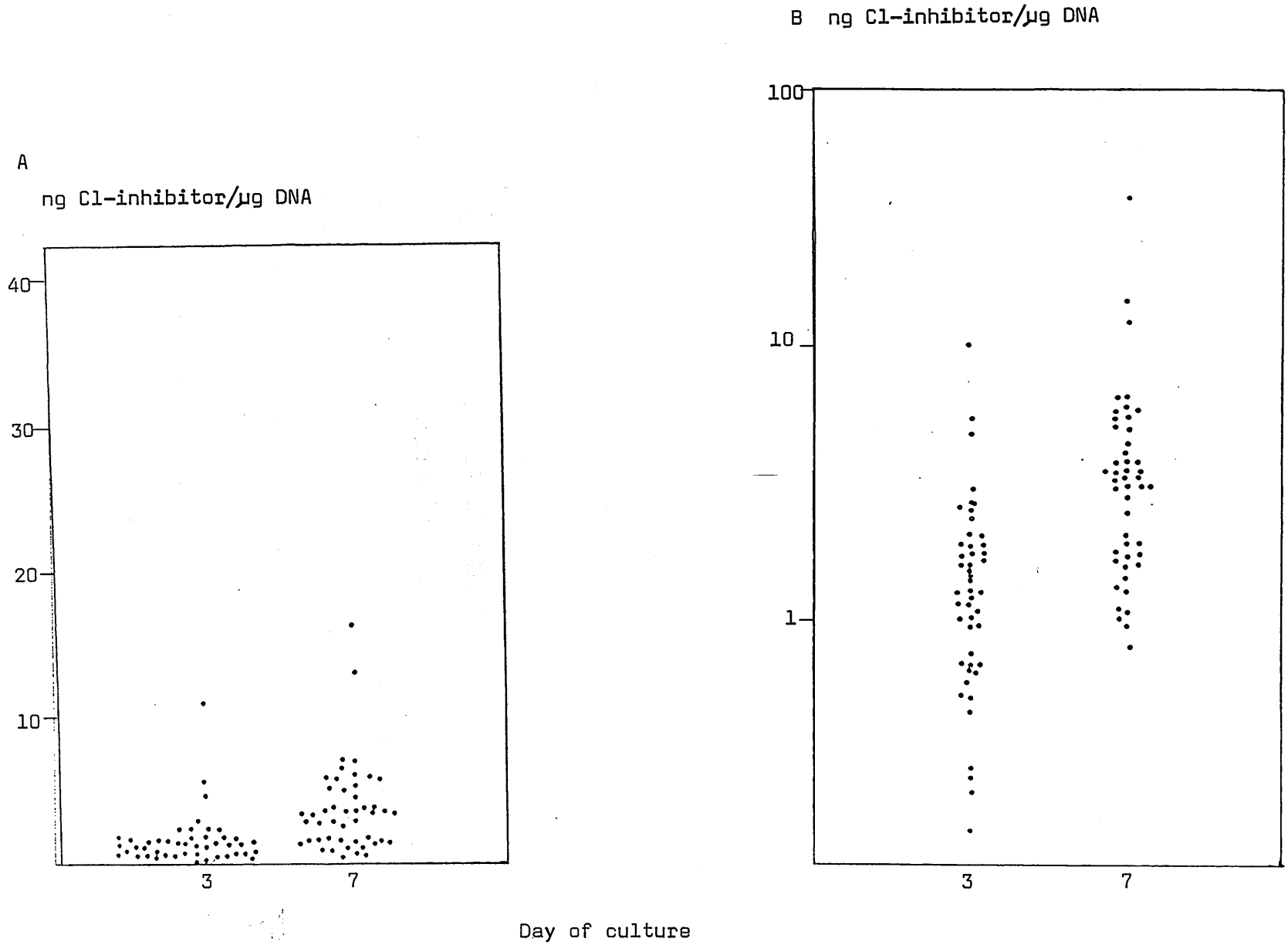
Thus, for enhancement of monocyte C2 synthesis to occur the glucocorticoids had to be present for a minimum of 48 hrs. For glucocorticoid-induced enhancement of C1-inhibitor synthesis, prednisolone had to be present for a minimum of 48 hrs while dexamethasone had to be present for 72 hrs to ensure its maximum stimulatory effect.

Fig. 34: Distribution of C1-inhibitor concentrations in normal monocyte culture supernatant.

A: Linear plot of C1-inhibitor concentrations on days 3, and 7 from 47 normal donors.

B: Logarithmic plot of C1-inhibitor concentrations on days 3, and 7 from 47 normal donors.

This demonstrates that the distribution of C1-inhibitor concentrations in normal monocyte culture supernatants is not normal but logarithmic.


A
ng Cl-inhibitor/µg DNA
40
30
20
10
3
7
B ng Cl-inhibitor/µg DNA
100
10
1
3
7
Day of culture

3.4.2 The Effect of Androgens, Anabolic Steroids, Oestrogens, Progesterones and Mineralocorticoids on Monocyte Complement Synthesis

The effect of the following groups of steroid hormones on monocyte C2 and C1-inhibitor synthesis was tested over the dose range 10^{-9} to 10^{-4}M. The androgens: testosterone benzoate, testosterone β-D-glucuronide, 17αmethyltestosterone and testosterone hemisuccinate. The anabolic steroids: danazol, stanozolol, fluoxymesterone and oxymethalone. The oestrogens: estrone-3-sulphate, estriol 17β glucuronide, 17αestradiol, diethylstilbestrol and Δ^4 androstene 3,17 dione. The progesterones: 11α hydroxyprogesterone, 17α hydroxyprogesterone and 17α hydroxypregnenolone. The mineralocorticoids: aldosterone and desoxycorticosterone.

In the three sets of cultures tested for each drug, no significant effects on monocyte C2 or C1-inhibitor synthesis were observed.

3.5 Study of Monocytes from C1-inhibitor Deficient Patients

3.5.1 Normal Range of Monocyte C1-inhibitor Synthesis

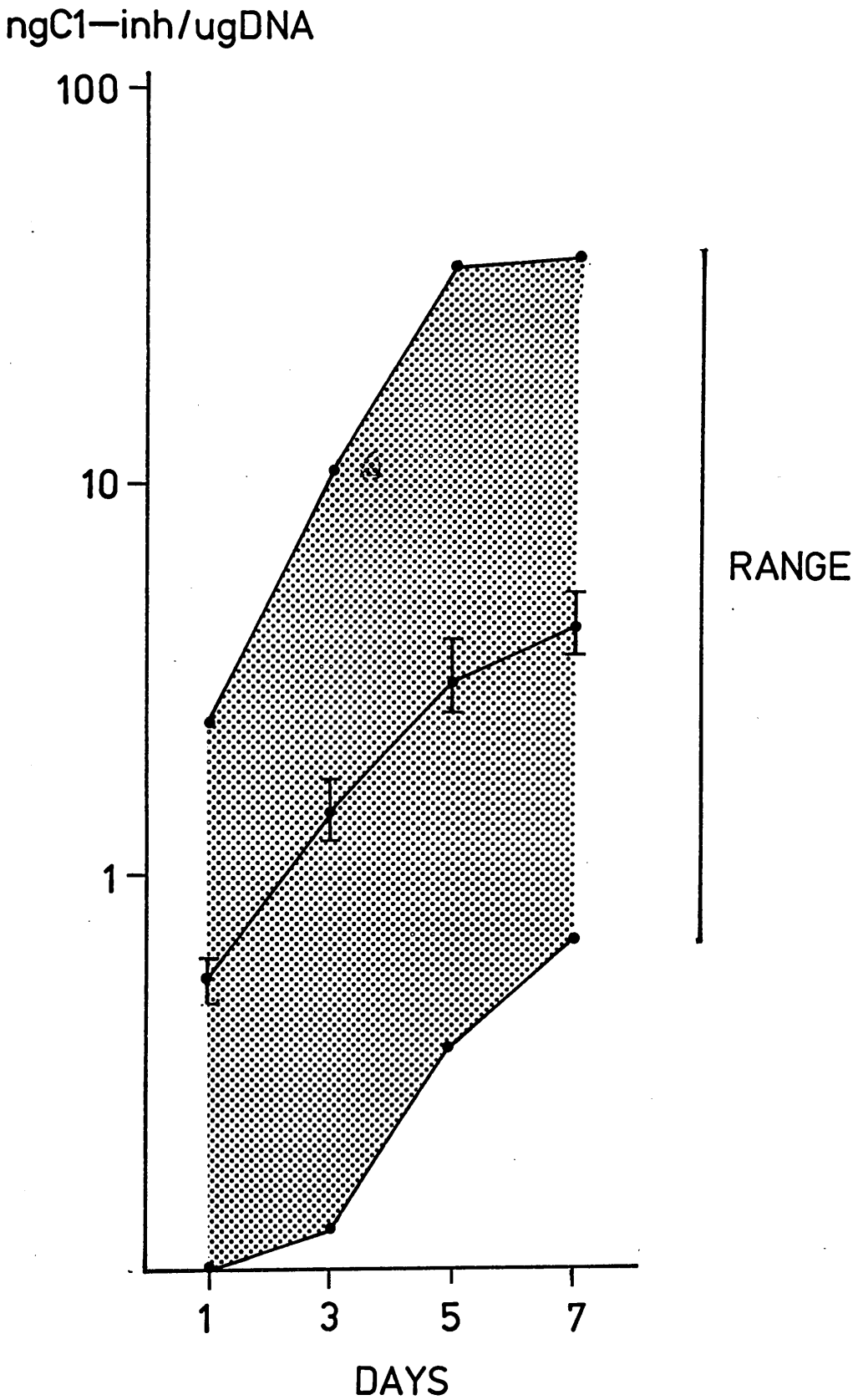
In order to study C1-inhibitor synthesis by monocytes from patients with various types of C1-inhibitor deficiency, a range for normal monocyte C1-inhibitor synthesis had to be established. These data were derived from monocyte cultures from 47 normal healthy donors.

The distribution of C1-inhibitor concentrations in the culture supernatants was logarithmic (Fig. 34), therefore the data were log transformed for statistical analysis. A logarithmic distribution was also demonstrated for C2 and

Fig. 35: Normal Monocyte C1-inhibitor Synthesis.

The shaded area represents the range of levels of C1-inhibitor (ng C1-inh/μg DNA) in monocyte supernatants from 47 normal donors on days 1, 3, 5 and 7.

$\overset{\pm}{\bullet}$ represents the mean ± SEM.


ngC1—inh/ugDNA
100
10
1
RANGE
1
3
5
7
DAYS

Patient	Age Yrs	Sex	Type	Treatment	Clinical Profile			
					C1-inh	C4	C3	CH50
JAn		F	I	None	75	391	1647	273
JA	47	F	I	None	90	106	1414	157
				Danazol	128	296	984	167
CB	28	F	I	None	79	60	1244	169
				Stanozolol	103	225	1066	218
DC		M	I	Danazol	208	186	1138	183
GC	28	M	I	Danazol	72	185	958	194
NC	57	M	I	Stanozolol	91	167	1020	23
CF	14	M	I	None	74	41	1114	18
EF	34	F	I	Stanozolol	116	160	1078	196
DH	40	M	I	None	26	79	978	81
				Danazol	88	183	1070	208
CA.McG	78	F	I	Stanozolol	116	115	970	218
CH.McG	76	F	I	Stanozolol	158	376	938	255
AM	71	M	I	Stanozolol	140	38	364	19
WM	27	M	I	None	37	41	732	ND
DP	28	M	I	Danazol	270	107	1363	214
CE		F	II	Danazol	510	314	1381	228
JH		F	II	Danazol	348	192	1125	129
JM		M	II	None	292	78	1217	115
JM		F	Acquired	None	27	0	1001	3
LP		F	Acquired	Danazol	325	86	834	68
GW		M	Acquired	None	90	0	1242	0

Table 23: Clinical details of patients involved in C1-inhibitor deficiency study.

C1-inh, C4 and C3 are in µg/ml; CH50 is units/ml.

Normal ranges are

C1-inh 160-370 µg/ml; C4 199-574 µg/ml;

C3 720-1800 µg/ml; CH50 150-250 units/ml.

Families: NC and GC are father and son; EF and CF are mother and son; Ca McG and Ch McG are sisters; CE and JH are sisters.

C3 concentrations in monocyte culture supernatant (data not shown), thus, these data were also log transformed for statistical analysis.

Figure 35 shows that the mean levels of C1-inhibitor for the 47 normal controls on days 1, 3, 5 and 7 were 550, 1504, 3239 and 4361 pg C1-inhibitor/μg DNA respectively. The shaded area represents the range, that is, the lowest and highest values obtained on each day. The wide range observed for the 47 normal donors demonstrates that C1-inhibitor synthesis rates vary widely throughout the normal population.

3.5.2 Patients Involved in Study

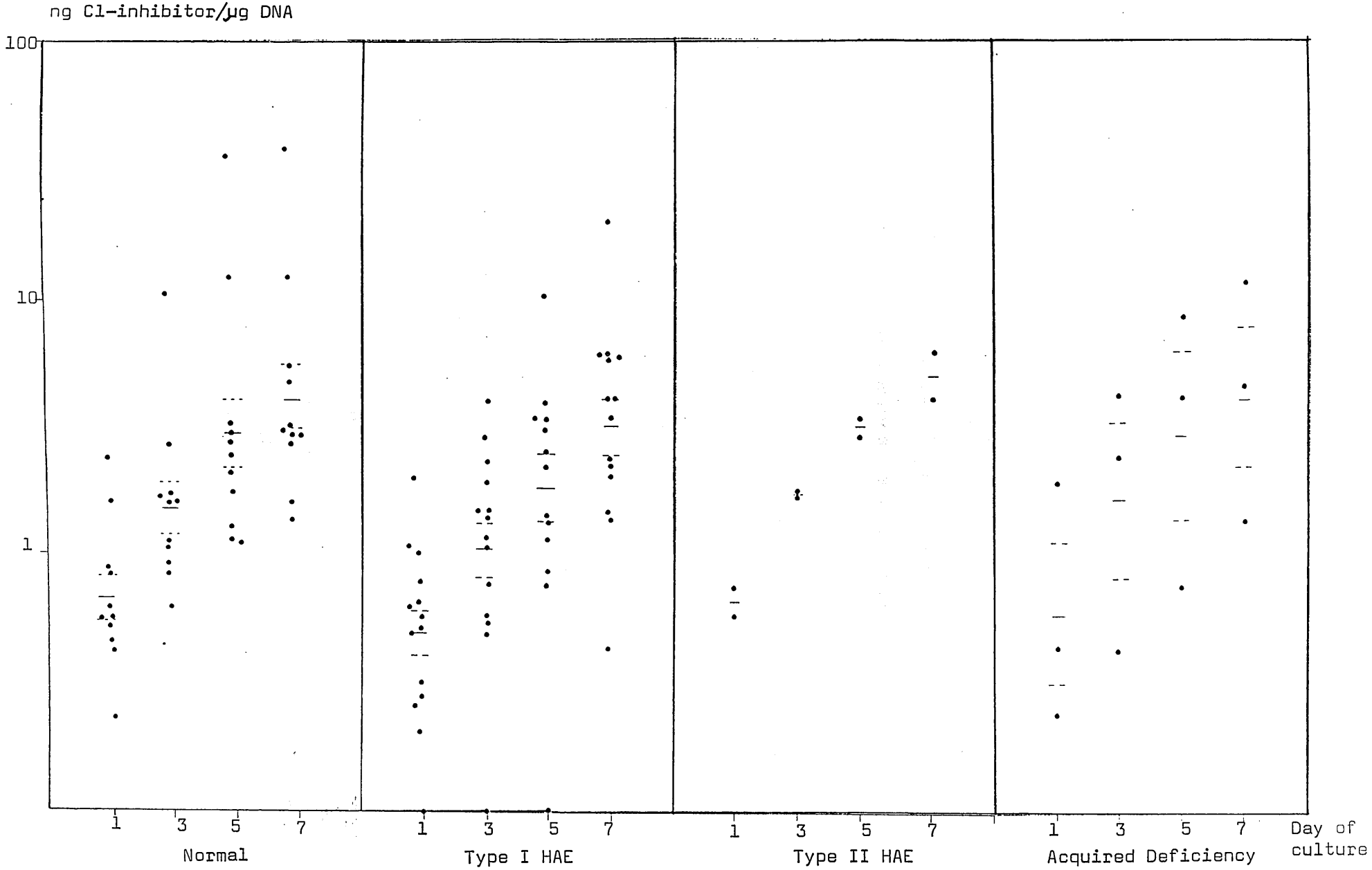
Table 23 lists all of the patients studied, showing age, sex, type of deficiency, treatment and clinical profile. The fourteen Type I HAE patients were diagnosed on the basis of previous family histories of HAE as well as a serum complement profile of low C1-inhibitor, C4 and CH50, with normal levels of C3. The three Type II HAE patients were diagnosed on the basis of normal or raised antigenic levels of C1-inhibitor which had reduced functional activity. The three patients with acquired C1-inhibitor deficiency were diagnosed on their serum complement profiles (low serum C1q, and occasionally low C3 levels, in addition to the abnormalities listed for Type I HAE). These patients developed symptoms later in life, did not have a previous family history, and all had lymphoproliferative disorders or paraproteinaemia.

Type	n	pg C1-inhibitor / µg DNA on Days			
		1	3	5	7
Normal	11	665 (547-808)	1561 (1240-1964)	3081 (2243-4234)	4299 (3244-5698)
Type I HAE	14	499 (405-613)	1071 (834-1376)	1872 (1393-2514)	3305 (2582-4230)
Type II HAE	2	660 (582-747)	1817 (1750-1887)	3243 (2983-3526)	5127 (4295-6120)
Acquired Deficiency	3	591 (314-1113)	1638 (803-3341)	2924 (1396-6125)	4252 (2294-7882)

Table 24: Levels of C1-inhibitor in Monocyte Culture Supernatants from Normals, patients with Type I HAE, Type II HAE and acquired C1-inhibitor deficiency.

C1-inhibitor levels are expressed as pg C1-inhibitor/ µg DNA on days 1, 3, 5 and 7 and represent the mean with the mean-SEM and mean + SEM values being bracketed thus e.g. 665 (547-808). n represents the number of individuals in each group.

Fig. 36: Cl-inhibitor levels in monocyte culture supernatant from normals (11), patients with Type I HAE (14), Type II HAE (2) and acquired (3) Cl-inhibitor deficiency. The levels are plotted as individual points (●) and the mean, (-), and the SEM (---) of log-transformed data on days 1, 3, 5 and 7.


ng C1-inhibitor/µg DNA
100
10
1
1 3 5 7
Normal
Type I HAE
Type II HAE
Acquired Deficiency
Day of culture

3.5.3 Synthesis of C1-inhibitor by Monocytes from C1-inhibitor deficient Patients

Monocyte cultures were prepared as described (Section 2.5.3) using blood samples from 14 Type I HAE, 2 Type II HAE patients and 3 patients with acquired C1-inhibitor deficiency, as well as 11 normal controls which were processed simultaneously. Each individual's levels and the mean levels of C1-inhibitor measured in the culture supernatants of these four groups are shown in Fig. 36 and Table 24.

Although the sample numbers were very small in the patient groups with Type II HAE and acquired C1-inhibitor deficiency, the mean levels of C1-inhibitor on days 1, 3, 5 and 7 were similar to that observed for the normal group (Table 24 and Fig. 36). The 14 Type I HAE patients showed lower mean levels of C1-inhibitor compared to normals (Fig. 36 and Table 24), however, upon statistical analysis there was no significant differences between the two groups, at any level using the Student 't' test or the Mann Whitney U test.

The monocytes of all Type I HAE patients appeared to synthesise C1-inhibitor, only a few of which had levels lower than the lowest observed normal level. Of the 14 Type I HAE patients, only one (DH) consistently showed very low levels of C1-inhibitor in his monocyte culture supernatants. Indeed, C1-inhibitor could not be detected before day 7 in culture. However, for the purpose of analysis (Tables 24 and 25) and graphic illustration (Fig. 36) this patient was assigned the lowest possible value on the log scale of 100 pg C1-inhibitor/μg DNA.

A

Type	n	value	pg C1-inhibitor/μg DNA on Days			
			1	3	5	7
Type I On Androgen Treatment	8	$\bar{x}$ $\bar{x} \pm$ SEM range	717 (579-888) 282-2085	1568 (1262-1949) 560-4101	2903 (2283-3691) 1155-10535	4731 (3683-6078) 2031-20457
Type I No Androgen Treatment	6	$\bar{x}$ $\bar{x} \pm$ SEM range	307 (222-416) 100-799	645 (411-1010) 100-2364	1043 (603-1804) 100-4002	2048 (1352-3102) 425-6252

Type	n	value	ng C2/μg DNA on Days			
			1	3	5	7
Type I On Androgen Treatment	8	$\bar{x}$ $\bar{x} \pm$ SEM range	2.2 (1.8-2.6) 1.1-4.7	5.6 (4.8-6.6) 3.5-9.3	8.8 (7.5-10.3) 5.1-15.7	12.0 (10.3-14.0) 7.0-21.7
Type I No Androgen Treatment	6	$\bar{x}$ $\bar{x} \pm$ SEM range	1.6 (1.2-2.0) 0.7-3.0	4.8 (3.4-6.7) 1.6-14.4	8.0 (6.2-10.4) 3.3-14.8	10.0 (7.8-12.9) 3.9-17.3

C

Type	n	value	ng C3/μg DNA on Days			
			1	3	5	7
Type I on Androgen Treatment	8	$\bar{x}$ $\bar{x} \pm$ SEM range	18.4 (15.9-21.3) 8.9-27.7	63.7 (53.5-75.9) 27.5-113.4	96.6 (82.0-113.7) 39.7-152.2	113.2 (96.0-133.5) 44.8-187.5
Type I No Androgen Treatment	6	$\bar{x}$ $\bar{x} \pm$ SEM range	9.1 (7.0-11.8) 5.0-23.5	28.1 (18.9-41.8) 13.4-154.2	38.2 (27.4-53.1) 17.8-131.2	49.5 (37.1-65.9) 25.1-145.9

Table 25: C1-inhibitor C2 and C3 levels in monocyte culture supernatants from Type I HAE patients receiving and not receiving androgen treatment. Values reported are mean, mean $\pm$ SEM and the range on days 1, 3, 5 and 7. n represents the number of individuals in each group.

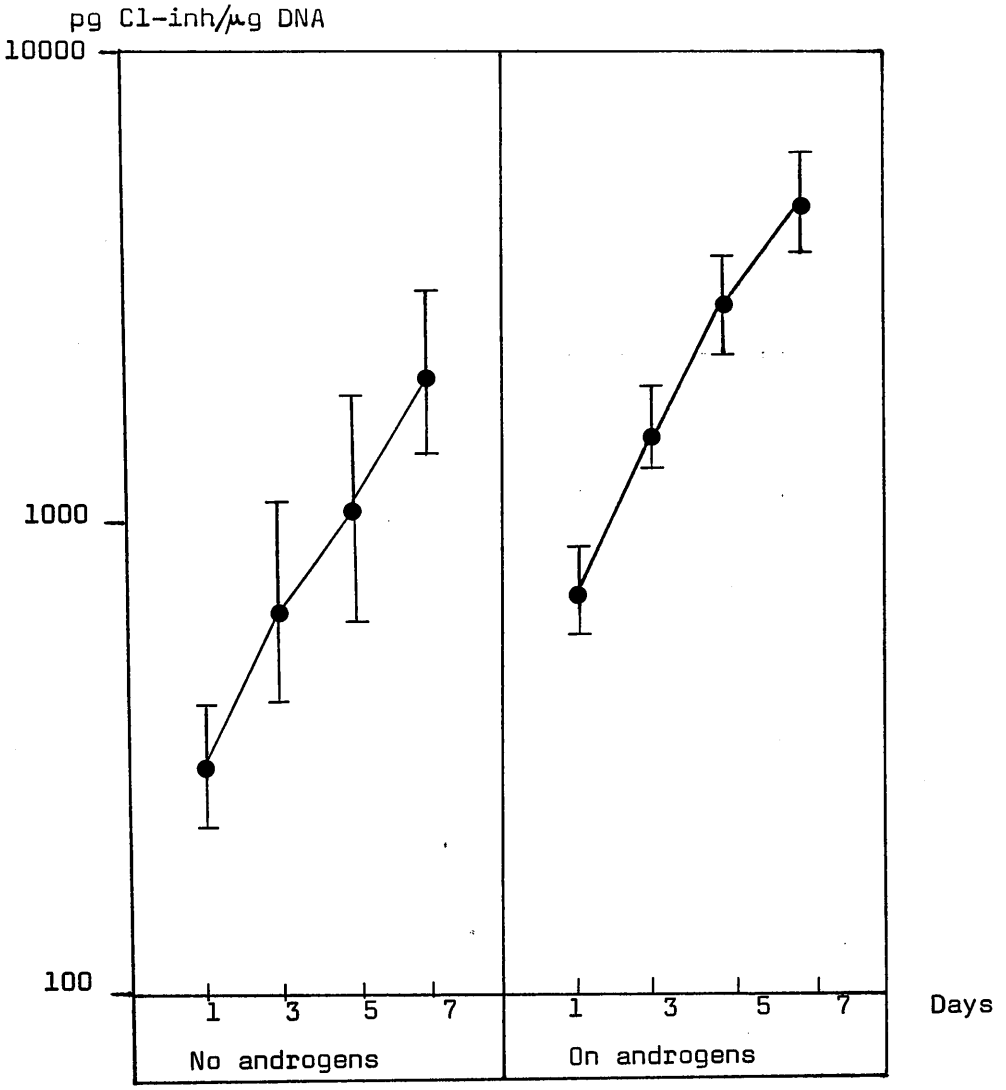


Fig. 37: Levels of Cl-inhibitor in monocyte culture supernatants of Type I HAE patients receiving and not receiving androgen treatment.

The values plotted represent the mean ± SEM of log transformed data. Patients receiving androgen treatment n = 8; patients not receiving androgen treatment, n = 6.

Type	ng C2/μg DNA on Days			
	1	3	5	7
Normal $\bar{x}$	1.6	3.3	6.1	8.2
$\bar{x}$ + SEM	1.8	3.9	7.3	9.9
$\bar{x}$ - SEM	1.4	2.8	5.1	6.8
n = 11, range	0.9 – 3.3	1.3 – 6.7	1.7 – 12.6	2.1 – 21.7
Type I $\bar{x}$	1.9	5.2	8.4	11.1
$\bar{x}$ + SEM	2.2	6.1	9.7	12.7
$\bar{x}$ - SEM	1.6	4.4	7.4	9.7
n = 14, range	0.7 – 4.7	1.6 – 14.4	3.3 – 15.7	3.9 – 21.7
Type II $\bar{x}$	0.8	2.1	4.1	5.9
$\bar{x}$ + SEM	1.0	2.5	4.6	6.8
$\bar{x}$ - SEM	0.6	1.8	3.7	5.2
n = 2, range	0.6 – 1.0	1.8 – 2.5	3.9 – 4.3	5.2 – 6.8
Acquired $\bar{x}$	0.8	2.1	3.0	4.1
$\bar{x}$ + SEM	1.2	2.9	4.4	6.1
$\bar{x}$ - SEM	0.6	1.5	2.0	2.7
n = 3, range	0.5 – 1.5	1.5 – 4.0	1.9 – 6.4	2.5 – 9.0

Table 26: Levels of C2 in monocyte culture supernatants from normals, patients with Type I HAE, Type II HAE and acquired C1-inhibitor deficiency.

C2 was measured by ELISA assay and the values are reported as the range, as the mean, and the mean ± SEM on days 1, 3, 5 and 7. n represents number of individuals in each group.

C1-inhibitor levels in monocyte culture supernatants from Type I HAE patients who were on androgen therapy (Danazol or Stanozolol, n = 8) were compared with those who were not receiving androgen therapy (n = 6); the results are shown in Table 25A and Figure 37. Although C1-inhibitor concentrations in the monocyte culture supernatants of androgen treated patients were higher than those that were not receiving androgen treatment (Table 25A and Fig. 37), these differences were not statistically significant using a Student's 't' test or a Mann Whitney U test. The mean levels of C1-inhibitor synthesised by the monocytes of Type I HAE patients who were receiving androgen treatment were similar to those observed for normal monocytes in culture. Thus the Type I HAE patients not receiving androgen therapy were the group synthesising less C1-inhibitor (Tables 24 and 25A).

3.5.4 Synthesis of C2 and C3 by Monocytes from C1-inhibitor Deficient Patients

C2 and C3 levels in monocyte culture supernatant from normals and the three patient groups were investigated as related parameters which should not have been affected by the C1-inhibitor deficiency. As was demonstrated with C1-inhibitor, the levels of C2 synthesised by normal monocytes were variable showing a very wide range; however, the mean of each of the three patient groups did not differ significantly from the normals (Table 26).

Type	ng C3/µg DNA on Days			
	1	3	5	7
Normals $\bar{x}$	14.2	46.6	73.3	90.3
$\bar{x}$ + SEM	17.7	60.1	92.1	113.4
$\bar{x}$ - SEM	11.3	36.2	58.3	71.9
n = 11, range	5.9 - 59.1	17.7-186.9	26.6-253.8	35.1-250.6
Type I $\bar{x}$	13.6	44.9	64.9	79.4
$\bar{x}$ + SEM	16.1	55.8	79.7	95.7
$\bar{x}$ - SEM	11.5	36.1	52.8	65.9
n = 14, range	5.0 - 27.7	13.4-159.2	17.8-152.2	25.2-181.5
Type II $\bar{x}$	17.9	54.7	79.0	103.5
$\bar{x}$ + SEM	38.1	114.3	175.2	220.9
$\bar{x}$ - SEM	8.4	26.2	35.6	48.5
n = 2, range	8.4 -38.1	26.2-114.3	35.6-175.2	48.5-220.9
Acquired $\bar{x}$	9.4	32.9	55.5	88.0
$\bar{x}$ + SEM	14.7	46.2	78.4	116.8
$\bar{x}$ - SEM	6.0	23.5	39.3	66.3
n = 3, range	4.1-18.4	16.8-46.0	28.9-93.3	40.2 - 129.8

Table 27: C3 levels in Monocyte Culture supernatants from normals, patients with HAE Type I, Type II HAE and Acquired C1-inhibitor deficiency.

C3 was measured by ELISA assay and the values are reported in ng/µg DNA as mean ($\bar{x}$), the mean + SEM ($\bar{x}$ + SEM), mean - SEM ($\bar{x}$ - SEM) and the ranges observed on days 1, 3, 5 and 7; n represents the number of individuals in each group.

The monocytes of Type II HAE patients and patients with acquired C1-inhibitor deficiency appeared to synthesise slightly lower mean levels of C2 compared to the monocytes from normal individuals (Table 26), however, their values fell within the range observed for the normal monocytes. The Type I HAE patients' monocytes appeared to synthesise slightly higher levels of C2 throughout the culture period (Table 26). However, again due to the wide ranges observed, these values were not significantly different from that observed in the normal monocytes.

In comparison to C1-inhibitor, when C2 synthesis by monocytes from Type I HAE patients who were receiving androgen therapy was compared to Type I HAE patients who were not receiving androgen therapy, there was very little difference observed in the ranges of levels synthesised by these two groups (Table 25B). Both groups of Type I HAE patients monocytes showed slightly higher, but not significantly different, levels of C2 from that observed with normal monocytes (Table 25B and 24).

The C3 levels in monocyte culture supernatant from normal monocytes were again distributed over a wide range (Table 27). The mean levels of C3 for the three patient groups were very similar to that observed for the normal group, however, monocytes from Type I HAE patients appeared to show a wider range (Table 27).

Comparison of C3 levels synthesised by monocytes from Type I HAE patients who were receiving androgen therapy and Type I HAE patients who were not receiving androgen therapy,

demonstrated that the mean levels of C3 in these two groups appeared to be different (Table 25C). The monocytes of patients not receiving androgen treatment synthesised mean levels of C3 lower than those of patients receiving androgen treatment. When the data was analysed by a Student's 't' test, the means of the two groups were shown to be significantly different at the 2% level on day 7 ($t = 2.988$), at the 5% level on days 1 and 5 ($t = 2.516$ and $t = 2.638$ respectively), and at the 10% level on day 3 ($t = 2.068$). When compared to normal monocytes, the monocytes from Type I HAE patients receiving androgen treatment synthesised slightly higher mean levels, whilst the monocytes of the patients not receiving androgen treatment synthesised lower mean levels than the normal monocytes (Tables 27 and 25C).

The mean level of C3 synthesis for the monocytes of Type II HAE patients appeared to be slightly higher than normals, however this was due to only two patients being studied: the supernatant of one contained small amounts of C3 while the other contained very high C3 levels (Table 27). The monocytes of patients with acquired C1-inhibitor deficiency appeared to synthesize lower levels of C3 compared to normal monocytes (Table 27), however the levels of two out of the three patients fell within the normal range (Table 27).

3.5.5 Response of C1-inhibitor Deficient Patient's Monocytes to γ interferon

The response of C1-inhibitor deficient patients' monocytes to γ interferon in culture at doses of 1 µg and 10 ng/ml was

Type	Molecules C1-inh/cell/hr. $\bar{x} \pm$ SEM (n)		
	Control	ɣIfn 1 µg/ml	ɣIfn 10 ng/ml
Normal	2374 ± 1184 (9)	57 960 ± 15 088 (9)	19016 ± 6233 (7)
Type I	1834 ± 570 (12)	42 278 ± 9095 (12)	16562 ± 7157 (9)
Type II	1958 (1)	63 583 (1)	21 690 (1)
Acquired	2721 ± 2360 (2)	60 144 ± 14 966 (2)	ND

Table 28: Monocyte C1-inhibitor secretion rates for normals, Patients with Type I HAE, Type II HAE and Acquired C1-inhibitor Deficiency under control and ɣ interferon-stimulated (1 µg/ml and 10 ng/ml) conditions.

The values are reported as molecules/cell/hr showing the mean ± SEM; (n) represents the number of individuals in each group. The values were calculated on the basis of 1 µg DNA approximating to 1 x 10^5 cells, and 105000 Daltons as the molecular weight of C1-inhibitor.

Type	Component	Molecules/cell/hr $\bar{x} \pm$ SEM (n)		
		Control	ɣ Ifn 1 µg/ml	ɣIfn 10 ng/ml
Type I On Androgen Treatment	C1-inh	2362 ± 902 (7)	43057 ± 10375 (7)	20783±10337 (6)
Type I No Androgen Treatment	C1-inh	1143 ± 462 (5)	41187 ± 17872 (5)	8122 ± 5875 (3)
Type I On Androgen Treatment	C2	4642 ± 1107 (7)	17905 ± 6128 (7)	11312± 3154 (6)
Type I No Androgen Treatment	C2	5219 ± 788 (5)	12042 ± 3217 (5)	10623± 4799 (3)
Type I On Androgen Treatment	C3	20777 ± 3641 (7)	17293 ± 2625 (7)	16631± 3093 (6)
Type I No Androgen Treatment	C3	10566 ± 3209 (5)	7962 ± 2994 (5)	12704 ±6696 (3)

Table 29: Monocyte C1-inhibitor C2 and C3 Secretion Rates for Type I HAE Patients Receiving and Not Receiving Androgen Therapy, under Control and ɣ interferon stimulated conditions.

The values are reported as molecules/cell/hr showing the mean ± SEM; (n) represents the number of individuals in each group. The values were calculated as described in legends to Tables 28, 30 and 31.

investigated. The results calculated as secretion rates (SR) for C1-inhibitor, C2 and C3 between days 3 and 5 were expressed as molecules/cell/hr (Tables 28-31), and were shown to be distributed in a linear fashion.

Normal monocytes stimulated with γ interferon (1 µg/ml) showed dramatic increases in their C1-inhibitor SRs, the mean increase being 24 times that observed in control monocytes (Table 28). A smaller increase was observed with the lower dose of γ interferon, the mean SR being increased 8 fold. The monocytes of patients with Type I HAE demonstrated a lower control SR as discussed previously (Section 3.5.3) but showed similar responses to γ interferon. At concentrations of 1 µg/ml and 10 ng/ml the mean C1-inhibitor SRs were increased 23 fold and 9 fold respectively (Table 28).

The lower control C1-inhibitor SR by monocytes of patients with Type I HAE compared to normal controls, was once again demonstrated to be due to a lower SR by those patients who were not receiving androgen therapy (danazol or stanozolol) (Table 29). Those Type I HAE patients who were receiving androgen therapy had a mean C1-inhibitor SR which was similar to that observed for normal monocytes (Tables 28 and 29), but statistical analysis of these two groups of Type I HAE patients' mean SRs using a Student's 't' test demonstrated that these means were not significantly different. Monocytes of Type I HAE patients not receiving androgen treatment showed a 36 fold increase in C1-inhibitor synthesis when stimulated with γ interferon (1 µg/ml), and a 7 fold increase when stimulated with γ interferon (10 ng/ml).

Type	Molecules C2/cell/hr $\bar{x} \pm$ SEM (n)		
	Control	γ Ifn 1 µg/ml	γ Ifn 10 ng/ml
Normal	4112 ± 725 (9)	10 441 ± 3201 (9)	10359 ± 2308(7)
Type I	4882 ± 701 (12)	15 462 ± 3778 (12)	11083 ± 2465(9)
Type II	2258 (1)	16 310 (1)	10 915 (1)
Acquired	2007 ± 1631 (2)	9 347 ± 7841 (2)	ND

Table 30: Monocyte C2 Secretion Rates for Normals, Patients with Type I HAE, Type II HAE and Acquired C1-inhibitor Deficiency under control and γ-interferon stimulated (1 µg/ml and 10 ng/ml) conditions.

The values are reported as molecules/cell/hr showing the mean ± SEM; (n) represents the number of individuals in each group. The values were calculated on the basis of 1 µg DNA approximating to 1 x 10^5 cells, and 100 000 Daltons as the molecular weight of C2.

Monocytes of Type I HAE patients on androgen therapy demonstrated an 18 fold and a 9 fold increase in C1-inhibitor synthesis when stimulated with γ interferon at 1 µg/ml and 10 ng/ml respectively (Table 29). Thus, although the mean basal SR of C1-inhibitor by Type I HAE patients' monocytes was slightly lower than that of normal monocytes, they still possess the capacity to respond equally well to stimulation by γ interferon.

The monocytes from one Type II HAE patient studied, demonstrated a slightly lower C1-inhibitor SR compared to normals (Table 28). However, this patient's monocytes appeared to respond equally well to stimulation by γ interferon, demonstrating a 32 fold increase, and an 11 fold increase over control with regards to the C1-inhibitor SR, when stimulated with γ interferon at 1 µg/ml and 10 ng/ml respectively (Table 28).

The monocytes from patients with acquired C1-inhibitor deficiency demonstrated slightly greater C1-inhibitor SR than normal monocytes (Table 28), however after stimulation with γ interferon (1 µg/ml) they increased to a similar level as the normal monocyte, demonstrating a 22 fold increase over control C1-inhibitor SR.

Secretion rates of monocyte C2 under control and γ-interferon stimulated conditions, for the normals and 3 groups of patients are shown in Table 30. The C2 SR under control conditions were similar in monocytes from normals and Type I HAE patients, although monocytes from patients with Type II HAE and acquired C1-inhibitor deficiency

Type	Molecules C3/cell/hr $\bar{x} \pm$ SEM (n)		
	Control	γ Ifn 1 µg/ml	γIfn 10 ng/ml
Normal	18 649 ± 5483 (9)	10 758 ± 4357 (9)	26 556 ± 8886 (6)
Type I	16 523 ± 2843 (12)	13 405 ± 2340 (12)	15 322 ± 2855 (9)
Type II	6247 (1)	11 747 (1)	5012 (1)
Acquired	23 042 ± 8324 (2)	10 205 ± 6639 (2)	ND

Table 31: Monocyte C3 Secretion Rates for Normals, Patients with Type I HAE, Type II HAE and Acquired C1-inhibitor Deficiency under control and γ-interferon stimulated (1 µg/ml and 10 ng/ml) conditions.

The values are reported as molecules/cell/hr showing the mean ± SEM; (n) represents the number of individuals in each group. The values were calculated on the basis of 1 µg DNA approximating to 1 x 10^5 cells, and 190 000 Daltons as the molecular weight of C3.

showed a lower mean C2 SR. However, this was probably due to the small sampling numbers in the latter two groups.

Stimulation of normal monocytes with ɣ interferon (1 µg/ml) resulted in a 2.5 fold increase in C2 SR, while monocytes from Type I and Type II HAE patients and patients with acquired C1-inhibitor deficiency increased their C2 SRs 3.2, 7.2 and 4.7 fold respectively (Table 30). Monocytes from Type I HAE patients receiving and not receiving androgen treatment, did not show any differences in mean C2 SR, and both groups responded as well as normal monocytes to stimulation by ɣ interferon (1 µg/ml) (Table 29); for patients receiving androgen treatment, a 3.9 fold increase was observed, and a 2.3 fold increase for patients not receiving androgen treatment.

C3 synthesis was inhibited by ɣ interferon in all of the four groups of monocytes, as demonstrated by a reduction in SR (Table 31). The control C3 SRs for monocytes isolated from normals, Type I HAE patients and patients with acquired C1-inhibitor deficiency were similar. The C3 SR of Type II HAE patient's monocytes was lower, possibly due to only one patient being studied (Table 31).

Normal monocytes C3 SR was reduced by 42% in the presence of ɣ interferon (1 µg/ml), whilst the C3SR of Type I HAE patients' monocytes under the same conditions was only reduced by 19% (Table 31). Monocytes from the Type II HAE patient did not show an inhibition of C3 SR with ɣ interferon at 1 µg/ml; it actually demonstrated enhancement. However, with ɣ interferon at 10 ng/ml an

inhibitory effect was observed, demonstrating 20% inhibition of the C3 SR. Monocytes from the patients with acquired C1-inhibitor deficiency showed an inhibition of C3 SR by 56% in the presence of γ interferon at 1 µg/ml (Table 31).

When the mean C3 SRs of monocytes from Type I HAE patients who were either receiving androgen treatment or not receiving androgen treatment were compared, they were shown to differ somewhat (Table 29). Those patients receiving androgen treatment had C3 SR (20777 molecules C3/cell/hr) comparable to normal monocytes (18 649 molecules C3/cell/hr), whilst those patients not receiving androgen treatment had much lower C3 SR (10 566 molecules C3/cell/hr) (Tables 29 and 31). However, when the basal C3 SR for these two groups of Type I HAE patients were analysed by a Student's 't' test, the means did not differ significantly. The ability of these two groups of Type I HAE patients monocytes to respond to γ interferon were similar: C3SR of patients on androgen treatment was inhibited by 17% in the presence of γ interferon (1 µg/ml), whilst those patients not receiving androgen treatment showed a level of inhibition of 25%.

The levels of inhibition of C3 SRs in the presence of γ interferon appeared to vary considerably within each group and between the normals and 3 patient groups, although there was always a decrease in C3 SR observed when γ interferon was present. This reflects the observations made earlier with regards to C3 levels in normal monocytes in the presence

Type	No. of individuals	C1-inhibitor Specific Activity units/ng		
		$\bar{x}$	SEM	Range
Normal	9	2.05	0.44	0.61 - 4.09
Type I	13	2.20	0.27	1.07 - 3.92
Type II	2	1.06	0.56	0.48 and 1.64
Acquired	2	1.74	0.21	1.53 and 1.95

Table 32: Specific Activity (units/ng) of Monocyte C1-inhibitor from Normals, Patients with Type I HAE, Type II HAE and Acquired C1-inhibitor Deficiency.

Values reported are the means, SEM and range for each group.

of γ interferon (Section 3.3.2) in that they showed a wide range of inhibitory levels. In this study the range of inhibitory levels for C3 SR for normals ranged from 17% to 97%, whereas for Type I HAE patients monocytes the range was from -14% to 92% in the presence of γ interferon at 1 µg/ml.

The overall picture from the effects of γ interferon, is that monocytes isolated from C1-inhibitor deficient patients respond similarly to normal monocytes when treated with γ interferon: demonstrating stimulation of C1-inhibitor and C2 synthesis, and inhibition of C3 synthesis.

3.5.6 Specific Activity of C1-inhibitor from Monocytes of C1-inhibitor Deficient Patients

The specific activity of C1-inhibitor synthesised by the normal monocytes and C1-inhibitor deficient patients monocytes was investigated using monocyte supernatants which had been stimulated with γ interferon (1 µg/ml). Due to the great enhancing effect of γ interferon on C1-inhibitor synthesis, there was no need to concentrate the samples as before (Section 3.2.4) when monocytes were cultured under serum free conditions. Table 32 summarises the results obtained showing the mean and range for the normals and the three patient groups.

The ranges observed with normal monocytes (0.61 to 4.09 units/ng) and monocytes from Type I HAE patients (1.07 to 3.92 units/ng) were very similar. The two patients with acquired C1-inhibitor deficiency showed monocyte C1-inhibitor

A

Type	N	% Enhancement of C1-inhibitor		
		$\bar{x}$	SEM	Range
Normals	7	167	50	22 to 381
Type I	11	84	31	-16 to 315

B

Type	N	% Enhancement of C2		
		$\bar{x}$	SEM	Range
Normals	6	63	23	0 to 168
Type I	11	43	16	-17 to 140

C

Type	N	% Inhibition of C3		
		$\bar{x}$	SEM	Range
Normals	6	53	5	37 to 72
Type I	11	46	6	2 to 64

Table 33: Enhancement of monocyte C1-inhibitor (A) and C2 (B) Synthesis and Inhibition of C3 (C) Synthesis by 10^{-5}M Hydrocortisone in Normals and Type I HAE patients.

The mean is represented by $\bar{x}$; the number of individuals in each group is represented by N.

with specific activities within the normal range (1.53 and 1.95 units/ng). However, of the two Type II HAE patients monocytes studied, one synthesised C1-inhibitor with a specific activity within the normal range (1.64 units/ng, patient J.M.), whilst the other Type II HAE patient's monocytes synthesised C1-inhibitor with a specific activity lower than the normal range (0.48 units/ng, patient C.E.) (Table 32).

3.5.7 The Effect of Steroids on Complement Synthesis by Monocytes from C1-inhibitor Deficient Patients

3.5.7a Anabolic Steroids and Androgens

The effects of the anabolic steroid danazol, and the androgen testosterone β-D-glucuronide, over the dose range of 10^{-9}M to 10^{-5}M, were investigated on monocytes isolated from two Type I HAE patients. These steroids had no effect on the synthesis of C1-inhibitor or C2, as reported previously for normal monocytes (Section 3.4.2).

3.5.7b Glucocorticoids

Monocytes isolated from C1-inhibitor deficient patients were treated with 10^{-5}M hydrocortisone and the effects on C1-inhibitor, C2 and C3 synthesis assessed. The results, shown in Table 33, represent the percentage change in synthesis, compared to control values; these changes were shown to be distributed in a linear fashion. The value chosen for each culture was representative of the maximum change obtained throughout the 7 day culture period. Only the effects of 10^{-5}M hydrocortisone on Type I HAE patients monocytes has been reported, due to the small sample numbers

tested in the groups of Type II HAE patients (1) and the patients with acquired C1-inhibitor deficiency (1).

As observed previously (Sections 3.4.1) the range of levels of enhancement and inhibition varied very much between normal individuals; this observation was again made with regards to normals, and also with regards to patients monocytes (Table 33).

The mean level of enhancement observed for C1-inhibitor with normal monocytes was 167%, whilst for Type I HAE patients' monocytes the corresponding mean enhancement was only 84% (Table 33A). When analysed by a Student's 't' test, the means were not found to be significantly different ('t' value of 1.46). The mean levels of enhancement of monocyte C1-inhibitor for Type I HAE patients receiving and not receiving androgen treatment were calculated ($61 \pm 39\%$ and $86 \pm 33\%$, respectively), and the results demonstrated that both groups of Type I HAE patients' monocytes responded similarly to hydrocortisone.

When hydrocortisone-induced enhancement of C2 synthesis was investigated the mean and range of enhancement values for normal monocytes was slightly higher than that observed for Type I HAE patient's monocytes. However, upon statistical analysis these values were shown not to be significantly different in a Student's 't' test (Table 33B).

The mean levels and ranges of inhibition of monocyte C3 synthesis in the presence of 10^{-5}M hydrocortisone in normals and Type I HAE patients were very similar (Table 33C).

Type	C1-inhibitor Molecular Weight kD		
	Serum precipitated	Extracellular precipitated	Intracellular precipitated
Normal	ND	103	81
Normal	ND	101	85
Normal	ND	96	82
Normal	ND	94	80
Normal	97	103	82
Normal	96.5	97.5	80
Normal	96	100	81
Normals $\bar{x}$	96.5	99.2	83
range	96 to 97	94 to 103	80 to 85
Type I	ND	94	ND
Type I	95	103	82
Type I	99	96.5	81
Type I	96	101	83
Type I $\bar{x}$	96.7	98.6	82
range	95 to 99	94 to 103	81 to 83
Type II	97.4	98	82
Type II	101	97	81
Acquired	ND	106	85
Acquired	97	97	81

Table 34: Molecular Weights of C1-inhibitor Precipitated from Serum, Monocyte Supernatants (extracellular) and Lysates (intracellular) of Normals, Patients with Type I HAE, Type II HAE and Acquired C1-inhibitor Deficiency.

Individual values are reported as well as means ($\bar{x}$) and ranges for each group, where the group contains more than 2 individuals.

From these results it would appear that hydrocortisone-induced enhancement of C1-inhibitor and C2 synthesis, was similar in normal monocytes and monocytes isolated from Type I HAE patients. Furthermore, the extent of inhibition of C3 synthesis was similar in monocytes from both groups.

3.5.8 ^{35}S-methionine Pulse-Chase Studies of C1-inhibitor Synthesised by Monocytes from C1-inhibitor Deficient Patients

γ interferon-treated (1 μg/ml) monocyte cultures from normals, Type I HAE, Type II HAE and patients with acquired C1-inhibitor deficiency were pulsed for 1 hr with ^{35}S-methionine and chased for 1.5 hr as described previously (Section 2.11). Immunoprecipitates of the lysates and supernatants were analysed by SDS-PAGE gels, and subjected to fluorography. The resulting autoradiographs were analysed and the molecular weight of C1-inhibitor from the different cultures calculated. These results are reported in Table 34 and Figures 38, 39 and 40, along with the molecular weights of autologous serum C1-inhibitor which were analysed in the same gels.

The mean molecular weight for extracellular C1-inhibitor from normal monocytes was 99.2 kD (range: 94 kD to 103 kD), whilst the intracellular form showed a mean of 83 kD (range: 80 kD to 85 kD) (Table 34). The C1-inhibitor immunoprecipitates from cultures of Type I HAE patients monocytes displayed similar ranges to those observed with normal monocytes: extracellular forms had a mean of 98.6 kD, with a range from 94 kD to 103 kD; the intracellular form had a mean of 82 kD

Fig. 38: Immunoprecipitation of C1-inhibitor from Serum (Coomassie-Blue Stained) and ^{35}S-methionine-pulsed monocyte supernatants and lysates (autoradiographs) of a normal, a patient with Type I and Type II C1-inhibitor deficiency.

Tracks A, N and O: Molecular weight standards

Track B: Purified C1-inhibitor

Track C: Immunoprecipitated purified C1-inhibitor

Track D: Immunoprecipitation of serum C1-inhibitor from normal (97 kD)

Track E: Immunoprecipitation of " " from Type II patient (97 kD)

Track F: Immunoprecipitation of " " from Type I patient (95 kD)

Track G, J, L, P, R and T: Non-specific immunoprecipitations

Track H: Immunoprecipitation of Extracellular Monocyte C1-inhibitor from Type I patient (103 kD)

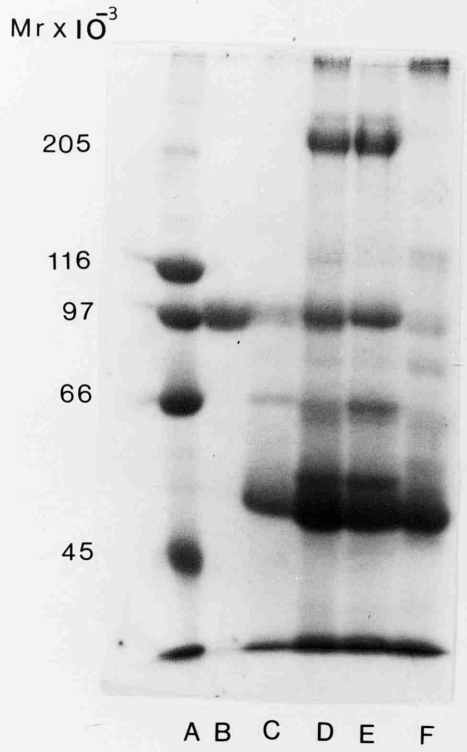
Track K: Immunoprecipitation of Extracellular Monocyte C1-inhibitor from Type II patient (98 kD)

Track M: Immunoprecipitation of Extracellular Monocyte C1-inhibitor from Normal (103 kD)

Track Q: Immunoprecipitation of Intracellular Monocyte C1-inhibitor from Type I patient (82 kD)

Track S: Immunoprecipitation of Intracellular Monocyte C1-inhibitor from Type II patient (82 kD)

Track U: Immunoprecipitation of Intracellular Monocyte C1-inhibitor from Normal (82 kD)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116
97
66
45
A B C D E F

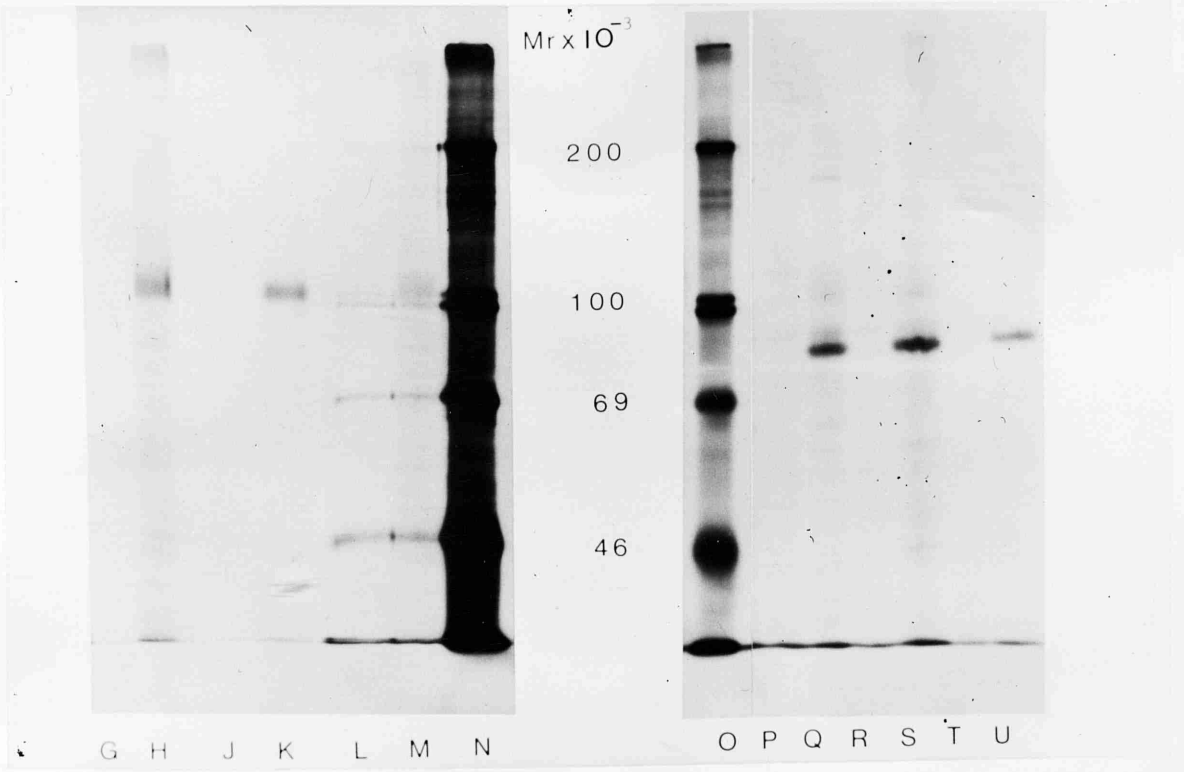

Mr x 10⁻³
200
100
69
46
G H J K L M N
O P Q R S T U

Fig. 39: Immunoprecipitations of C1-inhibitor from serum (Coomassie-blue stained) and ^{35}S-methionine-pulsed monocyte supernatants and lysates (autoradiographs) of a normal and a patient with Type I and Type II C1-inhibitor deficiency.

Tracks D and R: Molecular weight standards.

Track A: Immunoprecipitation of serum C1-inhibitor from Type II patient (101 kD)

Track B: Immunoprecipitation of serum C1-inhibitor from Type I patient (99 kD)

Track C: Immunoprecipitation of serum C1-inhibitor from Normal (97.5 kD)

Tracks E, G, J, L, N and P: Non-specific immunoprecipitations.

Track F: Immunoprecipitation of Extracellular Monocyte C1-inhibitor from Normal (96.5 kD)

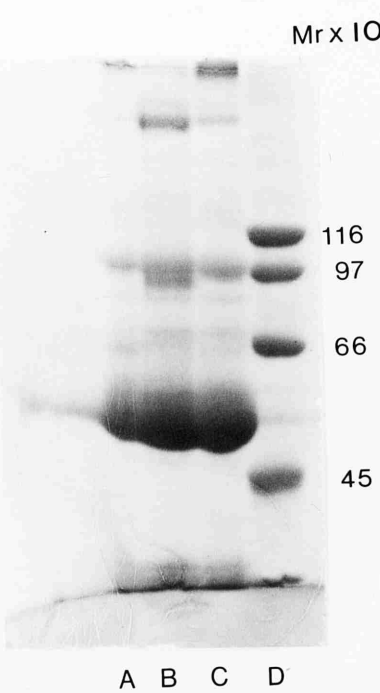
Track H: Immunoprecipitation of Extracellular Monocyte C1-inhibitor from Type I patient (96.5 kD)

Track K: Immunoprecipitation of Extracellular Monocyte C1-inhibitor from Type II patient (97 kD)

Track M: Immunoprecipitation of Intracellular Monocyte C1-inhibitor from Normal (80 kD)

Track O: Immunoprecipitation of Intracellular Monocyte C1-inhibitor from Type I patient (81 kD)

Track Q: Immunoprecipitation of Intracellular Monocyte C1-inhibitor from Type II patient (81 kD)


Mr x 10^{-3}
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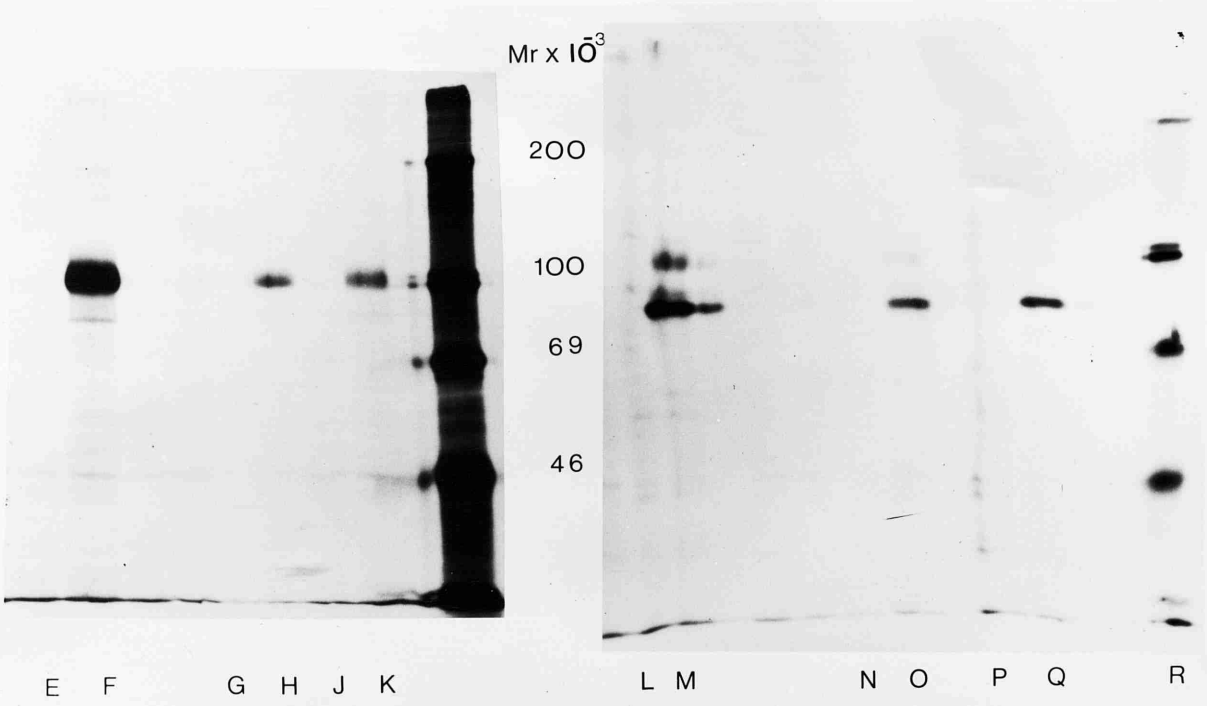
Mr x 10^{-3}
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46
E F G H J K
L M N O P Q R

Fig. 40: Immunoprecipitation of C1-inhibitor from serum (Coomassie-blue stained) and ^{35}S-methionine-pulsed monocyte supernatants and lysates (autoradiographs) of normal, and a patient with Type I HAE and Acquired C1-inhibitor deficiency.

Tracks E, F and N: Molecular weight standards.

Track A: Purified C1-inhibitor.

Track B: Immunoprecipitation of serum C1-inhibitor from Type I patient (96 kD)

Track C: Immunoprecipitation of serum C1-inhibitor from Normal (97 kD)

Track D: Immunoprecipitation of serum C1-inhibitor from Acquired patient (97 kD)

Tracks G, J, L, O, Q and S: Non-specific immunoprecipitations.

Track H: Immunoprecipitation of extracellular monocyte C1-inhibitor from acquired patient (97 kD)

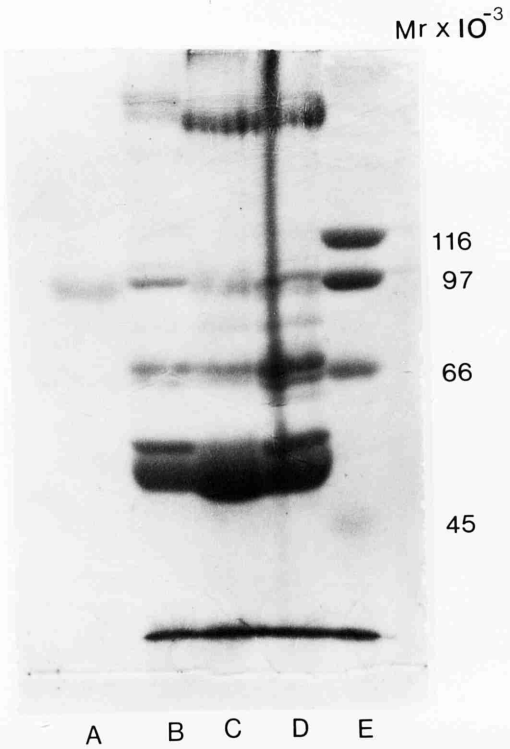
Track K: Immunoprecipitation of extracellular monocyte C1-inhibitor from normal (100 kD)

Track M: Immunoprecipitation of extracellular monocyte C1-inhibitor from Type I patient (101 kD)

Track P: Immunoprecipitation of intracellular monocyte C1-inhibitor from acquired patient (81.5 kD)

Track R: Immunoprecipitation of intracellular monocyte C1-inhibitor from normal (81 kD)

Track T: Immunoprecipitation of intracellular monocyte C1-inhibitor from Type I patient (83 kD)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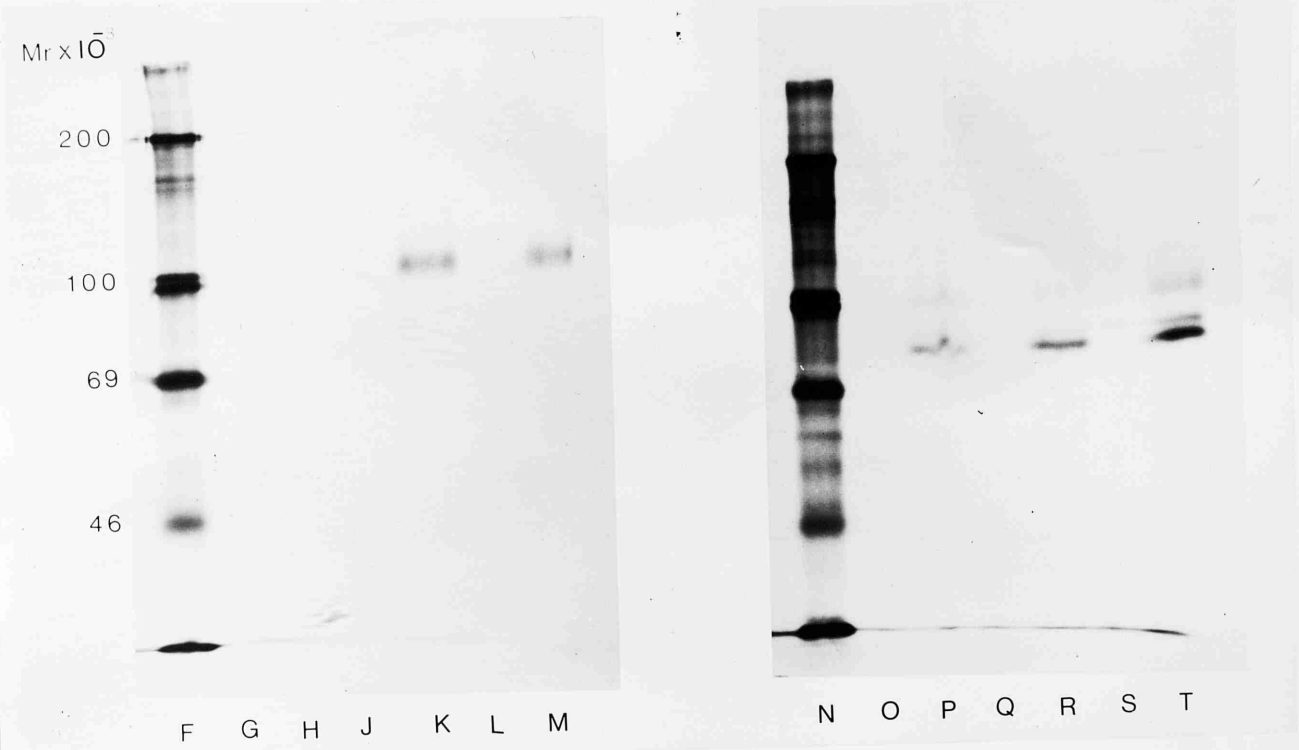
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with a range from 81 kD to 83 kD (Table 34). The two Type II HAE patients investigated showed molecular weights within the normal range; of the two patients with acquired C1-inhibitor deficiency studied, one showed a slightly higher extracellular molecular weight (106 kD) than was observed with the normals (94 kD to 103 kD, Table 34).

Upon investigation of the Coomassie blue-stained immunoprecipitates of serum C1-inhibitor from autologous serum samples from all four groups, a similar range of molecular weights to that of the extracellular form of monocyte C1-inhibitor were displayed (Table 34).

The photographs (Figs. 38, 39 and 40) of the immunoprecipitates from serum and monocyte supernatants were processed simultaneously, and photographed under the same magnification to enable direct visual comparison between serum C1-inhibitor and monocyte C1-inhibitor. Each set of photographs contains 1 normal and two sets of patient samples: Figures 38 and 39 show the results for a normal, a Type I and a Type II C1-inhibitor deficient patient. Figure 40 shows a normal, a Type I and a patient with acquired C1-inhibitor deficiency. The results which emerged from these experiments were, that the intracellular forms of C1-inhibitor from the four groups displayed very similar ranges as demonstrated previously (Table 34). The extracellular forms of C1-inhibitor were of the same or marginally higher molecular weight than the C1-inhibitor immunoprecipitated from serum in 7 out of 9 cases, the two exceptions being one Type I and one Type II patient. However, these differences

in molecular weights of a few kD, are in the area of the limits of resolution of SDS-PAGE. In this case it proved even more difficult, as C1-inhibitor migrated as a broad band due to the high proportion of carbohydrate present in the molecule.

Thus from this data it would appear that on the basis of molecular weight, the extracellular and intracellular C1-inhibitor synthesised by monocytes isolated from Type I HAE, Type II HAE patients and patients with acquired C1-inhibitor deficiency have a similar range of molecular weights to that synthesised by normal monocytes. The extracellular monocyte C1-inhibitor, and the C1-inhibitor precipitated from autologous serum samples appear to have similar molecular weights.

3.5.9 Study of Type I HAE Patient (D.H.) in Remission and Disease

A 38 year old male patient (D.H.) with Type I HAE was studied in several phases of his disease. Monocyte cultures were set up from blood withdrawn before he commenced Danazol treatment, both while in remission and during an acute attack, and after Danazol treatment had commenced. The levels of C1-inhibitor and C2 in culture supernatants were measured at all stages, and the results are reported in Figure 41.

With regards to the C2 levels, there was very little difference between the levels synthesised whilst in remission and disease, regardless of whether or not he was receiving danazol treatment (Fig. 41).

Whilst in remission, whether receiving danazol treatment or not, C1-inhibitor could not be detected in this patient's monocyte culture supernatant before day 12 whilst on danazol (350 pg C1-inhibitor/µg DNA) or day 7 on no treatment (425 pg C1-inhibitor/µg DNA); even this late on in culture the levels were very low indeed. This was in contrast to when monocytes were cultured from blood which was withdrawn during an acute attack phase: C1-inhibitor could be detected as early as day 2 in culture (280 pg C1-inhibitor/µg DNA), and continued to increase reaching levels (Day 9, 5552 pg C1-inhibitor/µg DNA) which were within the normal range later in culture (Fig. 41).

Unfortunately, this was the only patient whom we received blood from during disease exacerbation. Thus we have been unable to repeat these observations with any other patient. This Type I HAE patient was somewhat unusual, in that he was the only patient whom we could not detect any levels of C1-inhibitor in monocyte culture supernatant early on in culture, under resting conditions.

4: DISCUSSION.

4.1 Purification of Complement Components and Specificity of Antisera

4.1.1 Purification of C1-inhibitor

Analysis of the pools of C1-inhibitor from the Biogel column using SDS-PAGE, demonstrated that all six pools contained a protein with a molecular weight of 94 kD, which is consistent with the known molecular weight of C1-inhibitor (Reboul et al., 1977). Pools 1 and 2 contained minor contaminants with molecular weights of 65 kD and 35 kD. In the original purification method of Reboul et al. (1977), C1-inhibitor was reported to have a molecular weight of 98 kD, with some fractions having minor contaminants of 60 kD and 29 kD. They suggested that these may correspond to proteolytic fragments of C1-inhibitor. They noted that the quantity of these "contaminants" could be decreased by increasing the concentration of protease inhibitors in the buffers used during the purification procedure. However, their attempts to remove these minor contaminants by chromatography on anti-C1-inhibitor-IgG sepharose 6B were unsuccessful, leaving unresolved the nature of these contaminants.

When pools 3 and 4 from the C1-inhibitor preparation were analysed on IEP against whole normal human serum, only one arc was observed in each case. Thus, these pools were considered to be pure enough for the production of antisera.

4.1.2 Specificity of Antisera

The antisera which were obtained were tested in double diffusion in agarose gels; a single precipitin band between wells containing the antisera and purified C1-inhibitor or whole normal human serum was observed. These precipitins gave a reaction of complete identity with those produced using a commercially available anti-C1-inhibitor antiserum. Thus the specificity and monospecificity of these antisera were established; these results were confirmed by IEP analysis.

4.1.3 Purification of C2

In the purification of C2, the PEG supernatant was applied to the cation exchange resin Biorex 70; the C2 protein bound initially due to its overall negative charge at this pH. The bound C2 was then eluted at a conductivity greater than 8 mS. The resulting C2 pool was chromatographed over the anion exchange resin DEAE cellulose, where the protein was found in the exclusion peak, due to its overall negative charge.

Purity of the final pool of C2 was not established as it was only used in the preparation of haemolytic intermediates, and as a standard in haemolytic assays for C2.

4.2 The Study of Human Monocytes under Control Conditions

4.2.1 Characteristics of Human Monocytes

Identification of the population of cells which were being investigated was the first criterion, to ensure that a maximum proportion of the cells being studied were of the mononuclear phagocyte lineage. The results of the character-

isation study conclusively demonstrated that 95 to 98% of the cells were mononuclear phagocytes by virtue of their positive staining for non-specific esterase (Section 3.2.1), the most commonly used and best recognised staining marker for cells of this lineage (Horowitz et al., 1977). Fewer cells, 87 to 92%, expressed other well known markers for monocytes, such as the ability to phagocytose serum-treated zymosan particles. The expression of Fc and C3b receptors were demonstrated by rosette formation and phagocytosis of IgG coated sheep erythrocytes and rosette formation with EAC43b cells respectively.

4.2.2 Continuous and Cumulative Complement Synthesis by Monocytes

Monocytes in culture were shown to secrete C1-inhibitor in a linear fashion over 14 days, whilst C2 secretion was linear only over the first three days, and had reached a plateau after 7 days. It has been demonstrated that when functionally purified C2 was added to monocytes on day 7, the loss of C2 functional activity was not significantly different from that observed when functionally purified C2 was added to fresh RPMI/FCS (Lappin et al., 1986; Morrison and Whaley, 1983). These data suggest that the plateau reached after 7 days of continuous culture was not due to degradation of C2 haemolytic activity, but was probably due to decreased synthesis.

The plateau of C2 synthesis was abrogated when the whole culture supernatant was changed on alternative days, thus demonstrating that after three days of continuous culture there was a decrease in the rate of synthesis of C2. It has been shown in guinea pig macrophages that C4 synthesis was specifically inhibited by extracellular C4, but inhibition of C2 synthesis was not inhibited by extracellular C2 (Auerbach et al., 1984). The discrepancy between these observations in humans and guinea-pigs could be explained by a species difference.

Work by Lappin and Whaley (1982b) has demonstrated that C2 synthesis was inhibited by agents which caused an increase in intracellular cAMP, one such set of examples are the prostaglandins. Since monocytes are known to secrete large amounts of prostaglandins (Davis et al., 1980). Lappin and Whaley (1982) have suggested that this could act as a negative feedback mechanism to regulate the amount of C2 synthesised by the monocytes in culture.

4.2.3 Inhibition of Monocyte Complement Synthesis by Cycloheximidide

The reversible inhibition of monocyte C1-inhibitor and C2 synthesis by cycloheximide demonstrates that the cells were actively synthesising complement components, and they were not merely stored and secreted during culture. More convincing evidence to support the de novo synthesis of complement components comes from pulse-chase studies involving ^{35}S-methionine labelling, and immunoprecipitation

of specific proteins. In addition to C2 and C1-inhibitor which I have investigated, this has been demonstrated by other workers for C3, B and lysozyme (Cole *et al.*, 1985; Hamilton *et al.*, 1987; Strunk *et al.*, 1985). The identification of specific mRNA for C2 and B in monocytes, provides conclusive evidence that these complement components are synthesised by monocytes *in vitro* (Cole *et al.*, 1985).

4.2.4 Functional Activity of Monocyte C1-inhibitor

The other property investigated was the functional haemolytic activity of monocyte C1-inhibitor. The procedure used (detailed in Section 2.5.5) involved concentration and dialysis of serum free monocyte supernatant, from control and cycloheximide-treated cultures. Although cycloheximide reduced C1-inhibitor protein synthesis by over 90%, C1-inhibitor functional activity was only reduced by between 44% and 67%. As fresh culture medium to which cycloheximide had been added did not contain C1-inhibitory activity, this finding suggested that cultured monocytes release a second factor with C1-inhibitory activity, which was antigenically distinct from plasma C1-inhibitor. Using simultaneous equations the specific functional activity of monocyte C1-inhibitor was calculated and displayed a mean of 0.97 units/ng, with a range from 0.57 units/ng to 1.53 units/ng. Gigli *et al.* (1968) have reported that serum C1-inhibitor has a mean specific activity of 1.76 units/ng, which is in a similar range to that obtained for monocyte C1-inhibitor.

4.2.5 Pulse-Chase Studies Under Control Conditions

The findings that intracellular C2 and C1-inhibitor were of a lower molecular weight suggested that at this stage they were not completely glycosylated. Other workers report an intracellular C2 precursor of about 90 kD, with the secreted form of C2 having a slightly higher molecular weight (Cole et al., 1985). It was found that when tunicamycin, an antibiotic known to inhibit dolichol-phosphate-dependent N-linked glycosylation, was added to monocytes prior to a pulse chase, the intracellular form of C2 was reduced by approximately 12 kD (Cole et al., 1985). Harrison (1983) has deglycosylated purified C1-inhibitor and this PAS-negative staining band on SDS-PAGE had a molecular weight of 78 kD. This suggests that the intracellular form of monocyte C1-inhibitor was partially glycosylated, and that further carbohydrate residues were added prior to and during the secretory process.

Thus, it has been demonstrated that C1-inhibitor is synthesised by human monocytes in culture by demonstrating time-dependent increase in C1-inhibitor concentrations in culture supernatant, reversible inhibition of C1-inhibitor synthesis by cycloheximide, incorporation of radiolabelled amino acids into immunoprecipitable protein, and the presence of functional C1-inhibitor activity.

4.3 The Effects of γ Interferon on Monocyte Complement Synthesis

There are three possible effects of γ interferon on the expression of different genes:- 1) No effect: this was observed with lysozyme synthesis. 2) Stimulation of synthesis: this was observed with C1-inhibitor, C2 and B. 3) Reduced synthesis: this was observed with C3 and properdin.

Lysozyme secretion was not affected by γ interferon; however, my colleagues and I have shown that β interferon at doses of 100 units and 1000 units, reduced lysozyme synthesis from 87 ± 14 ng/ml to 55 ± 3 ng/ml, whilst α interferon, like γ , had no effect on monocyte lysozyme synthesis (Hamilton et al., 1987).

The stimulatory effect of γ interferon on monocytes varied depending on the dose and the component being investigated. Pulse chase studies confirmed the massive enhancement of C1-inhibitor synthesis by γ interferon. They also demonstrated that γ interferon did not alter the rate of secretion of C1-inhibitor, indicating that the effect of γ interferon was not post-translational. These findings are in agreement with those of Strunk et al. (1985) who demonstrated that the levels of mRNA for C2 and B were raised in γ interferon stimulated monocytes. This confirms that γ interferon stimulates C2 and B synthesis at the pre-translational level.

C1-inhibitor synthesis was most affected by γ interferon. Even at the lowest dose investigated (0.01 ng/ml) synthesis was increased by a greater extent than the maximum stimulation

observed for C2 or B. Furthermore, the dose response curve of C1-inhibitor synthesis to γ interferon was only just starting to reach a plateau at the highest dose used (10 000 ng/ml). The responses of C2 and B to γ interferon were of a similar magnitude in that the maximal enhancement for both was around 260% and 180% respectively. Hamilton et al. (1987) showed that the maximal stimulation of C2 and B synthesis was achieved when the concentration of γ-interferon was 100 ng/ml. They suggested that the sensitivity of the C2 gene to γ interferon was greater than that of the B gene, due to their observation that significant stimulation of C2 synthesis occurred at 0.01 ng γ interferon/ml, whereas stimulation of B synthesis only occurred at concentrations of γ interferon of 1 ng/ml and above. On the contrary, Strunk and colleagues (1985) have demonstrated that in their system 0.01 and 0.1 ng γ interferon/ml could stimulate B synthesis as well as C2 synthesis. The reasons for these differences is unknown, and is not due to differing potencies of γ interferon preparations (19 IU/ng, Strunk et al., 1985; 14 IU/ng Biogen preparation).

In the experiments performed in this thesis, γ-interferon was added on day 0, samples removed sequentially and replaced by fresh tissue culture medium. Thus, although the γ interferon was present all of the time, it was constantly being diluted due to sampling. Under these conditions stimulation of C1-inhibitor, C2 and B synthesis still occurred. In comparison, Strunk and his colleagues

(1985) noted that when γ interferon was removed from monocytes in culture, synthesis of C2 and B had returned towards basal levels within 24 hrs. Hamilton et al. (1987) have demonstrated that γ interferon could be removed after only 5 min. exposure to monocytes, and still produce enhanced C2, B and C1-inhibitor synthesis. Strunk et al. (1985) found that it took several hours before the effect of stimulation of C2 and B synthesis became apparent. They suggested that an initial rapid signal was translated into enhancement of C2 and B synthesis by a slower secondary process, which was not dependent on protein synthesis since it was unaffected by cycloheximide. In contrast, for a reduction of C3 synthesis it appeared that γ interferon had to be present for more than 8 hours, suggesting that the signal involved in the inhibition of C3 synthesis differed from that involved in the stimulation of C1-inhibitor, C2 and B synthesis (Hamilton et al., 1987).

Thus from my own work, the work of Hamilton et al. (1987) and Strunk et al. (1985), it would appear that γ interferon exerts differing effects on the C1-inhibitor, C2, B, P and C3 genes, and that these effects are most likely to be occurring at the pre-translational level. The greatest stimulatory effect was shown towards the C1-inhibitor gene, with less stimulation of the C2 and B genes, whilst an inhibitory effect was exerted on the expression of the C3 and properdin genes.

Interferons are a group of proteins which were first identified by their ability to protect cells against viral infection; however, recently it has been suggested that interferons may play a role in a variety of diseases (Reviewed by Clemens and McNurlan, 1985; Wilkinson and Morris, 1985).

Interferons are synthesised and secreted by many cell types in response to various inducers, and have been shown to exert their effects *in vivo*, as a result of interaction with cells in other areas of the body. Clemens and McNurlan (1985) have suggested a functional similarity with hormones, which was further emphasised when the mode of action and effects of interferons were considered. Receptors for the interferons are present on the surfaces of cells (Tomida, Yamamoto and Hozumi, 1982), the best characterised being the IFNα2 receptor on the Daudi human lymphoblastoid cell line. This cell has been shown to possess two types of interferon receptor, one which binds α and β specifically, and the other which preferentially binds γ interferon (Tomida, Yamamoto and Hozumi, 1982). Interaction of interferon with its cell surface receptor is followed by a series of, as yet poorly defined events. Various observations have been made which report transient increases in cAMP (Scheck *et al.*, 1982) and cGMP (Rochette-Egly and Tovey, 1982), changes in protein phosphorylation, as well as increased and decreased levels of mRNA for various gene products in various cells (Reviewed by Clemens and McNurlan, 1985).

The similarity of interferons to hormones is evident when one considers the multiplicity of biological effects which results from these interactions. These effects include the stimulation and suppression of differentiation and growth of macrophage precursors; stimulation of the expression of macrophage and monocyte surface markers such as the Fc receptor and MHC antigens; effects on phagocytosis, tumoricidal and microbicidal activity of macrophages (Reviewed by Wilkinson and Morris, 1985).

The roles of interferons and macrophages are very much interwoven: macrophages produce interferons (α and β) when stimulated by viruses or bacterial endotoxins; macrophages are involved in the T-cell production of γ interferon; γ interferon can stimulate the differentiation of precursors of monocytes and macrophages; γ interferon can increase the expression of Fc and MHC surface antigens; αβ interferon (a mixture of α interferon and β interferon) stimulates microbicidal activity of macrophages. Thus, it has been suggested that interferons (especially γ) play a major role in controlling the immune response due to its effects on the expression of class II MHC antigens on antigen-presenting cells (Unanue et al., 1984).

4.4 The Effect of Steroid Hormones on Monocyte Complement Synthesis

Steroid hormones are known to exert two types of effect, specific and non-specific, on the basis of their mechanism of action. Specific effects are the result of the interaction of the steroid with a specific receptor, which differs for

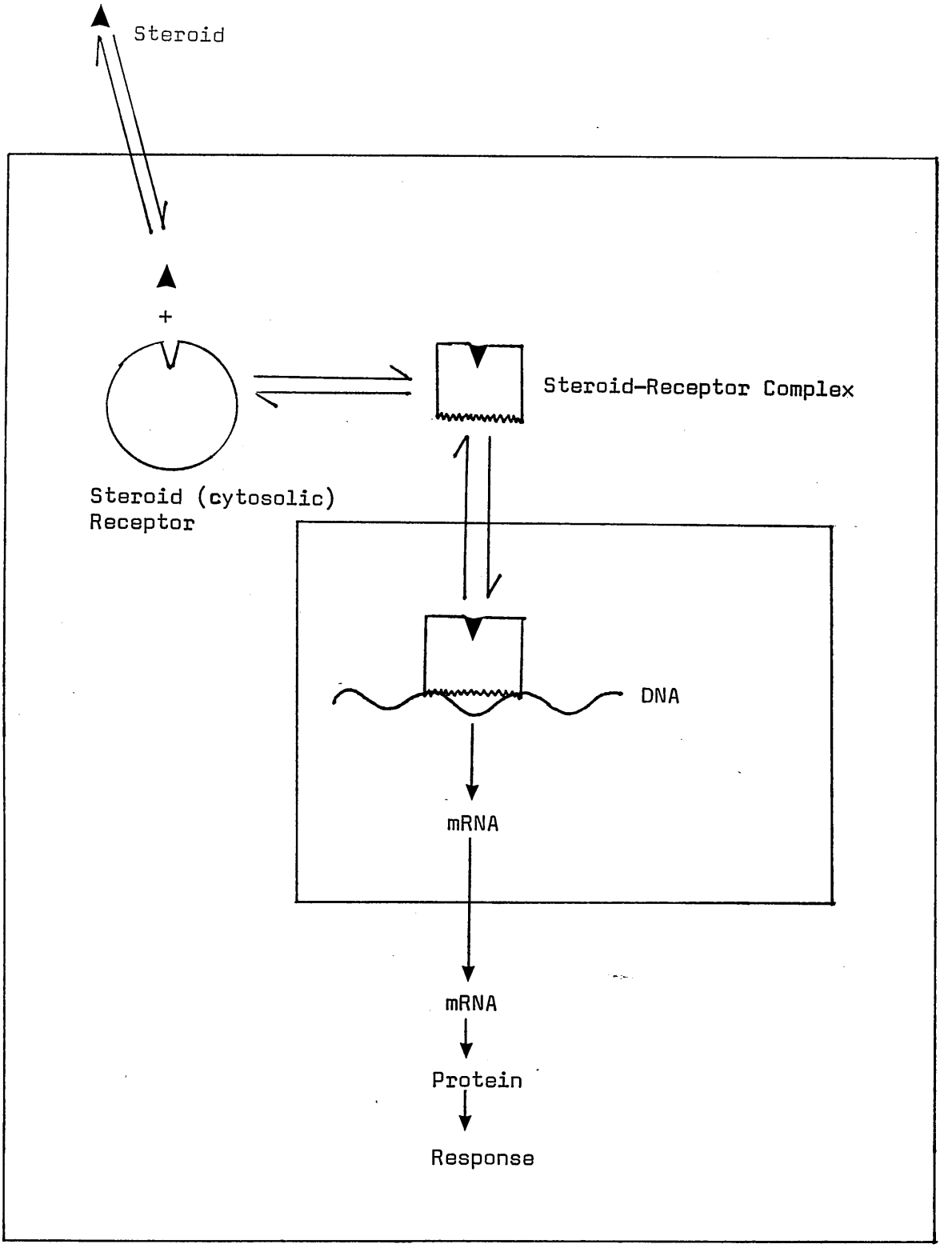

Fig. 42: A generalised scheme for the mechanism of action of steroid hormones. For details of explanation see text.

each class of steroid hormone. Non-specific effects are independent of receptor interaction and are usually exerted by insertion of the steroid into the lipids of the cell membrane, resulting in an alteration in the structure and properties of these membranes (Reviewed by Skidmore, 1981).

A generalised scheme of the mechanism of action of steroid hormones is outlined in Figure 42 (Jensen et al., 1968). Unlike most receptors which are plasma membrane bound, steroid receptors are proposed to be soluble cytoplasmic proteins. The steroids are thought to passively diffuse across the plasma membrane and combine with the cytoplasmic receptors which undergo a conformational change. This is thought to allow the steroid-receptor complex to be transported into the nucleus, where it binds with high affinity to selective sites on the chromatin. Interaction of the steroid-receptor complex with the DNA leads to the synthesis of specific mRNA which leaves the nucleus and is translated into proteins, through which the effect of the steroid is expressed.

Recently evidence has been accumulating to suggest that specific steroid receptors may be nuclear rather than cytoplasmic, as was originally proposed on the basis of the rat uterus model (Jensen et al., 1968). The results from these studies have demonstrated that the oestrogen receptor in uteri appeared to be associated with the particulate nuclear fraction (Pietras and Szego, 1979), and in avian liver preparations no cytoplasmic oestrogen receptor could be detected, but a nuclear receptor was suggested (Snow et al.,

1978). Another theory which has evolved is that the unoccupied receptor may be partitioned between the nucleus and cytoplasm (Martin and Sheridan, 1982), however, more recent work has cast considerable doubt as to whether there is ever any receptor found in the cytoplasm. This evidence has shown that using immunocytochemistry techniques which employ a monoclonal antibody to the oestrogen receptor, and investigating a variety of target tissues, immunoreactivity was confined to the nucleus (King and Greene, 1984). Hence these data will lead to the reconsideration of the action of steroid hormones, however, substantial evidence has still to be accumulated before a new model can be proposed. At present the evidence may only account for a nuclear-associated receptor, that is it may be attached to the outer nuclear membrane, or it may be present in the perinuclear space, and thus the model originally proposed (Fig. 42) may only indicate inaccurate compartmentation of the unoccupied steroid receptor.

The proteins which are synthesised in response to a steroid may be new to the cell, as a result of induction of synthesis of novel mRNA, or may merely represent enhanced synthesis of existing mRNA, leading to enhanced levels of previously existing proteins. Thus, steroids can induce the synthesis of new proteins which act as regulatory proteins and can in turn produce a steroid-induced effect, or they can directly increase the levels of previously existing proteins. (Reviewed by Rousseau, 1984). These two possibilities can often be distinguished on the basis of the lag period

observed between exposure to the steroid, and the appearance of the steroid-induced response. If a short lag period is involved, this would favour a direct effect on the production of previously existing proteins. However, a long lag period would favour the formation of steroid-induced intermediates, which in turn produce the response (Reviewed by Rousseau, 1984; Durant and Homo-delarche, 1983).

Observations made from my own research on the glucocorticoid time exposure experiments (Section 3.4.1f) demonstrated that in order to get maximum stimulation of C2 and C1-inhibitor synthesis, the glucocorticoids had to be present for 48 hr and in some experiments 72 hr. These findings appear to be more compatible with the latter mechanism of action. If so, then a steroid-induced intermediate is responsible for the final response of the monocytes to the glucocorticoid, rather than the glucocorticoid having a direct effect on C2 and C1-inhibitor synthesis. An experiment which would have confirmed this observation, would have been to determine whether or not the glucocorticoid effect was sensitive to cycloheximide. If it was shown to be sensitive to cycloheximide, it would support the theory that glucocorticoids act on monocyte C1-inhibitor and C2 synthesis via an intermediate protein, rather than directly affecting the C1-inhibitor and C2 genes.

An insight into the mode of action of steroids would have been resolved by performing Northern blot analysis and looking for increases in specific mRNA for C1-inhibitor and C2 in monocytes in response to stimulation by steroids. If

no increases in specific mRNA were observed and C1-inhibitor and C2 synthesis were enhanced, then it would be unlikely that the glucocorticoids were acting by directly enhancing C1-inhibitor and C2 synthesis, as was suggested by the time-exposure experiment results. Other effects which one would also have to rule out include translational effects, such as increasing the frequency of reading of the specific mRNA or increasing the stability of the specific mRNA. In addition pulse-chase experiments would have determined if the increased C2 and C1-inhibitor levels were due to an increase in secretion of intracellular stores of C2 and C1-inhibitor. However, this was unlikely since pulse-chase experiments under control conditions have shown that most of the intracellularly-labelled C2 and C1-inhibitor are secreted within a 4 hour chase period.

An outstanding property of all steroid receptors is their ability to recognise subtle minor differences in steroid structure. In a given tissue, receptors exhibit a high affinity for molecules that are biologically active in that tissue, a lower affinity for related steroids with low biological activity, and no binding of inactive steroids (Reviewed by Durant and Homo-delarche, 1983). On the basis of their biological activities and structures six major classes of hormonal steroids which bind to different receptors have been characterised: glucocorticoids, mineralocorticoids, oestrogens, androgens, progestins and vitamin D compounds. In general, despite possible overlap between receptor binding

profiles, each member of a given category binds essentially to its own specific receptors. (Reviewed by Durant and Homo-Delarche, 1983).

The observation that progesterones, oestrogens, mineralocorticoids, androgens and anabolic steroids had no effect on monocyte C2 or C1-inhibitor synthesis (Section 3.4.2) tends to suggest that either these specific steroid receptors are not present in monocytes, or if they are present the steroid-receptor complexes do not alter the expression of the C2 or C1-inhibitor genes, or genes which may produce products which would in turn control these genes. The observation that glucocorticoids increased monocyte synthesis of C1-inhibitor, C2, and properdin (unreported observation), and reduced synthesis of lysozyme and C3, suggests that monocytes possess glucocorticoid receptors, as has been shown previously by Werb and her colleagues (1978a). Corticosterone was the only glucocorticoid tested which did not have any effect on monocyte C1-inhibitor or C2 synthesis. Bray and Gordon (1978) found that in their system, unlike the other glucocorticoids tested, corticosterone did not inhibit macrophage prostaglandin synthesis. They concluded that this was probably due to macrophages being unable to convert corticosterone to hydrocortisone, its biologically active product, which would agree with my observations also.

Glucocorticoids, for example hydrocortisone produced in the human adrenal cortex, are known to suppress both acute and chronic inflammation; however they also possess weak mineralocorticoid properties. In the 1950s and 1960s chemists began to modify the molecule in order to maximise anti-inflammatory activity and attempt to reduce mineralocorticoid activity (Popper and Watnick, 1974). These modifications established that the Δ 4 double bond, the 11β and 17α hydroxyl groups and the side chains were required for significant anti-inflammatory activity. Introduction of the Δ 1 double bond enhanced anti-inflammatory potency whilst reducing electrolyte effects; the 19α halogenated groups, often a fluorine, enhanced potency, whilst substitution at C16 with hydroxyl or methyl groups eliminated electrolyte effects and enhanced anti-inflammatory potency. Thus, a league of relative potencies assigned hydrocortisone a potency of 1, prednisolone a potency of 4 and dexamethasone and betamethasone a potency of 25. (Reviewed by Skidmore, 1981).

From the experimental work performed, it proved difficult to assign an order of potency to the glucocorticoids as has been given with respect to their anti-inflammatory effects. This was due to the observation that the peak enhancing dose was not always the same in each set of cultures for each glucocorticoid that was tested. However, with regards to C1-inhibitor and C2 synthesis, hydrocortisone always showed a peak enhancement at 10^{-5}M, whereas prednisolone, dexamethasone and Org 6632 most often peaked at doses lower than 10^{-5}M. When the peak enhancement for prednisolone or

dexamethasone did occur at 10^{-5}M, the level of enhancement was usually higher than that observed for hydrocortisone. The peak enhancing dose for Org 6632 always occurred at 10^{-6}M or below. Thus, although in this system there was not a clearly defined order of potency, there did appear to be a ranking in that hydrocortisone was the least potent, prednisolone and dexamethasone were more potent, with Org 6632 being the most potent of all four tested. Although the relative order of potency of Org 6632 is unknown, it is known that in receptor studies using human and rodent leukocytes and lymphoid tissues, Org 6632 has a greater affinity for the dexamethasone receptor than does dexamethasone (four to twenty times greater) (Personal communication, Dr A. Campbell, Organon Laboratories, Newhouse). This correlated well with the observation in the monocyte system that Org 6632 was more potent than dexamethasone.

The basis for the anti-inflammatory effects of glucocorticoids encompass a very wide range of pharmacological and biochemical effects. One role of anti-inflammatory steroids is to cause vasoconstriction, counteracting the vasodilatory effects of histamine and bradykinin which are released during inflammation (Majno and Palade, 1961). Anti-inflammatory steroids are also known to produce a profound monocytopenia, and inhibit the accumulation of monocytes and macrophages at sites of inflammation, where the macrophage is known to be a key effector cell in causing the tissue damage associated with chronic inflammation

(Thompson and van Furth, 1970). This is partly due to the macrophage being a source of prostaglandins, lysosomal hydrolytic enzymes and neutral proteinases (Reviewed by Leoni, Dean and Jessup, 1985) as well as secreting complement components (McPhaden et al., 1985), factors which are chemotactic for neutrophils and soluble factors which stimulate both synovial cells and chondrocytes to produce prostaglandins and neutral proteinases (Englis et al., 1980).

Glucocorticoids are known to inhibit the release of arachidonic acid from macrophages (Bonney et al., 1978), inhibit the synthesis of prostaglandins (Bonney et al., 1978; Bray and Gordon, 1978) and inhibit the synthesis and release of neutral proteinases in vitro (Werb et al., 1978b). Since these effects occurred at low concentrations, were dose-related and displayed a consistent glucocorticoid rank order of potency, they can therefore be expected to occur in vivo in response to realistic doses of anti-inflammatory glucocorticoids.

Glucocorticoids inhibit prostaglandin synthesis, but the observation that this could be overcome by the addition of exogenously added arachidonate led to the suggestion that glucocorticoids operated mainly by decreasing the amount of precursor available for prostaglandin production (Hong and Levine, 1976). It is now known that anti-inflammatory glucocorticoids induce the synthesis of a novel protein called lipomodulin (formerly called macrocortin). This reduces arachidonic acid formation in cell membranes by inhibiting phospholipase A_2 activity. Thus, prostaglandin production is reduced (Blackwell et al., 1982).

This observation would link well with the observation in the system studied here where glucocorticoids stimulated monocyte C2 synthesis. Control of monocyte C2 synthesis was demonstrated to be dependent on several factors, one of the main ones of which was the intracellular levels of cAMP (Lappin and Whaley, 1984). Prostaglandins E_2, D_2 and 6 keto $PgF_{2\alpha}$ are known to stimulate adenyl cyclase activity leading to an increase in intracellular cAMP levels and hence a decrease in C2 synthesis (Lappin and Whaley, 1984). Thus in the presence of anti-inflammatory glucocorticoids, endogenous prostaglandin synthesis would be reduced by the mechanism involving lipomodulin. Consequently decreased levels of prostaglandins would lead to a reduced adenyl cyclase activity, lower cAMP levels, and greater C2 synthesis. Further evidence to support the theory that prostaglandins switch off C2 synthesis has come from the observation that prostaglandin synthetase inhibitors, such as indomethacin and ETYA, cause a reduction in monocyte cAMP and an increase in monocyte C2 synthesis (Lappin and Whaley, 1983). The theory that steroids, due to the synthesis of lipomodulin, inhibit prostaglandin synthesis, which in the monocyte system results in a decrease in cAMP and an increase in C2 synthesis, would appear to account for the mode of action of glucocorticoids on monocyte C2 synthesis.

The effect of prostaglandins and cAMP levels on the synthesis of monocyte C1-inhibitor and properdin is not known; thus it is not known whether the mode of action of glucocorticoids on C1-inhibitor and properdin synthesis, is

similar to that proposed for C2. It is somewhat unlikely that C1-inhibitor synthesis is controlled in a similar manner to monocyte C2 synthesis. The evidence of this was apparent when continuous and cumulative monocyte C1-inhibitor and C2 synthesis were studied (Section 3.2.2). This demonstrated that C1-inhibitor synthesis under continuous culture was not switched off as occurred with continuous C2 synthesis. The switching off of C2 synthesis, under these conditions, was attributed to accumulation of prostaglandins in the culture supernatant (Lappin and Whaley, 1984), since the prostaglandin synthetase inhibitor indomethacin could reverse the plateauing effect.

The effect of indomethacin on the synthesis of various complement components by monocytes was investigated. It was found that over the dose range 10^{-8}M to 10^{-4}M, C2, C1-inhibitor and C3 synthesis were enhanced, whilst there was no effect observed on lysozyme synthesis (Personal communication, Dr David Lappin). Thus, it is unlikely that the effect of glucocorticoids on C1-inhibitor, P, C3 and lysozyme synthesis occurred via a similar mechanism to that proposed for C2 which involved inhibition of prostaglandin synthesis, presumably via lipomodulin. However, the mechanism of stimulation of C1-inhibitor and properdin synthesis was not investigated further.

The biochemical basis for the observed inhibition of monocyte C3 synthesis by glucocorticoids is unknown but it has very important clinical implications with respect to the anti-inflammatory effects of glucocorticoids. Macrophages

are postulated to be very important for maintaining the local tissue levels of complement, especially at sites of inflammation where components are quickly depleted due to continuous complement turnover (Whaley, 1980; De Ceular, Papazoglou and Whaley, 1980). Glucocorticoid-induced reduction of C3 synthesis by macrophages at these sites would reduce the turnover of the complement pathways, due to quick depletion of C3 levels. This would result in a reduction in the production of the cleavage products of C3, C5 and B, all of which are known to be potent mediators of the inflammatory response (De Ceulaer, Papazoglou and Whaley, 1980). This in vitro effect of glucocorticoid-induced inhibition of monocyte C3 synthesis has another relevant clinical link, in that patients who are receiving long term treatment with steroids often suffer from recurrent infections. This could be as a direct result of reduced monocyte and macrophage C3 synthesis, hence a decrease in opsonisation of invading microorganisms, and thus a reduced capacity to phagocytose, and intracellularly kill these pathogens by tissue monocyte, macrophages and neutrophils.

The increase in levels of C1-inhibitor synthesised by glucocorticoid-stimulated monocytes, may also help to control activation of the classical pathway of complement, which is known to occur at sites of inflammation. C1-inhibitor is also known to be effective at controlling other plasma mediator systems such as the kinin generating system (which is important in inflammation) as well as the clotting and fibrinolytic systems.

The observation that glucocorticoids inhibited lysozyme secretion by monocytes provides more evidence to suggest that the theory of lysozyme being a constitutive product of mononuclear phagocytes, should be reviewed to include the observation that it can be regulated by various factors such as β interferon and glucocorticoids.

The role of glucocorticoid-inhibited secretion of lysozyme, would not appear to have any direct relevance to the anti-inflammatory properties of these drugs. However, the synthesis of other hydrolytic enzymes, such as lysosomal hydrolases and neutral proteases, are also inhibited by glucocorticoids (Werb, 1978). These hydrolytic enzymes are known to be involved in the degeneration which takes place in regions of inflammation. Inhibition of secretion of lysozyme, a protein which can account for up to 2.5% of the total cellular protein, may allow diversion and channelling of intracellular metabolites and precursors into other areas which may be beneficial to the role of the cell. Such an example would be the increase in synthesis of C1-inhibitor, a protein involved in the control of the complement cascade and the kinin generating system, both of which produce products which are involved in the generation and continuance of the inflammatory process.

Thus, the effects of glucocorticoids on monocyte <u>in vitro</u> complement synthesis could be postulated as a role which primarily makes attempts to reduce levels of, and decrease turnover of the complement pathways at sites of inflammation

where macrophages are abundant. Since these effects occurred *in vitro* at physiological concentrations of glucocorticoids, it could be expected to occur *in vivo* and this role of glucocorticoids would correlate well with the potent *in vivo* anti-inflammatory activity of this group of steroids.

4.5 Synthesis of Complement Components by Monocytes of Patients with C1-inhibitor Deficiency

C1-inhibitor synthesis by the monocytes from 47 normal donors was measured to establish a normal range. From these data, it was evident that the distribution of C1-inhibitor concentrations in the culture supernatants was not normally distributed, but the distribution approximated to normality following log transformation. This type of logarithmic distribution was also demonstrated for monocyte C2 and C3 synthesis, thus all data pertaining to C1-inhibitor, C2 and C3 were log-transformed prior to statistical analysis using a Student's 't' test. However, as a safeguard data were also analysed by the Mann Whitney U-test for non-parametric data. Interestingly, the concentrations of most plasma proteins appear to be distributed logarithmically (Ruddy and Austen, 1975).

4.5.1 Synthesis of C1-inhibitor, C2 and C3 by Monocytes from C1-inhibitor Deficient Patients

When C1-inhibitor synthesis by monocytes of patients with C1-inhibitor deficiency was investigated, some unexpected results were obtained. Monocytes isolated from the blood of patients with Type I HAE appeared to synthesise substantial levels of C1-inhibitor although the mean level was lower

than that for normal monocytes; this difference was not statistically significant. C1-inhibitor synthesis rates by monocytes from patients with Type II HAE or acquired C1-inhibitor deficiency were similar to those observed for normal monocytes.

The synthesis of C2 and C3 by monocyte cultures from the three groups of patients, were investigated to enable comparison of C1-inhibitor with proteins which should not be affected by the C1-inhibitor deficiency. The levels of C2 and C3 synthesised by monocytes of normals and each group of patients, on each day in culture, were shown to be distributed over wide ranges, as demonstrated with C1-inhibitor synthesis by normal monocytes. Monocytes from patients with Type I HAE, Type II HAE and acquired C1-inhibitor deficiency synthesised C2 and C3 mean levels very close to the mean levels observed with the group of monocytes from normal individuals which were processed simultaneously. Thus, as was expected, it appeared that C1-inhibitor deficient patients' monocytes synthesise C2 and C3 at similar rates to those of normal monocytes.

When the group of patients with Type I HAE were sub-divided into those patients receiving androgen treatment (8) and those patients not receiving androgen treatment (6), and the levels of C1-inhibitor in monocyte culture supernatant was investigated, interesting results emerged. Those patients receiving androgen treatment demonstrated mean C1-inhibitor levels almost identical to those observed for normal monocytes. However, those Type I HAE patients not receiving androgen

treatment showed lower mean levels of monocyte C1-inhibitor on all days in culture, compared to monocytes from normal donors or Type I HAE patients who were receiving androgen treatment. However, the means for the group of Type I HAE patients receiving androgen treatment were not statistically different from those of monocytes from patients not receiving androgens. The group of patients not receiving androgen treatment were probably responsible for the lower mean levels of C1-inhibitor observed in monocyte culture supernatant when Type I HAE patients were considered as a whole group.

When the mean levels of monocyte C2 in these two groups of Type I HAE patients was investigated, patients receiving androgens and those not receiving androgens had C2 levels similar to normals. However, C3 levels were shown to be significantly lower in monocyte culture supernatants from Type I HAE patients not receiving androgen treatment, compared to normals and Type I HAE patients receiving androgen treatment, which were similar.

Thus, monocytes from Type I HAE patients not receiving androgen treatment synthesise significantly less C3, but similar C2 and slightly less C1-inhibitor than monocytes from Type I HAE patients receiving androgen treatment. These data suggest that the monocytes from patients with Type I HAE who were not receiving androgen treatment differ in some way to those receiving androgen treatment and normals. It is unlikely that the difference between these two patient groups is due to a direct androgen effect. Firstly, in this system androgens *in vitro* were shown to have no effect on monocyte complement synthesis (Section 3.4.2). Secondly, there is no

evidence in the literature to suggest that monocytes possess androgen receptors. Thirdly, the monocytes of three different patients were studied before and after commencing androgen treatment: they showed no consistent change in the level of complement components synthesised by monocytes before and after treatment had commenced (unreported observations). Fourthly, Gelfand et al. (1976) demonstrated that administration of danazol to guinea pigs in vivo resulted in the stimulation of hepatic synthesis of C1-inhibitor leading to an increase in serum C1-inhibitor; however, no effect was observed on serum C3 levels.

The monocytes from patients not receiving androgen treatment were morphologically normal, and as viable as the normal monocytes and monocytes from patients receiving androgen treatment. Thus it is unlikely that their reduced synthesis of C3 was as a result of decreased viability. The condition of these cells would have been able to be assessed if lactate dehydrogenase or lysozyme levels had been measured; however, due to the limited number of monocytes and small volume of supernatant available, these assays could not be performed. The observation that these patients monocytes responded as well as normals to stimulation by hydrocortisone and γ interferon, is further evidence to suggest that decreased C3 synthesis was not due to reduced viability of the monocytes. Thus, the possible reasons as to why monocytes from Type I HAE patients not receiving androgen treatment synthesise less C3 compared to monocytes isolated from normal ind ividuals and Type I HAE patients

receiving androgen treatment, are as follows. Firstly, those patients receiving androgen treatment probably have increased well being. Secondly, due to their treatment with androgens, their disease process is halted or slowed considerably thus they do not experience the pathological effects of the disease, which may generate a factor(s) which may inhibit monocytes or reduce their capacity to synthesise proteins.

4.5.2 Response of C1-inhibitor Deficient Patients' Monocytes to γ Interferon

The effects of γ interferon on the secretion rates of C1-inhibitor, C2 and C3 between days 3 and 5 by monocytes from normals and the three patient groups was investigated. Calculation of the fold increase of SR for C1-inhibitor demonstrated that all three groups of patients possessed an equal capacity to normals to respond to γ interferon-induced enhancement of C1-inhibitor SR. Even the Type I HAE patients not receiving androgen treatment, who had a lower initial SR, responded equally well, and actually showed the greatest increase of 36 fold over control, when stimulated with 1 μg γ interferon/ml (Section 3.5.5).

With regards to enhancement of monocyte C2 SR by γ-interferon, all three groups of patients responded as well as, or better than normal monocytes. C3 SR of monocytes from Type I HAE patients not receiving androgen treatment were shown to be lower than that of monocytes from Type I HAE patients receiving treatment and normal monocytes. However, upon statistical analysis the difference was shown not to

be significant. The monocyte C3 SR of normal monocytes and monocytes isolated from all patient groups, were shown to be inhibited by γ interferon, although the dose and level of inhibition varied considerably between the groups.

Thus, the study of the effects of γ interferon on monocytes from patients with Type I HAE, Type II HAE and acquired C1-inhibitor deficiency, demonstrated that these patients monocytes respond as well to γ interferon as normal monocytes. This demonstrates that the DNA sequences responsible for γ interferon binding and effects on C1-inhibitor, C2 and C3 synthesis are present in monocytes of patients with the various types of C1-inhibitor deficiency.

4.5.3 Specific Activity of C1-inhibitor from Monocytes of C1-inhibitor Deficient Patients

Investigation of the specific functional activity of monocyte C1-inhibitor demonstrated that monocytes from normals, patients with Type I HAE and acquired C1-inhibitor deficiency all synthesise C1-inhibitor of a similar specific activity, the overall range being from 0.61 units/ng to 4.09 units/ng. The mean levels for these three groups were similar to that measured previously for monocytes cultured under serum-free conditions: 0.97 units/ng (Section 3.2.4).

Monocytes from one of the two patients with Type II HAE synthesised C1-inhibitor with a specific activity within the normal range, that is 1.64 units/ng (patient J.M.). The monocytes from the second patient with Type II HAE synthesised C1-inhibitor with a specific activity of 0.48 units/ng (patient C.E.) a level below that obtained for the

lowest normal monocyte specific functional activity (0.61 units/ng).

Unfortunately, only those two patients with Type II HAE were investigated for monocyte C1-inhibitor specific functional activity, and no clear cut result has been obtained. On the basis of the genetic defect of patients with Type II HAE the predicted results would have been that these patients' monocytes would have synthesised C1-inhibitor with a low specific activity. This is due to one of their genes synthesising a functionally abnormal protein, whilst the other synthesised a functionally normal protein. The result of this is normal antigenic levels of serum C1-inhibitor, which has a very low functional activity due to preferential consumption of the functionally active protein, in the control of complement turnover.

The results obtained suggest that the monocytes of one patient with Type II HAE (J.M.) have the ability to produce C1-inhibitor with a normal specific activity, whilst the other patient's monocytes (C.E.) do not. When normal monocyte C1-inhibitor specific functional activity was measured under serum free conditions (Section 3.2.4) it was found that upon addition of cycloheximide, C1-inhibitor protein synthesis (measured by RIA) was blocked by more than 90%; however the C1-inhibitor functional activity was only reduced by 44% to 67%. From this it was concluded that under serum-free conditions, monocytes released a second factor with C1-inhibitory activity, which was antigenically distinct from plasma C1-inhibitor. Thus, the observation that one patient with Type II HAE had

monocyte C1-inhibitor with a higher than predicted functional activity, could be due to this second C1-inhibitory factor. It might be argued that this background activity should also occur in normal monocytes. However the assumption would have to be made that this background activity was constant in all monocyte cultures, which may not be the case.

When considering the specific functional activity one must bear in mind that these monocyte cultures were stimulated with γ interferon, the mode of action of which on the C1-inhibitor gene is, as yet, undefined. It could be postulated that the γ interferon could preferentially stimulate transcription of only one gene, and not both. Thus in the case of patient J.M., stimulate transcription of the normal gene, resulting in C1-inhibitor with a normal specific activity, but patient C.E. may be stimulated to transcribe more of the abnormal gene, resulting in the synthesis of C1-inhibitor with a reduced specific activity. Investigations have demonstrated that C1-inhibitor purified from the plasma of patients with Type II HAE is very heterogenous in its ability to bind $\overline{C1s}$, plasmin, plasma kallikrein and activated forms of Hageman factor (Donaldson et al., 1985) as well as in its electrophoretic mobility (Rosen et al., 1971).

Donaldson and her colleagues (1985) have shown that out of eight patients with Type II HAE, each purified dysfunctional C1-inhibitor protein showed a unique spectrum of inhibitory activity against the above-mentioned enzymes. The patient of most interest with relevance to this work was a patient

T.A., whose purified dysfunctional C1-inhibitor could inhibit purified $\overline{C1s}$ to a level of 90% that observed with normal C1-inhibitor. The remaining seven patients showed a range from 4% up to 75%. In comparison C1-inhibitor from patient T.A. proved to be very poor at inhibiting kallikrein and activated Hageman factor. Thus, from this data it is evident that although this patient was a classical Type II HAE with dysfunctional C1-inhibitor activity, the purified C1-inhibitor from this patient was almost as effective as normal C1-inhibitor at inhibiting purified $\overline{C1s}$. Our patient J.M. may be of a similar type, in that his C1-inhibitor has the ability to inhibit purified C1 as was observed with a higher than predicted specific functional activity in the C1-inhibitor functional assay. The decreased activity of monocyte C1-inhibitor from patient C.E. allows one to conclude that the same gene is responsible for the synthesis of C1-inhibitor in the monocyte and in the liver, which is responsible for the synthesis of plasma C1-inhibitor.

4.5.4 The Effect of Steroids on Complement Synthesis by Monocytes from C1-inhibitor Deficient Patients

The effects of steroids (anabolic steroids, androgens and glucocorticoids) on the synthesis of C1-inhibitor, C2, and C3 by monocytes from Type I HAE patients was investigated. The anabolic steroid danazol, used in the treatment of HAE, was observed to have no effect on the synthesis of C1-inhibitor or C2 by monocytes from patients with Type I HAE, as observed previously with normal monocytes (Section 3.4.2). Thus, this demonstrates that danazol in the form it is administered has no stimulatory effect on monocyte C1-inhibitor synthesis; this is probably due to a lack of

androgen receptors in monocytes.

Similar negative results were observed with the androgen testosterone β-D-glucuronide, again demonstrating that the monocytes of patients with Type I HAE respond similarly to normal monocytes with regards to the effects of androgens (Section 3.4.2).

The effect of 10^{-5}M hydrocortisone on the synthesis of C1-inhibitor, C2 and C3 by monocytes from normals and Type I HAE patients was investigated. The results demonstrated that compared to normal monocytes, monocytes from Type I HAE patients responded equally well to stimulation of C2 synthesis and inhibition of C3 synthesis; their capacity to respond to hydrocortisone-induced enhancement of C1-inhibitor synthesis appeared to be less than normal , but this was shown not to be statistically significantly different. The reduced enhancement in Type I HAE patients was demonstrated not to be due to any differences in response between those patients receiving androgen treatment and those not receiving androgen treatment.

Thus, the observation that the monocytes of Type I HAE patients respond as well as normal monocytes to hydrocortisone-induced enhancement of C2 and C1-inhibitor synthesis, and inhibition of C3 synthesis, demonstrates that the patients monocytes possess the glucocorticoid receptor, and binding sequences on DNA responsible for initiating enhancement of C1-inhibitor and C2 synthesis and inhibition of C3 synthesis.

4.5.5 ^{35}S-Methionine Pulse-Chase Studies of C1-inhibitor Synthesised by Monocytes from C1-inhibitor Deficient Patients

The investigation of the molecular weight of C1-inhibitor synthesised by monocytes from normals, patients with Type I HAE, Type II HAE and acquired C1-inhibitor deficiency, demonstrated that the ranges for each of the four groups were very similar with regards to intracellular and extracellular forms. Intracellular pro C1-inhibitor forms displayed the lower molecular weights as described previously (Section 3.2.5) probably as a result of incomplete glycosylation (Range 80 kD to 85 kD). Extracellular forms of C1-inhibitor ranged from 94 kD to 103 kD, with only one exception of 106 kD, for the extracellular form of monocyte C1-inhibitor from one patient with acquired C1-inhibitor deficiency.

C1-inhibitor, precipitated from autologous serum, was analysed on the same gels as the extracellular monocyte C1-inhibitor: the range of molecular weights obtained for serum C1-inhibitor (95 kD to 101 kD) fell within the range for extracellular monocyte C1-inhibitor (94 kD to 103 kD). Upon examination of each individual's serum and extracellular monocyte C1-inhibitor, the majority (7 out of 9) demonstrated the same or a slightly higher molecular weight for extracellular monocyte C1-inhibitor compared to serum C1-inhibitor. The remaining two individuals (one patient with Type I HAE and one with Type II HAE) demonstrated higher serum C1-inhibitor molecular weights compared to monocyte extracellular C1-inhibitor.

Thus, from these results it would appear that there was very little difference in the molecular weights of extracellular C1-inhibitor from monocytes and that of C1-inhibitor precipitated from serum or purified plasma C1-inhibitor. This is further evidence to support the theory that monocyte C1-inhibitor and plasma C1-inhibitor, originating mainly from the liver, are products from the same gene, and not products from two different genes which control C1-inhibitor synthesis in these two different cell types.

The differences in molecular weight able to be resolved by SDS-PAGE may not be sensitive enough to detect any small changes in molecular weight between the C1-inhibitor proteins of the four groups investigated, for example as would occur with amino acid substitutions or small deletions and insertions. For more detailed analysis, the structure of the C1-inhibitor gene, and the amino acid sequence of C1-inhibitor synthesised by monocytes from normals and patients with C1-inhibitor deficiency would have to be studied.

4.5.6 The Study of a Type I HAE Patient in Remission and Disease

The study of patient D.H. (Type I HAE) during remission and disease attack resulted in a very interesting finding with regard to monocyte C1-inhibitor synthesis which appeared to change, whilst his C2 synthesis remained constant. This patient was unusual in that under resting conditions, whether receiving danazol treatment or not, his monocytes appeared to synthesise no or undetectable levels of C1-inhibitor early on in culture. These results were confirmed by Dr David

Lappin (personal communication, Pathology Department, Western Infirmary, Glasgow) who demonstrated that no specific C1-inhibitor mRNA was detectable on Northern blot analysis of an RNA extract of the monocytes of this patient. This was in comparison to Northern blots performed on monocyte RNA preparations from two other Type I HAE patients' monocytes (patients C.B. and J.A.) both of whom demonstrated the presence of specific C1-inhibitor mRNA. These latter two patients' monocytes were also shown in my own system to synthesise normal levels of C1-inhibitor. When D.H's monocytes were stimulated with γ interferon and the RNA extracted and subject to Northern blot analysis, amounts of specific C1-inhibitor mRNA similar to that observed with normals under the same conditions, and of a similar size to normals, was observed.

In contrast to monocyte C1-inhibitor synthesis during disease remission in D.H., when monocytes were isolated from blood which was withdrawn during an acute attack phase, C1-inhibitor could be detected as early as day 2 in culture supernatant, and reached normal monocyte C1-inhibitor levels later on in culture. This dramatic increase in C1-inhibitor synthesis during an acute attack phase, may be an inbuilt regulatory mechanism of monocytes, to supply C1-inhibitor at a local tissue level in an attempt to control complement activation, which is known to occur during angio-oedema attacks. Alternatively, there would appear to be a factor synthesised during disease attack phases which triggers the C1-inhibitor gene in the monocytes of this patient.

Consequently, during disease attacks at areas experiencing angio-oedema, C1-inhibitor would be synthesised locally by monocytes and macrophages, providing a replenishing source of C1-inhibitor. The C1-inhibitor provided can then attempt to block the uncontrolled fluid-phase activation of C1, known to occur locally during these angio-oedema attacks. This would also provide a source of C1-inhibitor to help control the other plasma mediator systems which may be involved in oedema and inflammation, such as the kinin-generating system, the clotting system and the fibrinolytic system. Thus, the monocyte during the disease exacerbation process would appear to be performing a similar role to that proposed for tissue macrophages in inflammatory joints, that is, as a source of local complement components.

Unfortunately, this was the only patient studied under remission and disease attacks, and since he did not appear to be a "normal" Type I HAE patient, he may not be representative of most "normal" Type HAE patients' monocytes during disease attacks. The unusual observation that the monocytes of D.H. synthesised very little specific C1-inhibitor mRNA and protein under resting conditions, compared to monocytes of other patients with Type I HAE, suggests that there may be further subclasses of Type I HAE patients yet to be defined. However, D.H's monocytes synthesise C1-inhibitor of a normal specific activity (when stimulated with γ interferon), they respond to glucocorticoid and γ interferon-induced enhancement of C1-inhibitor synthesis, under the latter conditions of which this patient's monocytes demonstrated full-sized specific C1-inhibitor mRNA on Northern

Blot analysis. These observations suggest that his structural gene encoding for C1-inhibitor is normal, but that his C1-inhibitor deficiency is due to a defect in his regulatory gene sequence, which controls the expression of the C1-inhibitor structural gene.

4.5.7 The Genetic Defect of C1-inhibitor Deficiency

Thus, to summarise, the results obtained in this study of monocytes from patients with various forms of C1-inhibitor deficiency demonstrated that all three groups of patients' monocytes synthesised normal levels of C1-inhibitor, C2 and C3. The patients' monocytes do not react to androgens with respect to C1-inhibitor or C2 synthesis, as was observed with normal monocytes. The patients monocytes responded similarly to normal monocytes when stimulated with hydrocortisone and γ interferon. Monocytes from patients with Type I HAE and acquired C1-inhibitor deficiency synthesise functionally normal C1-inhibitor . Monocytes from one of the two patients with Type II HAE synthesise functionally low specific activity C1-inhibitor, whilst the other synthesises functionally normal C1-inhibitor. The molecular weights for extracellular monocyte and serum C1-inhibitor were of similar ranges in normals and all patient groups.

From these results it would appear that in monocytes from patients with Type I HAE the structural gene for C1-inhibitor is present and functioning normally, and that this monocyte gene is the same one which is responsible for hepatic synthesis of C1-inhibitor and maintenance of plasma levels of C1-inhibitor. One must consider the possibility that in monocytes of Type I HAE patients which are heterozygous

transcription may be occurring only off the one normal copy of the C1-inhibitor gene. From this it would be predicted that Type I HAE patients monocytes would only synthesise half of the levels of normal monocytes. Since the distribution of C1-inhibitor synthesis by normal monocytes was so wide, it may be difficult to see this occurring in our system. The only patient whose monocytes synthesised very low levels of C1-inhibitor in culture was patient D.H.; thus if this prediction was true, we would have expected many more than one patient out of fourteen to be like this. The other point to be considered is that in order to synthesise the same levels as normal monocytes, transcription in patients monocytes would have to occur at twice the rate of normals. These theories are unlikely, however, since the patients monocytes have a similar capacity to normals to respond to δ interferon-induced enhancement of C1-inhibitor synthesis, which if they were only transcribing off one gene, would be unlikely to occur.

Another hypothesis which would account for the results obtained with the patients with Type I HAE is that there is a defect in the C1-inhibitor structural gene of the hepatocyte which is not present in the monocyte. This would suggest that either two different genes encode for C1-inhibitor in each of these cell types, of which there is no evidence thus far. Or, alternatively that there may be tissue specific mRNA processing which differs in the hepatocyte and the monocyte. These possibilities must be investigated, however, for this culture of human liver cells would have to be investigated in parallel with the same patient's monocytes.

The wide distribution of C1-inhibitor synthesis observed in the normal monocytes may be due to environmental influences, such as exposure to infection, during which acute phase proteins and interferons would be secreted, the latter of which are known to have profound effects on complement synthesis by monocytes in culture (Hamilton et al., 1987). Thus, retrospectively it would have been more logical to use normal family members or working colleagues of the patients who were exposed to the same environmental conditions as the patients, instead of hospital laboratory staff who may be exposed to extremely different environments.

The monocytes from patients with Type II HAE produced results which suggested that they have a structural gene defect since one patient's monocytes synthesised C1-inhibitor with an abnormally low specific functional activity, however, otherwise these monocytes were very similar to normal monocytes. This substantiates the hypothesis and findings of several groups of workers who have suggested that Type II HAE is a heterozygous state due to a lesion in the structural gene encoding for C1-inhibitor (Lachmann and Rosen, 1978; Curd et al., 1981).

Curd and his colleagues (1981) investigated the plasma of a Type II patient before and after stanozolol treatment, and demonstrated that the pretreatment plasma contained 94% dysfunctional C1-inhibitor and 6% functionally normal C1-inhibitor, whereas in post-treatment plasma the level of functionally normal C1-inhibitor had increased to 23%.

The purified functional and dysfunctional C1-inhibitors had identical or almost identical molecular sizes, charges, amino acid compositions and amino sugar contents, and could not be distinguished physicochemically from each other or from normal C1-inhibitor. Thus, they concluded that Type II HAE was due to a defect at the structural gene locus for one C1-inhibitor gene, and that the products of both genes were present in serum.

Lachman and Rosen (1978) have suggested that a structural gene lesion is responsible for both Type I and Type II HAE, with the affected subjects in Type I HAE being true hemizygotes and Type II HAE subjects being heterozygotes. This theory of Type I HAE patients being hemizygotes clearly conflicts with the evidence obtained from this study.

Tosi (personal communication, 1986) has studied all the affected members of two large independent families with Type I HAE. He has demonstrated that they have abnormal C1-inhibitor gene restriction maps, suggesting that there is a major defect in the C1-inhibitor gene of these Type I HAE patients. Unfortunately, in his preliminary studies he was unable to determine in which region of the C1-inhibitor gene the defect was occurring. However, contrary to this there is a report by Bock and her colleagues (1986) who observed no gross alteration in the C1-inhibitor structural gene of genomic DNA from patients of one Type I family, and three Type II families. This group therefore suggests that the mutations responsible for Type I HAE and Type II HAE involve small deletions, insertions or limited nucleotide

substitutions in the C1-inhibitor structural gene. Alternatively, there may be defects at other loci involved in the processing and modification of biologically active plasma C1-inhibitor. Further studies being carried out by this group are investigating isolation of mutant C1-inhibitor genes from patients with HAE; the identification of mutations in the dysfunctional C1-inhibitor genes will further the understanding of C1-inhibitor structure-function relationships.

In the light of the results I have obtained in this study, it would appear that my findings substantiate those of Bock et al. (1986) who suggest that the defects in HAE are not due to gross alterations in the C1-inhibitor structural gene. Conversely, the theory of Lachman and Rosen (1978) that Type I HAE patients are true hemizygotes, and the observation by Tosi (1986) that there were restriction fragment length polymorphisms in 3 out of 7 families with Type I HAE, are not upheld, since monocytes from patients with Type I HAE have the same capacity as normal monocytes to synthesise C1-inhibitor; this C1-inhibitor is indistinguishable from normal monocyte C1-inhibitor on the basis of molecular weight and specific functional activity.

A review by Al-Abdullah and Greally (1986) discusses the theory that C1-inhibitor synthesis may be controlled by a multi-component system of genes involving a structural gene, with an operator and promoter site, and a regulatory gene which need not be located close to the structural gene. The regulatory gene will code for a repressor protein, which blocks transcription of the structural gene when bound to the operator site. Interaction of an inducer with the

repressor protein would lead to derepression and allow transcription of the structural gene for C1-inhibitor. It was proposed that in Type I HAE, a mutation in the regulatory sequence would lead to the synthesis of an abnormal repressor which may have lost its affinity for the inducer, thus remaining bound to the operator site repressing transcription of the C1-inhibitor structural gene.

This model would account for the observations made in my study, that Type I HAE is unlikely to be due to a structural gene defect, and more likely to be a result of a regulatory gene defect. However, the defect would appear to be tissue specific in that it was not manifest in the monocyte, except possibly patient D.H., but it was manifest in the hepatocyte which is the major source of serum C1-inhibitor.

The observation that C1-inhibitor deficient patients monocytes *in vitro* synthesise C1-inhibitor is not unique, in that monocytes from homozygous C3 deficient humans synthesise C3 *in vitro* (Einstein et al., 1977). The two patients sera contained less than 1% of the normal C3 concentration, and demonstrated only slightly increased catabolic rates for C3. However, monocytes from these patients in culture produced C3 at about 25% the rate of normal human monocytes, which is substantially more than would have been predicted on the basis of their serum C3 levels. Einstein *et al.* (1977) proposed that C3 deficiency

may be a result of a defect in biosynthetic regulation, rather than in a structural gene defect.

A study of *in vitro* culture of macrophages from C2-deficient guinea pigs has demonstrated that 3 out of 4 cultures of macrophages from homozygous deficient guinea pigs did not secrete C2 protein, however one did appear to secrete C2 fragments of low molecular weight (Goldberger *et al.*, 1982). In contrast, low levels of an apparently reduced molecular weight intracellular C2 protein was detected in all four macrophage cultures, demonstrating that either the protein was not secreted due to its apparent structural abnormality, or that upon secretion it was rapidly degraded.

Thus, the observation that C1-inhibitor deficient patients monocytes synthesise and secrete C1-inhibitor *in vitro* is not a unique biochemical observation, especially with regard to complement component deficiencies.

From this study of complement component synthesis by monocytes from patients with Type I HAE, Type II HAE and acquired C1-inhibitor deficiency, it is apparent that these cells have a similar capacity to normal monocytes to synthesise C1-inhibitor, which is of a similar molecular weight to normal monocyte C1-inhibitor and serum C1-inhibitor. The specific functional activity of C1-inhibitor from monocytes of patients with Type I HAE and acquired C1-inhibitor deficiency are similar to that observed for normal monocyte C1-inhibitor. The monocytes of one of the patients with

Type II HAE synthesised C1-inhibitor with reduced specific functional activity. Monocytes from all three patient groups have a similar capacity to normals to respond to hydrocortisone and γ interferon-induced enhancement of C2 and C1-inhibitor synthesis and inhibition of C3 synthesis.

4.6 Conclusions and Future Work

The initial aims of this project were to investigate the synthesis of C1-inhibitor by normal human monocytes in culture. This was used as a model system, to allow the study of C1-inhibitor synthesis by monocytes from patients with the clinical syndrome of HAE, which is characterised by a deficiency of plasma C1-inhibitor functional activity. The other area originally proposed for study was the effects of steroids on monocyte complement synthesis. These investigations were initiated in an attempt to elucidate the mechanisms by which anabolic steroids correct C1-inhibitor deficiency, and to attempt to understand the underlying genetic defect in HAE.

The initial aims of the project have all been investigated, and have led to the following observations. Firstly, it has been firmly established that normal human monocytes in culture synthesise C1-inhibitor by demonstrating the following: there was an accumulation of C1-inhibitor in monocyte culture supernatant, detected using an RIA. The synthesis of C1-inhibitor by monocytes was reversibly blocked by cycloheximide; under serum-free conditions functional haemolytic C1-inhibitor activity was demonstrated.

^{35}S-Methionine-labelled C1-inhibitor was immunoprecipitated from lysates and supernatants of monocytes which were pulsed with ^{35}S-methionine and chased. Thus, yet again the blood monocyte has been demonstrated to be a mobile and local source of complement components, as has been previously demonstrated for C3, C2, Factor B, C1 and its subcomponents and properdin.

Secondly, it has been established that normal human monocytes respond to glucocorticoids by increasing their synthesis of C1-inhibitor, C2 and properdin and decreasing their synthesis of C3 and lysozyme. However, androgens, anabolic steroids, oestrogens, progesterones, and mineralocorticoids were shown not to have any effect on the synthesis of monocyte C1-inhibitor or C2. From these results it has been demonstrated that monocytes possess the glucocorticoid receptor, substantiating the work of Werb (1978a). However, the other groups of steroids investigated did not appear to have any effect on monocyte C2 or C1-inhibitor synthesis. The *in vitro* effect of glucocorticoids (increasing monocyte C1-inhibitor and decreasing C3 and lysozyme synthesis) is of great clinical relevance with regards to the observed *in vivo* anti-inflammatory activity of this group of steroids.

The third area which was investigated, although this was not originally proposed, was the effect of γ interferon on monocyte complement synthesis. This area proved to be very interesting, since γ interferon had its greatest effect on monocyte C1-inhibitor synthesis. It was also demonstrated that γ interferon inhibited the synthesis of

monocyte C3 and properdin, and had no effect on the synthesis of lysozyme. γ interferon is known to be synthesised in response to many different stimuli, thus under these conditions local C1-inhibitor levels would be increased dramatically, and may be important in controlling local classical pathway activation.

Fourthly, the investigation of complement synthesis by monocytes from patients with C1-inhibitor deficiency demonstrated that these patients' monocytes appear to have the same capacity as normal monocytes to synthesise C1-inhibitor, C2 and C3. The C1-inhibitor synthesised by monocytes from patients with Type I HAE was of a similar molecular weight and specific functional activity to that synthesised by normal monocytes under the same conditions. The monocytes of these patients responded as well as normal monocytes to γ interferon-induced enhancement of C1-inhibitor and C2 synthesis and inhibition of C3 synthesis. Similarly, they responded to hydrocortisone-induced enhancement of C1-inhibitor and C2 synthesis and inhibition of C3 synthesis. This demonstrates that patients' monocytes possess the specific binding regions on DNA for γ interferon and glucocorticoid-dependent effects on complement synthesis.

The C1-inhibitor synthesised by monocytes from patients with Type II HAE was similar in all respects to normals and patients with Type I HAE, with one exception. One of the two patients with Type II HAE investigated, synthesised monocyte C1-inhibitor with a lower specific activity than all of the normals and patients with Type I HAE.

From this study, it has been demonstrated that the monocytes isolated from patients with HAE have the capacity to synthesise C1-inhibitor which is very similar to normal plasma and monocyte C1-inhibitor. This provides evidence to suggest that it is the same gene encoding for C1-inhibitor synthesis in the monocyte and the liver, which is the major source of plasma C1-inhibitor. However, monocytes from patients with Type I HAE did not synthesise reduced levels of C1-inhibitor, which would have been expected on the basis that there is reduced hepatic C1-inhibitor synthesis in these patients. This would therefore suggest that in Type I HAE the genetic defect occurs in the regulatory gene, rather than the structural gene, and since this defect was not manifest in the monocytes of these patients, it would imply that it is a tissue specific regulatory defect. Unfortunately, only two patients with Type II HAE were studied in relation to specific functional activity of their monocyte C1-inhibitor. The results obtained were conflicting, and thus I was unable to propose and substantiate any valid conclusions about the nature of the genetic defect in patients with Type II HAE.

Due to the observations that anabolic steroids did not affect monocyte complement synthesis in normals or patients (probably as a result of the lack of androgen receptors), I was unable to investigate the mechanism by which anabolic steroids correct C1-inhibitor deficiencies.

Future investigations which could, and are at present being studied by colleagues at the Beatson Institute and the Pathology Department, Western Infirmary, Glasgow, are concentrating on the structure of genomic DNA encoding for C1-inhibitor in normals and patients with HAE. Also, the specific mRNA transcripts for C1-inhibitor produced by cultured monocytes isolated from normal individuals and patients with HAE, are being studied under resting conditions and under conditions of stimulation by steroids and γ interferon. Preliminary data from Dr David Lappin (personal communication) has suggested that on Northern blot analysis, monocytes from most patients with Type I HAE synthesise specific C1-inhibitor mRNA transcripts of a similar quantity and size, to that produced by monocytes from normal individuals.

Thus, the future work on DNA sequencing, restriction mapping and Northern blotting analysis, will eventually help to elucidate the underlying genetic defect in HAE. This may shed some light on the mechanisms by which anabolic steroids correct the C1-inhibitor deficiency in these patients.

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