COMPLEMENT BIOSYNTHESIS IN HUMAN SYNOVIAL TISSUE

by

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LIST OF ABBREVIATIONS

C1	First component of complement
C1q, C1r and C1s	Sub-components of C1
C1-INH	Inhibitor of activated C1
C2	Second component of complement
C2b and C2a	Fragments of C2
C3	Third component of complement
C3b	Largest fragment of C3
C3a	Anaphylatoxin fragment of C3
C3bi	Inactivated C3b
C3c, C3d and C3e	Fragments of C3b following trypsinisation
C3dg	Fragment of C3bi produced by control proteins
C4	Fourth component of complement
C4a and C4b	Fragments of C4
C5	Fifth component of complement
C5a	Anaphylatoxin fragment of C5
C5a des.arg	Desarginyl derivative of C5a
C5b	Largest fragment of C5
C6	Sixth component of complement
C7	Seventh component of complement
C8	Eighth component of complement
C9	Ninth component of complement
DAF	Decay accelerating factor
MCP	Membrane cofactor protein
CR1	Complement receptor type 1 (binds C3b and C4b)
CR2	Complement receptor type 2 (binds C3d, C3dg and C3bi)
CR3	Complement receptor type 3 (binds C3bi)

C4BP	C4 binding protein
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
MHC	Major histocompatibility complex
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
RNA	Ribonucleic acid
mRNA	messenger RNA
tRNA	transfer RNA
rRNA	ribosomal RNA
SCR	Short consensus repeat
RCA	Regulation of complement activation
cM	centiMorgans
kb	Kilobases
kDa	Kilodaltons
HSA	human serum albumin
PMN	polymorphonuclear
RA	rheumatoid arthritis
OA	osteoarthritis
AS	ankylosing spondylitis
CC	chondrocalcinosis
MOPS	sodium morpholino propanesulphonic acid
Tris	Tris hydroxymethyl-methylamine
w/v	weight per volume
v/v	volume per volume
C^{rat}	rat serum
FCS	foetal calf serum

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DEDICATION

I would like to dedicate this thesis to my grandparents, to whom it would have meant so much.

SUMMARY

The complement system, which comprises a group of at least 20 plasma proteins, plays a major role in host defence against bacterial infections and in immunologically-mediated inflammation. The pro-inflammatory biological activities generated as a result of complement activation include increased vascular permeability, chemotaxis of neutrophils and mononuclear phagocytes, enzyme secretion by phagocytic cells and cytolysis. Complement activation occurs within inflamed synovial joints of patients with rheumatoid arthritis and is thought to play a role in the pathogenesis of the condition.

Although plasma levels of most complement components are maintained by hepatic synthesis, the cell responsible being the hepatocyte, it is now well established that other cells including monocytes, macrophages, fibroblasts and epithelial cells of the gastrointestinal and genitourinary tracts synthesise complement components when cultured *in vitro*. Such cells are potential sources of extrahepatic synthesis of complement components *in vivo*. Synovial fluid macrophages and adherent synovial membrane cells have been shown to synthesise complement components when cultured *in vitro*, as have isolated fragments of synovial membrane from patients with either rheumatoid arthritis or osteoarthritis. However, the only evidence for extrahepatic synthesis occurring *in vivo* comes from metabolic studies with radiolabelled (^{125}I) C3, which indicated that up to 50% of C3 present in the rheumatoid joint was synthesised locally.

The principal aim of this study was to provide conclusive evidence that a large number of complement components including C4, C2, C3, C5, factor B and C1-inhibitor, were synthesised in synovial tissue *in vivo*. The availability of cDNA probes for these complement components allowed the

use of molecular biological technology, in addition to immunochemical techniques, to study complement biosynthesis by synovial tissue from patients with rheumatoid arthritis or osteoarthritis and by normal synovial tissue from a patient undergoing patellectomy.

Using Northern and dot-blot analyses, mRNAs coding for C1-inhibitor (2.1 kb), factor B (2.6 kb), C1q B-chain (1.4 kb), C2 (2.9 kb), C3 (5.2 kb) and C4 (5.5 kb) were detected in RNA isolated from both rheumatoid synovium and synovial membrane from osteoarthritis patients. Although the presence of C5 mRNA was also investigated, it was not detected in any sample. Dot-blot analysis showed the presence of mRNA coding for C1-inhibitor in RNA isolated from normal synovial membrane.

Quantitative analyses of the data using the students t-test did not show any statistically significant difference between the steady state levels of C3, C1-inhibitor or factor B mRNA in synovium from rheumatoid arthritis and osteoarthritis patients. Although the levels of C2 mRNA were significantly higher in rheumatoid synovium, and C4 mRNA levels were higher in osteoarthritis synovium, the P value was only marginally significant in both cases (<0.05).

When fragments of normal synovium or synovial membrane fragments from patients with rheumatoid arthritis or osteoarthritis, were cultured *in vitro*, synthesis of C1-inhibitor, C2, C3, C4 and factor B was detected by ELISA and C2, C3 and factor B were shown to be functionally active.

Following the demonstration of complement biosynthesis in synovial tissue *in vivo*, the next step would be to identify the cells within the synovium reponsible for complement biosynthesis. A number of cell-types that are present in synovial membrane have previously been shown to be capable of synthesising complement components *in vitro*. These include

mononuclear phagocytes and possibly the B-cells of the synovial membrane lining, fibroblasts and endothelial cells. However, the cells responsible for complement biosynthesis *in vivo* remain unidentified. This question may only be answered with the use of *in situ* hybridisation on frozen sections of synovial tissue.

As an initial step in this study, cells were isolated from synovial tissue of patients with rheumatoid arthritis and osteoarthritis and cultured *in vitro*. ELISA analyses of the culture supernatants showed synthesis of C4, C3, factor B and C1-inhibitor by both the adherent and non-adherent synovial cells. Immunohistochemistry showed that in both cell populations, fibroblasts were present in by far the greatest abundance (>95%), although a small proportion (<5%) of macrophages was identified.

This study thus provides conclusive evidence that synthesis of complement components occurs locally within normal and inflamed synovial tissue *in vivo*. The local synthesis of complement within normal synovial joints may be of importance in their defence against infection, whereas in inflamed joints it may contribute to the inflammatory response.

Further work is required to define the regulation of complement synthesis within the synovial joint as well as to identify the cells responsible for complement biosynthesis *in vivo*.

INTRODUCTION

THE COMPLEMENT SYSTEM

The principal role of the complement system is as an effector mechanism in host defence against infection by microorganisms. The activation products of the complement system cause lysis of cells, attract phagocytic cells to the site of activation and opsonise microorganisms which promotes their phagocytosis and killing by phagocytes.

The activation of complement is initiated by two pathways - the classical and alternative pathways - and is dependent on the sequential activation of zymogens to proteases, ie. the complement components circulate in an inactive form and as a result of their limited proteolysis, they acquire proteolytic activity, the substrate being the next protein in the activation sequence.

The classical pathway is activated mainly by the formation of antigen-antibody complexes, whereas activation of the alternative pathway is generally antibody independent ie. the alternative pathway can be activated by polysaccharides such as are present on bacterial cell walls.

A simplified view therefore is that the alternative pathway forms a first line of defence against infection prior to an immune response and the classical pathway becomes more important after antibody production has been stimulated. However, antibody-independent activation of the classical pathway has been described for bacterial lipid A (Cooper & Morrison, 1978), heart mitochondrial membranes (Giclas, Pinckhard & Olsen, 1979) and retrovirus membranes (Bartholomew & Esser, 1980). In addition, antibody-dependent activation of the alternative pathway has also been described and hence the true mechanism of how the

COMPONENT	MOLECULAR WEIGHT	SERUM CONC. (μg/ml)	POLYPEPTIDE CHAIN STRUCTURE
Classical pathway			
C1q	400,000	250	18 (6 x 3)
C1r	90,000	100	1
C1s	90,000	80	1
C4	204,000	430	3
C2	100,000	20	1
Alternative pathway			
Factor B	93,000	150	1
Factor D	25,000	2	1
Properdin	220,000	30	4
C3	190,000	1300	2
Terminal sequence			
C5	185,000	75	2
C6	128,000	60	1
C7	121,000	60	1
C8	153,000	80	3
C9	79,000	50	1

TABLE 1 - Complement proteins of the classical and alternative pathways and terminal sequence.

two pathways interact is likely to be more complex.

The human complement system is composed of at least 20 plasma proteins, 13 proteins of the classical and alternative pathways and at least 7 control proteins. From Fig.1, it can be seen that the end result of activation of either pathway is the generation of C3 convertases which activate C3. This is the key step in complement activation and initiates activation of the terminal sequence which is responsible for the cytolytic effects and other biological activities which are generated during activation.

The Classical Pathway

The classical pathway (Fig.2) consists of five proteins, C1q, C1r, C1s, C4 and C2, responsible for the assembly of the C3-cleaving enzyme (C4b2a) and three control proteins, C1-inhibitor (C1-INH), C4 binding protein (C4BP) and factor I (Whaley & Ferguson, 1981; Reid & Porter, 1981; Muller-Eberhard, 1988).

(a) C1 activation and its regulation

C1 is a macromolecule consisting of one molecule of C1q, two molecules of C1r and two molecules of C1s, held together by calcium ions (Ziccardi & Cooper, 1977). C1 is activated by interaction with antibody molecules of the IgM class or IgG1, IgG2 or IgG3 subclasses. Activation of C1 proceeds by three distinct steps: binding of C1q to antibody, activation of C1r to $C\bar{1}r$ and finally activation of C1s to $C\bar{1}s$.

C1q is the recognition unit of the C1 macromolecule and possesses a hexameric structure, with six globular heads being joined by collagen-like fibrillar stalks (Reid & Porter, 1975). Each of the heads is capable of binding to the CH2 domain of the IgG heavy chain or the CH3 domain of the IgM heavy chain. The affinity of C1q for monomeric

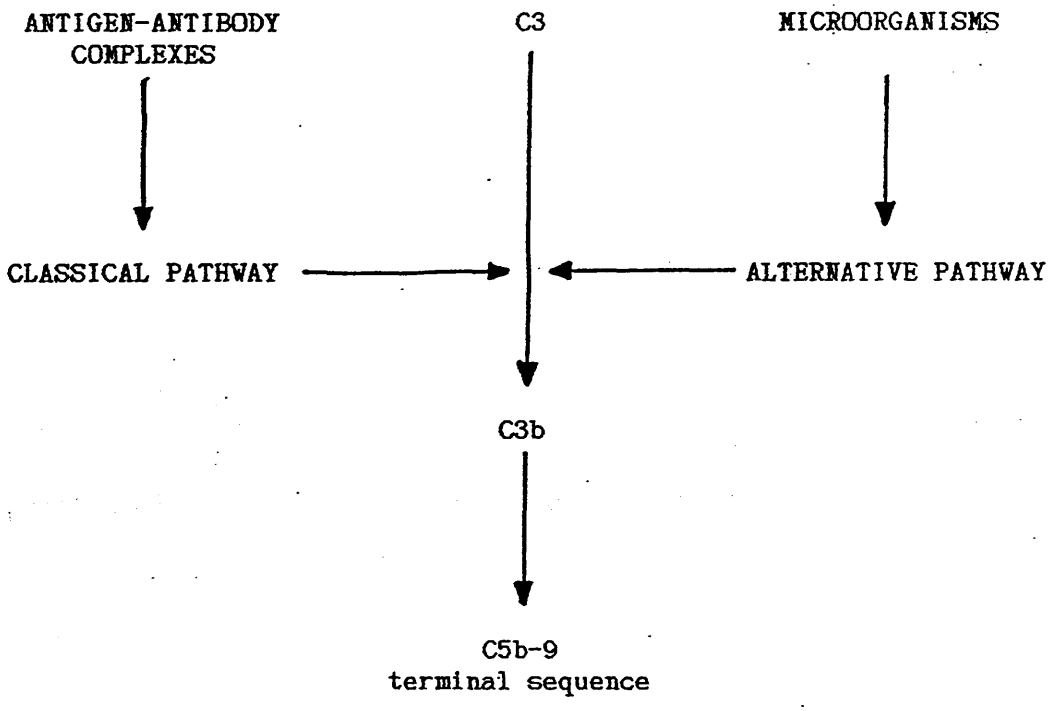


Figure 1 - *Activation of C3: the end result of the classical and alternative pathways.*

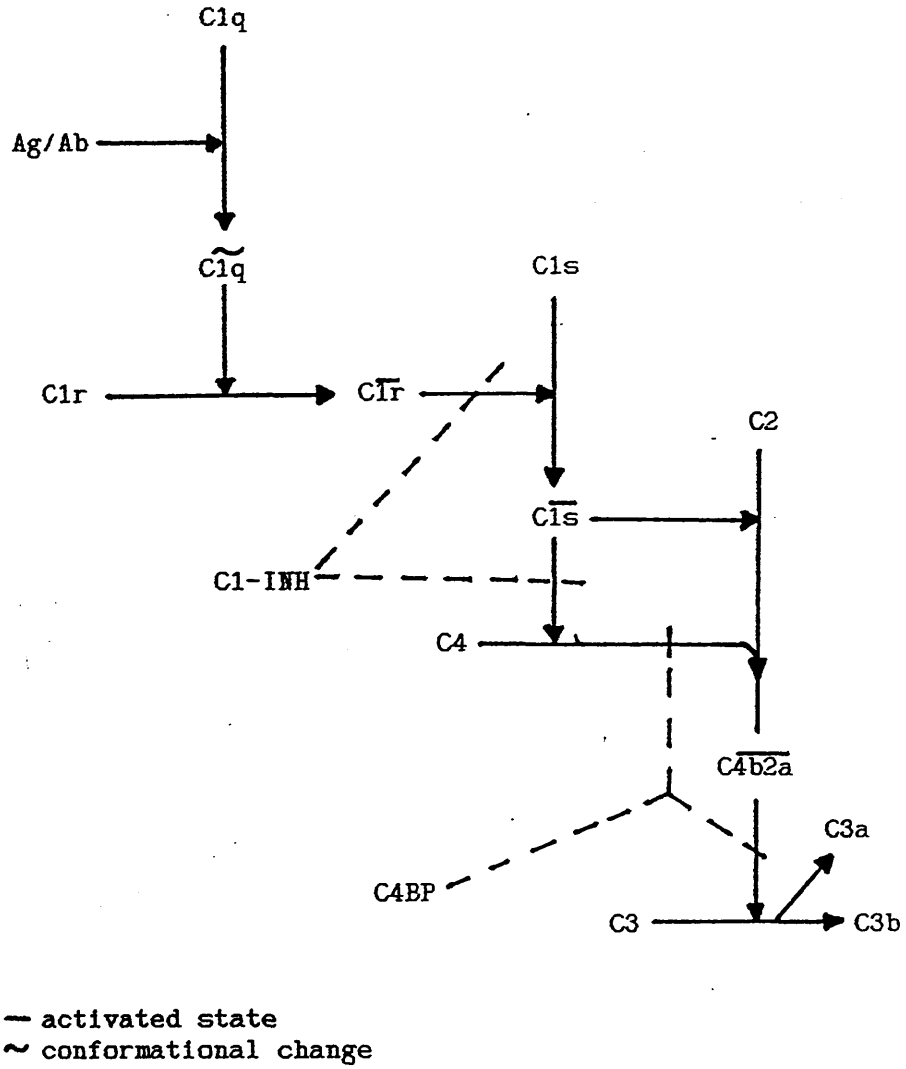


Figure 2 - *Classical pathway activation.*

IgG is weak but is enhanced when IgG has been aggregated in an immune complex (Ziccardi, 1984).

The $C1r_2$:$C1s_2$ tetramer binds to the collagen-like region of C1q (Strang et al., 1982). Both C1r and C1s have structural and functional similarities. They are single polypeptide chain zymogens which when activated by proteolytic cleavage, are converted to active enzymes consisting of disulphide-linked heavy and light chains. The enzymatic site of both is located on the light chains (Villiers, Arlaud & Colomb, 1985).

Upon binding with immunoglobulin, the heads of C1q undergo a conformational change (Hanson, Siegel & Schumaker, 1985) which is transmitted along the rigid collagen-like stalks to C1r. The shearing force presumably results in a realignment of the C1r molecule exposing a susceptible peptide bond to an enzymatic site at another part of the C1r molecule. Autocatalysis results in the generation of $\overline{C1r}$ (Ziccardi & Cooper, 1976) which has only one known natural substrate, C1s. It should be noted that activation of C1r can only occur in the presence of C1s. C1r produces a single proteolytic cleavage of C1s to convert it to $\overline{C1s}$ which carries the enzymatic site of C1 and thus cleaves C4 and C2 (Campbell, Dodds & Porter, 1980; Ziccardi, 1981).

Activated C1 has a half-life of only 13 seconds in the presence of C1-inhibitor (C1-INH) (Ziccardi, 1981). It has also been shown that in the absence of C1-INH, there is spontaneous activation of C1 even without any activator, (Ziccardi, 1982a,b). Two conformationally distinct forms of unactivated C1 have been hypothesised (Schumaker, Zavodsky & Poon, 1987): the "closed" complex which is incapable of autoactivation and the "open" complex in which catalytic sites of

unactivated C1r are exposed. Being in sevenfold molar excess over C1, C1-INH reversibly binds to C1 presumably when it is in the open complex state in which the C1r procatalytic sites are exposed. Two molecules of C1-INH bind to the two C1r sites, thus preventing autocatalytic activation (Muller-Eberhard, 1988). The active state of activated $\overline{C1}$ has a half life of only 13 sec because C1-INH rapidly enters into covalent interaction with $\overline{C1s}$, abrogating its enzyme activity. C1-INH also gains access to activated $\overline{C1r}$ which results in the release of two molecules of the fluid phase complex $C1rC1s(C1\text{-}INH)_2$ (Laurell et al., 1978; Ziccardi & Cooper, 1979).

(b) Formation of the classical pathway C3 convertase, C4b2a

C4 consists of three polypeptide chains - α, β and γ - held together by disulphide bonds. $\overline{C1}$ or $\overline{C1s}$ produce a limited proteolytic cleavage in the α-chain, separating a 6 kDa peptide, C4a from the N-terminus (Schreiber & Muller-Eberhard, 1974). The remainder of the C4 molecule, C4b possesses the ability to bind to antigen-antibody complexes. Failure to bind within 50msec results in loss of the labile binding site (by hydrolysis) and the molecule becomes fluid phase C4b. The ability of C4b to bind to surfaces is due to the presence of an internal thiolester group in C4 which upon cleavage by $\overline{C1s}$ is able to form amide or ester bonds with respective groups on the target surface. C4 occurs in two structurally and functionally distinct isotypes, C4A and C4B, each of which is encoded by a distinct gene (Carroll et al., 1984). C4A reacts preferentially with amino groups and C4B with hydroxyl groups.

C4b possesses a magnesium ion-dependent site for C2. C2 is cleaved by C1 or $\overline{C1s}$ to yield two fragments C2a (73 kDa) and C2b (34

kDa). Primarily because the molar concentration of C2 is about one twentieth of that of C4, only ≈4 C2 molecules are activated by one activated C1 molecule as opposed to ≈35 C4 molecules (Ziccardi, 1981). Although $\overline{C1s}$ can activate C2 in the absence of C4b, the reaction occurs more efficiently in the presence of the latter molecule (Gigli & Austen, 1969). C2 binds to C4b via the C2b fragment which remains bound to C2a by non-covalent bonds. C2a contains the enzymatic site of the C4b2a molecule (Nagasawa & Stroud, 1977).

C4b2a is an unstable enzyme, having a very short half-life at 37°C. C2 decays from the complex, which can be regenerated by fresh C2 in the presence of C1 or $\overline{C1s}$.

(c) Regulation of the classical pathway C3 convertase formation

The control of C4b activity is performed by the action of factor I and its cofactor C4BP (Fujita, Gigli & Nussenzweig, 1978; Gigli, Fujita & Nussenzweig, 1979). C4BP binds to C4b and renders it susceptible to cleavage by factor I. Two cleavages occur in the α'-chain while the β and γ subunits remain intact. Situated 15KDa from the N-term of the α'-chain of C4b is C4d (47 kDa), the remainder of the chain and the intact β and γ chains comprise C4c (Fujita et al., 1978). C4c is released into the fluid phase, the C4d fragment remains bound to the immune complexes which can no longer bind to C3b/C4b receptors (CR1) on erythrocytes or leukocytes.

C4BP will also displace C2a from C4b by binding to the latter protein. The rate of decay of this already labile enzyme is therefore accelerated (Gigli et al., 1979).

C4BP is found with two different molecular weights. The smaller is only found in free form, while the larger is found in two forms,

either free or bound in a complex with protein S (Dahlback & Stenflo, 1981; Dahlback, 1985) (the most important function of protein S is in the coagulation system (Comp et al., 1984).) C4BP retains its C4 binding property in complex with protein S, but the importance of protein S together with C4BP is unknown.

The Alternative Pathway

When polysaccharides e.g endotoxin, are added to C4 or C2 deficient serum, C3 consumption is still observed. Furthermore, when these polysaccharides are added to normal serum, C3 consumption occurs without significant utilisation of C1, C4 or C2. Therefore an alternative pathway for C3 cleavage and activation must exist (Reid & Porter, 1981; Whaley & Ferguson, 1981; Pangburn, 1983; Muller-Eberhard, 1988).

(a) Activation and amplification of the alternative pathway

The alternative pathway exists in its activated state at all times due to the spontaneous reaction of C3 with water. Native C3 possesses an internal thiolester that undergoes low-grade hydrolysis giving rise to a continuous supply of C3(H_2O) which possesses all the functional properties of C3b (Isenman et al., 1981; Pangburn, Schreiber & Muller-Eberhard, 1981; von Zabern, Nolte & Vogt, 1981; Muller-Eberhard, 1988). When as a result of formation of the initial enzyme, native C3 is cleaved and the fragment C3b deposited on the surface of particles, the alternative pathway is able to distinguish between self and non-self. Only on particles recognised as foreign will amplification of C3b deposition occur and membrane attack be initiated (Muller-Eberhard, 1988).

In the presence of magnesium ions, C3b will complex with factor

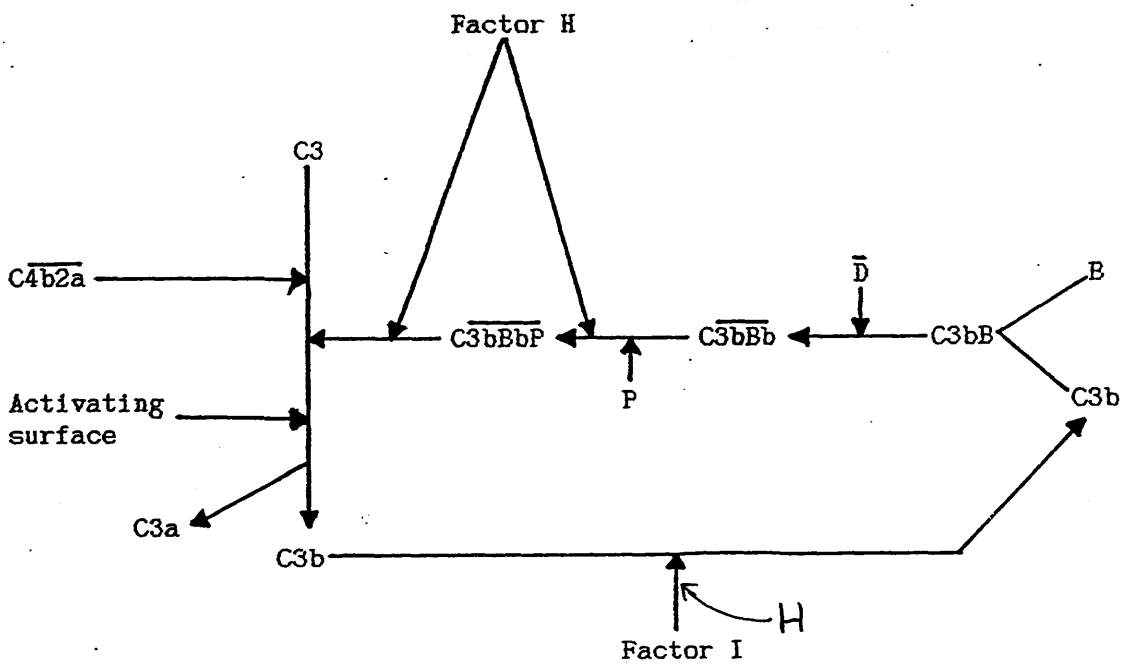


Figure 3 - *Alternative pathway activation.*

	CLASSICAL PATHWAY		ALTERNATIVE PATHWAY
C4b	1. Binds C2, Mg^{2+} dependent 2. Degraded by I + C4BP	C3b	1. Binds B, Mg^{2+} dependent 2. Degraded by I + H.
C2	1. Single polypeptide chain 2. Cleaved by C1s → C2a + C2b 3. Enzymatic site for C3 on larger fragment (C2a) 4. Decays from C42 5. Structural gene on chromosome 6	B	1. Single polypeptide chain 2. Cleaved by D → Ba + Bb 3. Enzymatic site for C3 on larger fragment (Bb) 4. Decays from C3bBb 5. Sructural gene on chromosome 6
$C\bar{1}\bar{s}$	1. Serine protease cleaves C4 and C2	$\bar{D}$	1. Serine protease cleaves factor B
I	1. Enzymatically inactivates C4b in the presence of C4BP	I	1. Enzymatically inactivates C3b in the presence of H
C4BP	1. Binds to C4b 2. Cofactor for I in C4b cleavage 3. Accelerates decay of C4b2a	H	1. Binds to C3b 2. Cofactor for I in C3b cleavage 3. Accelerates decay of C3bBb

TABLE 2 - Similarities between the classical and alternative pathways.

B which is itself cleaved by factor D resulting in an unstable C3bBb complex and a fluid phase Ba fragment. The complex is stabilised by the binding of properdin (P) to C3b forming the stable C3 convertase, C3bBbP (Fearon, Austen & Ruddy, 1973; Fearon & Austen, 1975).

(b) Regulation of the alternative pathway

Activation of the alternative pathway is controlled by factor I but in this pathway the cofactor is factor H (Whaley & Ruddy, 1976a,b). Factor H can bind to C3b alone or in convertases and competes for the binding site for factor B (Pangburn & Muller-Eberhard, 1978). It is a cofactor for factor I in the enzymatic inactivation of C3b (Pangburn, Schreiber & Muller-Eberhard, 1977) and accelerates the decay of the alternative pathway C3 convertase, C3bBb (Weiler et al., 1976).

Membrane-phase regulation of the classical and alternative pathways

Cofactors for factor I in the membrane-phase are CR1, which is found on many erythrocytes and leukocytes and binds C3b and C4b alone or in the convertases (Fearon, 1979; Iida & Nussenzweig, 1981; Medof et al., 1982; Medof & Nussenzweig, 1984) and membrane cofactor protein (MCP) which binds C3b and C4b alone or in the convertases (Cole et al., 1985; Holers et al., 1985; Yu et al., 1986). CR1 also increases the dissociation of both C3 convertases (Seya, Holers & Atkinson, 1985) while no decay accelerating activity has been found for MCP. It has been shown there is reciprocity between the amount of CR1 and MCP on particular cells (Holers et al., 1985) suggesting that these two proteins have similar functions. However, CR1 also serves as a receptor which in response to certain stimuli can increase its expression on neutrophil membranes (Lee, Hakim & Fearon, 1984; Yancey et al., 1985) while MCP has no such receptor function.

	MOLECULAR WEIGHT	SERUM CONC. (μg/ml)
C1-inhibitor	105,000	180
Anaphlatoxin inactivator	300,000	35
C4 binding protein	540,000	250
Factor I	90,000	50
Factor H	150,000	300
Properdin	220,000	30
S-protein	88,000	100-500
CR1	200,000	?
Decay accelerating factor	70,000	?
Membrane cofactor protein	60,000	?
Homologous restriction factor	80,000	?

TABLE 3 - The major regulatory proteins of the complement system.

Decay accelerating factor (DAF) is found on erythrocytes, platelets and all types of leukocytes (Kinoshita et al., 1985) and accelerates the decay of the C3 convertases by binding to the catalytic subunits Bb and C2b (Pangburn, 1986). DAF also binds to C3b and C4b alone and may therefore prevent the formation of convertases (Medof, Kinoshita & Nussenzweig, 1984). Hence CR1 and DAF work together to protect autologous cells against attack by complement.

C3 activation

Both C4b2a and C3bBbP cleave the α-chain of C3 to release a small peptide C3a (9 kDa) from the N-terminus. The remainder of the molecule, C3b comprises the rest of the α-chain and the β-chain. Cleavage of the α-chain of C3 by either of the C3 convertases exposes a labile binding site by which C3b binds to immune complexes or polysaccharides. The thiolester in the α-chain of C3 is exposed on activation and a covalent bond is formed between the thiolester and hydroxyl groups (ester bond) or amino groups (amide bond) (Tack et al., 1980; Law, Lichtenberg & Levine 1980). Failure to bind quickly to a suitable acceptor molecule results in the loss of binding activity because the thiolester reacts with water to form inactive fluid-phase C3b.

C3b, which is a product of both pathways, is a constituent of the alternative pathway C3 convertase and hence a positive feedback loop mechanism is in operation.

The Terminal Sequence

The terminal sequence is activated when C3 is cleaved by either of the two C3 convertases, C4b2a and C3bBbP. As described above, C3b

binds covalently to immune complexes and cell membranes. Some of the C3b will bind close to the C3 convertase enzyme thus changing the specificity of the enzyme to a C5 convertase (Cooper & Muller-Eberhard, 1970; Medicus, Gotze & Muller-Eberhard, 1976).

Following classical pathway activation, the change in substrate specificity of C4b2a is accomplished by activation of C5 (Vogt, Schmidt & von Buttlar, 1978). High-affinity C5-binding sites were demonstrated on C3b-C4b dimers in which C3b was linked covalently to the α-chain of C4b through an ester bond (Takata et al., 1987). The high-affinity binding may allow selective binding of C5 to the convertase in spite of surrounding monomeric C3b molecules generated by the C3 convertase.

The role of the second C3b molecule following alternative pathway activation is to bind C5 and modify it for cleavage by Bb (Isenman, Podack & Cooper, 1980; Vogt et al., 1978).

C5 has several structural and functional similarities to C3. It consists of a double polypeptide chain (185 kDa), the α-chain of which is cleaved by the C5 convertases releasing a small peptide, C5a (10 kDa) from the N-terminus, and a larger fragment, C5b which comprises the remainder of the α-chain and the β-chain.

The C5a peptide is a potent anaphylatoxin which is inactivated, along with C3a and C4a by anaphylatoxin inactivator (Bokisch & Muller-Eberhard, 1970). By splitting off the terminal arginine residue, C4a and C3a are completely inactivated, while C5a is partly inactivated (Huey et al., 1983; Marder et al., 1985).

The labile binding site of C5b has specificity for C6 and bimolecular C5b6 remains loosely bound to C3b on the target cell surface

until it reacts with C7. The trimolecular complex undergoes hydrophilic-amphiphilic transition (Preissner, Podack & Muller-Eberhard, 1985a), and anchors itself firmly in the lipid bilayer. It would appear that C5b6 converts C7 to an activated state in which it expresses hydrophobic binding sites that are concealed in the native molecule (Preissner et al., 1985a,b). This leads to C5b-7 membrane insertion which commits membrane attack complex (MAC) assembly to a discrete site. This step is controlled by S-protein which competes with membranes for binding to C5b-7 (Podack et al., 1978; Bhakdi & Tranum-Jensen, 1982), thus preventing its attachment to the cell surface.

Inserted C5b-7 constitutes an integral membrane protein that functions as a receptor for C8. C8 binds to C5b-7 with its β-chain which possesses a recognition site for C5b (Stewart & Sodetz, 1985). While C5b-7 interacted strongly with the ionic part of the bilayer and penetrated only slightly into the hydrophobic region, C5b-8 penetrated into the hydrophobic phase more deeply (Esser, Kolb & Podack, 1979).

Binding of C9 to the C5b-8 complex accelerates the cytolytic process. The C5b-9 complex consists of one molecule each of C5b, C6, C7, C8 and up to 18 molecules of C9. C5b-8 forms a small functional channel of ≈30A diameter (Mayer, 1982; Martin et al., 1987). As increasing numbers of C9 molecules are incorporated into the channel structure, its functional size increases to ≈100A (Martin et al., 1987). Hence, the hydrophobic exterior of the complex binds to the membrane lipid and the hydrophilic interior facilitates the passage of water and electrolytes. Once the loss of ion flux occurs the cell is doomed to osmotic lysis (Mayer, 1972).

Homologous restriction factor (HRF), which is found on the

membranes of erythrocytes, and peripheral blood leukocytes, acts to control the final assembly of the terminal complement complex to protect autologous cells against complement mediated lysis (Schonermark et al., 1986; Shin et al., 1986; Zalman, Wood & Muller-Eberhard, 1986). HRF binds to C8 and C9, inhibiting the trans-membrane channel formation.

Biological Activities of Complement

A major effect of the complement system is the cytolytic effect of the C5b-9 complex but there are many other biological activities generated during complement activation, particularly opsonisation.

(a) Increased vascular permeability

C4a, C3a and C5a are anaphylatoxins, C5a being the most potent and C4a the least. C3a and C5a act on mast cells and basophils to release histamine while C3a also causes platelets to release serotonin (Johnson, Hugli & Muller-Eberhard, 1975). C3a and C5a also induce smooth muscle contraction which presumably has a direct effect on the endothelial cells of the post-capillary venules, opening up inter-cellular gaps and increasing exudation of fluid.

As described above, the effects of C3a and C5a are inhibited by the action of anaphylatoxin inactivator which cleaves the C-terminal arginine residues from these potent peptides (Bokisch & Muller-Eberhard, 1970).

(b) Chemotaxis

C5a and C5a which has been degraded by anaphylatoxin inactivator ($C5a_{des\ Arg}$) are powerful chemotactic agents, attracting neutrophils, eosinophils and monocytes (Fernandez et al., 1978). C3bBb is also chemotactic for neutrophils (Ruddy, Austen & Goetzl, 1975).

ACTIVITY	PRODUCT
Pro-inflammatory	
1. Increased vascular permeability	C4a, C3a, C5a, C2-kinin
2. Chemotaxis	C5a, $C5a_{desarg}$, C5b67, C3bBb
3. Lysosomal enzyme secretion	C3b
Cytolitic	C5b-9
Bystander lysis	C5b-7
Opsonic	
1. Adherence to receptors	C4b, C3b, C3bi, C3d
2. Enhancement of phagocytosis	C3b
3. Increased intracellular killing of bacteria	C3b
4. Increased intracellular degradation of immune complexes	C3b
Solubilisation of complexes	C3b C4b
Antibody production	
1. T-dependent responses	C3
2. Generation of B memory cells	C3

TABLE 4 - Biological activities of complement.

(c) Leukocytosis

C3e, a peptide from the α-chain of C3, causes initial leukopenia, followed by leukocytosis. This is probably achieved by mobilising neutrophils from the bone marrow (McCall et al., 1974; Rother, 1972).

(d) Phagocytosis

Cells such as polymorphonuclear leukocytes, mononuclear phagocytes and B lymphocytes possess C3b receptors on their membranes, which can bind C3b-coated immune complexes or particulate material. C3b by itself promotes adherence to monocytes but not ingestion, whereas when IgG antibody also coats organisms ingestion is enhanced. It has been shown that activated macrophages are capable of binding and ingesting particles coated only with C3b while resting macrophages require IgG for ingestion to occur (Griffin, Bianco & Silverstein, 1975). C3b acting on macrophage C3b receptors enhances the rate of intracellular killing of bacteria (Leigh et al., 1979).

It should be noted there is more than one type of C3b receptor. Receptors for C3b/C4b (CR1) and C3bi (CR3) are found on the surface of neutrophils and monocytes. Receptors for C3bi and C3d (CR2) as well as CR1 are present on B-lymphocytes (Abrahamson & Fearon, 1983). CR1 alone is found on erythrocytes and CR1, CR2 and CR3 are all found on dendritic cells.

(e) Solubility of immune complexes

Complement-mediated prevention of immune precipitation (PIP) occurs when immune complexes are formed in the presence of complement (Schifferli, Bartolotti & Peters, 1981). The phenomenon depends upon an intact classical pathway (Schifferli, Woo & Peters, 1982; Naama et al.,

1984) with C3 convertase formation leading to C3 activation and the binding of C3b to immune complexes (Naama et al., 1984,1985). The alternative pathway plays a secondary role in PIP, helping to retain in solution, immune complexes which have already been rendered soluble by complement (Naama et al., 1985). In contrast, another complement-dependent function, the solubilisation of immune precipitates, displays an absolute dependency upon the alternative pathway (Czop & Nussenzweig, 1976) although optimal solubilisation also requires classical pathway activation (Takahashi et al., 1978).

(f) Lymphocyte function

C3b is required for the generation of B-memory cells (Klaus & Humphrey, 1977) and T-dependent antibody responses (Pepys, 1974). C3a and possibly other C3 derived peptides suppress antibody production (Ochs et al., 1983).

The numerous biological activities generated during complement activation emphasise the importance of the complement system as a defence mechanism against infection of the host by microorganisms and helps explain why deficiency of complement componenents can lead, in some cases, to serious disease states.

THE MOLECULAR BIOLOGY OF COMPLEMENT

The isolation of cDNA and genomic clones for nearly all the complement components has helped scientists gain a clearer understanding of the complement system at the molecular level. These powerful tools of molecular biology have been used in a wide range of applications:

(a) deriving the primary structure of complement proteins

(b) establishing the precise molecular defect in deficiency states

(c) detailed examination of polymorphic variants of a particular component and their possible relationship to disease states

(d) examination of tissue specific expression and hormonal control of expression

(e) *in vitro* expression studies by transfection of mammalian cells with cloned complement genes

(f) control of expression levels and biosynthesis

(g) establishing chromosomal localisation

(h) examination of the processing of precursor molecules

(i) provision of insight into the evolution of protein families

Studies with these clones have divided the complement proteins into six separate structurally and functionally related groups, many of which are coded on the same chromosomes and are closely linked: (1) the C1 complex and C1-INH, (2) the class III region of the major histocompatibility complex (MHC) ie. C2, factor B and C4, (3) C3, C4 and C5, (4) the regulators of complement activation ie. factor I, C4BP, factor H, CR1, CR2, DAF and MCP, (5) CR3 as a member of the cell adhesion glycoprotein family, and (6) the membrane attack complex, C5b-9. (This summary is based on the review by Campbell et al., 1988)

1. THE C1-COMPLEX AND C1-INHIBITOR

(a) C1q

Macrophages may be a major source of C1q (Tenner & Volkin, 1986; Rabs et al., 1986). Both the C1q B-chain and the A-chain have been assigned to chromosome 1p (Reid, 1985; Sellar et al., 1987) and the B-chain gene, which has been studied more extensively, is approximately 2.6 kb long (Reid, 1985). The B-chain gene contains a single intron of 1.1 kb located within the codon where the triple helical chains of C1q appear to bend. It is not yet known whether the A- and C-chain genes have an intron at the equivalent position.

(b) C1r and C1s

Amino acid sequences show 40% homology between these two proteins. In the activated form of both enzymes, the A- or heavy chain which does not possess the active site, contains two different pairs of internal repeating units. The first set has not been found on any other proteins and hence may account for the control of specificity of C1r and C1s. The second set of repeating units, found toward the C-terminal end of the A-chain, are units of approximately 60 amino acids and are homologous to the 60 amino acid repeats (short consensus repeat, SCR) found in the C3b/C4b binding proteins such as C4BP and factor H. The C1r and C1s genes are both found in the region p13 on human chromosome 12 and studies with pulse field gel electrophoresis (PFGE) have indicated the genes are tightly linked within a 50 kb stretch of DNA (Tosi et al., 1987).

(c) C1-inhibitor

The isolation and characterisation of cDNA clones encoding C1-INH together with sequence analysis of the protein (Bock et al., 1986)

show that C1-INH is a member of the serpin "superfamily" of serine protease inhibitors (Carrell, 1984; Davis et al., 1986). This "superfamily" includes α-1-antitrypsin, antithrombin III, α-1-anti-chymotrypsin, α-2-antiplasmin and heparin cofactor II.

The entire C1-INH gene is 17 kb long (which is larger than those encoding the other serpins) and gives rise to a 2.1 kb transcript. The gene contains eight exons and seven introns and has been mapped to subregion p11.2-q13 of human chromosome 11 (Bock et al., 1986; Carter & Fothergill, 1987).

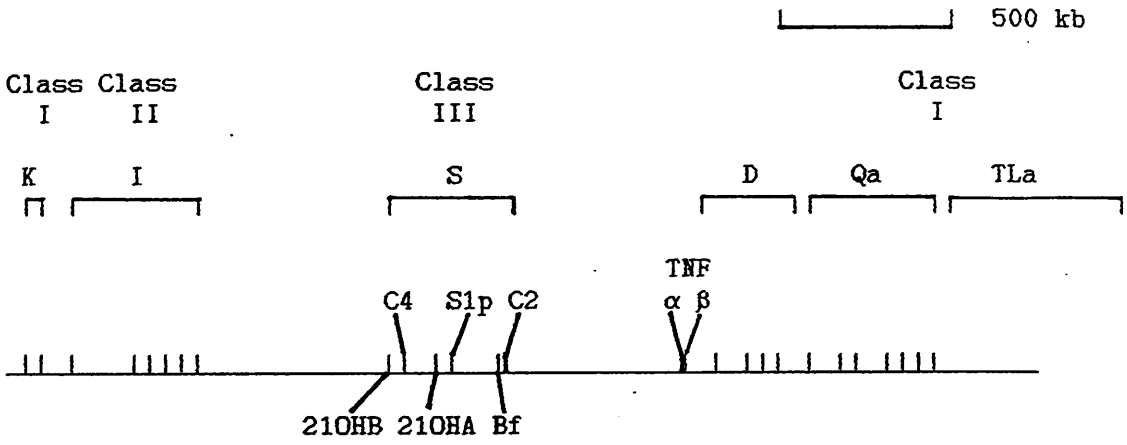
2. THE HLA CLASS III COMPLEMENT GENES

Using the powerful technique of PFGE, the orientation and position of the complement genes in both the human and mouse MHC class III region have been determined (Spies et al., 1986; Hardy et al., 1986) (see figure 4). These studies indicated that the MHC could span up to 3800 kb and that the class III region spans approximately 1100 kb on the short arm of human chromosome 6, and contains two C4 loci as well as single C2 and factor B loci. In the mouse these genes have been mapped to the S region of the H-2 complex on chromosome 17 (Chaplin, 1985) and also to the MHC of a number of other species (Alper, 1981).

In common with the MHC class I and class II genes, the class III region genes, particularly C4, are highly polymorphic, with more than 35 alleles described for the two C4 isotypes (Mauff et al., 1983).

The C2 and factor B genes are 421 bp apart (Wu, Morley & Campbell, 1987) and lie about 30 kb from the C4A locus, which is separated from the C4B locus by about 10 kb. Further mapping identified a gene encoding the cytochrome P450 21-hydroxylase (21-OHase), approximately 3 kb from the 3' end of each C4 gene (Carroll, Campbell &

MOUSE (Balb/c) H-2 COMPLEX



HUMAN HLA-COMPLEX

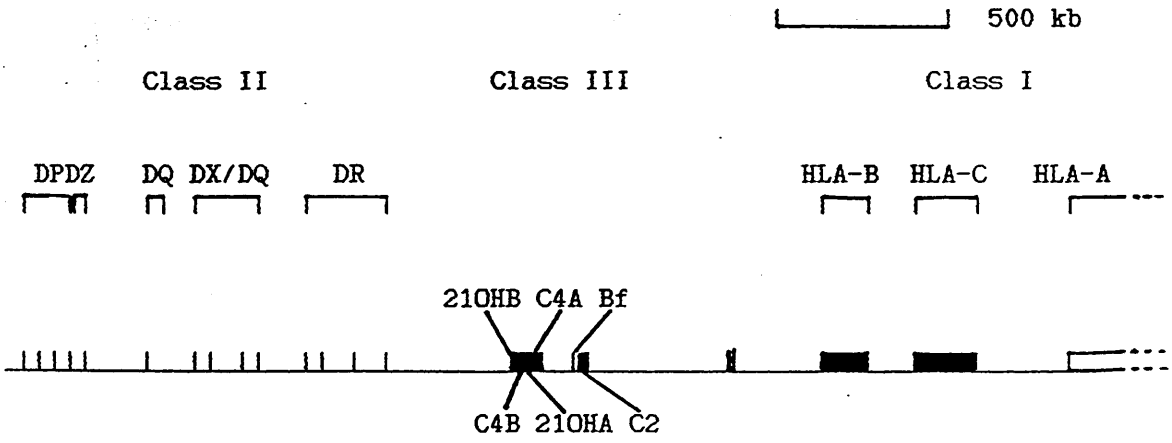


Figure 4 - *Molecular map of the HLA-region on human chromosome 6 and the H-2 complex on mouse chromosome 17.* (From Campbell et al, 1988)

Porter, 1985; White et al., 1985). The DRα and C4 genes were found to be separated by at least 300 kb. A similar organisation was established for the mouse where single C2 and factor B genes were found to be closely linked and lie about 50 kb from two C4-like genes separated by 80 kb (Chaplin, 1985). It was later established that the gene closest to factor B encoded S1p.

(a) C2

Characterisation of the gene encoding C2 has shown that it spans 18 kb of DNA (Campbell, 1987), but no exon-intron organisation has yet been reported. The C2 gene gives rise to a 2.9 kb transcript which directs the synthesis of C2 protein. C2, like several other complement and non-complement proteins, possesses three 60 amino acid internal repeats (SCRs), which are found at the N-terminal end of the protein.

(b) Factor B

The factor B gene is 6 kb long and is split into 18 exons (Campbell et al., 1985). Three of the exons at the 5' end encode the three SCRs within the Ba fragment that show significant homology to similar repeating units in a number of other proteins. The eight exons in the 3' one third of the gene encode the serine protease domain.

The homology in structure and function between factor B and C2 proteins and the close linkage of the genes suggests they arose from an ancestral gene by duplication. However, although the mRNA molecules encoding C2 and factor B are similar in size (2.9 kb and 2.6 kb), the C2 gene is three times longer than the factor B gene.

DNA sequencing, S1 mapping and primer extension experiments have established the transcription initiation site of the factor B gene lies only 421 bp from the poly A site of the C2 gene (Wu, Morley & Campbell,

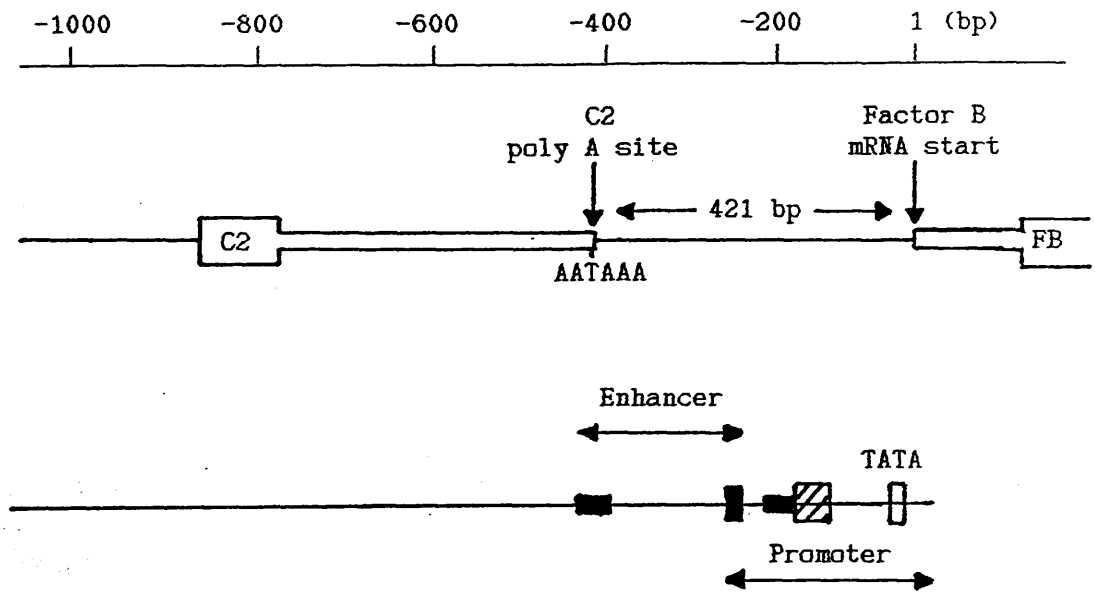


Figure 5 - *Close linkage of the human C2 and factor B genes on chromosome 6.* (From Campbell et al., 1988)

	DNA SEQUENCE
TATA Box consensus	G-GTATA$\overset{\blacktriangle}{T}A\overset{\blacktriangle}{T}$-G--G
	|||||||| | |
Factor B	TTGTATAAAAGGCTG
	-31 -17
α-Interferon consensus	TTC-$\overset{\blacktriangle}{C}$-ACCTC-GCAGTTTCTC$\overset{\blacktriangle}{T}$TCTCT
	| || || |||||||| || ||
Factor B	GGTGGGACTTCTGCAGTTTCTGTTTCCT
	-154 -127
Enhancer core consensus	GTGG$\overset{\blacktriangle}{T}\overset{\blacktriangle}{T}\overset{\blacktriangle}{T}$G
	||||||||
Factor B	GTGGTTTG
	-234 -227
MCMV enhancer 18bp consensus	TCAATAGGGACTTTCCA$\overset{\blacktriangle}{T}$
	| | | ||| |||| |
Factor B	TTCACATGGAATTTCCCA
	-472 -455
HCMV enhancer 19bp consensus	CACCATTGACGTCAATGGG
	||| ||| | ||| |||
Factor B	GACCTTTGGCAGCAAAGGG
	-171 -153

TABLE 5 - Homologies found in the factor B 5'-flanking region with known regulatory sequence. (from Wu et al., 1987a).

1987).

The presence of *cis*-acting DNA elements, essential for the cell specific expression of the factor B gene has been suggested by deletion analysis of the factor B 5'-flanking region (Wu, Morley & Campbell, 1987). A transcriptional enhancer was defined between nucleotides -496 to -201, and a promoter was found to extend up to 260 bp from the factor B CAP site.

The factor B enhancer is found at the 5' end of the gene, but is also at the 3' end of the C2 gene. It was thought it may also act as a C2 enhancer although it may not be able to work over a distance of 18 kb ie. the distance to the C2 promoter. However recent studies have demonstrated the presence of a transcriptional enhancer in the 5'-flanking region of the C2 gene (Wu & Campbell, unpublished).

(c) C4

In humans there are two C4 genes, C4A and C4B, which lie about 10 kb apart. C4 genes can differ in size (Yu et al., 1986; Schneider et al., 1986; Palsdottir et al., 1987) so that C4B genes can be either 22 kb or 16 kb depending on the presence of a 6-7 kb intron situated about 2.5 kb from the 5' end of the gene (Yu et al., 1986). An unusual phenomenon is the high frequency of null alleles at either locus (Schendel et al., 1984; Partanen & Koskimies, 1986). These null alleles may be clinically important as they occur at high frequency in patients suffering from some HLA-related diseases such as SLE (Fielder et al., 1983).

Both human C4 genes give rise to 5.5 kb transcripts and comparison of cDNA sequences has revealed less than 1% nucleotide variation between C4A and C4B (Belt, Carroll & Porter, 1984).

Several *cis*-acting DNA elements have been defined within 1 kb of the transcriptional start point of C4 (Wu et al., 1987). The C4 gene contains a non-TATA box promoter within nucleotides -147 to +13 as well as a transcriptional enhancer. In the 5'-flanking region there appears to be quite a complex arrangement of regulatory elements with a positive regulator within less than 200 bp which includes also the promoter and the CAP site, and a distal positive regulator separated by a negative regulator.

In mouse the two C4-like genes, C4 and S1p, are both 16 kb in size (Chaplin, 1985) but they are not as conserved as the two human C4 genes and share only 94% homology (Ogata & Sepich, 1985; Nonaka et al., 1986). However, S1p is not activated by C1s and extensive studies have led to the suggestion that S1p has no functional role (Sepich, Rosa & Ogata, 1987).

3. C3, C4 and C5

Complete cDNA and derived amino acid sequences are available for human C3 (de Bruijn & Fey, 1985) and C4 (Belt et al., 1984,1985) and mouse C3 (Lundwall et al., 1984; Wetsel et al., 1984), C4 (Nonaka et al., 1985; Sepich, Noonan & Ogata, 1985) and C5 (Wetsel, Ogata & Tack, 1987). Partial cDNA sequences are also available for human C5 (Lundwall et al., 1985; Wetsel et al., 1988) and rabbit C3 (Kusano et al., 1986). Striking homology has been shown between these proteins. C3, C4 and C5 within each species show 25% identity while the same protein from different species shows 75-80% identity.

The human C3 gene was mapped to chromosome 19 (Whitehead et al., 1982) while although the mouse C3 gene was mapped to chromosome 17, it is about 12cM from the H-2 region (da Silva et al., 1978; Natsuume-

Sakai, Hayakawa & Takahashi, 1978) and was estimated to be 24 kb long (Wiebauer et al., 1982). The human C5 gene was located on chromosome 9 (Wetsel et al., 1988) while the mouse C5 gene was mapped to chromosome 2 (D'Eustachio et al., 1986).

4. FACTOR I AND THE C3b/C4b BINDING PROTEINS

(a) Factor I

The factor I gene has been mapped to human chromosome 4 (Goldberger et al., 1987) and gives rise to a 2.4 kb transcript in liver (Catterall et al., 1987). The heavy chain of factor I contains several recognisable homology units but agreement with the consensus sequence of SCRs is poor. So far, factor I polymorphisms have only been detected in the Japanese population but in no other racial group (Nishimukai & Tamaki, 1986).

Primary structure studies of the C3b/C4b binding proteins, factor H, C4BP, CR1 and MCP, all of which act as cofactors for factor I mediated degradation of C3b or C4b, have shown that factor H, C4BP and CR1 are highly homologous. They all contain the 60 amino acid repeat units referred to as SCRs (Klickstein et al., 1987a). Two other C3b/C4b binding proteins, DAF and CR2 contain these SCRs but lack cofactor activity. The genes for CR1, DAF, factor H, C4BP and MCP have been shown to be closely linked while studies with cDNA probes have mapped the CR1, MCP, C4BP, DAF, CR2 and factor H genes to chromosome 1 (Holers et al., 1985; Reid et al., 1986; Kristensen et al., 1987a; Rey-Campos, Rubinstein & Rodriguez de Cordoba, 1987; Rodriguez de Cordoba & Rubinstein, 1986,1987). Using PFGE, a single 950 kb human genomic DNA fragment has been identified which hybridises to cDNA probes for MCP, CR1, CR2, C4BP and DAF but not factor H (Rey-Campos, Rubinstein &

Rodriguez de Cordoba, 1988; Carroll et al., 1988; Bora et al., 1989). This fragment can be split into two pieces, one of 500 kb, containing DAF and C4BP genes and the other 450 kb which contains CR1, CR2 and MCP. These studies have resulted in the identification of a major gene cluster known as the regulation of complement activation (RCA) cluster. The order of the genes in this RCA cluster is MCP-CR1-CR2-DAF-C4BP (Rey-Campos et al., 1988; Carroll et al., 1988; Bora et al., 1989).

(b) Factor H

Factor H is the most abundant cofactor protein and the human and mouse factor H show 61% sequence identity. This identity is made up entirely of SCRs of which there are 20 in each protein (Ripoche et al., 1988a; Kristensen & Tack, 1986) with individual SCRs showing 25-45% identity with each other. The C3b-binding site is likely to be found within SCR 4 or 5 (Alsenz et al., 1985).

Initial studies have suggested the factor H gene may be as large as 90 kb, with another gene or pseudogene also present (McAleer et al., 1987). Murine studies have revealed a 90 kb factor H gene with at least two H-related genes or pseudogenes of 60 and 120 kb (Vik et al., 1987).

Three factor H related mRNA transcripts have been identified with Northern analysis of human liver poly A^+ mRNA (Ripoche et al., 1988a; Schwaeble et al., 1987). The longest transcript of 4.4 kb codes for the whole 155 kDa protein, while the 1.8 kb transcript codes for the 43 kDa shorter form of factor H seen in plasma. This short-form factor H corresponds to the N-terminal region of the whole protein, and only hybridises to probes from the 5' end of the factor H cDNA probe. The function of this truncated form of factor H is as yet unknown, although it does possess the C3b-binding site. The 43 kDa protein translated from

the 1.8 kb transcript is distinct in size from the normal proteolytic cleavage products of factor H. This has led to the suggestion that the two factor H transcripts arise from separate genes rather than splicing of the 4.4 kb transcript (Schwaeble et al., 1987).

A third factor H transcript of 1.0 kb has also been detected and corresponds to the C-terminal portion of the cofactor (Schwaeble et al., 1987; Ripoche et al., 1988a).

(c) C4BP

The human C4BP gene is about 30 kb long, while the mouse gene is 20-25 kb long (Barnum et al., 1987; Lintin & Reid, 1986). Unlike factor H, there is no evidence of C4BP-related genes or pseudogenes.

There is only one mRNA species of 2.5 kb detectable in human liver (Chung, Bentley & Reid, 1985) while the mouse C4BP liver transcript is 1.8 kb long (Kristensen et al., 1987b).

The N-terminal 491 residues of the seven human C4BP chains are in the form of eight SCRs while the C-terminal 57 residues do not follow this pattern (Chung et al., 1985). Structure-function studies suggest that the C4b-binding site is likely to be in the fourth SCR (Kristensen et al., 1987a,b). Comparison of the human and mouse C4BP amino acid sequence suggest SCRs 5 and 6 of the human C4BP are missing from the mouse protein (Kristensen et al., 1987b), although the human and mouse sequences show 52% identity when the appropriate regions are aligned.

(d) CR1 and CR2

CR1 exists as four allotypes, the most common being CR1-A (Klickstein et al., 1987a). The sequence of CR1 is much more repetitive than factor H and C4BP and contains at least 33 SCRs, most arranged into four groups of seven (Klickstein et al., 1987a). These 450 amino acid

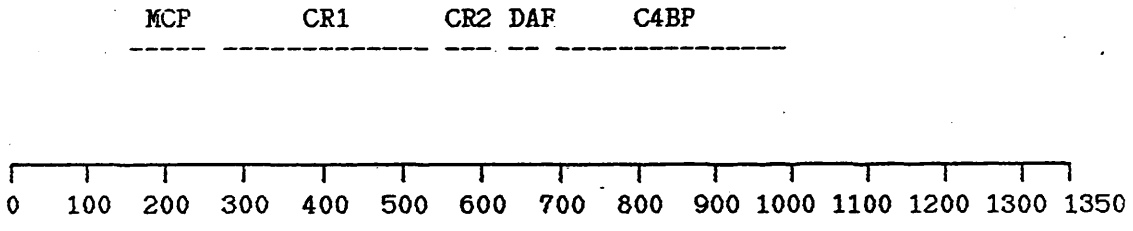


Figure 6 - *Arrangement of the genes in the RCA cluster on human chromosome 1.* (From Campbell et al., 1988)

segments are termed long homologous repeats (LHR) which have a very high degree of homology ie. as high as 99% identity (Klickstein et al., 1987a). This high level of homology shows good evidence for duplication and conversion events in the evolution of CR1.

cDNA sequencing has revealed a sequence of 5.5 kb, containing an open reading frame of 4.7 kb (Klickstein et al., 1987a). Northern blotting has suggested that each allele has two transcripts of unequal amount. In the case of CR1-A, the major transcript is 8.6 kb while for B is 11.6 kb, for C is 7.3 kb and for D is 12.8 kb (Hourcade et al., 1987). As for factor H smaller transcripts corresponding to different regions of the protein have also been detected (Holers et al., 1987; Klickstein et al., 1987b).

CR1 cDNA probes hybridise to CR2 cDNA at low stringency thus revealing a close sequence similarity. The CR2 mRNA has been shown to be 5 kb long (Weis et al., 1986) while cDNA sequencing has indicated CR2 made up of 15 SCRs. Human CR1 cDNA probes also cross-hybridise to some mouse DNA sequences which most likely correspond to mouse CR1 and CR2 (Weis et al., 1987).

(e) DAF and MCP

The human DAF gene is 35 kb long (Stafford et al., 1987) and several mRNA transcripts have been reported (Caras et al., 1987; Medof et al., 1987). The shorter transcript, which is the major form found in HeLa cells, lacks the 118 bp intron seen in the longer, minor transcript. The shorter mRNA is thought to be responsible for producing the mature membrane bound DAF while the longer transcript is translated into the soluble form of DAF.

MCP, the C3b/C4b binding protein most recently mapped to the RCA

gene cluster on chromosome 1 (Bora et al., 1989), produces a transcript 1.5 kb long (Lublin et al., 1988).

5. CR3

CR3 is composed of two non-covalently linked subunits, α and β, with this same β-subunit also present on two other cell surface adhesion antigens, p150,95 and LFA-1 (Sanchez-Madrid et al., 1983). The gene encoding this β-subunit has been mapped to chromosome 21 (Marlin et al., 1986) and is estimated to be about 32 kb long (Kishimoto et al., 1987). A species of mRNA has been detected and is about 3.2 kb long.

An interesting finding was that patients deficient in CR3 are also deficient in p150,95 and LFA-1, thus suggesting that it is their β-subunit that is defective (Springer et al., 1987).

6. THE TERMINAL PATHWAY COMPONENTS

Components of the membrane attack complex, C6, C7, C8 and C9 are structurally, antigenically and functionally related. For example, C6 and C7 are both single chain glycoproteins of about 115 kDa and have been shown to be closely linked.

C7, C8α, C8β and C9 have been cloned and show 21-26% identity, while studies on the human C9 gene indicate a coding region of 12 exons covering at least 80 kb of DNA (Muller-Eberhard, 1986; DiScipio et al., 1987; Howard, Rao & Sodetz, 1987; Marazziti et al., 1987). Studies with cDNA probes for the three C8 chains have localised them all to chromosome 1 (Rogde et al., 1986).

Homology with a number of other membrane-disrupting proteins such as perforin (Young, Cohn & Podack, 1986), has led to the suggestion that the terminal complement components are members of a large family of membrane attack proteins.

COMPLEMENT BIOSYNTHESIS

Although the complement system was first recognised more than one hundred years ago, the sites of complement biosynthesis remained unknown for a further eighty years. Various cell types have been shown to be capable of synthesising functionally active complement components *in vitro* but indications are that the full list of sites of complement biosynthesis is yet to be completed.

The study of complement biosynthesis by tissues *in vivo* has been revolutionised by the use of molecular hybridisation techniques to detect mRNAs coding for complement components. However, evidence using these powerful techniques is thus far extremely limited and nearly all complement biosynthesis work has been performed using *in vitro* culture systems.

The study of complement biosynthesis was greatly enhanced with the development of a number of well defined sensitive assays (Rapp & Borsos, 1970). However, failure to detect components using these assays does not necessarily mean that the cells are not producing them. Instead the level of the component in the culture medium may be less than the lower limit of sensitivity of the assay. Secondly, the component may not have been converted from its precursor state to its active form and hence not detected by the assay system. Finally, the component may have been degraded in the culture medium thus avoiding detection.

Detection of complement component biosynthesis may be first approached using an enzyme linked immunosorbent assay (ELISA) or radio-immunoassay. Although these two assays are very sensitive neither give any indication of functional activity of the component.

To detect functionally active complement components, the use of

the haemolytic assay system is essential, where the ability of the complement component to lyse antibody coated sheep erythrocyte cells is measured. The haemolytic plaque assay may also be used, which allows single cell synthesis to be studied.

The incorporation of radioactively labelled amino acids into immunoprecipitable protein is another important technique used for confirming synthesis of complement components with ^{35}S-methionine being the most commonly used label. Analysis of the immunoprecipitable protein by SDS-PAGE and fluorography confirms the subunit structure of the protein and enables the precursor form to be identified. Inhibition of synthesis by cycloheximide is a further requirement to prove complement biosynthesis by cultured tissue or cells.

As stated above, the isolation of cDNA clones for nearly all the complement components and the use of molecular hybridisation techniques have revolutionised the study of sites of complement biosynthesis *in vivo*. Northern and dot-blot analyses of RNA isolated from tissue that had been snap-frozen immediately after removal from the patient, have been used to detect mRNAs coding for complement components. These techniques have emerged as the most powerful tools for determining the sites of complement biosynthesis *in vivo*.

However, thus far much of the evidence for locating the sites of complement biosynthesis has been derived from *in vitro* culture techniques and the other methods outlined above. Combinations of these techniques have demonstrated the following tissues and cell types to be capable of synthesising complement components.

(a) LIVER

The major site of synthesis of complement proteins found within

blood is the liver or more specifically the hepatocyte. This conclusion is based on the observation made in patients undergoing orthotopic liver transplantation where their C3, C6, B and C8 allotypes quickly changed to that of the donor following the operation (Alper et al., 1969; Alper & Nathan, 1981). Other work has also shown that human fetal livers are capable of synthesising *in vitro*, biologically active C2, C4, C3 and C1-INH (Colten, 1972).

(b) Hep G2 CELLS

Further work with the human hepatoma cell line Hep G2 emphasised the liver as being the major site of complement synthesis. The Hep G2 cell line was shown to synthesise and secrete functional C1r, C1s, C2, C3, C4, C5, factor B, C1-INH, factor H, C6 and C8 but failed to produce C1q, C7 or C9 (Morris et al., 1982a,b). Work with this cell line, which was derived from a human hepatocellular carcinoma, also showed that human C5, like C3 and C4, is synthesised as a single chain precursor that is converted by limited proteolysis to the native two chain molecule and established the precursor-product relationship for human pro-C4 and native C4 and pro-C5 and native C5 (Morris et al., 1982b).

(c) MONONUCLEAR PHAGOCYTES

Biosynthesis of complement components by mononuclear phagocytes was first reported by Stecher and coworkers (1967). Human studies have revealed that human mononuclear phagocytes synthesise the following complement components: C1 and its subcomponents (Stecher, Morse & Thorbecke, 1967; Muller, Hanauske-Abel & Loos, 1978), C2 (Einstein, Schneeberger & Colten, 1976; Littman & Ruddy, 1977; Ackerman et al., 1978; Whaley, 1980; De Ceulaer, Papazoglou & Whaley, 1980; Cole et al.,

1982; Lappin et al., 1986), C3 (Stecher et al., 1967; Cole et al., 1982,1983; Strunk, Kunke & Ciclas, 1983), factor B (De Ceulaer et al., 1980; Whaley, 1980; Cole et al., 1982,1983), factor D, properdin, factor I, factor H (De Ceulaer et al., 1980; Whaley, 1980) and C1-INH (Yeung-Laiwah et al., 1985).

Although it has been suggested that synthesis of C4 (De Ceulaer et al., 1980; Whaley, 1980; Hetland et al., 1987) and the terminal complement components (C5-C9) occurs (Hetland, Johnson & Aaseb, 1986; Petterson, Johnson & Hetland, 1987), these reports have not been substantiated using the more conventional techniques listed above.

A number of complement components exist intracellularly as precursor molecules, namely, C4 and C3 in guinea pig peritoneal macrophages (Brade & Kreuzpainter, 1982; Colten, 1982) and C4 and C5 in mouse peritoneal and splenic macrophages (Colten, 1982), while pro-C3 has been identified in human monocytes.

Processing of complement precursor molecules occurs intracellularly (Fey & Colten, 1981) and several studies have examined the mechanism involved in the processing of these molecules. Pulse-chase experiments have shown that the precursor molecule appears intracellularly before the native form appears in the supernatant. Furthermore, the disappearance of the intracellular precursor coincides with the appearance of the secreted form (Parker, Roos & Schreffler, 1979). Finally, the conversion of pro-C4 to C4 by plasmin (Goldberger & Colten, 1980) confirms the precursor-product relationship. The process would appear to involve limited proteolysis since the precursor proteins are all single chain molecules whereas the native forms are composed of three chains in the case of C4 and two chains each for C3 and C5.

Most of the complement components are glycoproteins but the importance of the carbohydrate moieties is not fully understood. Secretion of C4, C2 and factor B by guinea pig macrophages is greatly inhibited by the addition of tunicamycin, an inhibitor of dolichyl-phosphate-dependent glycosylation (Matthews et al., 1982) and there is an increased intracellular catabolism of under-glycosylated pro-C4. However the C4 secreted by these tunicamycin treated guinea pig macrophages has virtually the same functional activity as normal C4. Hence the carbohydrate moieties may be important in restricting intracellular proteolytic cleavage of the precursors and also in ensuring that the appropriate tertiary structure formation is achieved to facilitate secretion.

It has been shown that changes in the profile of complement component biosynthesis by mononuclear phagocytes occurs as they undergo maturation from monocytes to macrophages. Freshly isolated peripheral blood monocytes only start to produce C2 and factor B after 3 days in culture after which synthesis increases with time (Einstein et al., 1976; Whaley, 1980). In contrast, macrophages do not show this lag phase but produce complement proteins immediately (De Ceulaer et al., 1980; Cole et al., 1982). Furthermore, the ratio of C2 to factor B secretion in breast milk, bronchoalveolar and synovial fluid macrophages differs from the relative secretion rates of these components in monocytes (De Ceulaer et al., 1980; Cole et al., 1982) thus demonstrating that mononuclear phagocytes are able to alter the synthesis rate of individual components selectively as well as acting as a marker of monocyte-macrophage maturation. It has also been shown that the proportion of macrophages synthesising complement depends on the tissue

of origin. Using a haemolytic plaque assay, only 2% of guinea pig bronchoalveolar macrophages synthesised C2 compared with 45% of peritoneal and splenic macrophages from the same animals (Alpert et al., 1983). Similar variations in human macrophage populations have also been reported (Cole et al., 1983).

Many studies have shown that complement protein secretion increases as the macrophage becomes more activated (Cole et al., 1980; Zimmer et al., 1982) but studies using murine peritoneal macrophages have produced conflicting results, in that C4 production by the activated cells fell despite an increase in total protein production and increased factor B secretion (Newell & Atkinson, 1983). However this may be due to inhibition of C4 synthesis by secreted C4 (Matthews et al., 1979).

It is now well established that functions of mononuclear phagocytes are modulated by various factors in their environment. For example there is enhanced complement production *in vitro* by macrophages from sites of inflammation such as active rheumatoid arthritis joints (De Ceulaer et al., 1980). Phagocytosis of bacteria or bacterial products such as lipopolysaccharide, stimulates complement production by mouse peritoneal macrophages although the ingestion of zymosan by human monocytes has the opposite effect (Morrison & Whaley, 1983). T-lymphocyte derived lymphokines stimulate C2 production by monocytes (Littman & Ruddy, 1977) and the binding of immune complexes and polymerised IgG_1 and IgG_3 to monocytes has been shown to increase complement production (McPhaden, Lappin & Whaley, 1981). This effect was shown to be Fc-receptor mediated as $F(ab)_2$ fragments had no effect.

While the liver is responsible for the maintenance of plasma

levels of most complement components, it is possible that mononuclear phagocytes play a role in the local production of complement in peripheral tissues. In other words, as a result of synthesis of complement components mononuclear phagocytes may play a key role in mounting the initial response to many infectious agents and hence act as an important mobile source of complement.

(d) SYNOVIAL MEMBRANE AND SYNOVIAL FLUID

Human synovial tissue from rheumatoid arthritis patients has been shown to be able to synthesise C2, C3, C4 and C5 when cultured *in vitro* (Ruddy & Colten, 1974). This study also provided the only evidence thus far for *in vivo* synthesis of complement by synovial tissue. Radiolabelled (^{125}-I) C3 was injected intravenously into a man suffering from rheumatoid arthritis. The specific activity (cpm/μg C3) of the radiolabelled C3 was then followed in both the plasma and synovial fluid. The results showed the specific activity of C3 in the synovial fluid never reached that of the plasma, and suggested that 50% of the C3 found within the inflamed joint was synthesised locally. *In vitro* studies showed cultured fragments of synovial tissue synthesised functionally active C2, C3, C4 and C5 and also showed incorporation of ^{14}C-labelled amino acids into C3, C4 and factor B but not C5. These studies also demonstrated that synthesis of C2, C3, C4 and C5 was frequently observed in rheumatoid synovium but only rarely in degenerative or traumatic joint disease.

De Ceulaer et al (1980) demonstrated increased biosynthesis of complement components by cultured monocytes, synovial fluid macrophages and synovial membrane macrophages from patients with rheumatoid arthritis and degenerative joint disease. Synthesis of functionally

active C2, factor B, factor D, properdin, factor I and factor H was observed in all three cell types while ^{14}C-labelled amino acids were incorporated into immunoprecipitable, but functionally inactive C3, C4 and C5. This final observation is particularly interesting because Ruddy and Colten (1974) showed synovial tissue fragments synthesised functionally active C3, C4 and C5, thus suggesting that other cells in the synovial membrane synthesise these components or that other cells in the cultured tissue are synthesising an enzyme (or enzymes) capable of activating precursor complement proteins.

Recently, fibroblast-like cells were isolated from normal synovial membrane and cultured *in vitro*. After several passages, these cells synthesised C1r, C1s, C1-INH, C2, C3, factor B and factor H (Katz & Strunk, 1988b). These data suggest that cells in normal synovial tissue may also synthesise complement components.

Thus, *in vitro* work has led to the suggestion that synovial tissue in arthritic, and possibly normal individuals may be capable of synthesising a number of complement components. However the *in vivo* evidence is very limited but could be substantially expanded by using the powerful techniques of molecular biology. The isolation of RNA from synovial tissue that has been snap-frozen in liquid nitrogen immediately after removal from the joint would allow the detection of mRNAs coding for various complement components by Northern and dot-blot analyses. Detection of these mRNAs would be strong evidence for *in vivo* synthesis of complement components by the synovial tissue. Such evidence could be reinforced by the *in vitro* synthesis of these components by cultured fragments of synovial membrane from the same patient.

SITE	COMPLEMENT COMPONENTS
LIVER	Maintains serum levels of the vast majority of complement components.
MONONUCLEAR PHAGOCYTES	Mobile source of most of the complement components.
EPITHELIAL CELLS OF THE GASTRO-INTESTINAL AND GENITOURINARY TRACTS	Main site of C1 synthesis.

Table 6 - Major sites of complement biosynthesis.

(e) EPITHELIAL AND ENDOTHELIAL CELLS

The evidence now is that the main site of human C1 synthesis *in vivo* is the gastrointestinal and genitourinary epithelial cell (Colten, 1976). *In vitro* studies with human umbilical vein endothelial cells have shown these cells to be capable of synthesising and secreting C3 protein as well as expressing a C3 transcript of the correct size, 5.3Kb (Warren, Pantazis & Davies, 1987). These data suggest that C3 may be synthesised locally within the vascular bed. Recent studies have shown that γ-interferon induces synthesis of alternative pathway components by cultured human endothelial cells (Ripoche et al., 1988b).

(f) FIBROBLASTS

Al-Adnani & McGee first showed *in vitro* synthesis of C1q by human and rat fibroblasts in 1976. Since then, fibroblasts have been shown to be capable of synthesising C3, factor B, C2 (Katz, Cole & Strunk, 1988) and factor H (Katz & Strunk, 1988a). As described above, fibroblast-like cells isolated from normal synovial membrane have been shown to be capable of synthesising seven complement components *in vitro* (Katz & Strunk, 1988b).

CONCLUSION

The above evidence shows that many different cell types are capable of complement component synthesis and secretion. It is likely the list of tissues and cell types responsible for complement biosynthesis will continue to grow.

INFLAMMATION

Inflammation can be defined as the response of living tissue to any injury. It functions as a protective mechanism against injurious agents and acts to remove these agents and repair damaged tissue. Inflammation is characterised by dilatation of blood vessels, invasion of the tissue by leukocytes from the blood and the passage of blood proteins and fluid through capillary walls into the tissue space.

The effects of inflammation are usually beneficial by helping to eliminate microorganisms, limiting the injurious effects of irritating chemicals and bacterial toxins and participating in the removal of necrotic cells and tissue debris. However some effects of inflammation may be harmful such as the impairment of movement, constriction of airways, interference with blood flow (causing ischaemia and cell death) and allergic reactions (Anderson, 1985; Ryan & Majno, 1977; Hurley, 1972; Bessis, 1964).

There are two main types of inflammation: acute and chronic inflammation. A relatively intense injury over a short period of time stimulates an acute inflammatory reaction which frequently subsides without leaving residual effects, although the formation of granulation tissue with consequent fibrosis or scarring is possible (Gabbiani et al., 1972). Other agents which may not produce severe initial tissue injury may persist for long periods and result in chronic inflammation.

1. ACUTE INFLAMMATION

(a) Active hyperaemia

Following injury, arteriolar contraction causes a transient blanching of the tissue which within a few minutes is followed by

arteriolar dilatation in and around the injury site. Capillaries and post-capillary venules become engorged with rapidly flowing blood which accounts for the redness and heat seen in inflamed tissues.

The autonomic nervous system and in particular the sympathetic adrenergic nerves regulate changes in tone of the smooth muscle of arterioles which in turn controls the flow of blood through a tissue. Depending upon the metabolic state of the tissue there will also be a variety of local factors exerting their effects on the blood vessels. Hence active hyperaemia in acute inflammation is probably due to a predominance of local factors over the general arteriolar control mechanisms (Anderson, 1985).

(b) Exudation

As described above, the onset of an inflammatory reaction is marked by swelling caused by an exudation of fluid from the blood vessels into the surrounding tissues.

Landis (1927) first showed that the balance between the internal hydrostatic pressure and osmotic pressure of the plasma is disrupted by arteriolar dilatation at inflammatory sites, resulting in a net loss of fluid from the terminal vascular bed. Loss of fluid from the blood vessels causes an increase in the concentration of proteins and cells which results in a slowing of the blood flow due to an increase in blood viscosity (Anderson, 1985).

(c) Exudation of plasma proteins

Exudation of plasma proteins has been shown to occur in two phases: one immediate and transient and the other delayed and prolonged. (i) the immediate phase: due to the release of mediators and in particular histamine, which cause opening of gaps between endothelial

cells by causing their contraction resulting in escape of fluid and proteins (Sevitt, 1958).

(ii) the delayed phase: mainly due to the direct effect of the injury but may be amplified by inflammatory mediators exerting their effects on surrounding tissues (Lewis, 1927).

(d) Endogenous mediators

Endogenous mediators may be derived from plasma or released by cells.

(i) Plasma: there are four related groups of proteins capable of producing mediators; the kinin system, the clotting system, the fibrinolytic system and the complement system. Contact to basement membrane and several other substances activates Hageman factor which results in activation of the kinin system (Miles, 1969; Anderson, 1985). Once activated the kinin system leads to activation of the clotting cascade and the fibrinolytic system, following the release of kinins such as bradykinin (Anderson, 1985). Activation of Hageman factor releases plasmin (from plasminogen), an enzyme capable of digesting fibrin, activating Hageman factor (thus feeding back to trigger the kinin system) and which may also activate C1 and cleave C3 of the complement system (Margolis, 1959; Willoughby, Cooke & Turk, 1969).

(ii) cells: a number of mediators are known to be released by cells but only a few have been characterised chemically. Mast cell granules, basophil leukocytes and platelets store an inactive form of histamine which is released on activation by many stimuli, e.g. heat irradiation, toxins, anaphylatoxins and IgE antibody complexed with an allergen binding to mast cells, as in the case of hay fever (Ryan, 1974). Factors promoting platelet aggregation also release histamine from platelets

(Majno, Palade & Schoefl, 1961). 5-hydroxytryptamine (seretonin) is present in some tissues and may cause a brief increase in vascular permeability when it is released (Majno et al., 1961). Oxidation of polyunsaturated fatty acids such as arachidonic acid leads to release of prostaglandins (Majno et al., 1961). Prostaglandins are potent stimulators of smooth muscle contraction thus resulting in active hyperaemia. Although prostaglandins do not cause pain directly, they lower the pain threshold of nerve endings to histamine, seretonin and bradykinin.

(e) Emigration of leukocytes

Initially, leukocytes stick to the endothelium and then pass through the vessel wall. Emigration of leukocytes appears to be independent of gaps between endothelial cells which are responsible for increased vascular permeability (Marchesi, 1961).

In acute inflammatory lesions neutrophil polymorphs migrate earlier and in much greater numbers than monocytes (Anderson, 1985). The polymorphonuclear (PMN) leukocytes take only a few minutes to pass through venule walls and then assume their scavenger role as they wander through tissues. Emigration of PMN leukocytes is thought to be mediated by chemotaxis and many substances have been shown to be chemotactic for polymorphs *in vitro* e.g. products of the activated complement, clotting, fibrinolytic and kinin systems, substances released by injured tissues and microorganisms (Wilkinson, 1974; Wilkinson, Russel & Allen, 1977).

In acute inflammation the peak of polymorph emigration has passed before monocytes migrate in sufficient numbers. Hence it would appear as though different factors control the infiltration of polymorphs and monocytes (Ryan, 1967). Although mononuclear phagocytes

are attracted by some of the agents which are chemotactic for polymorphs such as C5a and $C5a_{des.arg}$, a number of substances are known to be predominantly chemotactic for monocytes.

2. CHRONIC INFLAMMATION

Chronic inflammation, which may not have an acute onset, is elicited when the injurious stimulus persists resulting in a continuation of the inflammatory process.

Some bacteria are remarkably non-toxic and can survive and multiply within macrophages without being destroyed. The infected tissues become heavily infiltrated with macrophages containing huge numbers of the organisms and although little fibrosis occurs with such lesions the infiltrating cells are typical of a chronic inflammatory reaction (Ryan & Spector, 1970; Warren, 1972; Anderson, 1985). Other bacteria are equally non-toxic but features of the lesion are altered by the development of delayed hypersensitivity by the host which not only promotes killing of the bacteria by macrophages but also causes tissue injury, necrosis granuloma formation and scarring. Hypersensitivity reactions develop as a result of the host's immune response (Warren, 1972; Unanue & Benaceraff, 1973) and is characterised by infiltration of the inflammatory site with lymphocytes and macrophages. In some chronic inflammatory diseases with features of hypersensitivity reactions, the causal agent remains obscure, e.g. Crohn's disease, sarcoidosis, rheumatoid arthritis (see later) and systemic lupus erythematosus.

There are considerable variations seen at chronic inflammatory sites and these variations are determined by the causal agent. The features include foci of acute exudative inflammation with PMN leukocytes, macrophages, lymphocytes and plasma cells, necrosis,

formation of granulation tissue and dense fibrous tissue. The variations result from the relative prominence of each of these features (Anderson, 1985; Ryan & Majno, 1977).

Effects of chronic inflammation

Chronic inflammation either results from a hypersensitivity reaction to an otherwise harmless substance or occurs in autoimmune diseases and seems to serve no useful function (Unanue & Benaceraff, 1973; Anderson, 1985). When tissue has been lost in an inflammatory process, healing occurs by fibrosis but this fibrous tissue may induce serious effects by blocking or constricting, e.g. the mitral valve in chronic rheumatic fever or the small intestine in Crohn's disease, and destruction of tissues by scarring and shrinkage.

3. TYPES OF CELLS IN INFLAMMATORY LESIONS

(a) Polymorphonuclear leukocytes: the neutrophil polymorph is the predominant phagocytic cell at sites of acute inflammation. They are highly specialised cells, actively motile, rich in lysosomal enzymes and respond readily to chemotaxins. Eosinophil polymorphs are also seen to accumulate at sites of inflammation when hypersensitivity reactions are involved and may play a regulatory role (Ryan, 1967; Hurley, 1972).

(b) Monocytes: are less actively motile and phagocytic than neutrophil polymorphs but provide a reserve of cells, which on infiltration of an inflammatory site mature into macrophages (Ryan, 1967; Van Furth, 1979; Ebert & Florey, 1939; Cronkite et al., 1960; Spector, Walter & Willoughby, 1965; Van Furth, 1970). Monocytes develop an increase in lysosomal enzymes, metabolic activity, motility and phagocytic and microbicidal capacity.

(c) Macrophages: ingest and destroy inflammatory debris and indigestable

material may also be taken up by macrophages and sequestered for long periods. Macrophages may be capable of limited division and long survival after phagocytic activity. They are able to synthesise plasma membrane, lysosomal enzymes and lysosomes. Such properties make macrophages particularly suited to sustained function in prolonged inflammatory reactions where they gradually replace neutrophils as the predominant phagocytic cell (Hurley, 1972; Ryan & Spector, 1970; Spector, 1974).

(d) Lymphocytes: when present in large numbers are suggestive of either a delayed hypersensitivity reaction or of antibody-dependent lymphocyte cytotoxicity. The presence of plasma cells are indicative of antibody production (Hurley, 1972; Unanue & Benaceraff, 1973; Anderson, 1985).

(e) Fibroblasts: are seen in most inflammatory lesions but their presence in large numbers is indicative of chronic inflammation and repair (Hurley, 1972; Ryan et al., 1974; Anderson, 1985).

(f) Serosal cells: which include endothelial lining cells and macrophages often pass into the exudate at an early stage of an inflammatory reaction (Hurley, 1972; Ryan, 1967; Anderson, 1985).

RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a classical example of chronic inflammation which due to its high incidence worldwide, its frequent onset in early adult life and its chronic and disabling effects, is one of the most important diseases of mankind.

(a) Course of the disease

Rheumatoid arthritis (RA) is one of the connective tissue diseases and is characterised by a subacute or chronic non-suppurative

arthritis usually affecting several joints. The course of the disease is punctuated with periods of relapse and remission with the joint suffering further damage each time.

RA affects approximately 3% of the female and 1% of the male population. Those between the ages of 25 years and 55 years are most susceptible, although the disease is not restricted to this age group. Any synovial joint may be affected but those of the hands and feet are most often involved, the disease often being bilateral and symmetrical.

The onset of RA is often insidious but can be acute and during this early stage and during relapse, the joints become inflamed, hot, swollen (partly due to synovial effusion) and tender and the patient often suffers from fatigue, weight loss and fever. The inflammatory process occurs within the synovial membrane resulting in cartilage damage and subsequent bone erosion. The surrounding muscles become subject to wasting and weakness, and the tendons may be ruptured causing even further deformity (Calto , 1985; Harris, 1984).

Synovitis in RA begins with a non-specific increase in the number of small capillaries and synovial blood flow associated with an accumulation of mononuclear cells around these blood vessels. The stimulus to new blood vessel formation in RA is probably related to the inflammatory response. Synovial cells then proliferate and the synovium thickens. After a while there is an infiltration of lymphocytes (mostly T-cells) (Van Boxel & Paget, 1975), around the blood vessels. Three main types of infiltration areas have been described: lymphocyte-rich, plasma cell rich and transitional areas (Ishikawa & Ziff, 1976). Transitional areas contain what are likely to be dendritic cells which may act as antigen presenting cells for the T-lymphocytes.

Changes in other tissues can also be seen including subcutaneous lesions known as rheumatoid nodules which develop over pressure sites in about 20% of patients. The nodules persist throughout life and are a helpful clinical and histological diagnostic aid. They tend to occur in more severely affected patients and are associated with a worse prognosis.

In severe RA, vasculitis may occur which results in skin ulceration and occasionally gangrene, bowel perforation and myocardial infarction (Anderson, 1985).

(b) Pathogenesis and aetiology of RA

The cause of RA is unknown but the disease presents features which suggest the lesions in the joints and elsewhere are secondary hypersensitivity reactions.

(i) Complement activation

Complement activation is now accepted as playing a major role in the pathogenesis of RA. Complement was first suspected of being involved when levels in serum were observed to be normal while complement levels in synovial fluid were depressed, leading to the suggestion that there was a high turnover of complement within the joint space (Pekin & Zvaifler, 1964; Ruddy & Austen, 1970). More recent work has identified complement breakdown products within the synovial joint (Berkowicz et al., 1983; Mollnes et al., 1986) confirming that complement activation does occur. Ruddy and Colten, (1974) have also shown that rheumatoid synovial tissues are capable of synthesising the complement components C2, C3, C4 and C5 *in vitro* as well as demonstrating local C3 synthesis *in vivo*.

(ii) Rheumatoid factors

Rheumatoid factors (RFs), are autoantibodies mainly of the IgM and IgG classes, which are present in the serum of most cases and react with antigenic components of the Fc region of IgG molecules, forming immune complexes. Patients with RF in their sera are said to be seropositive distinguishing their disease from a group of seronegative arthritides (RF is absent) which includes ankylosing spondylitis (Fong, Carson & Vaughan, 1986; Carson et al., 1987).

Rheumatoid factor activity was first recognised by the ability of the sera of RA patients to agglutinate erythrocytes coated with small amounts of IgG antibody (Waaler, 1940; Rose et al., 1948).

RFs will bind more avidly with IgG that has already bound to an antigen than free monomeric IgG and hence may act to further aggregate immune complexes (Fong et al., 1986; Carson et al., 1987; Anderson, 1985).

However, rheumatoid factors (RFs) have only been found in approximately 80% of RA patients and have also been found in patients suffering from various other diseases and in a small proportion of healthy individuals, very few of whom subsequently develop RA (Anderson, 1985; Harris, 1984; Carson et al., 1987).

RF in serum has a lower avidity than RF found in affected synovium, and serum RF in RA patients has been shown to be capable of activating complement whereas the RF present in the serum of some individuals without RA does not activate complement (Zvaifler & Schur, 1968; Anderson, 1985). These findings could help to explain why serum RF is not always associated with disease but a high titre in patients with RA and particularly in the presence of IgG RF, is associated with severe

disease .

Even if RFs are suspected of being involved in the development of RA, it is still not known what initiates the production of RF. Various infective agents have been considered e.g. exogenous antigens such as bacteria and viruses and endogenous antigens such as collagen, have all elicited RF but the results are inconsistent and no one antigen or group of antigens have been identified as being involved in all cases (Harris, 1984; Anderson, 1985).

The features of RA are consistent with the view that rheumatoid factor-IgG immune complexes within the joint, activate complement thus inducing inflammation by the emigration and activation of cells such as PMN leukocytes and monocytes which phagocytose the immune complexes, releasing agents such as lysosomal enzymes which can injure the cartilage and synovium.

(iii) Involvement of T-cells

Hence while immune complex disease could explain some of the major features of RA, there is some evidence that T-cells are also involved. The majority of lymphocytes found in the joint fluid of RA patients are T-helper cells which have been shown to be activated and secrete lymphokines which play a role in the emigration and activation of several inflammatory mediators (Van Boxel & Paget, 1975).

(iv) Genetic predisposition

The major immunogenetic association with RA is the histo-compatibility locus, HLA-Dw4 (Stastny, 1978; Panayi, Wooley & Batchelor, 1979). The association however is by no means as strong as the HLA-B27 locus for ankylosing spondylitis. The incidence of HLA-Dw4 in sero-positive patients has been shown to be 52%, while seronegative patients

had a frequency of 24% and control subjects had a frequency of 25% (Stastny, 1982). However it has also been shown that the HLA-Dw4 locus does not appear to be associated with rheumatoid factor because patients who have rheumatoid factor but do not have RA, do not have a higher demonstrated frequency of HLA-Dw4 (Harris, 1984).

2. OSTEOARTHRITIS

Osteoarthritis (OA) is the most common rheumatic disease (Lawrence, Bremener & Beir, 1966; Peyron, 1986) with the greatest increase in occurence between 40 and 50 years of age. Radiologic surveys have shown that more than 80% of the population over 55 years of age have OA, with the hands and feet most often affected, although the overall frequency of OA is equal in men and women (Hamerman, 1989).

(a) Pathogenesis and aetiology

While RA may be regarded as a chronic inflammatory disease, OA is looked upon more as a wear and tear disease, although features of inflammation may be seen. OA may be defined as a clinical syndrome resulting from defects in articular cartilage and related changes in subchondral bone, joint margins, synovium and para-articular structures (Howell, 1985).

There are no commonly accepted criteria for diagnosis of OA and diagnosis is often based on the presence of symptoms when other arthritic diseases have been excluded.

The synovial fluid from an OA patient is usually clear, may be yellow-tinged but is often colourless and highly viscous. It usually contains between 200 and 2000 white blood cells, mostly mononuclear, but if significant inflammation is present the synovial fluid white cell count may be 20000 (Altman, 1987).

Several causative mechanisms have been postulated for OA but any single patient may have a different pathogenic mechanism or set of mechanisms all of which may be affected by aging.

Although joint damage may lead to OA (Lawrence et al., 1966), it is now considered that the disease has a biochemical basis. Histological examination of the synovium reveals proliferation of the synovial membrane lining cells, with occassionally perivascular foci of chronic inflammatory cells in the deeper layers.

A relationship between obesity and OA has been suggested but the evidence is rather controversial (Leach, Baumgrad & Broom, 1973). However it is generally accepted that decreasing the body weight will relieve the symptoms and pain of OA.

Although complement activation has been demonstrated in rheumatoid joints and is thought to play a role in the pathogenesis of rheumatoid arthritis, it remains unclear as to whether complement components are synthesised locally in the rheumatoid synovial joint *in vivo*. Metabolic studies using radiolabelled (^{125}-I) C3 (Ruddy & Colten, 1974) have thus far provided the only evidence for *in vivo* biosynthesis of complement in rheumatoid synovium.

Northern and dot-blot analyses of RNA isolated from rheumatoid synovial tissue have been used in these studies to detect mRNAs coding for complement components as a powerful means of determining *in vivo* biosynthesis of C4, C2, C3, C5, factor B and C1-inhibitor in the rheumatoid synovial joint. In addition to the molecular hybridisation studies, *in vitro* cultures of synovial membrane fragments were also used to measure the synthesis of these complement components. The results of

these studies were compared with those obtained from synovial tissue from osteoarthritis patients and normal synovium to investigate whether local synthesis of complement by synovium was specific to rheumatoid arthritis patients or a constitutive property of all synovial tissues.

MATERIALS

AND

METHODS

BUFFERS AND REAGENTS

The buffers and reagents listed below are arranged under the headings in which they first appear in the text. The 'water' used in each solution refers to sterile deionised water. Unless otherwise stated, all chemicals, acids and alcohols were supplied by BDH Chemicals, Poole, Dorset, UK..

1. Preparation of plasmid DNA

(a) ψa medium - 5g yeast extract (Difco), 20g Bactotryptone (Difco) and 5g magnesium sulphate were dissolved in water and the pH was adjusted to 7.6 with concentrated KOH. The volume was adjusted to 1 litre with water and 14g Bactoragar (Difco) was added before autoclaving.

(b) TfbI buffer - This buffer contained 30mM potassium acetate, 100mM rubidium chloride, 10mM calcium chloride, 50mM manganese chloride and 15% (v/v/) glycerol. The pH was adjusted to 5.8 with 0.2M acetic acid and the volume was made up to 500ml with water. TfbI was filtered through 0.45μm filter units (Millipore) and stored at room temperature.

(c) TfbII buffer - This buffer contained 10mM MOPS (sodium morpholino propanesulphonic acid; Sigma), 75mM calcium chloride, 10mM rubidium chloride and 15% (v/v) glycerol. The pH was adjusted to 6.5 with concentrated KOH and the volume was made up to 100ml with water. TfbII was filtered through 0.45μm filter units (Millipore) and stored at room temperature.

(d) L-broth medium - 10g Bactotryptone (Difco), 10g sodium chloride and 5g yeast extract (Difco) were dissolved in 800ml water and the volume made up to 1 litre. The medium was then autoclaved and stored at room temperature. When the L-broth had cooled, ampicillin (50μg/ml; Sigma)

[or tetracycline (12.5μg/ml; Sigma) for the β_2-microglobulin plasmid] was added.

(e) Bactoagar - 10g Bactotryptone (Difco), 10g sodium chloride and 5g yeast extract (Difco) were dissolved in water and the volume made up to 1 litre. Bactoagar (Difco; 20g) was added prior to autoclaving during which time the agar dissolved. When cool, the appropriate antibiotic was added to the same final concentration as above. The medium was then poured into 9cm Petri dishes (Sterilin) and allowed to set. The plates were stored at 4°C for up to two weeks.

(f) Lysosyme mix - Lysosyme (Sigma) was dissolved to a final concentratiom of 2mg/ml in 25mM Tris.HCl pH8.0 containing 50mM glucose and 10mM EDTA. The lysosyme mix was stored at 4°C.

(g) TE buffer - 0.5M TrisHCl pH8.0 (2ml) and 0.5M EDTA pH8.0 (20μl) were mixed and the volume was made up to 100ml with water. The solution was autoclaved and stored at room temperature.

2. Characterisation of the plasmid DNA

(a) 1 x Tris Borate (TBE) - Tris (10.8g) and boric acid (5.5g) were dissolved in 500ml water after which 0.5M EDTA (4ml) was added. The pH was adjusted to 8.0 before making the volume up to 1 litre with water.

(b) 10 x ligation buffer - This buffer was composed of 100mM Tris.HCl pH7.6 containing 100mM $MgCl_2$, 10mM dithiothreitol (Sigma) and 1mg/ml bovine serum albumin (Sigma).

3. Isolation of RNA

(a) 5M guanidinium isothiocyanate - A 6M stock solution was prepared by dissolving 141.8g guanidinium isothiocyanate (Bethesda Research Laboratories) in heated water and the volume made up to 200ml with

water. 24ml 0.5M Tris.HCl pH7.0 containing 0.5M EDTA and 4ml of water were added and the pH of the resultant solution was adjusted to 7.0 if necessary. Several drops of diethylpyrocarbonate* (Sigma) were added and the solution was autoclaved. Once cooled, 12ml mercaptoethanol (Sigma) was added and the final solution was filtered through 0.45μM filter units (Millipore) to ensure freedom from bacterial contamination. The sterile solution was stored in 20ml aliq. ts at 4°C for up to two weeks.

* Diethylpyrocarbonate (DEPC or 'Baycovin') is commonly used as an inhibitor of exogenous ribonucleases. DEPC inactivates all proteins as a result of an esterification reaction. It is particularly useful for removing ribonucleases from solutions and glassware and any excess can be quickly hydrolysed to CO_2 and ethanol by heating. This last step is important because DEPC reacts with RNA and DNA and hence excess must be removed from any extraction buffer (Birnie & Graham, 1984).

(b) 5.7M cesium chloride in 50mM EDTA (pH7.0) - 95.97g CsCl (Bethesda Research Laboratories) were dissolved in 60ml water and 12.5ml 0.4M EDTA were added. The pH of the solution was adjusted to 7.0 before the volume was made up to 100ml with water and the refractive index adjusted to 1.3995 by the addition of more CsCl or water. The final solution was filtered through 0.45μM filter units (Millipore) and stored at room temperature in foil covered bottles to protect it from the light.

4. Northern and dot-blot analyses

(a) 10 x MOPS pH7.0 - 42g sodium morpholino propanesulphonic acid (Sigma), 6.8g sodium acetate and 3.7g EDTA were dissolved in water and the pH was adjusted to 7.0 with glacial acetic acid. The volume was made up to 1 litre and the final solution was stored in the dark at 4°C.

(b) 20 x SSC - 175.2g of sodium chloride and 88.2g of sodium citrate

were dissolved in water and the pH adjused to 7.0. The volume was made up to 1 litre and the final solution was stored at room temperature.

(c) 20 x SSPE - This buffer used in prehybridisation and hybridisation buffers was composed of 0.2M sodium phosphate buffer, pH7.7 containing 3M NaCl and 0.02M EDTA. 20 x SSPE was stored at room temperature.

(d) 100 x Denhardts solution - 2g Ficoll (Sigma) were dissolved in 75ml water and then 2g polyvinyl pyrollidine (Sigma) and 2g BSA (Sigma) were added. The volume was made up to 100ml and the solution was stored at 4°C in the dark.

(e) Salmon sperm DNA (1mg/ml) - 0.1g salmon sperm DNA (Sigma) was added to 100ml of water. The DNA was dissolved by autoclaving and the final solution was stored at 4°C in the dark.

5. Synovial tissue explant cultures

(a) Transport medium - Sodium bicarbonate (70mg) was dissolved in water to which was added 20ml 10xHank's solution (Gibco), 4ml fetal calf serum (heat inactivated for two hours at 56°C) (Gibco) and 10ml penicillin/streptomycin (5000units/ml;5000µg/ml). The volume was made up to 200ml with water and the final solution was filtered through 0.2µM filter units (Swinnex) into 10ml aliquots which were stored at -20°C.

(b) Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum - 20ml 10 x DMEM (Gibco), 8ml 7.5% sodium bicarbonate (Gibco), 20ml fetal calf serum (Gibco) (heat inactivated for 2 hours at 56°C), 2ml 0.2M glutamine (Gibco) and 2ml penicillin/streptomycin (5000units/ml; 5000µg/ml) were mixed together and the volume made up to 200ml with water. The final medium was filtered through 0.2µM filter units (Swinnex) and stored at 4°C for up to two weeks.

6. Enzyme linked immunosorbent assays (ELISAs)

(a) Coating buffer - Sodium bicarbonate (Na_2CO_3; 0.79g) and sodium hydrogen bicarbonate ($NaHCO_3$; 1.46g) were dissolved in water and the pH was adjusted to 9.6 if necessary. The volume was made up to 500ml with water and stored at 4°C for up to one week.

(b) PBS/0.05% Tween (PBST) - 20 x PBS was prepared by dissolving 320g sodium chloride, 48.4g dipotassium hydrogen phosphate and 13.6g potassium dihydrogen phosphate in water and the volume made up to 2 litres. PBS/0.05% (v/v) Tween was prepared by adding 1ml Tween 20 (Sigma) to 100ml 20 x PBS and making the volume up to 2 litres with water.

(c) Blocking buffer - Bovine serum albumin (0.5g; Sigma) was dissolved in 500ml 1 x PBS. Blocking buffer was stored at 4°C for up to one week.

(d) Peroxide substrate - 28ml 0.2M disodium hydrogen phosphate were added to 44ml 0.1M citric acid and the pH adjusted to 5.6 by the addition of either of these solutions. The volume was made up to 100ml with water and the solution was covered from the light. 34mg o-phenylenediamine (Sigma) were dissolved in the solution which was then kept in the dark. Immediately before its use as the substrate, 40µl hydrogen peroxide was added to the solution.

7. Haemolytic assays of complement components

(a) Alsever's solution - D-glucose (41g), sodium citrate (16g), sodium chloride (8.4g) and citric acid (0.8g) were dissolved in a final volume of 1 litre of water (the final pH was between 6.0 and 6.5). Alsever's solution was stored at 4°C for two weeks.

(b) Veronal buffered saline, 5 x VBS - Sodium chloride (85g), sodium barbitone (3.75g; Sigma) and barbitone (5.75g; Sigma) were dissolved in

water and the volume made up to 2 litres. 5 x VBS was stored at 4°C.

(c) Isotonic veronal-buffered saline containing gelatin and cations, GVB^{2+} - 5ml 10% gelatin was added to 100ml 5 x VBS, 2.5ml 0.03M calcium chloride and 5ml 0.1M magnesium chloride and the volume made up to 500ml with water. GVB^{2+} was stored at 4°C for up to 3 days.

GVB^{2-} is GVB^{2+} without $MgCl_2$ and $CaCl_2$.

(d) Isotonic dextrose containing cations, $D5W^{2+}$ - D-glucose (25g) was added to 2.5ml 0.03M calcium chloride and 5ml 0.1M magnesium chloride and the volume made up to 500ml with water. $D5W^{2+}$ was stored at 4°C for up to 3 days.

$D5W^{2-}$ used in the preparation of Mg-EGTA is $D5W^{2+}$ without $MgCl_2$ and $CaCl_2$.

(e) $DGVB^{2+}$ - equal volumes of GVB^{2+} and $D5W^{2+}$ were mixed together. $DGVB^{2+}$ was prepared fresh daily.

(f) EDTA (10mmol/l) GVB^{2-} - 58ml isotonic 86mM $EDTA_{pH7.5}$ was mixed with 442ml GVB^{2-} and stored at 4°C.

(g) D50S - Glucose (50g) and sodium chloride (5.95g) were dissolved in water and the volume made up to 100ml. D50S was stored at 4°C.

(h) Mannitol:GVB^{2+} - Three parts 20% (w/v) mannitol were mixed with 1 part GVB^{2+} and the mixture was stored at 4°C.

(i) C2 oxidising reagent - Potassium iodide (8.25g) and iodine (300mg; Sigma) were dissolved in 100ml 0.1M phosphate buffer (pH6.0). This solution was stored at 4°C in the dark for several weeks and was diluted 1/200 in 0.1M phosphate buffer (pH6.0) immediately prior to use.

(j) EDTA (40mmol/l) GVB^{2-} - 232.5ml isotonic 86mM EDTA and 267.5ml GVB^{2-} were mixed and stored at 4°C. This reagent chelates calcium and magnesium ions, preventing the formation of the classical and

alternative pathway C3 and C5 convertases and thus provides a source of C3, C5, C6, C7, C8 and C9, when used to dilute rat serum (C^{rat}).

(k) Mg-EGTA - 10ml 100mM EGTA, 7ml 100mM magnesium chloride and 83ml $DGVB^{2-}$ (3 parts $D5W^{2-}$:1 part GVB^{2-}) were mixed together and the pH was adjusted to 7.4-7.6. Mg-EGTA was prepared fresh daily. EGTA selectively chelates Ca^{2+} ions, to prevent C1 activation and thus classical pathway activation. The Mg^{2+} ion content permits alternative pathway activation as it is required for the formation of C3bBb.

8. Incorporation of ^{35}[S]-methionine into complement proteins

(a) Solution A, pH7.5 - This buffer was made up to a volume of 100ml with water and contained 50mM NaCl, 100mM KCl, 50mM Tris, 5mM EDTA, 0.5% (w/v) BSA (Sigma), 0.5% (w/v) sodium deoxycholate (Sigma), 1% (w/v) Triton X100, 5mM PMSF (final conc.) (Sigma) in dimethylsulphoxide (Sigma), 0.02% (w/v) sodium azide (Sigma) and 10mM benzamidine (Sigma). Solution A was protected from the light and stored at room temperature.

(b) Solution B - This buffer was composed of 63.8mM Tris.HCl, pH6.8 containing 3% (w/v) SDS, 5% (v/v) mercaptoethanol (Sigma) and 10% glycerol. This solution was protected from the light and stored at room temperature.

9. Isolation of synovial cells from synovial tissue

(a) Collagenase/DNase 1 - 100mg collagenase (Type 1, Sigma), 10mg deoxyribonuclease 1 (DN-25, Sigma) and 35mg sodium bicarbonate were dissolved in 10ml 10 x Hank's balanced salt solution (Gibco) and 2ml penicillin/streptomycin (5000units/ml;5000µg/ml). The volume was made up to 100ml with water. The final solution was filtered through 0.2µM filter units (Swinnex) into 25ml aliquots and stored at -20°C.

(b) Trypsin/EDTA - 100mg trypsin (Type 111, Sigma) and 40mg EDTA were dissolved in 20ml 10 x phosphate buffered saline (Gibco) and the volume was made up to 200ml with water. The final solution was filtered through 0.2μM filter units (Swinnex) into 25ml aliqouts and stored at -20°C.

10. Immunohistochemical analysis of cultured synovial cells

(a) Veronal acetate buffer, VAB - Sodium acetate trihydrate (1.94g) and sodium barbitone (2.94g; Sigma) were dissolved in 100ml water. 5ml of this solution was added to 16ml water and the pH adjusted to 9.2 with 0.1N hydrochloric acid.

(b) Preparation of phosphate substrate - Naphthol AS-MX phosphate (80mg; Sigma) (dissolved in a drop of dimethyl formamide), fast red violet LB salt (80mg; Sigma) and levamisole (8mg; Sigma) were added to 20ml VAB and filtered through Whatman filter paper (qualitative 1) in the dark immediately before use.

MATERIALS AND METHODS

1. Preparation of plasmid DNA

1.1 - Preparation of competent JM101 cells

(i) *E. coli* JM101 cells were streaked from frozen stock onto a ½a plate and incubated at 37°C overnight.

(ii) A single colony was picked and used to inoculate 5ml ½b medium (½b is ½a without the agar). The culture was then incubated at 37°C overnight.

(iii) 5ml of the overnight culture was subcultured into 100ml ½b and grown for a further 2 hours until the optimal OD_{550} of 0.48, had been reached.

(iv) The culture was chilled on ice for 5 min and then centrifuged (3000rpm for 5 min at 4°C) in a Sorvall centrifuge.

(v) The cells were resuspended in 40ml of TfbI, left on ice for 5 min and then centrifuged (3000rpm for 5 min at 4°C) in a Sorvall centrifuge

(vi) The cells were resuspended in 4ml of TfbII and left on ice for 15 min.

(vii) The competent cells were dispensed into 200µl aliquots using a prechilled pipette and tubes. The cells were snap frozen in dry ice and stored at -70°C.

1.2 - Transformation of competent JM101 cells with plasmid DNA

(i) 50ng plasmid DNA was added to 100µl competent JM101 cells, mixed well and left on ice for 45 min.

(ii) The sample was incubated at 42°C for 90 sec and returned to ice for 2 min.

(iii) 400µl L-broth was added and incubated at 37°C on a shaker for 1

hour.

(iv) 100µl of the sample was plated onto each of 3 agar (Bactoagar) plates containing ampicillin (50 µg/ml) (or tetracycline (12.5 µg/ml) for the β_2-microglobulin plasmid only), which were then incubated at 37°C overnight.

1.3 - Small-scale preparation (miniprep) of plasmid DNA

(i) 5ml L-broth containing ampicillin (50 µg/ml) (or tetracycline (12.5 µg/ml) for β_2-microglobulin) was inoculated with one colony picked from the above plates. This liquid culture was then incubated at 37°C in an orbital incubator overnight.

(ii) 2ml of the liquid culture was removed and centrifuged (10000rpm for 5 min at room temperature) in an Eppendorf microfuge. [The 2ml medium removed from the 5ml culture in this step was replaced with 2ml L-broth containing ampicillin (50 µg/ml) or tetracycline (12.5 µg/ml) and incubation of the culture was continued at 37°C.]

(iii) After centrifugation, the pellet was resuspended in 200µl lysosyme mix and incubated at room temperature for 5 min.

(iv) 400µl 0.2M sodium hydroxide containing 0.1% SDS was added and the tube was inverted gently until lysis had occurred.

(v) 300µl 3M sodium acetate pH4.8 were added to the sample which was then left on ice for 15 min before centrifugation at 10000rpm for 5 min at room temperature.

(vi) 800µl of the supernatant was transferred to a fresh tube and 480µl isopropanol were added. The sample was then incubated at -20°C for 1 hour to precipitate the DNA.

(vii) The sample was centrifuged at 10000rpm for 10 min at room temperature and the pellet was washed in 1ml 100% ethanol, freeze-dried

and resuspended in 30µl TE buffer.

Restriction enzyme digests were performed on the plasmid minipreps to ensure the correct restriction map of the plasmid was obtained thus indicating that the correct plasmid had been amplified. Once this had been confirmed, 500ml L-broth containing ampicillin (50 µg/ml) or tetracycline (12.5 µg/ml) was inoculated with the 5ml culture [from step (ii)] and incubated at 37°C overnight.

1.4 - Large-scale preparation of plasmid DNA

(i) The 500ml culture was centrifuged (5000rpm for 5 min at 4°C) in a Sorvall centrifuge and the pellet was resuspended in 10ml 50mM Tris.HCl (pH8.0) containing 25% (w/v) sucrose

(ii) 100mg lysosyme was added and the sample stored on ice for 20 min.

(iii) 5ml 0.2M EDTA pH8.0 were added, mixed well and the sample stored on ice for 20 min.

(iii) 600µl 10% (v/v) NP-40 detergent in 50mM Tris.HCl (pH8.0) containing 25% (w/v) sucrose was added and the sample inverted until the mixture became viscous. The sample was then centrifuged (15000rpm for 30 min at 4°C) in a Sorvall centrifuge.

(iv) The supernatant was collected and an equal volume of phenol (equilibrated with TE buffer) was added. The sample was mixed well for 15 min and centrifuged (2500rpm for 20 min at room temperature) in an MSE 4L centrifuge.

(v) An equal volume of chloroform:isoamyl alcohol (24:1 mixture) was added to the aqueous layer and the sample was centrifuged (2500rpm for 5 min at room temperature) in an MSE 4L centrifuge.

(vi) The aqueous layer was collected and the following ribonucleases: 10µl RNase A (10mg/ml) and 20µl T1 RNase (20mg/ml) were added. [Both

RNase enzymes had previously been boiled for 10 min to remove any deoxyribonuclease activity.] The sample was then incubated at 37°C for 30 min with constant mixing.

(vii) 1/2 volume phenol was added, mixed well for 2 min and the sample was centrifuged (2500rpm for 5 min at room temperature) in an MSE 4L centrifuge.

(viii) An equal volume chloroform:isoamyl alcohol was added to the aqueous layer and mixed well for 2 min. The sample was then centrifuged (2500rpm for 5 min at room temperature) in an MSE 4L centrifuge. The aqueous layer was collected and half of its volume of 3M sodium acetate pH6.8 and an equal volume of isopropanol were added to the aqueous layer. After mixing, the tube was incubated at room temperature for 90 min to precipitate the DNA.

(ix) The sample was centrifuged (10000rpm for 10 min at 4°C) in a Sorvall centrifuge, after which the pellet was washed in 100% ethanol and freeze-dried. The plasmid DNA was resuspended in 1ml TE buffer and the absorbances at 260nm (OD_{260}) and 280nm (OD_{280}) were measured.

Extensive restriction enzyme digests were performed on the plasmid to ensure the correct plasmid had been prepared before it could be used to prepare radioactivelly-labelled cDNA probes for Northern and dot-blot analyses.

2. Characterisation of the plasmid DNA

2.1 - Restriction mapping of the plasmid DNA

Table 7 summarises the cDNA probes used in this study. *E.coli* JM101 cells were used as the host for amplifying all of these plasmids.

cDNA PROBE (plasmid vector)	INSERT	Region to which the cDNA probe corresponds	Reference
C2 (pAT153)	BamH1 — 400bp — Hind111	A region containing the active site serine residue and the secondary substrate binding of the serine protease.	Bentley and Porter (1984)
C3 (pAT153)	EcoR1 — 2400bp — EcoR1	The N-terminal region of C3 - containing the entire β-chain and a segment of the α-chain.	De Bruijn and Fey (1985)
C4 (pAT153)	BamH1 — 200bp — Hind111	A segment of the C4d region within the α-chain.	Carroll and Porter (1983)
C5 (pBR322)	BamH1 — 940bp — Hind111	The C-terminal region of the β-chain.	Lundwall et al (1985)
Factor B (pAT153)	BamH1 — 540bp — Hind111	Virtually the entire Ba fragment.	Morley and Campbell (1984)
C1-INH (pAT153)	BamH1 — 1250bp — Hind111	The 3' end of the C1-INH gene.	Lappin et al (1989)
Actin (pBR322)	Pst1 — 630bp — Pst1	Muscle actin amino acids of the region AA 162 to the C-term.	Katcoff et al (1980)
β2-microglobulin (pBR322)	Pst1 — 545bp — Pst1	328bp(97%) of the coding region and 217bp of the 3' untranslated region of the mRNA.	Suggs et al (1981)

Table 7 - A summary of the cDNA probes used in the Northern and dot-blot analyses of total RNA isolated from synovial membrane.

2.2 - Preparation of the cloned cDNA inserts

(i) The appropriate restriction enzyme digest was performed on the plasmid at 37°C for 3 hours to remove the cloned cDNA insert from plasmid vector DNA

(ii) Electrophoresis on a 1% low melting point agarose (Bethesda Research Laboratories) gel in the cold room at 50 V for 30 min in 1 x TBE buffer, separated the excised cDNA insert from plasmid vector DNA.

(iii) The cDNA insert was then cut out of the gel, 500µl water were added and the agarose block melted completely at 65°C for 10 min.

(iv) 500µl phenol (equlibrated with TE buffer) were added and after mixing, the sample was stored on ice for 15 min and then centrifuged at 10000rpm for 5 min (all centrifugations in this procedure were performed at room temperature in an Eppendorf microcentrifuge).

(v) 500µl chloroform:isoamyl alcohol was added to the aqueous layer and mixed well. The sample was then stored on ice for 5 min and centrifuged at 10000rpm for 5 min.

(vi) 1/10 volume 3M sodium acetate pH6.8 and 2 volumes 100% ethanol were added to the aqueous layer and mixed well. The cDNA insert was then precipitated overnight at -20°C.

(vii) After centrifugation at 10000rpm for 10 min, the cDNA insert pellet was washed sequentially in 70% and absolute ethanol, freeze dried and resuspended in TE buffer to give a DNA concentration of 5ng/µl.

2.3 Recloning of the cDNA inserts into pGem 1 plasmid vector

Several of the cDNA inserts (C1-INH, factor B, C2, C4 and C5) were recloned into the pGem-1 (Promega Biotec) transcription vector. The 2.9 kb pGem-1 vector was constructed using the bacteriophage SP6 promoter-containing plasmid pSP64 (Melton et al., 1984) and a

bacteriophage T7 promoter. Each plasmid was digested with BamH1 and Hind111. The pGem-1 vector was also digested with BamH1 and Hind111 and then treated with 1 unit of phosphatase enzyme for 1 hour at 37°C to prevent religation of the plasmid vector. The ligation reaction was performed in the following way:

(i) 10µl (0.3µg) cDNA insert was added to 1µl phosphatased-digested (0.1µg) pGem-1

(ii) To this mixture the following were added: 2µl DNA ligase, 2µl 10 x ligation buffer and 4µl 3mM ATP. The volume was then made up to 20µl with water and incubated at room temperature for 48 hours.

(iii) The resultant plasmid was used to transform JM101 cells as described above.

3. Radioactive labelling of the cloned cDNA inserts

As described above, cDNA inserts were excised by restriction enzyme digestion and isolated by electrophoresis through a 1% low melting point agarose gel. In all cases radioactive labelling of the cDNA probes was performed using the Random Primed Labelling Kit (Boehringer Mannheim) and high specific activity (3000Ci/mmol) α-^{32}P-dCTP (Amersham International). 25ng DNA (denatured by boiling for 10 min then cooling rapidly on ice) was added to a mixture of 1µl 0.5mM dATP, 1µl 0.5mM dGTP, 1µl 0.5mM dTTP and 2µl reaction mixture (hexanucleotide mixture in 10 x concentrated reaction buffer). 5µl (50µCi) α-^{32}P-dCTP were added to this mixture and the volume made up to 19µl with water. 1µl DNA polymerase (Klenow enzyme; Boehringer Mannheim) (2 units/µl) was introduced into the sample which was then incubated at 37°C for 30 min. The unincorporated deoxyribonucleosides were removed by gel filtration on a BioGel (A1.5m) column. Before hybridisation, the radioactive cDNA probe was denatured by boiling for 15 min and cooling rapidly on ice.

4. Collection of synovial tissue specimens for RNA analysis

Specimens of synovial tissue were collected at the time of surgery from 20 patients with rheumatoid arthritis, 23 with osteo-arthritis and 5 other patients, 1 with chondrocalcinosis, 2 with psoriatic arthritis and 2 with ankylosing spondylitis. In addition, normal synovium was obtained from a patient who underwent patellectomy for recurrent patellar dislocation. In all but 7 patients (four elbow and three shoulder) the knee or hip joints were the source of synovium. Immediately after collection the tissue specimens were snap-frozen in liquid nitrogen and stored at -70°C for analysis. Tissue fragments were removed from 12 of these synovium specimens immediately after surgery, for use in organ culture studies (see Materials and Methods, section 7).

5. Preparation of RNA

Total RNA was prepared much as described (Chirgwin et al, 1979; Birnie and Graham, 1984), with all solutions being pre-treated with diethylpyrocarbonate (DEPC). 300mg of synovial tissue was pulverised under liquid nitrogen and transferred to 20ml of 5M guanidinium thiocyanate (Bethesda Research Laboratories), pH7.0, containing 5% (v/v) mercaptoethanol and 50mM Tris/50mM EDTA. After sonication, 2ml of a 20% (w/v) solution of sodium-N-lauroyl sarcosine was added and the mixture was heated to 65°C for 2 min before cooling to room temperature. Following centrifugation (35000rpm for 40 hours at 15°C, using a 6x14ml rotor in a Centrikon T-2070 ultracentrifuge) over 2.5 ml of 5.7M CsCl containing 50mM EDTA (pH7.0, refractive index 1.3995). The RNA pellet was resuspended in 200µl of water and precipitated overnight at -20°C using 1/10 volume 0.3M sodium acetate (pH7.0) and 2.5 volumes of 100% ethanol. The pellet was then washed sequentially in 70% and 100% ethanol

and resuspended in 500µl of TE buffer. The OD_{260} and OD_{280} were measured and when necessary, contaminating protein was removed by a phenol/chloroform extraction (Maniatis, Fritsch & Sambrook, 1982).

6. Northern and dot-blot analyses

6.1 - Northern blot analysis:

Mini-gels (50ml) were used for Northern blot analysis. The gels were prepared by melting 0.5g agarose (Bethesda Research Laboratories) in 43.5ml water. When the molten agarose had cooled to approximately 50°C, 5ml 10 x MOPS and 2.6ml 37% (v/v) formaldehyde were added. The molten agarose was poured into the gel mould and allowed to set at room temperature. Total RNA (20µg) was resuspended in 8µl of freshly prepared sample buffer: 750µl formamide (Fluka), 150µl 10 x MOPS, 240µl formaldehyde, 100µl water, 100µl glycerol and 80µl (10% w/v) bromophenol blue, and denatured at 65°C for 15 min, then rapidly cooled on ice. Before loading, ethidium bromide (1µl of a 1mg/ml solution) was introduced into each sample. Electrophoresis was performed at 100 V for 90 min at room temperature in 1 x MOPS, pH 7.0. Immediately after electrophoresis, the gels were visualised on a UV transilluminator. The gels were then blotted onto Hybond-N nylon membrane (Amersham International) using 20 x SSC for at least 24 hours after which time the filter was fixed with UV light for 10 mins and the gels viewed for efficiency of transfer on a UV transilluminator.

6.2 - Prehybridisation and hybridisation

The prehybridisation and hybridisation buffer was composed of 62.5ml 20 x SSPE, 12.5ml 100 x Denhardt's solution, 5ml of a 1mg/ml solution of salmon sperm DNA, 12.5ml 10% (w/v) SDS and 125ml formamide,

made up to 250ml with water. All prehybridisation and hybridisation reactions were performed overnight at 42°C. Hybridisation was performed with 1 x 10^6 counts/min of ^{32}P-labelled probe per ml, and all filters were washed sequentially in 2 x SSC, 0.5 x SSC and finally in 0.1 x SSC, each containing 0.1% (w/v) SDS. Each wash was performed at 65°C and lasted for 30 min. Filters were stripped for rehybridisation by boiling in 0.1% (w/v) SDS for 20 min.

Autoradiography was performed at -70°C using Kodak X-Omat AR film for 1-15 days with an intensifying screen.

6.3 - Dot-blot analysis

Total RNA (10µg) dissolved in 2.5µl water was applied as a single drop onto Hybond-N nylon membrane, dried in air and fixed with UV light for 10 min. Duplicate dots of each specimen were tested. Prehybridisation, hybridisation and autoradiography were performed as described for Northern blot analysis. Autoradiographs were scanned using a densitometer (AutoScanner, Helena Laboratories). Results were standardised using a rat actin cDNA probe (approximately 80% homologous to human actin) to control for the amount of RNA loaded onto the dot blots. Yeast tRNA was used as a negative control on all dot blots.

7. Synovial tissue explant cultures

Synovial tissue specimens were collected at the time of surgery from a total of 12 patients (3 with rheumatoid arthritis, 8 with osteoarthritis and one undergoing patellectomy for recurrent patellar dislocation) and transported to the laboratory in transport medium. The tissue was then washed thoroughly in Dulbecco's calcium and magnesium free phosphate buffered saline (1 x PBS) (Gibco). Each culture was set

up with fragments of tissue from different areas of the specimen, with 40-80mg tissue used per culture. The tissue was cultured in 1.5ml of Dulbecco's Modified Eagle Medium (Gibco) with 10% heat inactivated (2 hours at 56°C) fetal calf serum (Gibco) (DMEM.FCS) at 37°C in a humidified atmosphere consisting of 5% CO_2 in air. The culture medium was changed daily for 6 days. The culture supernatants were divided into aliquots and stored at -70°C for analysis. Six cultures were set up for each patient, with two cultures being treated with 2.5μg/ml cyclo-heximide to inhibit protein synthesis. Complement synthesis was expressed as μg complement component produced per 100mg tissue.

8. Enzyme linked immunosorbent assays (ELISAs)

Double antibody sandwich ELISAs were used to measure the synthesis of complement components by cultured fragments of human synovial membrane. The IgG fraction of the goat anti-human C1-INH, factor B, C4, C3, C5 (Atlantic Antibodies) and C2 (SciPac) antibodies were used. One complement component was studied per ELISA plate and the procedure outlined below was followed:

(i) Each well of the ELISA plate [either Dynatech Immunolon A (C1-INH, factor B, C3 and C5) or Nunc (C2 and C4)], was coated with 100μl of the antibody diluted in coating buffer. The concentration of each antibody is listed below:

anti-C1-INH	10μg/ml	anti-C3	5μg/ml
anti-factor B	5μg/ml	anti-C4	2.5μg/ml
anti-C2	10μg/ml	anti-C5	5μg/ml

The plates were then covered and incubated at 4°C overnight.

(ii) Each well was washed five times with PBS containing 0.05% (v/v) Tween (PBST) using a Dynatech plate washer, which was used for washes at every step. Blocking buffer (250µl) was then added to each well and the plates were incubated in a moist atmosphere at room temperature for 1 hour.

(iii) Each well was washed five times in PBST and then 100µl of a 1:5 dilution (in PBST) of each culture supernatant was added to duplicate wells. A pool of normal human serum which contained known concentrations of the complement components under investigation, was used as a standard. The pool was diluted serially in PBST and 100µl of each dilution was added to a duplicate series of wells. The following range of complement component concentrations was used to plot the standard curves:

C1-INH (60ng/ml to 0.06ng/ml)	C3 (299ng/ml to 0.3ng/ml)
factor B (412ng/ml to 0.4ng/ml)	C4 (12ng/ml to 0.01ng/ml)
C2 (800ng/ml to 0.8ng/ml)	C5 (75ng/ml to 0.07ng/ml)

Negative controls used on each plate were 100µl of PBST and 100µl of DMEM.FCS, both of which were added in duplicate.

After addition of the samples the plates were incubated in a moist atmosphere at room temperature for 2 hours.

(iv) Each well was washed five times in PBST and then 100µl of biotin conjugated antibody (Bonnard, Papermaster & Krachenbuhl, 1986) were added to each well. Each conjugate antibody* was diluted as follows;

C1-INH	1/1600	C3	1/1000
factor B	1/2000	C4	1/10000
C2	1/2000	C5	1/750

* These conjugates were prepared in our laboratory by Drs E. Holme & G. Plummer.

The plates were then incubated in a moist atmosphere at room temperature for 1 hour.

(v) Each well was washed five times in PBST and then 100µl of a 1/5000 dilution (in PBST) of avidin-linked horseradish peroxidase (Vector Laboratories) were added to each well. The plates were then incubated in a moist atmosphere at room temperature for 1 hour.

(vi) Each well was washed five times in PBST and then 100µl of peroxide substrate was added to each well. The plates were left in the dark until the colour reaction had developed sufficiently and the reaction was then stopped by the addition of 25µl 4N sulphuric acid. The intensity of colour in each well was then measured on a microplate reader (Dynatech, MR700 microplate reader).

A standard curve was constructed for each plate by plotting the optical density readings of each standard against the concentration of the complement component in the standard. The concentration of each complement component in the culture supernatants was determined by interpolation of the standard curve. A comparison of the levels of complement found in the cycloheximide-treated cultures with the untreated cultures made it possible to determine that the complement components detected in the medium had been synthesised by the tissue and not simply released into the medium as a result of contamination with blood or inflammatory exudate.

In addition to the ELISAs for the complement components an ELISA to detect albumin was also performed (on Dynatech Immunolon A ELISA plates) as a control for protein being released into the medium as a result of contamination with blood or inflammatory exudate. The procedure used was the same as described above except for two steps;

(1) the blocking buffer used was PBST

(2) the second antibody was conjugated directly to the horseradish peroxidase enzyme and hence the biotin-avidin binding step was omitted.

The concentrations of antibody, culture supernatants, standards and conjugate antibody used were as follows;

(1) anti-albumin antibody 5µg/ml (in coating buffer)

(2) culture supernatants diluted 1:20

(3) standard range 500ng/ml to 2ng/ml

(4) conjugate antibody diluted 1/2000 (concentration of stock = 1mg/ml)

All dilutions were performed in PBST.

9. Haemolytic assays of complement components

Haemolytic assays were performed on the synovial membrane culture supernatants for C2, C3, C4, C5 and factor B in order to determine that the complement components detected by ELISA were functionally active. The haemolytic assays were performed as described by Whaley, (1985) using erythrocytes as the target. The cell suspensions were standardised by lysing 100µl of suspension in 2.9ml of water and measuring the optical density of the released haemoglobin in lysates. Oxyhaemoglobin has three major light absorption peaks at 576, 541 and 414 nm. For the standardisation of erythrocyte suspensions used in complement assays, the optical densities of cell lysates were determined at either 541 nm (for suspensions of 5 x 10^8 cells/ml or 1 x 10^9 cells/ml) or 414 nm (for suspensions of 1 x 10^8 cells/ml or 2 x 10^8 cells/ml) (see Table 8).

ERYTHROCYTE	CONCENTRATION	OPTICAL DENSITY
SHEEP	1 x 10^9/ml	0.385 (541 nm)
	5 x 10^8/ml	0.192 (541 nm)
	2 x 10^8/ml	0.654 (414 nm)
	1 x 10^8/ml	0.327 (414 nm)
RABBIT	1 x 10^8/ml	0.294 (414 nm)

Table 8 - Standardisation of erythrocyte suspensions. The optical density is measured on lysate prepared by the addition of 100µl suspension to 2.9ml water.

9.1 - Preparation of cells for haemolytic assays

(a) Sheep erythrocytes, E

Sheep blood was drawn aseptically into an equal volume of sterile Alsever's solution. The cells were stored in Alsever's solution at 4°C for up to two weeks.

(b) Antibody-sensitised sheep erythrocytes, EA

(i) Sheep erythrocytes, E were washed in 10mM EDTA.GVB^{2-} and resuspended to 1 x 10^9/ml in the same buffer.

(ii) Antibody to sheep E was diluted in 10mM EDTA.GVB^{2-} and prewarmed to 37°C as were the cells.

(iii) The antiserum was then added to the erythrocyte suspension and the mixture incubated at 37°C for 30 min in a shaking water bath.

(iv) The antibody-sensitised cells were washed once in 10mM EDTA.GVB^{2-}, twice in GVB^{2+} and resuspended to their original volume in GVB^{2+}. The cells were stored at 4°C for up to one week.

The dilution of antiserum used in the preparation of EA was the minimum dilution which did not agglutinate an equal volume of sheep E at a concentration of 1 x 10^9/ml.

(c) EAC4 cells (Borsos & Rapp, 1967)

(i) 500ml EA cells were washed and resuspended to 5 x 10^8/ml in $DGVB^{2+}$ and prewarmed to 30°C.

(ii) C1 (400units/ml) (Lachmann et al., 1973) was added slowly to the EA suspension which was shaken continuously. After 15 min at 30°C, the EAC1 cells were centrifuged (2000rpm for 5 min at 2°C) and resuspended in 500ml ice-cold $DGVB^{2+}$ and cooled to 0°C.

(iii) 100ml fresh human serum was mixed with 400ml 10mM EDTA.GVB^{2-} and

cooled to 0°C. When the serum and EAC1 had been cooled to 0°C, they were mixed and shaken continuously at 0°C for 15 min. The EAC4 were washed three times in 10mM EDTA.GVB^{2-}, then incubated at 37°C in 200ml 10mM EDTA.GVB^{2-} to decay off any C2 which may have been incorporated into C4b2a. The EAC4 were washed once in GVB^{2+} and stored at 4°C. Alternatively the EAC4 could be stored frozen at -70°C.

(d) Freezing EAC4 cells

(i) EAC4 cells (50% suspension in $DGVB^{2+}$) and glycerol buffer$_A$ (pH 6.8) were prewarmed to 37°C.

(ii) The glycerol buffer was added (dropwise) to the cells in three aliquots and the cells were shaken at 37°C for 10 min after each addition. The sizes of the aliquots were 0.15, 0.45 and 1.2 times the initial volume of the 50% suspension. The cells were dispensed into 0.8ml aliquots, snap-frozen and stored at -70°C.

(e) Reconstitution of EAC4 cells

(i) An aliquot of EAC4 cells was thawed at 37°C and D50S, mannitol:GVB^{2+} and $DGVB^{2+}$ were prewarmed to 37°C.

(ii) 230µl D50S was added to the EAC4 cells and incubated for 5 min at 37°C.

(iii) Mannitol:GVB^{2+} was added in three aliquots; 1, 2 and 8ml. Between the first and second and second and third aliquots, the cells were incubated at 37°C for 2 min. Following the addition of the third aliquot, the cells were incubated at 37°C for 5 min.

(iv) $DGVB^{2+}$ was added in four aliquots, the first being 5ml and the remaining three, 10ml. Following the addition of the first three aliquots, the cells were incubated at 37°C for 2 min but after the

fourth aliquot the incubation time at 37°C was 5 min.

(v) The cells were then centrifuged (2000rpm for 10 min at 2°C), washed three times in $DGVB^{2+}$ and resuspended in $DGVB^{2+}$ to give a final concentration of $1x10^8$ cells/ml.

(f) $EAC\overline{14}$ cells

(i) EAC4 cells were washed three times in $DGVB^{2+}$ and resuspended to a concentration of $1x10^8$ cells/ml in $DGVB^{2+}$. The cells were then prewarmed to 37°C.

(ii) Complement component C1, at an initial concentration of 100 units/ml in $DGVB^{2+}$ was prewarmed to 37°C.

(iii) Equal volumes of EAC4 cells and C1 were mixed together and incubated for 15 min at 37°C.

(iv) The $EAC\overline{14}$ cells were then centrifuged (2000rpm for 5 min at 2°C) and the supernatant was discarded. The cells were resuspended in $DGVB^{2+}$ to a concentration of $1x10^8$ cells/ml and were used immediately.

T_{max} test - used to measure the functional activity of C4b on $EAC\overline{14}$. A batch of EAC4 is accepted if t_{max} is less than 6 min.

2ml EAC4 ($1x10^8$/ml in $DGVB^{2+}$) were converted to EAC14 and resuspended in 2ml $DGVB^{2+}$. $EAC\overline{14}$ and 2ml $DGVB^{2+}$ containing C2 (1unit/ml) were prewarmed to 30°C in a shaking water bath. After 5 min, the C2 and $EAC\overline{14}$ were mixed and at 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7.5, 10 and 15 min, 200µl of cells were transferred to test tubes containing 300µl C^{rat}-$EDTA_3$ (rat serum diluted 1/15 in 40mM EDTA.GVB^{2-}) and incubated at 37°C for 1 hour. Saline (2ml) was then added to each tube (except 100% lysis to which was added 2ml water) after the one hour incubation and the tubes were centrifuged at 2000rpm for 5 min at 2°C. The haemoglobin released into the supernatants was measured

spectrophotometrically (OD_{414}).

Controls:	$DGVB^{2+}$	$EAC\overline{142}$	$EAC\overline{14}$	Ca^{++}-EDTA
Cell blank	300µl	200µl	-	-
Reagent blank	100µl	-	100µl	300µl
100% lysis	-	200µl	-	300µl
Complement colour	200µl	-	-	300µl

(EAC142 at 5 x 10^7/ml and EAC14 at 1 x 10^8/ml)

(g) $EAC\overline{14^{oxy}2}$ cells

C2 was oxidised by mixing equal volumes of C2 (50 units/ml) and oxidising reagent and incubating at room temperature for 5 min until the solution paled. (Polley & Muller-Eberhard, 1967)

Oxidised C2 was prepared fresh each time it was required. The oxidation of SH groups in the C2 molecule increases the haemolytic activity of the molecule and stabilises the C4b2a enzyme by preventing the decay of C2a from the complex.

(i) $EAC\overline{14}$ (1 x 10^8/ml in $DGVB^{2+}$) and an equal volume of $DGVB^{2+}$ containing oxidised human C2 ($C^{oxy}2$) at 50 units/ml were prewarmed to 30°C.

(ii) The $EAC\overline{14}$ cells were mixed with the oxidised C2 and incubated at 30°C for 15 min. The $EAC\overline{14^{oxy}2}$ were resuspended to 1 x 10^8/ml in $DGVB^{2+}$ and used immediately.

(h) EAC43b cells

(i) EA cells were washed three times in GVB^{2+} and resuspended to a concentration of 2 x 10^8 cells/ml in GVB^{2+}.

(ii) 1ml R3 (see below) and 19ml EA cells were prewarmed to 37°C, mixed rapidly and incubated at 37°C for 75 seconds.

(iii) Antrypol was added to a concentration of 1mg/ml and left for 2 min at 37°C to stop the reaction.

(iv) The cells were centrifuged at 2000rpm for 5 min at 2°C.

(v) The cells were then resuspended in 20ml 10mM EDTA:GVB^{2-} and incubated for 2 hours at 37°C in a shaking water bath.

(vi) The cells were washed twice in 10mM EDTA:GVB^{2+}, twice in $DGVB^{2+}$ and resuspended to a concentration of 1 x 10^8 cells/ml in $DGVB^{2+}$. These EAC43b cells were stored at 4°C for up to 2 weeks.

Preparation of R3:

(i) Zymosan (10mg/ml) was washed three times in 1xPBS.

(ii) 1ml packed zymosan was added to 10ml normal human serum (to give a final zymosan concentration of 1mg/ml) and then incubated at 37°C for 1 hour with regular mixing.

(iii) After centrifugation to remove the zymosan pellet, the supernatant (R3) was kept and stored in aliquots at -70°C.

Several different concentrations of zymosan were tested: 0.5mg/ml, 1mg/ml, 5mg/ml and 10mg/ml. The concentration of zymosan used in the preparation of R3 was the dilution immediately preceding that which produced 5-10% lysis.

Titration of R3: (see figure 7)

(i) The following dilutions of each R3 were made in GVB^{2+};

1/10, 1/20, 1/40, 1/80 and 1/160.

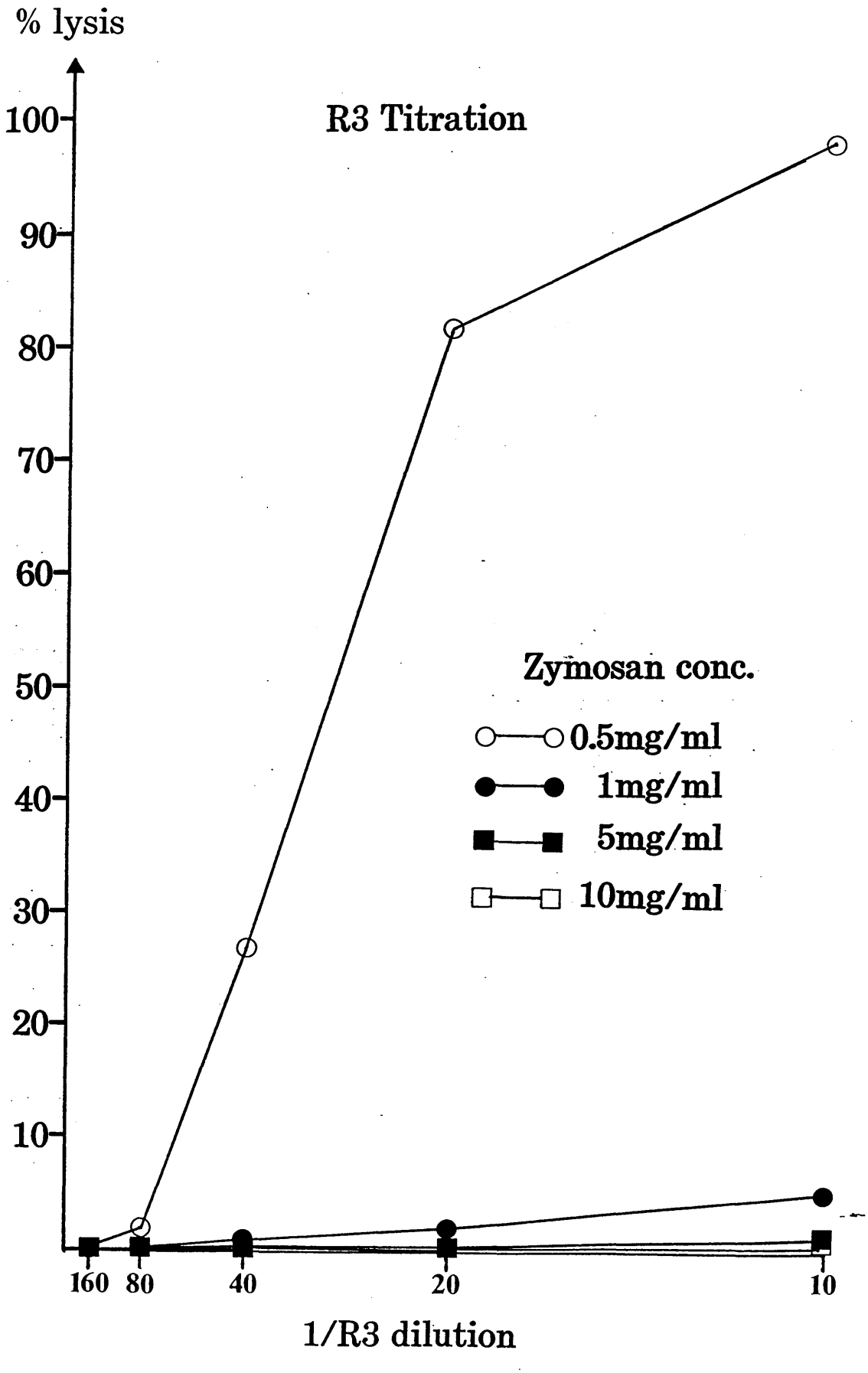
(ii) 100µl of each of the above dilutions were added to 100µl EA (2x10^8 cells/ml) and incubated for 1 hour at 37°C in a shaking water bath.

Controls:

	GVB^{2+}	EA
Cell blank	100µl	100µl
100% lysis	100µl	100µl

(iii) The reaction was stopped with 2ml saline (0.9% sodium chloride in

Figure 7 - *Titration of R3 reagent. A 1/20 dilution of the 1mg/ml zymosan preparation was used.*


% lysis
R3 Titration
100
90
80
70
60
50
40
30
20
10
Zymosan conc.
0.5mg/ml
1mg/ml
5mg/ml
10mg/ml
160
80
40
20
10
1/R3 dilution

distilled water), except 100% lysis to which was added 2ml water), the assays centrifuged (2000rpm for 5 min at 2°C) and the OD_{414} measured.

(i) Rabbit erythrocytes, E^{rabbit}

Rabbit blood was obtained fron the marginal ear vein. The blood was collected in an equal volume of Alsever's solution and stored at 4°C.

9.2 - Protocols for haemolytic assays

In all cases, the assays were stopped by the addition of 2ml saline to each tube (except 100% lysis to which was added 2ml water). The tubes were then centrifuged (2000rpm for 5 min at 2°C) and then measured the OD_{414} to determine the haemoglobin release.

(a) C2 assay

Reagents:

(i) $EAC\overline{1}4$ cells at $1x10^8$ cells/ml in $DGVB^{2+}$

(ii) C2 standard diluted in $DGVB^{2+}$ from 1/5000 to 1/80000.

(iii) C^{rat} (rat serum) diluted 1/15 in 40mM EDTA/GVB^{2-}

Procedure:

(i) 100µl culture supernatant diluted 1/4 in $DGVB^{2+}$ was added to assay tubes. 100µl C2 standard dilutions were also added to a series of assay tubes.

(ii) The assay tubes, $EAC\overline{1}4$ cells and C^{rat} were prewarmed to 30°C.

(iii) 100µl $EAC\overline{1}4$ cells were added to each assay tube (except complement colour, CC) and incubated for 2 min at 37°C.

(iv) 300µl C^{rat} was added to each tube (except cell blank, CB) and incubated for 1 hour at 37°C, after which the assay was stopped.

Controls:

	$DGVB^{2+}$	$EAC\overline{14}$	C^{treat}
Reagent blank, RB	100µl	100µl	300µl
Cell blank, CB	400µl	100µl	-
Complement colour, CC	200µl	-	300µl
100% lysis	100µl	100µl	300µl

Note: a medium blank (MB) was also set up for all assays using the same dilution of DMEM.FCS as the sample dilution.

(b) C4 assay

Reagents:

(i) EA cells at $1x10^8$ cells/ml in $DGVB^{2+}$

(ii) C4-deficient guinea pig serum diluted 1/50 in $DGVB^{2+}$

(iii) Pooled human serum standards with doubling dilutions in $DGVB^{2+}$ from 1/8000 to 1/1024000

Procedure:

(i) 100µl culture supernatant diluted 1/4 in $DGVB^{2+}$ were added to the assay tubes, followed by 100µl C4-deficient guinea pig serum and 100µl EA cells.

(ii) The assay was then incubated for 1 hour at 37°C in a shaking water bath, after which the assay was stopped.

Controls:

	$DGVB^{2+}$	C4 deficient guinea pig serum	EA cells
Reagent blank	100µl	100µl	100µl
Cell blank	200µl	-	100µl
Complement colour	200µl	100µl	-
100% lysis	100µl	100µl	100µl

(c) C3 assay

Reagents:

(i) EAC$\overline{14^{cxy}2}$ cells at 1x10^{8} cells/ml in $DGVB^{2+}$.

(ii) $DGVB^{2+}$ containing C5, C6 and C7 (Cordis reagents), each at a concentration of 100units/ml.

(iii) $DGVB^{2+}$ containing C8 and C9 (Cordis reagents), each at a concentration of 50units/ml.

(iv) Serum standards in doubling dilutions from 1/25 to 1/8000 in $DGVB^{2+}$.

Procedure:

(i) The culture supernatants were diluted 1/2 in $DGVB^{2+}$ and 100µl were transferred to a series of assay tubes.

(ii) 100µl $DGVB^{2+}$ containing C5, C6 and C7 and 100µl EAC$\overline{14^{cxy}2}$ cells were added to each tube and incubated for 30 min at 37°C in a shaking water bath to form EAC1-7.

(iii) 100µl $DGVB^{2+}$ containing C8 and C9 were then added and incubation was continued at 37°C for a further hour, before the assay was stopped.

Controls:

	$DGVB^{2+}$	C5,C6,C7	EAC$\overline{14^{cxy}2}$	C8,C9
Reagent blank	100µl	100µl	100µl	100µl
Cell blank	300µl	-	100µl	-
100% lysis	100µl	100µl	100µl	100µl

(d) C5 assay

Titration of C5 followed the same procedure as for C3 but different Cordis reagents were used in the initial mixture ie. $DGVB^{2+}$ containing C3, C6 and C7 (instead of C5,C6,C7) each at a concentration of 100units/ml.

(e) Titration of D activity for use in the factor B assay

The D used initially for these studies was contaminated with factor B which meant it could not be used in factor B assays. As a result more D was purified from human serum (see below). When the D was removed from the serum by Biorex 70 cation-exchange chromatography, the exclusion peak represented serum that contained no D and hence was termed the RD reagent. In order to titrate the purified D, reconstitution of the RD reagent was used in the following assay.

Reagents:

(i) E^{rabbit} cells at $1x10^8$ cells/ml in Mg-EGTA.

(ii) The RD reagent diluted 1/5 in Mg-EGTA.

Procedure:

(i) The purified D was serially diluted from 1/5 to 1/5120 in Mg-EGTA and 50µl of each dilution was added to a series of assay tubes that contained 50µl of RD reagent.

(ii) 100µl E^{rabbit} cells were added and the assay incubated at 37°C for 30 min in a shaking water bath, after which the assay was stopped.

Controls:

	Mg-EGTA	E^{rabbit}
Reagent blank	100µl	100µl
100% lysis	100µl	100µl

(f) Factor B assay

Reagents:

(i) EAC43b cells at $1x10^8$ cells/ml in $DGVB^{2+}$

(ii) D (19.3 units/ml) diluted 1/40 in $DGVB^{2+}$

(iii) C^{rat} diluted 1/15 in 40mM EDTA.GVB^{2-}

Procedure:

(i) 50µl culture supernatant diluted 1/2 in $DGVB^{2+}$ were added to a series of assay tubes containing 50µl D (0.5 units/ml).

(ii) 100µl EAC43b cells were added to each tube which were then incubated for 30 min at 37°C in a shaking water bath.

(iii) 300µl Cr^{mt} EDTA were added and incubation was continued at 37°C for a further hour, after which the assay was stopped.

Controls:

	$DGVB^{2+}$	D	EAC43b	Cr^{mt} EDTA
Reagent blank	100µl	-	100µl	300µl
Cell blank	400µl	-	100µl	-
D alone	50µl	50µl	100µl	300µl
Complement colour	200µl	-	-	300µl
100% lysis	100µl	-	100µl	300µl

9.3 - Calculation of haemolytic assay results

In each of the above assays, the amount of lysis was measured by reading the optical density of the sample at 414 nm. To convert the optical density measurement into per cent lysis the following calculations were performed:

$$y = \frac{\text{OD sample} - \text{OD reagent blank}}{\text{OD 100 \% lysis} - \text{OD reagent blank}}$$

$$\text{Per cent lysis} = 100 \times y$$

When y is plotted against serum dilution (following the haemolytic titration of complement components e.g. D above), the curve is concave to the x-axis. The shape of the curve led Mayer and his colleagues (Borsos et al., 1961) to postulate that complement-mediated

lysis was due to a single effective hit by a complement molecule (one-hit theory). Thus, if the number of effective molecules offered per cell = Z then the proportion of unlysed cells (cells with nil hits) will be e^{-z}.

Therefore,

$$-Z = \text{In } (1-y)$$

$$\text{or } Z = -\text{In } (1-y)$$

In all the above component assays, 100µl of sample dilution and 100µl of cell suspension (containing 1 x 10^6cells/ml ie. 10^7 cells/tube) were used.

(a) Calculation of the haemolytic activity of complement components in the synovial membrane culture supernatants

The number of effective molecules per 100mg tissue was calculated by:

$$Z \text{ value} \times \frac{100}{\text{weight of tissue}} \times \frac{\text{total volume of culture}}{\text{sample volume used in assay}}$$

The resultant haemolytic activity was expressed as:

effective molecules (e.m.) x 10^7/100mg tissue

(b) Haemolytic titration of complement components e.g. D

When haemolytic titration of a component is performed (e.g. D, see below), Z is plotted against the concentration of that particular component. A straight line, passing through the origin is obtained. When Z = 1, then -In (1-y) = 1, and y = 0.632. In other words, 63.2% of the cells have been lysed. To calculate the number of effective molecules of a component in a given volume, a vertical line is drawn from the point on the graph which passes through Z = 1 to the x-axis. The number

of units of component were calculated by the:

$$\frac{\text{distance along x-axis to initial dilution}}{\text{distance along x-axis to Z = 1}} \times \text{initial dilution}$$

Therefore, the concentration of effective molecules = units x 10^7/ml, which is usually expressed as units/ml.

10. Partial purification of D from human serum

D was purified from human serum as described by Tenner, Lesavre & Cooper, (1981).

Ion exchange chromatography

Biorex-70 cation exchange chromatography was performed at 4°C. Using a 2.6cm x 80cm column which had been equilibrated with starting buffer (50mM phosphate buffer, pH 7.3 containing 82mM NaCl, 2mM EDTA). Human serum (180ml) was loaded onto the column at a flow rate of 10ml/hour and 10ml fractions were collected. Following the application of the sample, the column was washed with starting buffer at a flow rate of 50ml/hour until the protein content (OD_{280}) of the eluate was zero. The fractions of the exclusion peak were pooled and retained for use as an RD reagent for the D haemolytic assay (see above).

The column was then eluted using a gradient consisting of 300ml starting buffer and 300ml starting buffer containing 300mM NaCl at a flow rate of 30ml/hour. Fractions were screened for OD_{280} and D haemolytic activity by their ability to reconstitute the haemolytic activity of the RD reagent. Fractions containing D activity were pooled and concentrated using an Amicon YM-10 filter.

11. Incorporation of ^{35}S-methionine into complement proteins

11.1 - Synovial tissue explant cultures

Synovial tissue explant cultures were set up as described above but DMEM containing 10% (v/v) fetal calf serum and methionine at a concentration of 6μg/ml was used. After the addition of 500 μCi ^{35}S-methionine (Amersham International) the cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 hours. The medium was removed, treated with protease inhibitors (5mM PMSF, 10mM benzamidine and 10mM EDTA) and stored at -70°C prior to analysis.

11.2 - Measuring acid-precipitable counts

GF/A filters (Whatman) were soaked in 2mM unlabelled methionine for 10 min and then air dried. Culture supernatant (5μl) was dotted on the filter, which was then subjected to the following washing procedure;

(i) 10% trichloroacetic acid (TCA) for 1 min

(ii) 5% TCA for 1 min

(iii) 100% acetone for 1 min

(iv) 50% acetone for 1 min

(v) 50% ethanol for 1 min

(vi) 100% ethanol for 1 min

The filters were then air dried and the radioactivity measured on a scintillation counter using 2.5ml scintillation fluid (Ecoscint). The percentage incorporation of ^{35}[S]-methionine into acid-precipitable protein was calculated.

11.3 - Immunoprecipitation of ^{35}S-methionine labelled complement components

(i) Formalin fixed *Staph. aureus* protein A was washed three times in PBS

containing Triton X100 (1% w/v). Following the final wash, 100µl of packed *Staph. aureus* protein A was added to each culture supernatant (1ml) as a preabsorption step to remove excess immunoglobulin and immune complexes. The samples were incubated at room temperature for 1 hour on a rotating mixer and then centrifuged at 6000rpm for 2 min. The pellets were discarded and the supernatants transferred to fresh tubes.

(ii) The supernatants were diluted 1/10 in solution A and split into ten 1ml aliquots.

(iii) 10µl IgG fraction of the following antibodies at 1mg/ml (in PBS) were added to the appropriate culture supernatant aliquot:

anti-C1-INH	anti-C4
anti-factor B	anti-C5
anti-C2	anti-human serum albumin
anti-C3	

The samples were then incubated overnight at 4°C.

(iv) 20µl packed *Staph. aureus* protein A was added to each sample which were then incubated at room temperature for 1 hour on a rotating mixer.

(v) The samples were centrifuged (6000rpm for 2 min at room temperature) and the supernatants were removed and stored at -70°C.

(vi) The pellets were washed five times in PBS containing 0.1% (w/v) SDS and 1% (v/v) Triton and then the complexes were eluted with solution B (50µl) for 15 min at 37°C.

(vii) The radioactivity of the samples was determined using a scintillation counter to ensure that the same number of counts/min were loaded onto each track for SDS-polyacrylamide gel electrophoresis.

11.4 - SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The percentage of gel used in SDS-PAGE was determined by the

size of the proteins being studied - larger proteins require lower percentage gels. All the proteins being investigated had molecular weights of approximately 50-100 kDa and 10% reducing gels were used.

Reagents:

(i) 10% SDS

(ii) 0.75 M Tris

(iii) 0.75M Tris.HCl pH6.8

(iv) 0.75M Tris.HCl pH8.8

(v) 30% acrylamide, 0.8% bis-acrylamide

Procedure:

(i) The glass plates and spacers were cleaned and greased with isobutanol and the following 10% separating gel was poured: 10ml 30% acrylamide, 4.7ml water, 15ml 0.75M Tris.HCl pH8.8, 300µl 10% SDS, 30mg ammonium persulphate (Sigma) and 15µl TEMED (Sigma).

(ii) Once the separating gel had polymerised the following stacking gel was poured: 1.8ml 30% acrylamide, 13ml water, 3ml 0.75M Tris.HCl pH6.8, 180µl 10% SDS, 18mg ammonium persulphate and 9µl TEMED.

(iii) 5µl bromophenol blue was added to each sample which were boiled 2 min before loading. The sizes of the proteins were determined by including a series of radioactively-labelled (^{14}C) proteins of known molecular weights (Sigma); 14 kDa, 30 kDa, 46 kDa, 69 kDa, 92.5 kDa and 200 kDa. The gels were run at 50 V for 3 hours.

(iv) The gels were stained (0.1% (w/v) Coomassie blue (Sigma), 50% methanol, 10% acetic acid) for one hour and destained (10% methanol, 5% acetic acid) overnight.

(v) The gels were impregnated with 1M salicylic acid (Sigma) for 1 hour and dried for 3 hours. Fluorography was performed at -70°C with Kodak X-Omat AR film and intensifying screens for 1-4 weeks

12. Isolation of synovial cells from synovial tissue

Synovial cells were isolated from synovial tissue much as described by Dayer et al., (1976). Synovial tissue specimens were collected at the time of surgery from 10 patients (5 with rheumatoid arthritis and 5 with osteoarthritis) and transported to the laboratory in transport medium. The tissue was then washed thoroughly in Dulbecco's calcium and magnesium free 1 x PBS, chopped into small 1-2mm^3 fragments and washed a further three times in 1 x PBS. The tissue was then digested with 25ml Hanks balanced salt solution containing collagenase (1mg/ml) (Type 1, Sigma) and DNase 1 (0.1mg/ml) (Sigma) for 3-4 hours at 37°C in a shaking water bath. Cells and debris were removed by centrifugation at 1500rpm for 10 min in a MSE swing-out centrifuge. The supernatant was discarded and the cells and tissue were resuspended in 50ml 1xPBS containing trypsin (0.5mg/ml) (Type 111, Sigma) and EDTA (0.2mg/ml) and incubated for a further hour at 37°C in a shaking water bath. The cells were separated from the undigested tissue by standing at room temperature for 5 min. The supernatant containing the cell suspension was removed and the cells washed three times in DMEM.FCS. A cell count was taken and after appropriate dilution in DMEM.FCS, the cells were plated onto 35x10mm petri dishes (Nunc) at a cell density of 1x10^6 cells/dish in a volume of 1ml/dish. The cultures were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

After 24 hours, the medium was removed and centrifuged for 5 min at 1500rpm to precipitate the non-adherent cells. The non-adherent cells from each culture were resuspended in 1ml DMEM.FCS and plated onto fresh 35x10mm petri dishes. The adherent cells on the original dishes were supplemented with 1ml DMEM containing 10% fetal calf serum. All cultures

were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Each day the culture medium was removed from the adherent cell cultures which were then supplemented with 1ml DMEM.FCS. The culture medium was also removed daily from the non-adherent cultures and the supernatants were separated from the non-adherent cells by centrifugation (1500rpm for 5 min at room temperature). The supernatants were collected and the cells were resuspended in 1ml DMEM.FCS before being replated onto the same dishes from which they had been removed. In each case, two cultures were treated with 2.5µg/ml cycloheximide which inhibits protein synthesis and therefore can be used as negative control cultures when synthesis of complement components is being investigated. The medium removed from the cultures was stored in aliquots at -20°C prior to analysis.

13. Immunohistochemical analysis of cultured synovial cells

13.1 - Cell fixation

The culture medium was removed from the cells which were then washed three times in 1 x PBS. The cells were fixed in 10% neutral buffered formalin (100ml formaldehyde and 900ml water containing 4g $NaH_2PO_4.H_2O$ and 6.5g Na_2HPO_4 - the pH was adjusted to 7.0 if necessary) for 1 hour at room temperature. The cells were then washed four times in 1 x PBS and if not used immediately were stored in 1 x PBS at 4°C overnight.

13.2 - Immunohistochemistry

(i) The cells, which had been grown on plastic coverslips (Thermanox), were flooded for 10 min with a 1/5 dilution of normal swine serum as a blocking agent.

(ii) The cells were washed thoroughly in 1 x PBS and a predetermined dilution of rabbit antiserum (Hoechst; diluted in a 1/25 dilution of normal swine serum), was added. After incubation for 1 hour at room temperature, the monolayers were washed extensively in 1 x PBS. Normal rabbit serum was used as a negative control for all cells.
(iii) A 1/20 dilution of swine anti-rabbit IgG alkaline phosphatase (Dako-Patts; in 1/25 normal swine serum) was then applied to the cells which were then incubated at room temperature for a further 90 min.

A mouse monoclonal anti-human vimentin antibody (Amersham) was also used as a fibroblast marker and was diluted 1/40 in 5% bovine serum albumin. Rabbit anti-mouse IgG conjugated to alkaline phosphatase (Dako-Patts) at a dilution of 1/20 in 5% BSA was used on the anti-vimentin treated cells.

(iv) After the cells were washed extensively in 1 x PBS they were incubated with the phosphate substrate for 10 min at room temperature.
(v) The cells were sequentially washed in 1 x PBS and tap water and then fixed in 1% neutral buffered formalin (contains 1/10 of the formaldehyde concentration in 10% neutral buffered formalin) for 10 min at room temperature.
(vi) The cells were washed in tap water, counterstained in haematoxylin and alkaline tap water substitute, mounted on slides with Aquamount (BDH) and stored at 4°C until examined by light microscopy.

RESULTS

RESULTS

1. CHARACTERISATION OF PLASMID DNA

After preparation of the cDNA probes used in these studies (see Table 7), it was necessary to characterise each plasmid using the appropriate restriction enzymes. Figures 8 - 12 show the restriction enzyme digests performed on the plasmids.

Figure 8 shows the restriction digests of the pAT153.Pvu11 8.C3 plasmid. The C3 insert (2400bp) was excised from the plasmid vector using the restriction enzyme EcoR1.

Figure 9 shows the restriction digests of the pAT153.Pvu11 8.C2, pAT153.Pvu11 8.factor B and pGem.C1-INH plasmids. The C2 insert (400 bp), factor B insert (540 bp) and C1-INH insert (1250 bp) were each excised from their plasmid vectors with the restriction enzymes BamH1 and Hind 111.

Figure 10 shows the restriction digests of the pAT153.Pvu11 8.C4 and pGem.C5 plasmids. The C4 insert (300 bp) and C5 insert (940 bp) were excised from their plasmid vectors using the restriction enzymes BamH1 and Hind 111.

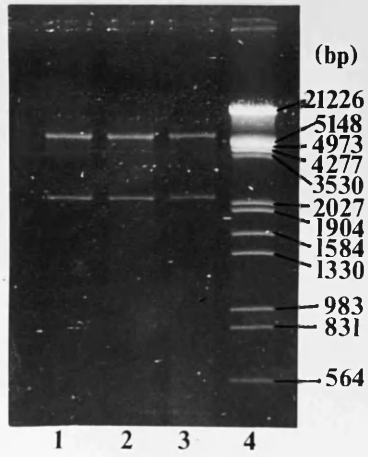
Figure 11 shows the restriction digests of the pAT153.Pvu11 8. C4BP and pBR322.β_2-microglobulin plasmids. The C4BP insert (1400bp) was excised from the plasmid vector using the restriction enzymes BamH1 and Hind 111 while the β_2-microglobulin insert (525 bp) was excised from the pBR322 vector using the restriction enzyme Pst1.

Figure 12 shows the restriction digests of the pAT153.Pvu11 8. C1q B-chain plasmid. The C1q B-chain insert (1000 bp) was excised from the plasmid vector using the restriction enzymes BamH1 and Hind 111.

Several of the cDNA inserts (C1-INH, factor B, C2, C4 and C5)

pAT153.C3

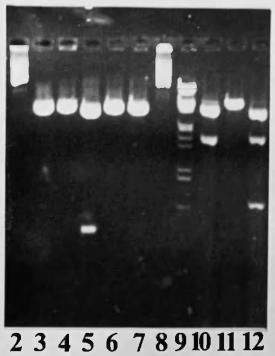
LANE	RESTRICTION DIGEST
1	pC3.11a + Cla1
2	pC3.11a + Sal1
3	pC3.11a + Cla1 + Sal1
4	pC3.11a + EcoR1
5	pC3.11b + Cla1
6	pC3.11b + Sal1
7	pC3.11b + Cla1 + Sal1
8	pC3.11b + EcoR1
9	Lambda DNA markers



LANE	RESTRICTION DIGEST
1-3	pC3.11a + EcoR1
4	Lambda DNA markers

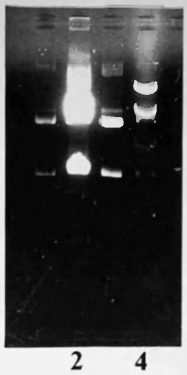
Figure 8 - Restriction enzyme digests of pAT153.C3. The top gel shows the various restriction enzyme digests performed on the pAT153.C3 plasmid. The lower gel represents the EcoR1 digest of pAT153.C3 which excised the 2400bp cDNA insert which could then be extracted from this low melting point agarose gel. In all cases, the gels were calibrated with λ markers (λ phage DNA digested with EcoR1 and Hind111; Boehringer Mannheim).

pAT153.C2 and pAT153.factor B



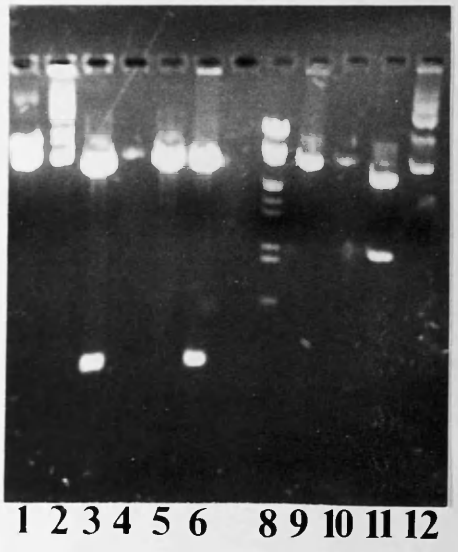
LANE	RESTRICTION DIGEST
2	Undigested pAT153.C2
3	pAT153.C2 + BamH1
4	pAT153.C2 + Hind111
5	pAT153.C2 + BamH1 + Hind111
6	pAT153.C2 + Hind111
7	pAT153.C2 + BamH1
8	Undigested pAT153.C2
9	Lambda DNA markers
10	pAT153.FB + BamH1
11	pAT153.FB + Hind111
12	pAT153.FB + BamH1 + Hind111

pGem.C1-INH



LANE	RESTRICTION DIGEST
2	pGem.C1-INH + BamH1 + Hind111
4	Lambda DNA markers

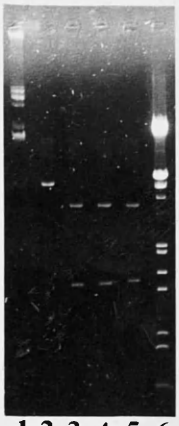
Figure 9 - Restriction enzyme digests of pAT153.C2, pAT153.factor B and pGem.C1-INH.



LANE	RESTRICTION DIGEST
1	pAT153.C4 + BamH1
2	Undigested pAT153.C4
3	pAT153.C4 + BamH1 + Hind111
5	pAT153.C4 + Hind111
6	pAT153.C4 + BamH1 + Hind111
8	Lambda DNA markers
9	pGem.C5 + BamH1
10	pGem.C5 + Hind111
11	pGem.C5 + BamH1 + Hind111
12	Undigested pGem.C5

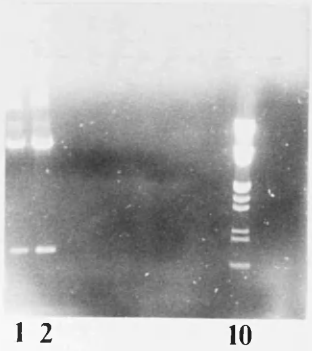
Figure 10 - Restriction enzyme digests of pAT153.C4 and pGem.C5.

pAT153.C4BP



LANE	RESTRICTION DIGEST
1	Undigested pAT153.C4BP
2	pAT153.C4BP + BamH1
3	pAT153.C4BP + BamH1 + HindIII
4	pAT153.C4BP + BamH1 + ClaI
5	pAT153.C4BP + BamH1 + EcoRI
6	Lambda DNA markers

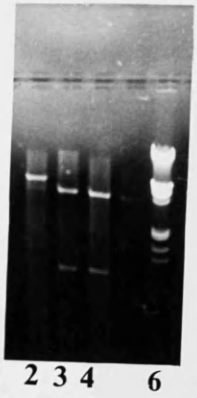
pBR322.B_2-microglobulin



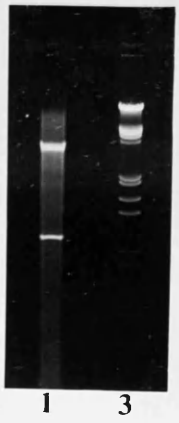
LANE	RESTRICTION DIGEST
1	pBR322.β_2M + PstI
2	pBR322.β_2M + PstI
10	Lambda DNA markers

Figure 11 - Restriction enzyme digests of pAT153.C4BP and pBR322.β_2-microglobulin.

pAT153.C1q B-chain



LANE	RESTRICTION DIGEST
2	pAT153.C1qB + BamH1
3	pAT153.C1qB + BamH1 + Hind111
4	pAT153.C1qB + BamH1 + C1a1
6	Lambda DNA markers



LANE	RESTRICTION DIGEST
1	pAT153.C1qB + BamH1 + Hind111
3	Lambda DNA markers

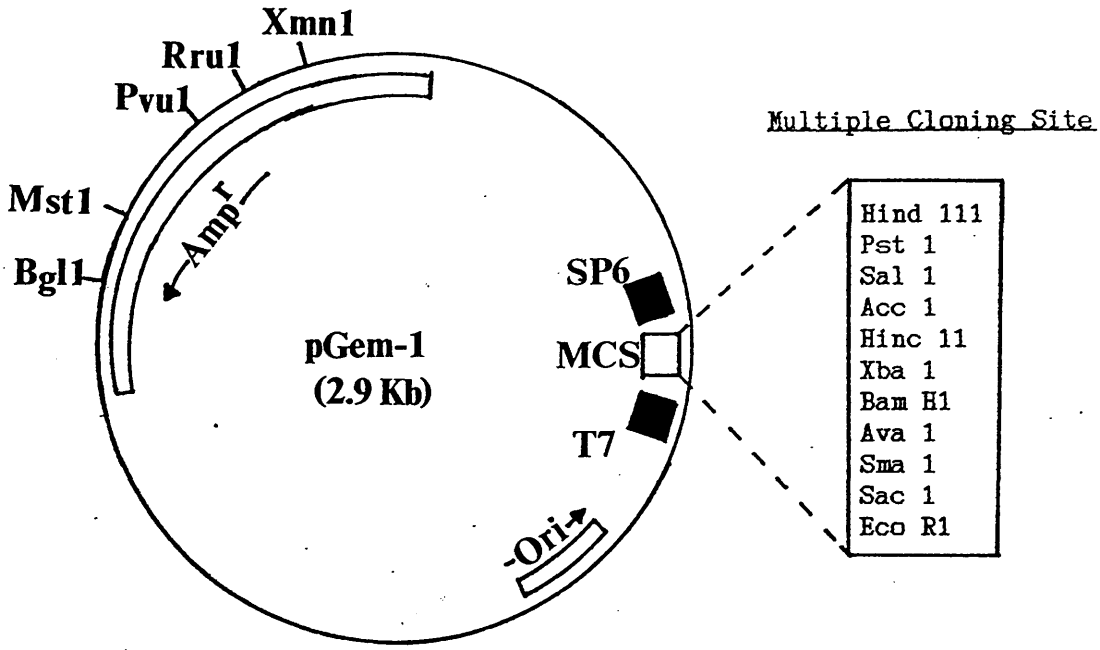
Figure 12 - Restriction enzyme digests of pAT153.C1q B-chain.

were recloned into the pGem-1 (Promega Biotec) transcription vector. The 2.9 kb pGem-1 vector was constructed using the bacteriophage SP6 promoter-containing plasmid pSP64 (Melton et al., 1984) and a bacteriophage T7 promoter. The resulting plasmid has SP6 and T7 promoters separated by a short length of DNA containing multiple cloning sites (Figure 13). The single stranded RNA probes produced by this system were reported to yield greater sensitivity in Northern and dot-blot analyses because RNA/RNA duplexes are more stable than the corresponding DNA/RNA hybrids obtained with DNA probes. Hybridisation with RNA probes allows background on blots to be further reduced by RNase A treatment which is not possible with DNA probes.

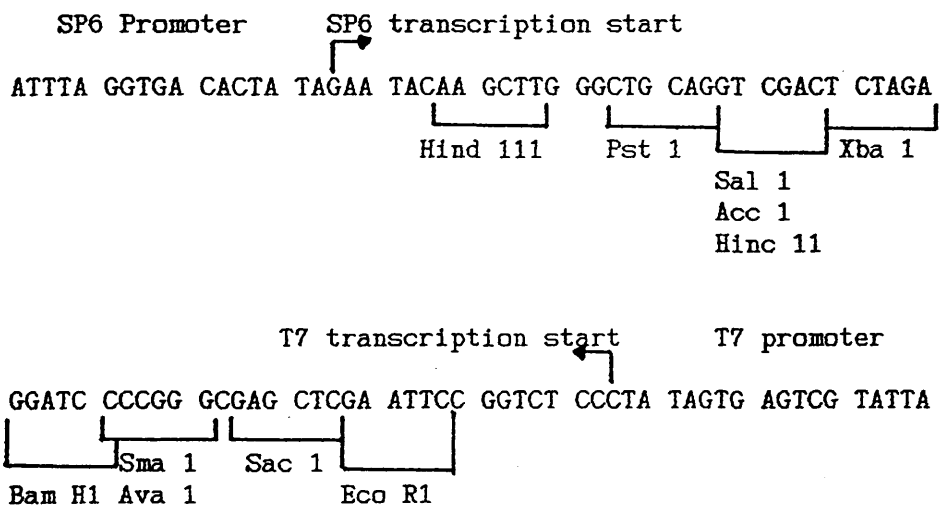
In each case, the C1-INH (pAT153.Pvu11 8), factor B (pAT153. Pvu11 8), C2 (pAT153.Pvu11 8), C4 (pAT153.Pvu11 8) and C5 (pBR322) plasmids were digested with BamH1 and Hind111 to excise the cDNA insert which was then ligated into the BamH1/Hind111 digested pGem-1 vector.

However these RNA probes gave rather disappointing results. During optimisation of the Northern blot analysis procedure, most workers in this laboratory started to use random primed labelled cDNA probes which gave much clearer results than the conventional nick translated cDNA probes. These random primed cDNA probes, like the RNA probes described above, involved labelling the specific insert sequence only and not the plasmid vector DNA, which decreases the risk of non-specific background hybridisation.

In an attempt to improve the Northern blot analysis of the synovial tissue mRNA, random primed cDNA probes were used and were found to give much better results than the RNA probes. However, when the Northern blots were analysed with the β_2 microglobulin random primed



pGem-1 plasmid promoter and multiple cloning site sequence:



The sequence shown is the coding strand for T7 RNA polymerase and the non-coding strand for SP6 RNA polymerase.

Figure 13 - *Restriction map and DNA sequence of the promoters and multiple cloning site of the pGem-1 transcription vector.*

cDNA probe (β_2 microglobulin mRNA is a relatively intact mRNA *in vivo*), the β_2-microglobulin mRNA on the Northern blots analysed with the RNA probes was shown to be substantially more degraded than the β_2-microglobulin mRNA on the Northern blots analysed with random primed cDNA probes. Therefore, the better results achieved with the random primed cDNA probes are more likely due to the better quality of mRNA on the Northern blots rather than any inherent property of the cDNA probes being superior to the RNA probes. However, because satisfactory results were being obtained with the random primed cDNA probes it was decided to continue using them instead of reverting to the RNA probes.

2. ISOLATION OF RNA FROM SYNOVIAL TISSUE

Optimising the procedure for the isolation of undegraded RNA from synovial tissue required many detailed modifications of the conventional techniques of RNA isolation to be tested, which resulted in this aspect of the study becoming rather time-consuming.

As stated in the Materials and Methods section, the guanidinium thiocyanate procedure for RNA isolation was used. This was the standard procedure used in this laboratory and had previously been shown to isolate undegraded RNA from ribonuclease-rich tissues such as pancreas. Many other studies had also shown this to be the best method available for the extraction of intact RNA from both tissues and cells (Birnie & Graham, 1984).

Isolation of RNA from rat liver was initially used in order to gain experience in the technique. Liver contains a large number of endogenous ribonuclease-inhibitors which should ensure that the isolation of RNA from liver is relatively straightforward. Therefore, rat liver was a good model system to ensure that, in my hands, the method being used was working satisfactorily. Using the rat actin cDNA probe the actin mRNA was shown to be undegraded which shows that guanidinium thiocyanate can be used to isolate intact RNA from rat liver.

However, when the same technique was used to isolate RNA from human synovial tissue, several technical problems were encountered.

The first problem that had to be overcome was the sheer toughness of the synovial tissue which made it extremely difficult to pulverise. On a number of occasions the mortar and pestle broke before the tissue, so a solid steel mortar and pestle was used. This allowed me

to pulverise the frozen synovial tissue under liquid nitrogen using a large hammer. This procedure resulted in the formation of a coarse synovial tissue powder. However, the tissue tended to thaw very quickly in this steel mortar and hence the time taken to pulverise the tissue had to be minimised. After the initial pulverisation, the tissue was ground into a fine powder in a conventional mortar and pestle under liquid nitrogen before being transferred to guanidinium thiocyanate.

The next (and most important problem) was the inconsistent quality of RNA being isolated from the synovial tissue. Some specimens consistently yielded undegraded RNA free of contaminating protein while other specimens always yielded degraded RNA. Because undegraded RNA was isolated from a number of specimens, the guanidinium thiocyanate method appeared to be suitable for this type of tissue and some other factor is required to explain why the RNA was degraded in some cases. Over a period of time, it became obvious that the most important factor was to minimise the delay between the removal of the synovium from the patient and the tissue being snap-frozen in liquid nitrogen. Although there was no strict rule, the isolation of undegraded RNA usually became impossible if the tissue was left at room temperature for longer than 10 minutes. Thus in order to minimise RNA degradation, synovial tissue was snap frozen in liquid nitrogen within 10 minutes of being removed from the patient.

Another method for RNA isolation, RNAzol, was recently reported to be much faster and more reliable than the guanidinium thiocyanate method (Chomczynski & Sacchi, 1987). To investigate this claim further, I pulverised some synovial tissue and added half of the powder to guanidinium thiocyanate and half to the RNAzol solution and proceeded to

prepare RNA by these two separate methods. The experiment was repeated with rat liver. Staining the gel with ethidium bromide followed by Northern blot analysis showed that liver mRNA was undegraded when prepared by either method, while synovial tissue RNA was only undegraded when it had been prepared by the guanidinium thiocyanate method. The RNAzol method always produced totally degraded synovial tissue mRNA. Thus it appears that RNAzol is suitable for RNA extraction from tissues such as liver and is also now being used routinely in this laboratory for the isolation of RNA from cultured cells such as monocytes. However, it was clear that RNAzol was not suitable for the extraction of RNA from human synovial tissue and the original guanidinium thiocyanate method was retained as the most suitable for this purpose.

3. NORTHERN BLOT ANALYSIS OF SYNOVIAL TISSUE RNA

In addition to the modifications to the RNA isolation procedures, the Northern blot analysis procedure was also optimised. The major modification in these analyses was the use of nylon membranes (Hybond-N) in both Northern blots and dot-blots instead of the more widely used nitrocellulose. It was shown that the nylon membranes had two major advantages over the nitrocellulose:

(a) in almost every case, non-specific background hybridisation was not present when Northern and dot-blot analyses was performed on nylon membranes. This however, was a major problem when nitrocellulose was used. Both types of filters were washed to the same stringency (0.1 x SSC containing 0.1% SDS).

(b) the nylon membranes were stripped and reprobed up to six times without significantly affecting either the intensity of the radioactive signal after reprobing or the physical structure of the membrane itself. However, nitrocellulose becomes brittle even after being stripped once.

In order to optimise the hybridisation conditions, several buffers and incubation conditions were tested. Both prehybridisation and hybridisation were found to be most efficient when performed at 42°C for at least 16 hours, although prehybridising at 65°C for only two hours seemed to have no effect on the quality of the end result. The buffer used for both prehybridisation and hybridisation is described in detail in the Materials and Methods section 6.2.

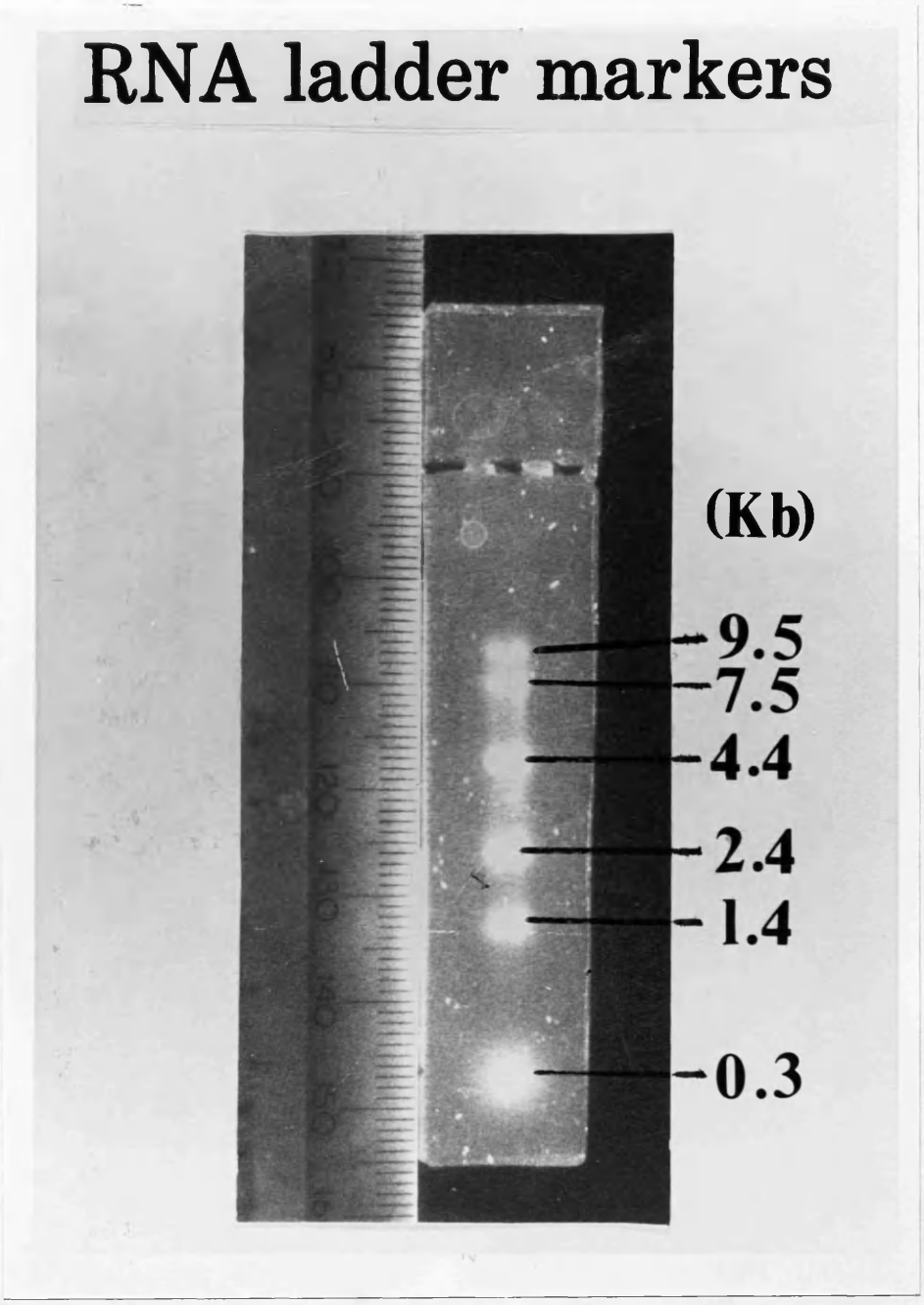


Figure 14 - RNA sized ladder markers (Bethesda Research Laboratories) used to calibrate Northern blot analysis.

PATIENT	SYNOVIAL JOINT	TYPE OF ANALYSIS						
		RNA STUDIES		SYNOVIAL MEMBRANE CULTURES			SYNOVIAL CELL CULTURES	
		Northern blot	Dot blot	ELISA	Haemolytic assay	SDS-PAGE	ELISA	Immuno histochem
RA1	Knee	+	+	−	−	−	−	−
RA2	Knee	+	+	−	−	−	−	−
RA3	Hip	+	+	+	−	−	−	−
RA4	Shoulder	+	+	+	−	−	−	−
RA5	Knee	+	+	−	−	−	−	−
RA6	Knee	+	+	−	−	−	−	−
RA7	Knee	+	+	−	−	−	−	−
RA8	Knee	+	+	+	−	−	−	−
RA9	Hip	+	+	+	−	−	−	−
RA10	Shoulder	+	+	−	−	−	−	−
RA11	Hip	+	+	−	−	−	−	−
RA12	Hip	+	+	−	−	−	−	−
RA13	Knee	−	−	−	−	+	−	−
RA14	Knee	−	−	−	−	−	+	+
RA15	Knee	−	−	−	−	−	+	+
OA1	Knee	+	+	−	−	−	−	−
OA2	Knee	+	+	−	−	−	−	−
OA3	Hip	+	+	−	−	−	−	−
OA4	Knee	+	+	−	−	−	−	−
OA5	Knee	+	+	+	−	−	−	−
OA6	Hip	+	−	−	−	−	−	−
OA7	Hip	+	+	+	−	−	−	−
OA8	Hip	+	−	+	−	−	−	−
OA9	Knee	+	+	+	−	−	−	−
OA10	Hip	+	+	−	−	−	−	−
OA11	Knee	+	+	−	−	−	−	−
OA12	Hip	+	+	−	−	−	−	−
OA13	Hip	+	+	−	−	−	−	−
OA14	Hip	+	+	−	−	−	−	−
OA15	Knee	−	−	+	+	−	−	−
OA16	Knee	−	−	−	−	+	−	−
OA17	Knee	−	−	−	−	+	−	−
OA18	Hip	−	−	−	−	+	−	−
OA19	Knee	−	−	−	−	−	+	+
OA20	Knee	−	−	−	−	−	+	+
AS1	Knee	+	+	−	−	−	−	−
CC1	Knee	+	+	−	−	−	−	−
N1	Knee	−	+	+	+	−	−	−

TABLE 9 - List of patients used in these studies (RA - rheumatoid arthritis, OA - osteoarthritis, AS - ankylosing spondylitis, CC - chondrocalcinosis, N - normal synovium).

RNA was prepared from the synovial membrane of 49 patients, 20 with rheumatoid arthritis, 23 with osteoarthritis, 1 with chondrocalcinosis, 2 with psoriatic arthritis and 2 with ankylosing spondylitis. In addition, RNA was isolated from one specimen of normal synovium from a patient who underwent patellectomy for recurrent patellar dislocation.

However, due to some patients' RNA being consistently degraded or insufficient RNA being prepared for analyses, only 28 patients were included in the Northern blot analysis (20μg RNA/track). Of the 26 patients examined in this study, there were 12 with rheumatoid arthritis, 12 with osteoarthritis, 1 with chondrocalcinosis and 1 with ankylosing spondylitis (see Table 9). However, even within this group, RNA isolated from a small number of patients was consistently shown to be degraded.

These 26 patient samples were divided into three groups: two groups containing nine patients and another group of eight patients. Each group was analysed in sets of four identical Northern blots; A - D (group 1), E - H (group 2) and I - L (group 3).

Figure 14 shows the RNA sized ladder markers (Bethesda Research Laboratories) used to calibrate the Northern gels while figures 15 - 20 show the results from Northern blot analysis of RNA isolated from the synovial membrane of the 26 patients.

Northern blots A - D (figures 15 and 16) contain RNA isolated from the synovial membrane of 5 rheumatoid arthritis patients (RA1 - RA5) and four osteoarthritis patients (OA1 - OA4).

Figure 15 (a) shows the results of Northern blot A which was initially analysed with the factor B cDNA probe. Factor B mRNA (2.6 kb)

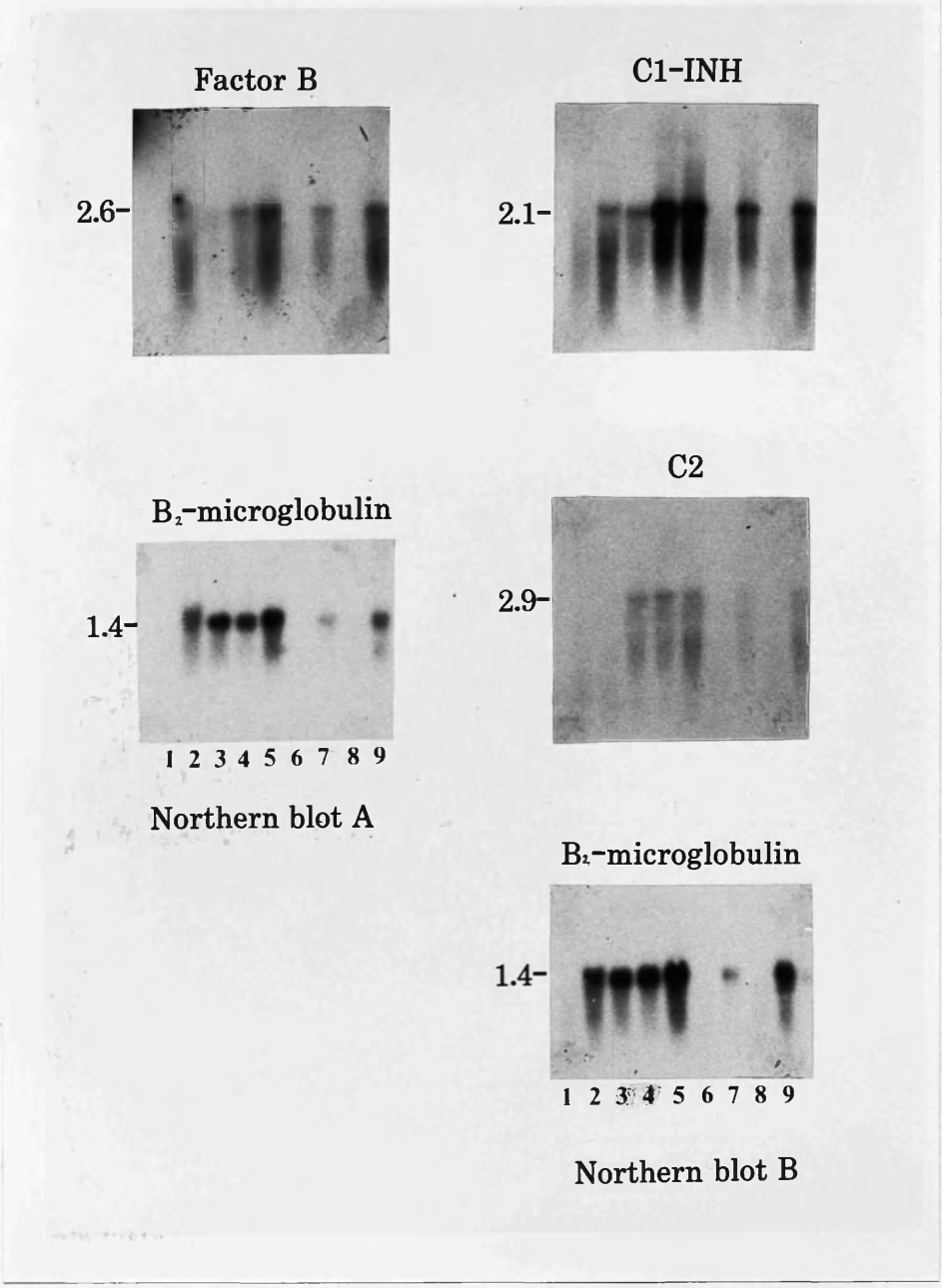


Figure 15 - Northern blot analysis of RNA isolated from the synovial membrane of 9 patients (group 1). Both Northern blot A and B contain RNA from the same patients loaded in the following order: left to right, lanes 1-9; RA1, RA2, RA3, RA4, RA5, OA1, OA2, OA3 and OA4. Northern blot A was probed for factor B mRNA, stripped and then reprobed for β_2-microglobulin mRNA. Northern blot B was probed, stripped and reprobed with different cDNA inserts in the following order; C1-INH, C2 and β_2-microglobulin.

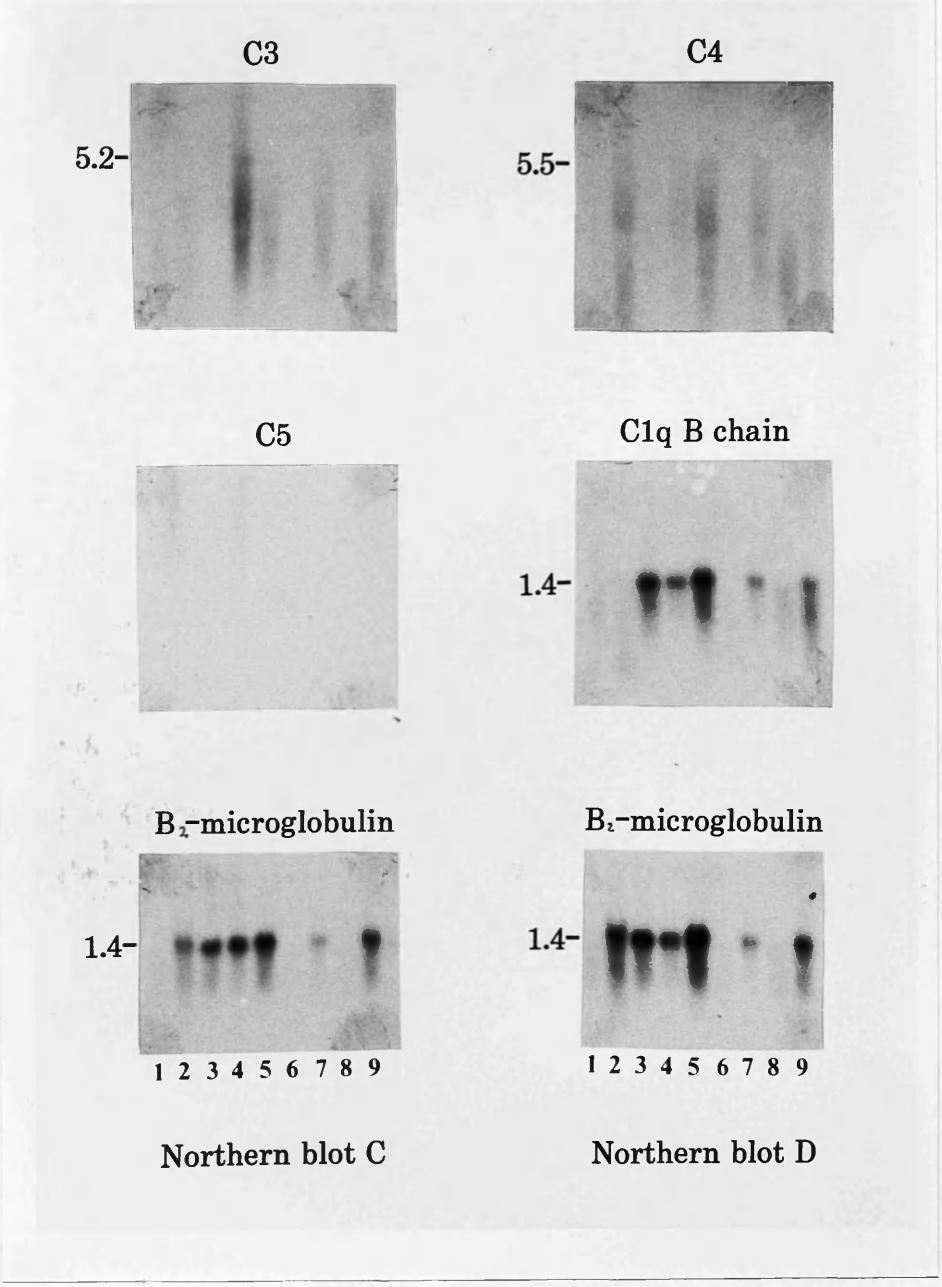


Figure 16 - Northern blot analysis of RNA isolated from the synovial membrane of 9 patients (group 1). Both Northern blot C and D contain RNA from the same patients as Northern blots A and B and were loaded in the following order: left to right, lanes 1-9; RA1, RA2, RA3, RA4, RA5, OA1, OA2, OA3 and OA4. Northern blot C was probed, stripped and reprobed with different cDNA inserts in the following order; C3, C5 and β_2-microglobulin. Northern blot D was probed, stripped and reprobed with different cDNA inserts in the following order; C4, C1q B-chain β_2-microglobulin.

was detected in all samples except RA1, OA1 and OA3. However when this filter was stripped and reprobed for β_2-microglobulin mRNA (1.4 kb) (a relatively stable mRNA *in vivo*), intact mRNA was detected for samples RA2, RA3, RA4, RA5, OA2 and OA4. However the mRNA from samples RA1, OA1 and OA3 was totally degraded. This observation, which was repeated on Northern blots B, C and D, explains the negative results obtained for these three patients with all cDNA probes.

Figure 15 (b) shows the results of Northern blot B which was initially analysed with the C1-INH cDNA probe. C1-INH mRNA (2.1 kb) was detected in all samples (except RA1, OA1 and OA3). This filter was stripped and reprobed with the C2 cDNA probe which revealed C2 mRNA (2.9 kb) in all samples although only very weak signals were detected for RA2 and OA2. However, when the filter was reprobed with the β_2-microglobulin cDNA probe substantial amounts of mRNA were detected in these two lanes.

Figure 16 (a) shows the results of Northern blot C which was initially analysed with the C3 cDNA probe. A weak band corresponding to intact C3 mRNA was detected in only one sample (RA4). Only low molecular weight material (degraded C3 mRNA) was detected in the other samples, RA2, RA3, RA5, OA2 and OA4. C5 mRNA was not detected in any sample but when the filter was reprobed for β_2-microglobulin mRNA, not only was mRNA present in all six lanes but it did not show any sign of degradation.

Figure 16 (b) shows the results of Northern blot D which was initially analysed with the C4 cDNA probe. Similar results were obtained as for C3 mRNA although a faint band corresponding to intact C4 mRNA (5.5 kb) was detected in three samples RA2, RA5 and OA2. Low molecular weight material was detected in all other samples. The filter was

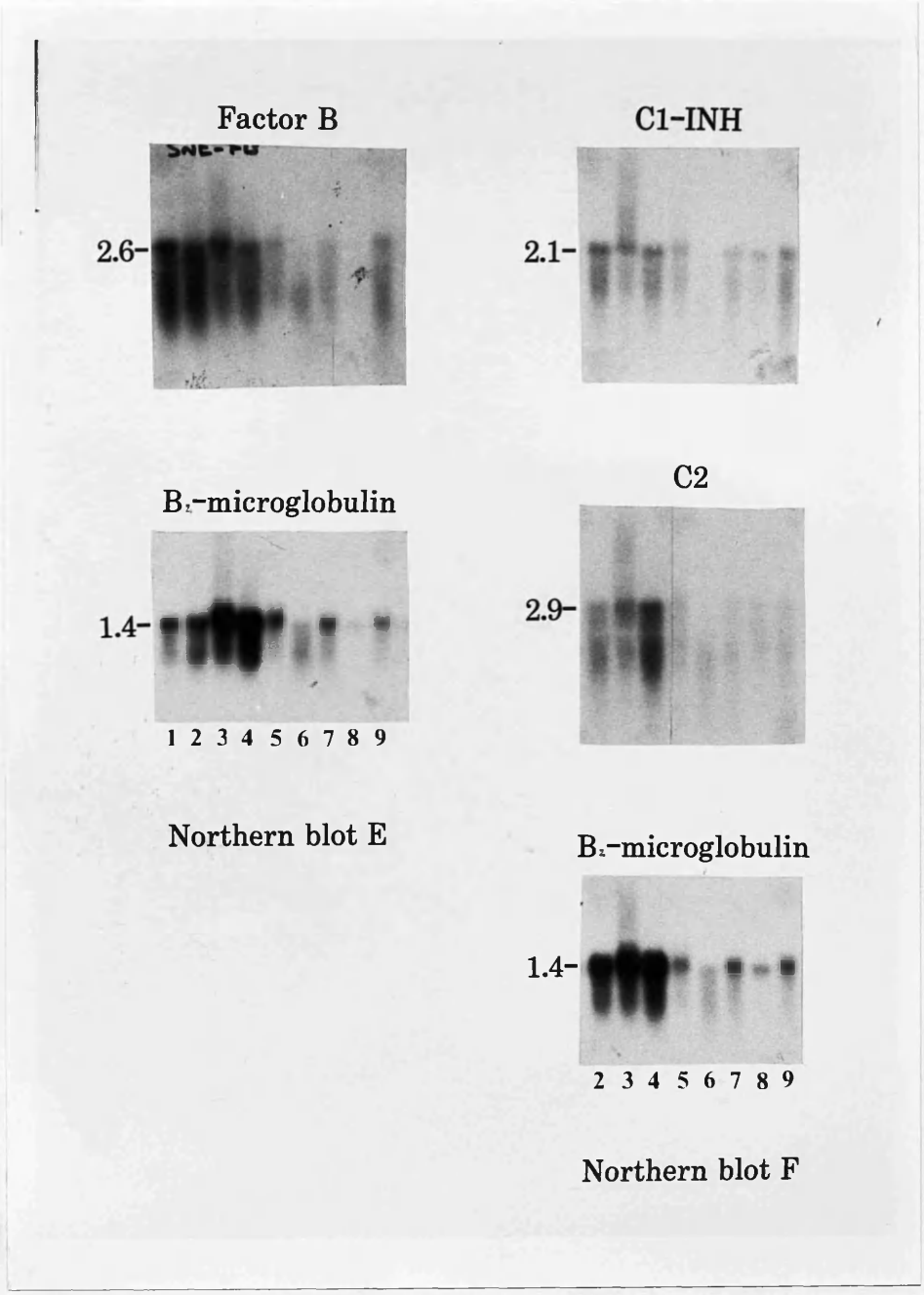


Figure 17 - Northern blot analysis of RNA isolated from the synovial membrane of 9 patients (group 2). Northern blot E and F contain RNA from the same patients (except lane 1 is omitted in Northern blot F) loaded in the following order: left to right, lanes 1-9; RA6, RA7, RA8, RA9, OA5, RA10, RA11, OA6, AS1. Northern blot E was probed for factor B mRNA and then stripped and reprobed for β_2-microglobulin mRNA. Northern blot F was probed, stripped and reprobed with different cDNA inserts in the following order; C1-INH, C2 and β_2-microglobulin.

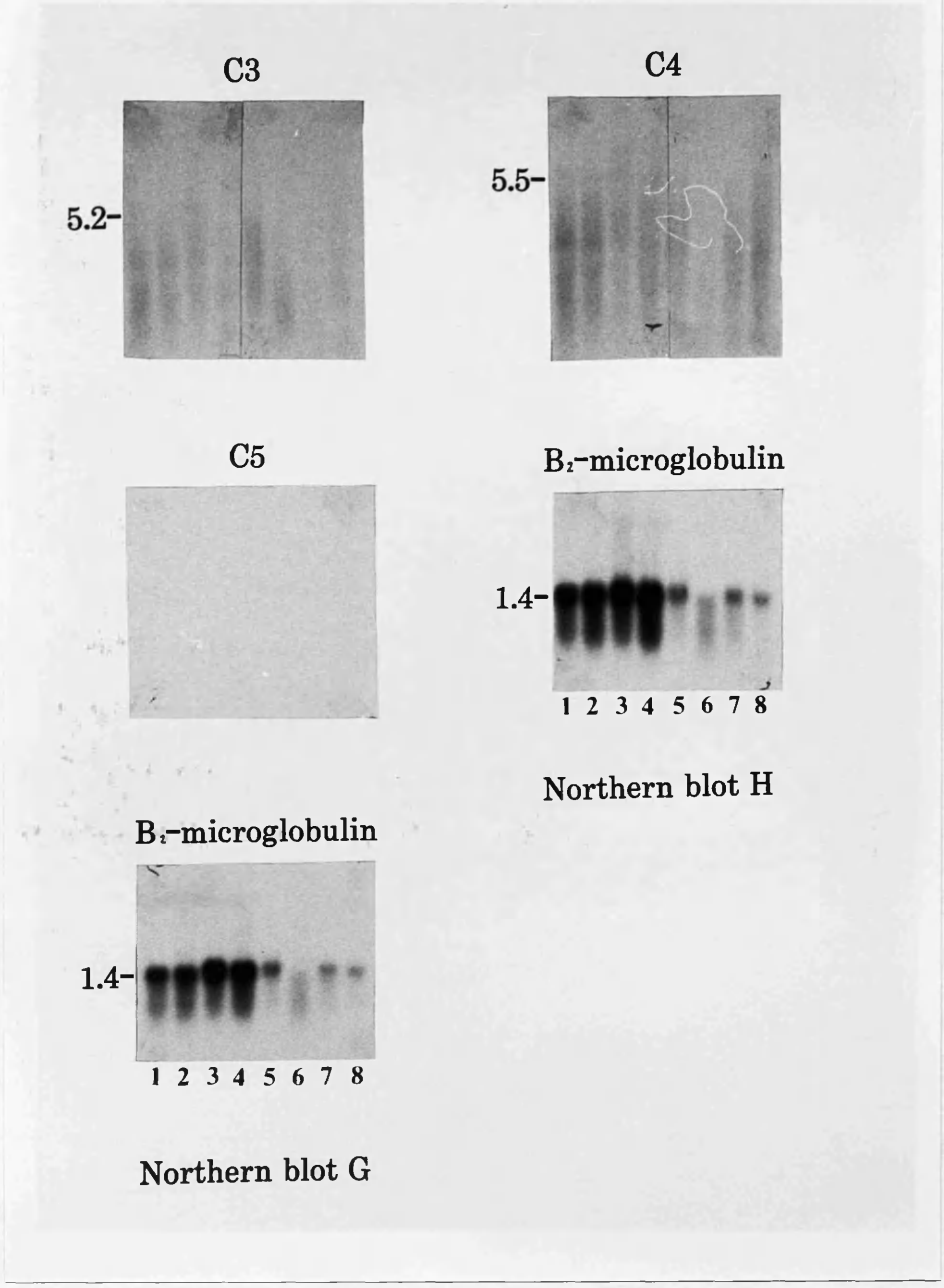


Figure 18 - Northern blot analysis of RNA isolated from the synovial membrane of 8 patients (group 2 - AS1 is omitted). Both Northern blot G and H contain RNA from the same patients loaded in the following order: left to right, lanes 1-8; RA6, RA7, RA8, RA9, OA5, RA10, RA11, OA6. Northern blot G was probed, stripped and reprobed with different cDNA inserts in the following order; C3, C5 and β_2-microglobulin. Northern blot H was probed for C4 mRNA and then stripped and reprobed for β_2-microglobulin mRNA.

reprobed with the C1q B-chain cDNA probe and intact C1q B-chain mRNA (1.4 kb) was detected in samples RA3, RA4, RA5, OA2 and OA4. Only lower molecular weight material was detected in sample RA2. Reprobing the filter with the β_2-microglobulin cDNA probe showed undegraded mRNA in all six lanes.

Northern blots E-H (figures 17 and 18) contain RNA isolated from the synovial membrane of 6 rheumatoid arthritis patients (RA6 - RA11), 2 osteoarthritis patients (OA5 and OA6) and 1 patient suffering from ankylosing spondylitis (AS1).

Figure 17 (a) shows the results of Northern blot E which was initially analysed with the factor B cDNA probe. Factor B mRNA (2.6 kb) was detected in all samples although only very weak signals were detected in RA10 and OA6. Reprobing this filter with the β_2-microglobulin cDNA probe showed there was a much smaller amount of mRNA in the OA6 track and the RA10 mRNA was more degraded than the other samples.

Figure 17 (b) shows the results of Northern blot F on which sample RA6 was omitted. This filter was initially analysed with the C1-INH cDNA probe. C1-INH mRNA (2.1 kb) was detected in all samples although only a very weak signal was obtained for OA6. When the filter was reprobed for C2 mRNA, a band at 2.9 kb was detected in all samples with a more intense signal for OA6 than that obtained with the C1-INH probe. Reprobing the filter for β_2-microglobulin mRNA showed intact mRNA in all lanes except OA6.

Figure 18 (a) shows the results of Northern blot G on which sample AS1 was omitted. This filter was initially analysed with the C3 cDNA probe and similar results to Northern blot C were obtained.

Degraded C3 mRNA was present in all samples and when the filter was reprobed with the C5 cDNA probe, C5 mRNA was not detected in any sample. However, analysis with the β_2-microglobulin cDNA probe showed undegraded mRNA in each track except OA6.

Figure 18 (b) shows the results of Northern blot H on which sample AS1 was omitted. This filter was initially analysed with the C4 cDNA probe and similar results to Northern blot D were obtained. A faint band corresponding to intact C4 mRNA (5.5 kb) was detected in samples RA6, RA7, RA8 and OA6 and low molecular weight material (degraded C4 mRNA) was detected in all other samples. Reprobing with the β_2-microglobulin cDNA probe showed intact mRNA in each track except OA6.

Northern blots I-L (figures 19 and 20) contain RNA isolated from the synovial membrane of 1 rheumatoid arthritis patient (RA12), 6 osteoarthritis patients (OA7 - OA12) and 1 chondrocalcinosis patient (CC1).

Figure 19 (a) shows the results of Northern blot I which was initially analysed with the factor B cDNA probe. Factor B mRNA (2.6 kb) was detected in all samples except OA8 but when the filter was reprobed for β_2-microglobulin mRNA, the mRNA in this track was shown to be more degraded than the other samples.

Figure 19 (b) shows the results of Northern blot J which was initially analysed with the C1-INH cDNA probe. C1-INH mRNA (2.1 kb) was detected in all samples although OA8 RNA was degraded and only low molecular weight material was detected. Reprobing this filter with the C2 cDNA probe showed intact C2 mRNA (2.9 kb) in samples RA12, OA7, OA9 and OA10. RNA samples OA8, OA11, OA12 and CC1 only showed low molecular weight material corresponding to degraded C2 mRNA. When the filter was

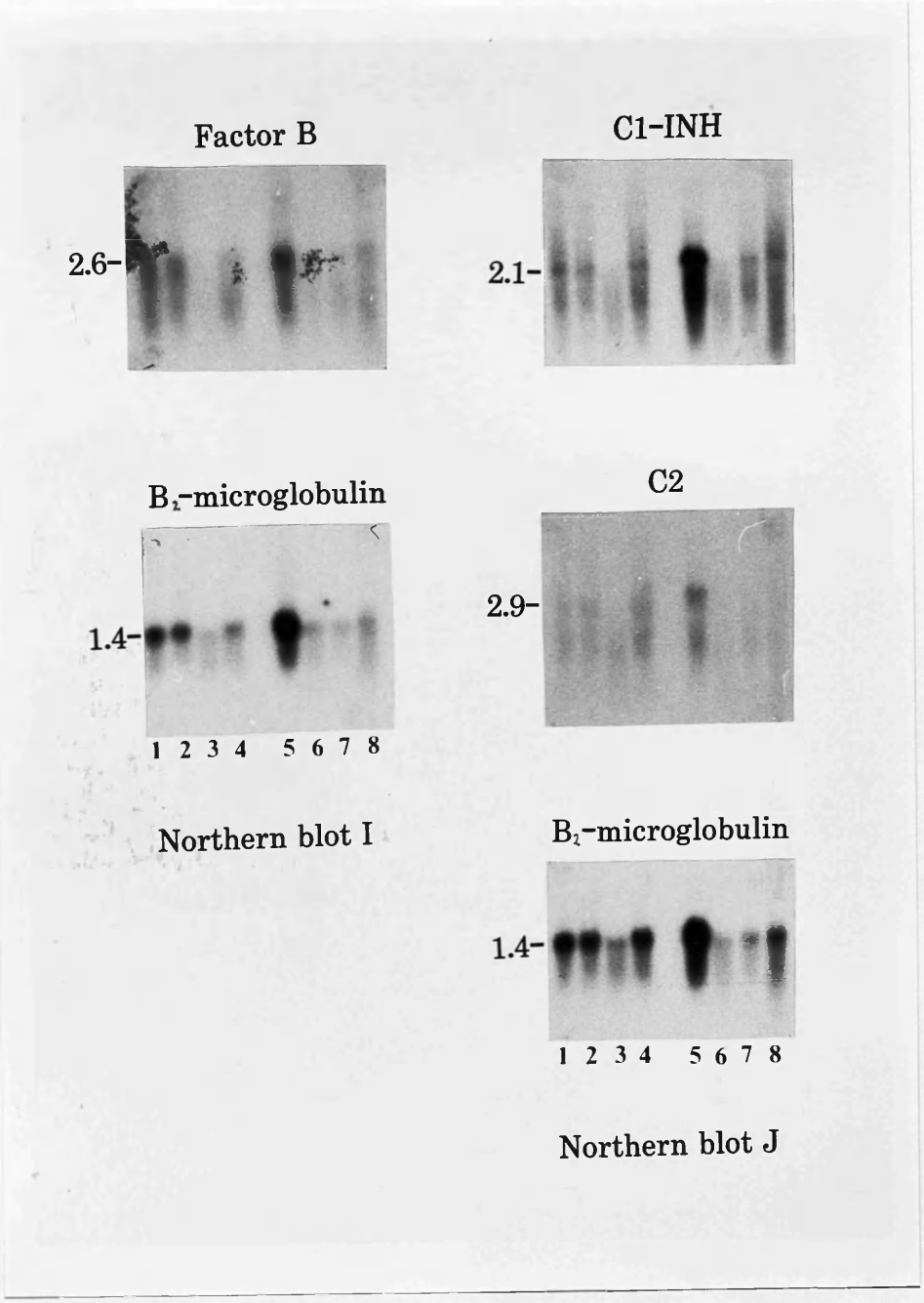


Figure 19 - Northern blot analysis of RNA isolated from the synovial membrane of 8 patients (group 3). Both Northern blots I and J contain RNA from the same patients loaded in the following order: left to right, lanes 1-8; RA12, OA7, OA8, OA9, OA10, OA11, OA12, CC1. Northern blot I was probed for factor B mRNA and then stripped and reprobed for β_2-microglobulin mRNA. Northern blot J was probed, stripped and reprobed with different cDNA inserts in the following order; C1-INH, C2 and β_2-microglobulin.

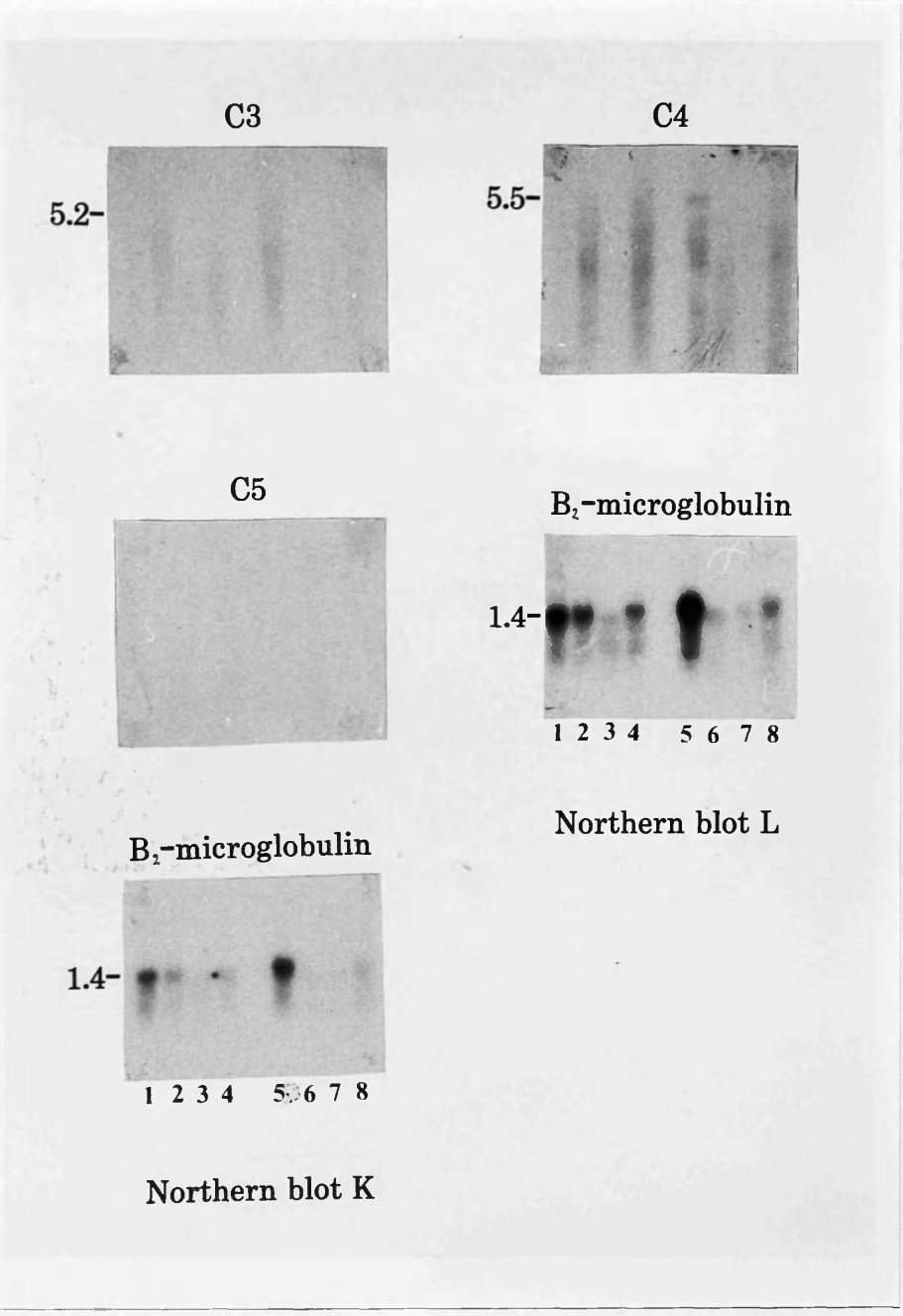


Figure 20 - Northern blot analysis of RNA isolated from the synovial membrane of 8 patients (group 3). Both Northern blots K and L contain RNA from the same patients as Northern blots I and J, loaded in the following order: left to right, lanes 1-8; RA12, OA7, OA8, OA9, OA10, OA11, OA12, CC1. Northern blot K was probed, stripped and reprobed with different cDNA inserts in the following order; C3, C5 and β_2-microglobulin. Northern blot L was probed for C4 mRNA and then stripped and reprobed for β_2-microglobulin mRNA.

reprobed with β_2-microglobulin cDNA, intact mRNA was detected in all samples except OA8 and OA11 in which the mRNA was more degraded than other samples.

Figure 20 (a) shows the results of Northern blot K which was initially analysed with the C3 cDNA probe. Extremely weak signals were obtained from this analysis but on reprobing the filter for β_2-microglobulin mRNA, weak signals were also obtained. It would appear likely that a substantial amount of the RNA had either not been properly fixed on the filter or had been removed during the stripping procedure. Once again, C5 mRNA was not detected in any sample.

Figure 20 (b) shows the results of Northern blot L which was initially analysed with the C4 cDNA probe. A band corresponding to C4 mRNA (5.5 kb) was detected in sample OA10. Low molecular weight material was detected in samples OA7, OA9, OA11 and CC1 while only an extremely weak signal was demonstrated for samples RA12, OA8 and OA12. Reprobing for β_2-microglobulin mRNA showed undegraded mRNA in all tracks, although OA8 mRNA was slightly more degraded than the other samples.

In summary, mRNAs corresponding to factor B (2.6 kb), C1-INH (2.1 kb), C2 (2.9 kb), C3 (5.2 kb) and C4 (5.5 kb) were detected in all intact RNA samples using the appropriate cDNA probe, but C5 mRNA was not detected in any sample. C1q B-chain mRNA (1.4 kb) was also detected in all intact RNA samples (except RA2) analysed with the C1q B-chain cDNA probe. Low molecular weight material was present in most samples suggesting that degradation of the mRNA had occurred. The proportion of degraded RNA varied: C1-INH, factor B and C2 mRNAs showed less degradation while mRNAs coding for C3 and C4 were usually largely degraded,

although a faint band of undegraded mRNA could be detected in some samples. However, it is notable that when these same filters were stripped and reprobed with β_2-microglobulin cDNA, the β_2-microglobulin mRNA showed no signs of degradation.

4. DOT-BLOT ANALYSIS OF SYNOVIAL TISSUE RNA

Dot-blots were used to compare the relative abundances of C1-INH, factor B, C2, C3 and C4 mRNAs between RNA isolated from different synovial tissue specimens. Although the presence of C5 mRNA was also sought, it was not detected in any sample.

RNA (10μg/dot) isolated from the synovial membrane of 26 patients (12 with rheumatoid arthritis, 12 with osteoarthritis, 1 with ankylosing spondylitis and 1 with chondrocalcinosis) were investigated. The specimens studied in these dot-blots were the same as for the Northern blot analysis except for samples OA6 and OA8 which were replaced by samples OA13 and OA14. Three identical dot blots are shown in figure 21, each containing RNA from the same 26 patients. Row A contains RNA from 8 rheumatoid arthritis patients (left to right: RA10, RA6, RA11, RA1, RA12, RA7, RA9 and RA2). Row B contains RNA from 9 osteoarthritis patients and 1 patient suffering from ankylosing spondylitis (left to right: OA13, OA10, OA14, OA4, OA3, OA11, OA1, OA12, OA2 and AS1). Row C contains RNA from 4 patients suffering from rheumatoid arthritis, 3 osteoarthritis patients and 1 chondrocalcinosis patient (left to right: OA7, OA5, CC1, RA3, RA8, OA9, RA5, blank and RA4). Each RNA sample was analysed in duplicate. The three dot blots were stripped and reprobed with different cDNA inserts in the following order: factor B, C3 and actin (dot blot I); C1-INH, C2 and actin (dot blot II) and C4, C5 and actin (dot blot III).

Each dot blot was analysed with a complement component cDNA probe in the order described above. The radioactive signal detected from each sample was measured by densitometric examination of the auto-radiographs. Using one row of patients as a reference, the arbitrary

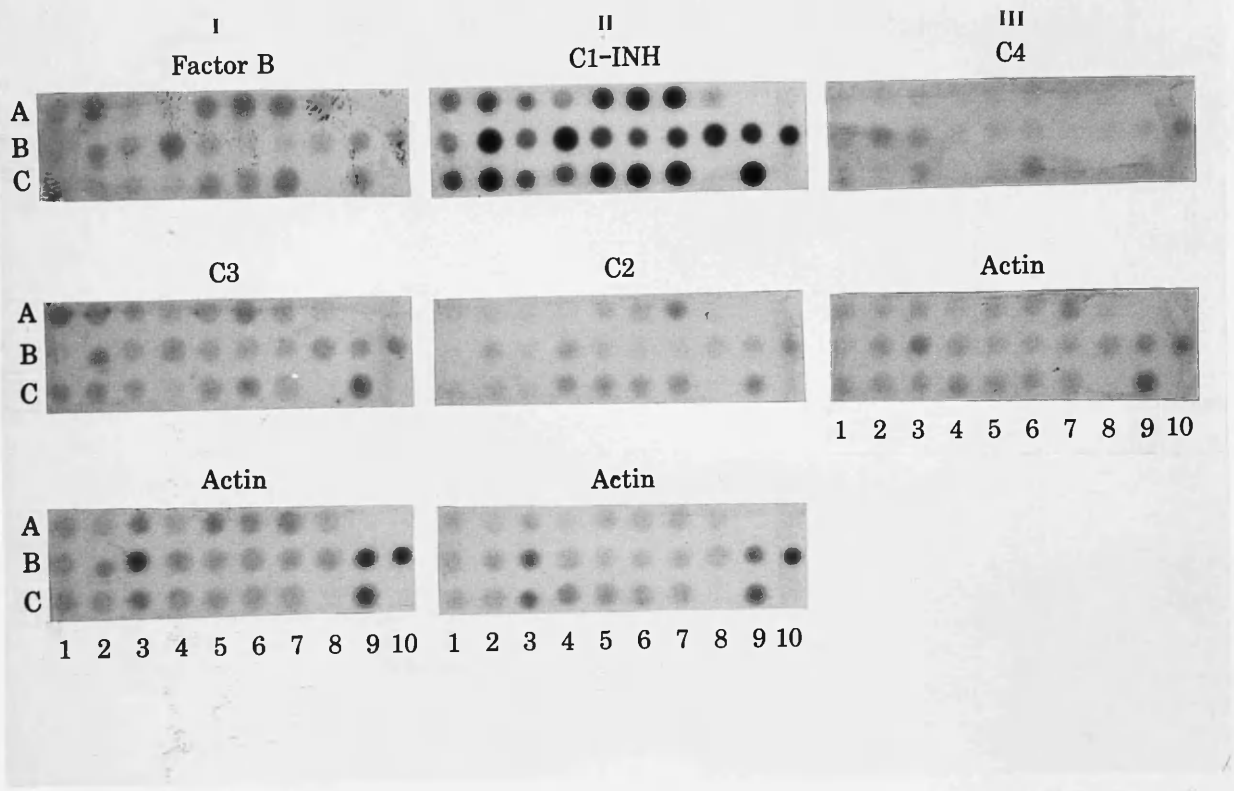


Figure 21 - Dot-blot analysis of synovial membrane RNA (10µg/dot). Three identical dot-blots (I, II and III) are shown, each containing RNA from the same 26 patients. Row A contains RNA from 8 rheumatoid arthritis (RA) patients loaded in the following order (left to right): RA10, RA6, RA11, RA1, RA12, RA7, RA9 and RA2. Row B contains RNA from 9 osteoarthritis (OA) patients and 1 patient suffering from ankylosing spondylitis (AS) loaded in the following order (left to right): OA13, OA10, OA14, OA4, OA3, OA11, OA1, OA12, OA2 and AS1. Row C contains RNA from 4 RA patients, 3 OA patients and 1 chondrocalcinosis (CC) patient loaded in the following order (left to right): OA7, OA5, CC1, RA3, RA8, OA9, RA5, blank, and RA4. The three dot blots were stripped and reprobed with different cDNA inserts in the following order: factor B, C3 and actin (dot-blot I); C1-INH, C2 and actin (dot blot II); C4, C5 and actin (dot-blot III). C5 mRNA was not detected in any sample and hence the C5 autoradiograph is not shown. (All samples were analysed in duplicate).

units obtained from this analysis were used to measure the relative intensity of the signal detected from each sample.

In order to standardise the dot-blots, the filters were stripped after analysis with a complement component cDNA probe and reprobed with a rat actin cDNA. Actin is a constitutively expressed protein and hence was used to control for the amount of RNA loaded onto each dot. Measurements from densitometric examination of a dot-blot analysed with a complement component cDNA probe were divided by the measurements obtained from the same filter that had been stripped and reprobed with the actin cDNA.

Table 10 summarises the results obtained from densitometric examination of the dot-blots. As with the Northern blot analysis, all patients were positive for each mRNA but to varying degrees. The mean densitometric measurement of each complement component mRNA from the rheumatoid arthritis patients was compared with the mean value for the osteoarthritis patients. Although there appears to be a trend towards higher levels of factor B, C3 and C1-INH mRNA in rheumatoid arthritis synovium than osteoarthritis synovium the differences were not statistically significant, when analysed by the student's t-test. However, the level of C2 mRNA in rheumatoid synovium was shown to be significantly higher ($0.05 > P > 0.02$) than the level found in osteoarthritis synovium while C4 mRNA was found in significantly greater abundance ($0.05 > P > 0.02$) in osteoathritis synovium. Comparison of the levels of different mRNAs cannot be made due to the varying specific activities of each cDNA probe.

However, it was possible to determine whether the ratio of a patient's steady state level of one particular complement mRNA to the

mRNA	DIAGNOSIS	MEAN±SD	t-test	Probability, P
Factor B	RA	1.60±1.36	1.630	0.2 > P > 0.1
	OA	0.85±0.72		
C3	RA	1.46±1.19	0.667	0.6 > P > 0.5
	OA	1.18±0.72		
C1-INH	RA	2.18±1.13	0.775	0.5 > P > 0.4
	OA	1.87±0.70		
C2	RA	0.80±0.57	2.105	0.05 > P > 0.02
	OA	0.40±0.28		
C4	RA	0.42±0.39	2.111	0.05 > P > 0.02
	OA	0.99±0.81		

TABLE 10 - Comparison of the levels of each complement mRNA between 12 rheumatoid arthritis (RA) and 12 osteoarthritis (OA) patients. Levels are expressed as arbitrary units obtained from densitometry measurements.

PATIENT	C2	Factor B	C4	C3	C1-INH
RA1	51%	-	14%	63%	158%
RA2	13%	17%	104%	20%	58%
RA3	78%	48%	-	9%	35%
RA4	76%	24%	36%	81%	40%
RA5	205%	117%	190%	101%	99%
RA6	91%	295%	283%	238%	208%
RA7	120%	188%	171%	158%	139%
RA8	120%	90%	118%	105%	80%
RA9	260%	19%	131%	75%	97%
RA10	60%	55%	44%	263%	106%
RA11	19%	295%	283%	38%	51%
RA12	104%	129%	95%	50%	108%

TABLE 11 - Differential regulation of the expression of C2, factor B, C4, C3 and C1-INH in rheumatoid synovium. The percentages refer to the C2, factor B, C4, C3 or C1-INH mRNA densitometric measurement for each patient compared to the mean value for the same component in all 12 rheumatoid arthritis patients, as obtained by dot-blot analysis (figure 21).

PATIENT	C2	Factor B	C4	C3	C1-INH
OA1	70%	-	24%	76%	111%
OA2	35%	24%	45%	25%	39%
OA3	140%	153%	84%	64%	134%
OA4	198%	41%	261%	85%	104%
OA5	73%	71%	82%	162%	156%
OA7	68%	261%	128%	197%	96%
OA9	253%	198%	242%	180%	116%
OA10	145%	121%	179%	160%	98%
OA11	88%	102%	51%	64%	113%
OA12	103%	-	56%	104%	127%
OA13	20%	162%	29%	72%	81%
OA14	15%	63%	20%	10%	25%

TABLE 12 - Differential regulation of the expression of C2, factor B, C4, C3 and C1-INH in osteoarthritis synovium. The percentages refer to the C2, factor B, C4, C3 or C1-INH mRNA densitometric measurement for each patient compared to the mean value for the same component in all 12 osteoarthritis patients, as obtained by dot-blot analysis (figure 21).

mean level for that component is the same for all the complement components studied. In other words, if the steady state level of C2 mRNA in one patient is twice the mean C2 value for patients in the same disease group are the steady state levels of C4 mRNA, C3 mRNA, factor B mRNA and C1-INH mRNA also twice their own mean values, ie. if steady state level of y mRNA is 2 times mean for y, are all complement mRNAs steady state levels 2 x their mean value?

The patients were divided into two groups - those with rheumatoid arthritis and those with osteoarthritis. To answer the above question, the ratio of the densitometric measurement of one complement component mRNA for each patient to the mean value for that component within the same disease group was calculated e.g. the ratio of the densitometric measurement of C1-INH mRNA in RA1 to the mean RA value for C1-INH mRNA calculated from all 12 rheumatoid arthritis patients. Tables 11 and 12 show the results of these calculations. In almost every patient, the ratios calculated for each complement component mRNA show a marked difference from one another. These calculations show that in each patient the steady state levels of all the complement component mRNAs do not increase or decrease to the same extent when compared to the mean values for all patients suffering from the same disease. The data also suggests that there may be different regulatory mechanisms for the expression of each of the five complement components studied, C4, C2, C3, factor B and C1-INH.

In addition, normal synovium was obtained from a 19 year old patient who underwent patellectomy for recurrent patellar dislocation. RNA isolated from this normal synovium was also examined by dot-blot analysis (figure 22) using the C1-INH cDNA probe. RNA from 10 patients

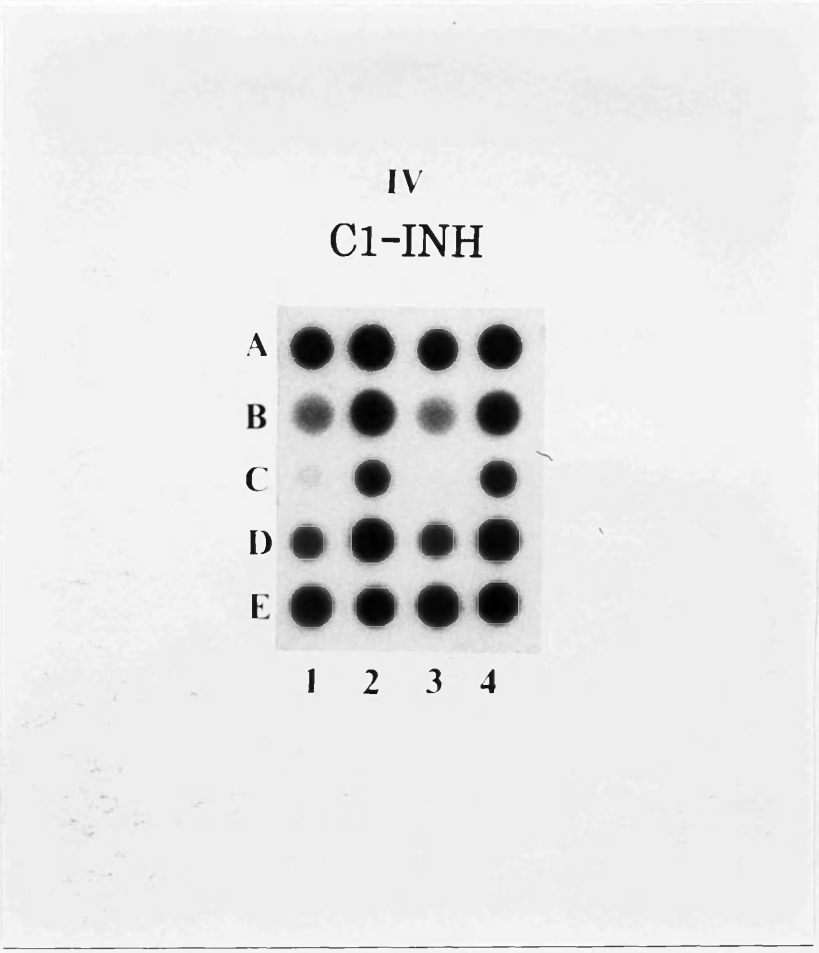


Figure 22 - Dot-blot analysis of synovial membrane RNA (10µg/dot). Dot-blot IV contains RNA from 10 patients loaded in duplicate in the following order: column 1 - RA12, RA2, N1, RA11 and RA7; column 2 - OA10, RA8, CC1, OA4 and RA4. The filter was analysed with the C1-INH cDNA probe. N1 produces an extremely weak signal but less than 2µg of RNA from this sample was available for use in this dot-blot analysis. Columns 3 and 4 are duplicates of columns 1 and 2 respectively but no N1 RNA is in position 3C, which was left blank.

was loaded in duplicate onto this dot blot in the following order: column 1; RA12, RA2, N1, RA11 and RA7 and column 2; OA10, RA8, CC1, OA4 and RA4, column 3; RA12, RA2, blank, RA11 and RA7 and column 4: OA10, RA8, CC1, OA4 and RA4. mRNA coding for C1-INH was detected in all samples including N1. However, the low amount of RNA (less than 2μg) isolated from the small piece of tissue received from this patient, resulted in a weak signal being detected on dot-blot analysis, thus not enabling this patient's results to be analysed alongside those described in Table 10.

5. DETECTION OF COMPLEMENT PROTEINS IN SYNOVIAL MEMBRANE CULTURE SUPERNATANTS

Figures 23 - 28 show the results of ELISA analysis of synovial membrane culture supernatants from 6 patient samples (2 with rheumatoid arthritis (RA8 and RA9), 3 with osteoarthritis (OA5, OA8 and OA15) and 1 normal synovium specimen (N1) from a patient who underwent patellectomy for recurrent patellar dislocation). Synthesis of each complement protein was measured as μg complement component/ml culture supernatant per 100mg tissue.

(a) C4 synthesis

The concentration of C4 in the culture supernatants from each patient sample increased with time although the rate of increase diminished towards the end of the culture period. After six days in culture, the total concentration of C4 in the culture supernatants of the six synovial tissue specimens studied was: RA8 (3.07μg C4), RA9 (7.91μg C4), OA5 (11.21μg C4), OA8 (2.52μg C4), OA15 (8.08μg C4) and N1 (5.93μg C4). When cycloheximide (2.5 μg/ml) was present in cultures RA8, RA9, OA5 and OA8, the concentration of C4 was only reduced after the third day in culture. However, the concentration of C4 in culture supernatants OA15 and N1 was reduced after the second day in culture. In all cases, the accumulation of C4 in the culture supernatants not treated with cycloheximide, continued to increase throughout the period of culture (although accumulation of C4 was less in the OA8 culture). However, after 2-3 days C4 accumulation did not increase in the cyclo-heximide-treated cultures.

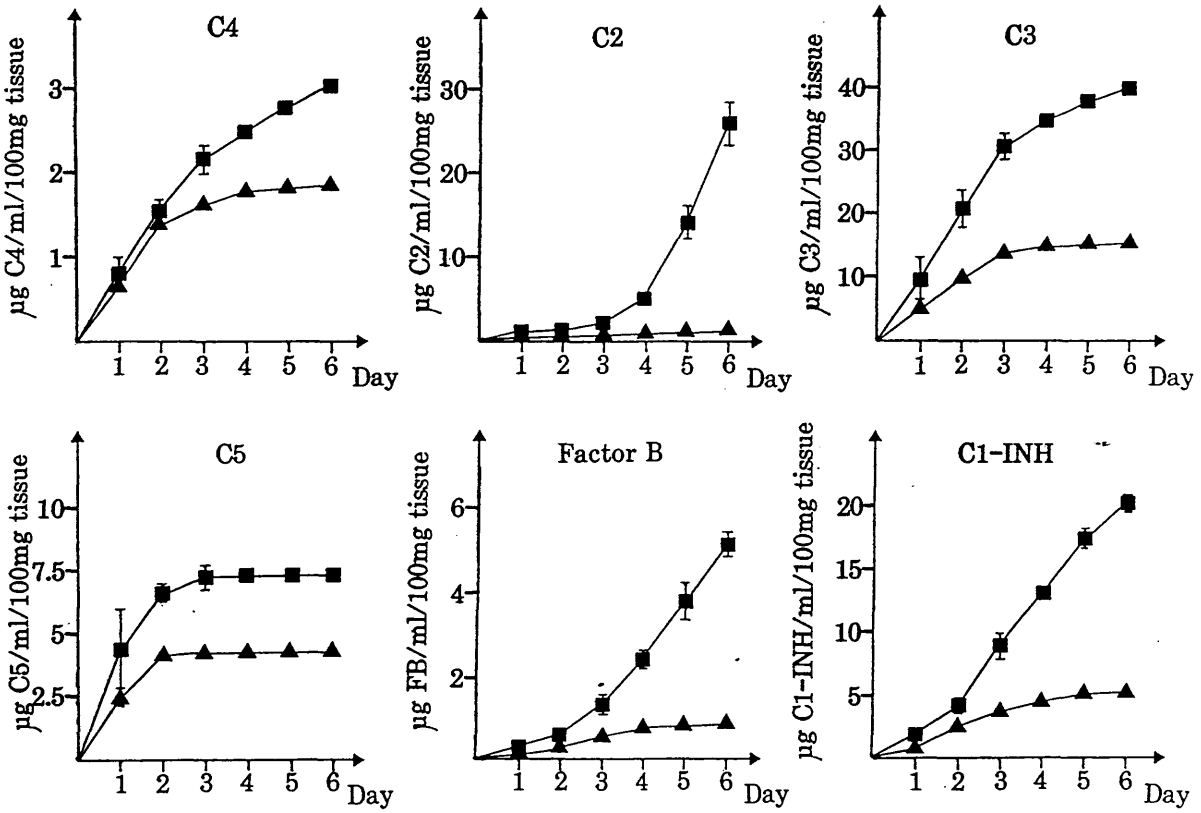


Figure 23 - ELISA analysis of supernatants from cultured fragments of synovial membrane from rheumatoid arthritis patient, RA8. The culture medium was changed completely every 24 hours and the amount of each complement component secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and complement component levels are expressed as µg complement component produced by 100mg tissue (mean±SD). Synovium fragment cultures were treated with 2.5µg/ml cycloheximide (▲—▲) or were left untreated and cultured in medium alone (■—■).

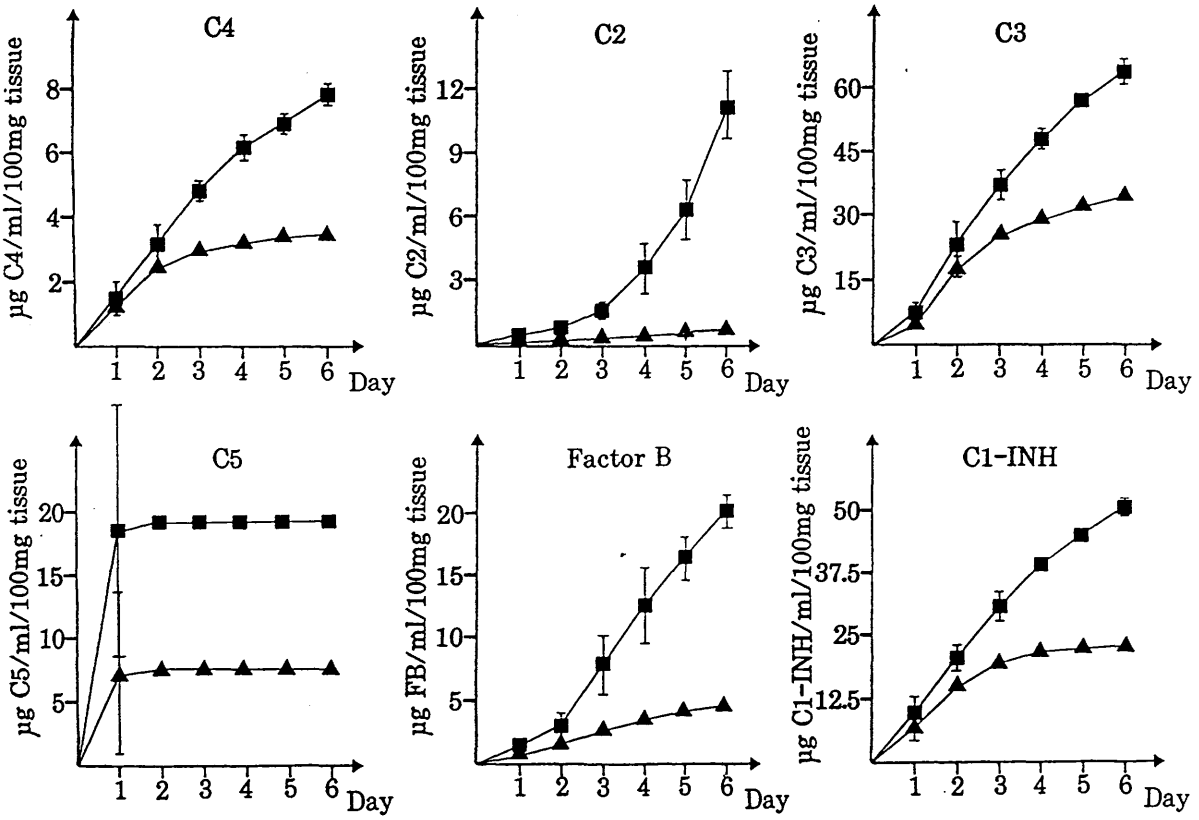


Figure 24 - ELISA analysis of supernatants from cultured fragments of synovial membrane from rheumatoid arthritis patient, RA9. The culture medium was changed completely every 24 hours and the amount of each complement component secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and complement component levels are expressed as µg complement component produced by 100mg tissue (mean±SD). Synovium fragment cultures were treated with 2.5µg/ml cycloheximide (▲—▲) or were left untreated and cultured in medium alone (■—■).

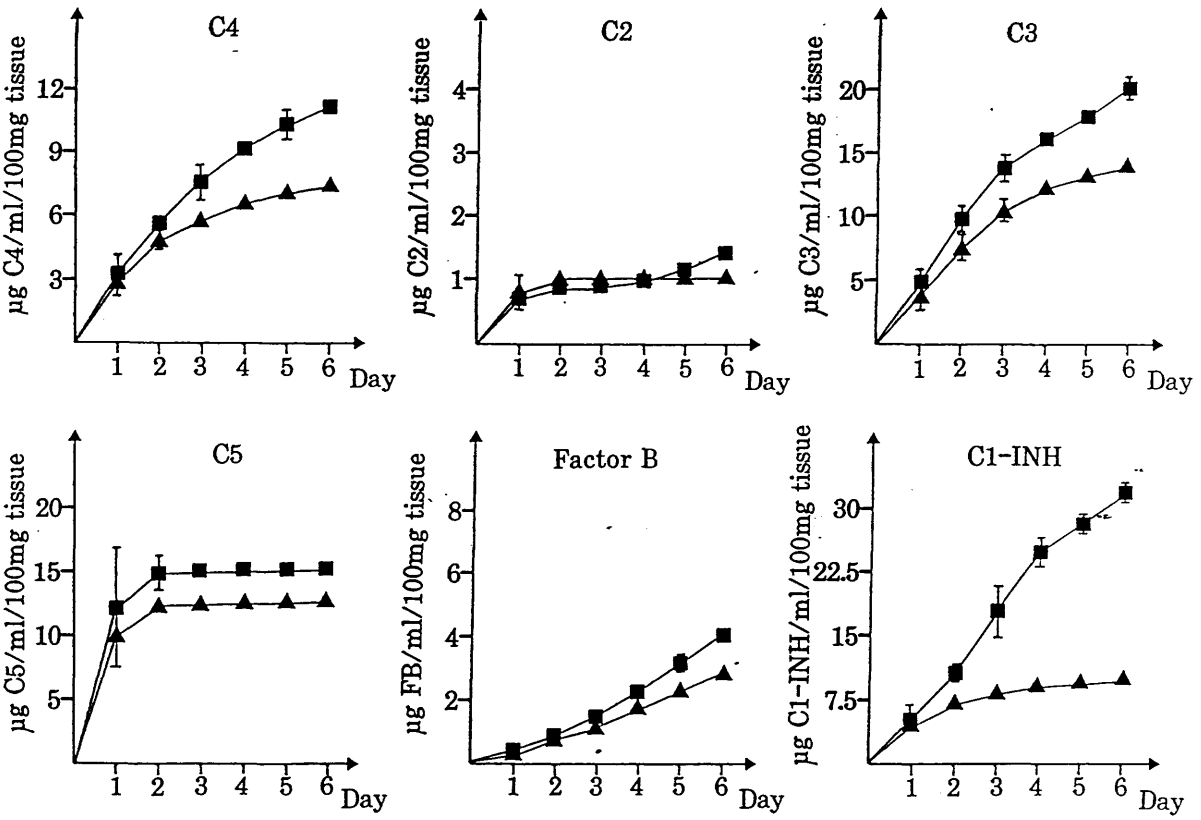


Figure 25 - ELISA analysis of supernatants from cultured fragments of synovial membrane from osteoarthritis patient, OA5. The culture medium was changed completely every 24 hours and the amount of each complement component secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and complement component levels are expressed as µg complement component produced by 100mg tissue (mean±SD). Synovium fragment cultures were treated with 2.5µg/ml cycloheximide (▲—▲) or were left untreated and cultured in medium alone (■—■).

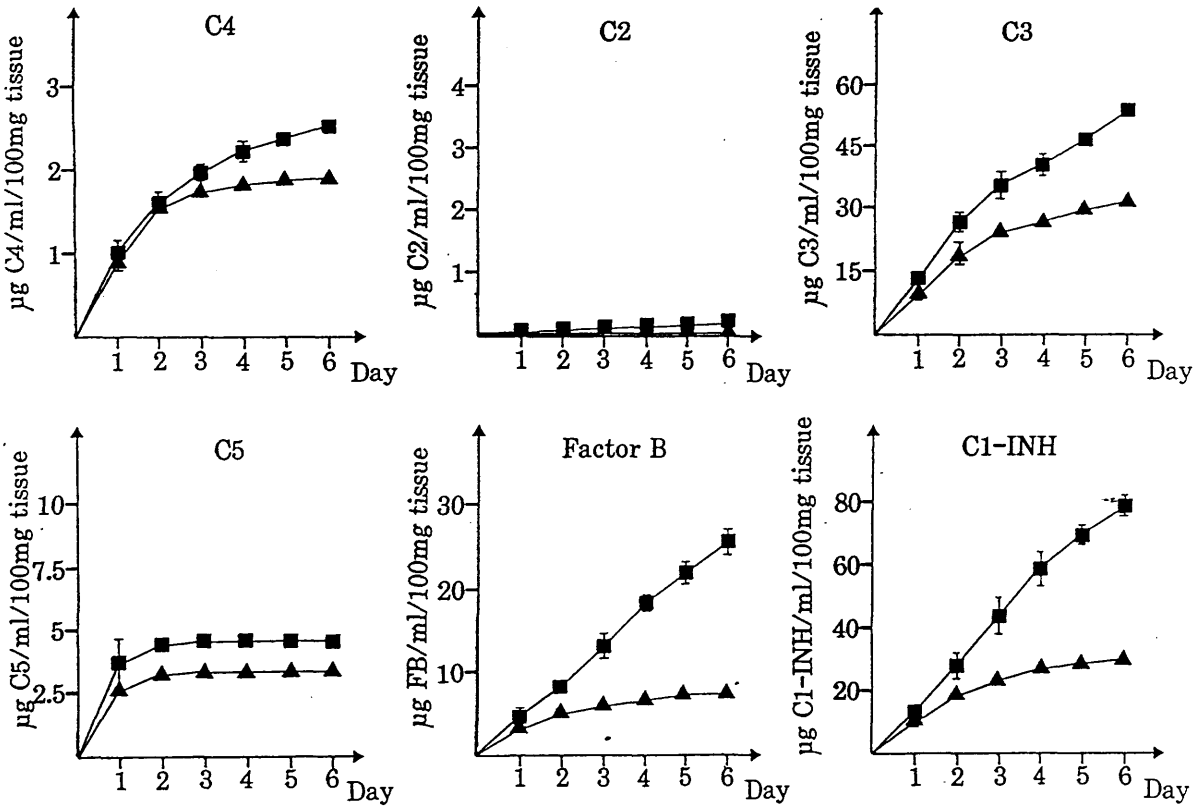


Figure 26 - ELISA analysis of supernatants from cultured fragments of synovial membrane from osteoarthritis patient, OA8. The culture medium was changed completely every 24 hours and the amount of each complement component secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and complement component levels are expressed as µg complement component produced by 100mg tissue (mean±SD). Synovium fragment cultures were treated with 2.5µg/ml cycloheximide (▲—▲) or were left untreated and cultured in medium alone (■—■).

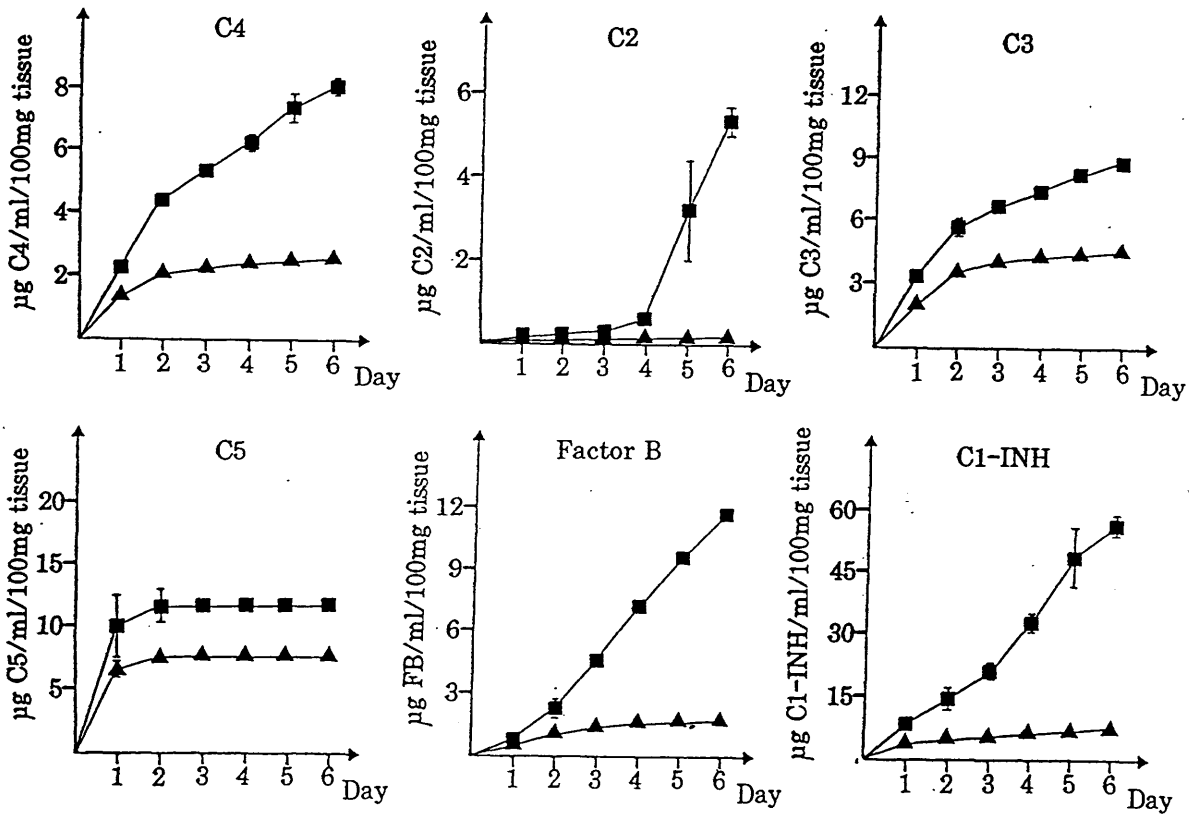


Figure 27 - ELISA analysis of supernatants from cultured fragments of synovial membrane from osteoarthritis patient, OA15. The culture medium was changed completely every 24 hours and the amount of each complement component secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and complement component levels are expressed as µg complement component produced by 100mg tissue (mean±SD). Synovium fragment cultures were treated with 2.5µg/ml cycloheximide (▲—▲) or were left untreated and cultured in medium alone (■—■).

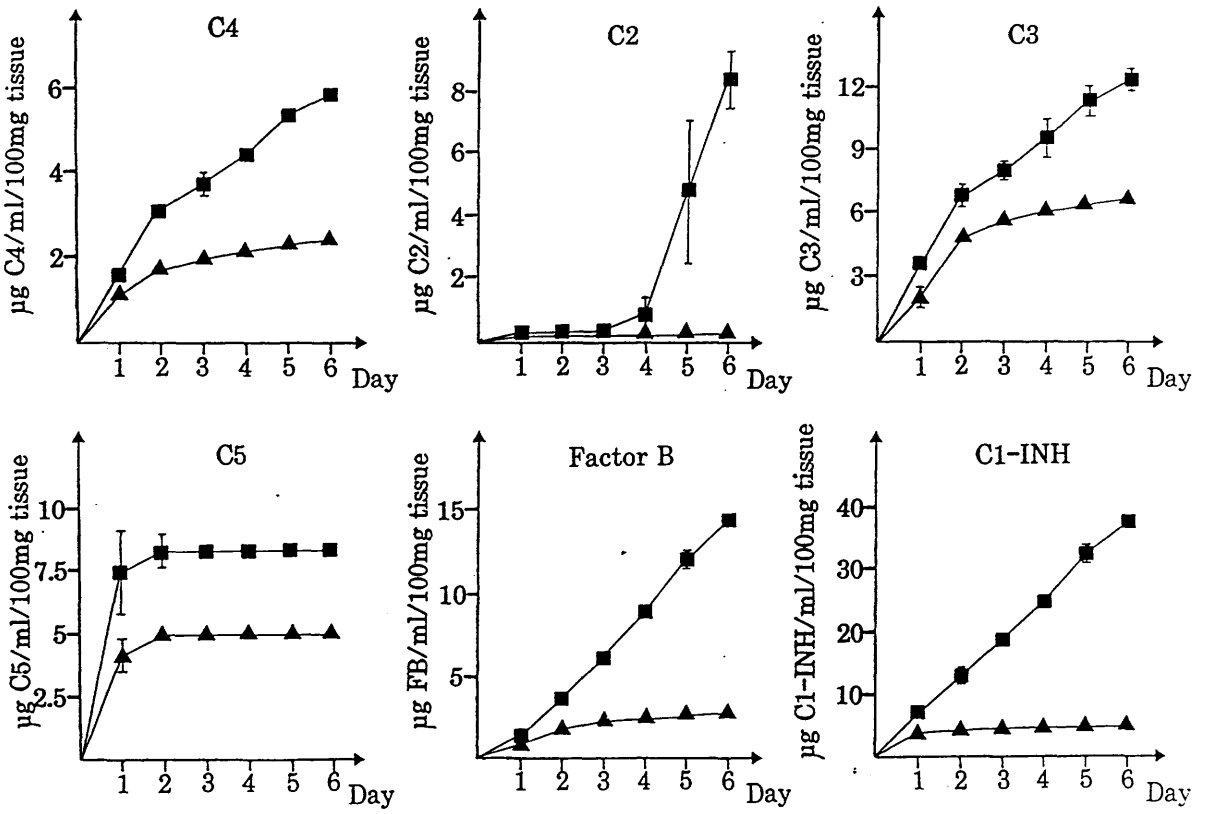


Figure 28 - ELISA analysis of supernatants from cultured fragments of normal synovial membrane from a patient who underwent patellectomy, N1. The culture medium was changed completely every 24 hours and the amount of each complement component secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and complement component levels are expressed as µg complement component produced by 100mg tissue (mean±SD). Synovium fragment cultures were treated with 2.5µg/ml cycloheximide (▲—▲) or were left untreated and cultured in medium alone (■—■).

(b) C2 synthesis

Over the six day period of culture, the total amount of C2 released into the culture medium in the six patient samples was: RA8 (25.78μg C2), RA9 (11.33μg C2), OA5 (1.44μg C2), OA15 (5.38μg C2) and N1 (8.39μg C2). Only trace amounts of C2 were detected in the OA8 culture supernatants, even although C2 mRNA had been detected for this sample in Northern and dot-blot analyses. The concentration of C2 was not significantly reduced by the presence of cycloheximide in cultures OA5 and OA8, whereas the introduction of cycloheximide into the culture medium caused a significant reduction in C2 accumulation in RA8, RA9, OA15 and N1. Patient samples RA8, RA9, OA15 and N1 all showed a delay in the initiation of C2 synthesis. In patients RA8 and RA9 this delay lasted 3 days while a delay of 4 days was observed for OA15 and N1. Thereafter, the rate of C2 synthesis increased markedly for the remaining period of culture.

(c) C3 synthesis

The pattern of C3 accumulation in the culture supernatants was similar to that seen for C4, ie. the concentration of C3 increases throughout the period of culture but the rate of increase diminishes with time. No delay in C3 synthesis was observed. In the presence of cycloheximide, the concentration of C3 was sigificantly reduced in all cases. After six days in culture, the concentration of C3 in the culture supernatants of the six patient samples was: RA8 (39.36μg C3), RA9 (64.35μg C3), OA5 (20.42μg C3), OA8 (53.53μg C3), OA15 (9.03μg C3) and N1 (12.42μg C3). All six samples showed similar patterns of C3 release into the culture medium, although the reduction in C3 concentration by cycloheximide was less marked in OA5 and OA8.

(d) C5 synthesis

Although C5 was detected in all culture supernatants, its concentration did not increase after the second or third day in culture, although in the presence of cycloheximide a reduction in C5 concentration was observed. At the end of the culture period, the total amount of C5 released into the culture medium for the six samples was: RA8 (7.33μg C5), RA9 (19.29μg C5), OA5 (16.3μg C5), OA8 (4.67μg C5), OA15 (11.62μg C5) and N1 (8.12μg C5). It is possible that the C5 detected was due to contamination of the synovial tissue with plasma or inflammatory exudate or from *in vitro* release of C5 from synovial cells following pinocytic uptake *in vivo*.

(e) Factor B synthesis

After six days in culture, the total concentration of factor B in the culture supernatants of the six synovium specimens was: RA8 (5.02μg factor B), RA9 (19.69μg factor B), OA5 (3.98μg factor B), OA8 (25.07μg factor B), OA15 (11.55μg factor B) and N1 (14.18μg factor B). The pattern of factor B synthesis by the synovial membrane cultures shows more similarity to C2 accumulation than C3 or C4 synthesis. In samples RA8 and RA9 there was a 2 day delay in factor B accumulation followed by a steady daily increase in factor B synthesis. This delay was not so marked in cultures OA8, OA15 and N1, although like RA8 and RA9 the rate of accumulation of factor B in the culture supernatants did not appear to significantly increase or diminish during the period of culture. Unlike the cultures RA8, RA9, OA8, OA15 and N1, factor B synthesis by sample OA5 was not significantly reduced by the presence of cycloheximide although factor B mRNA had been detected in this sample by Northern and dot-blot analyses.

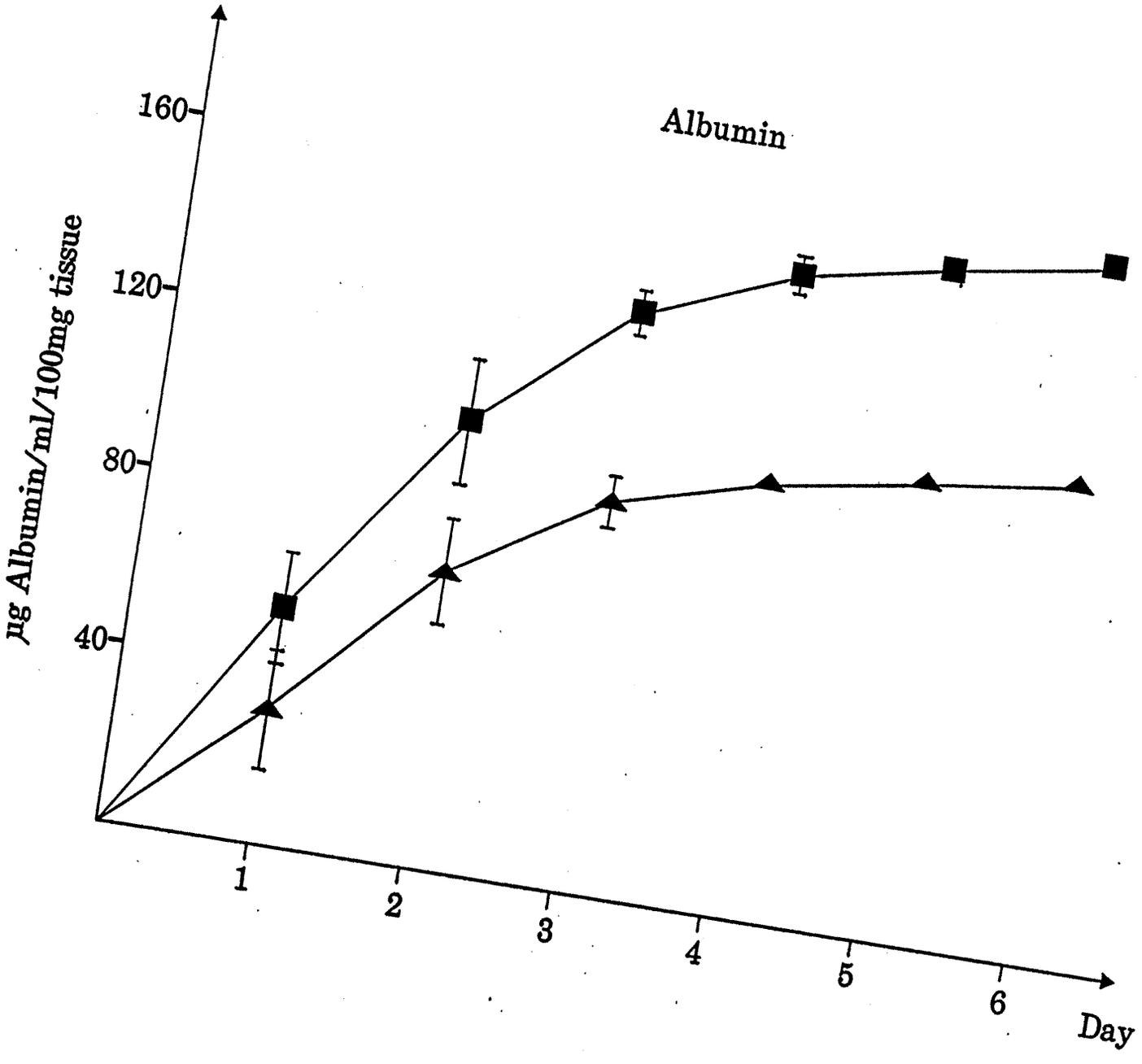
(f) C1-INH synthesis

The pattern of C1-INH accumulation in the culture supernatants was similar to that seen for factor B and C2. A slight delay in C1-INH synthesis was observed for samples RA8 and OA15. All six samples showed a steady increase of C1-INH concentration in the culture supernatants although the rate of accumulation did show a slight decline towards the end of the period of culture. In all cultures the concentration of C1-INH was significantly reduced by the presence of cycloheximide. The concentration of C1-INH in the culture supernatants at the end of the period of culture was: RA8 (19.97μg C1-INH), RA9 (50.31μg C1-INH), OA5 (31.73μg C1-INH), OA8 (78.16μg C1-INH), OA15 (56.61μg C1-INH) and N1 (38.05μg C1-INH). Of the six complement components investigated, C1-INH and C3 were detected in the greatest abundance in the culture supernatants.

(g) Albumin release from cultured fragments of synovial membrane

As stated above, the accumulation of C5 in the culture supernatants did not increase after the second or third day in culture. As a result it was thought that the C5 being detected by ELISA was due to contamination of the synovial tissue with plasma or inflammatory exudate or from *in vitro* release of C5 from synovial cells following pinocytic uptake *in vivo*. Levels of albumin in the culture supernatants were measured as a control for either of these events. Figure 29 shows the results of this analysis on the RA8 culture supernatants but the other samples all showed similar results. Release of albumin into the culture supernatants followed a similar pattern to C5 release with the concentration of albumin not increasing significantly more than the cycloheximide-treated cultures after the second day in culture. However, like

Figure 29 - ELISA analysis for albumin release by synovial membrane cultures of rheumatoid arthritis patient, RA8. The culture medium was changed completely every 24 hours and the amount of albumin secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and albumin levels are expressed as μg albumin produced by 100mg tissue (mean±SD). Synovium fragment cultures were treated with 2.5μg/ml cycloheximide (▲—▲) or were left untreated and cultured in medium alone (■—■).


Albumin
160
120
80
40
μg Albumin/ml/100mg tissue
1
2
3
4
5
6
Day

C5 accumulation, in the presence of cycloheximide a reduction in albumin concentration was observed in the early period of culture.

In summary, C4, C2, C3, factor B and C1-INH were detected in the culture supernatants and their levels increased throughout the period of culture. When cycloheximide was present the concentrations of these components were reduced markedly. In contrast, although C5 was detected by ELISA, its concentration did not increase after the second or third day of culture.

Levels of albumin were measured as a control for contamination of the synovial tissue with plasma or inflammatory exudate, or for release of protein from synovial cells following pinocytic uptake *in vivo*. The pattern of albumin accumulation in the culture supernatants was similar to that seen for C5.

All samples (except OA5 and OA8) showed a delay in C2 synthesis. This delay in accumulation (although less marked) was also observed for factor B and C1-INH synthesis in samples RA8, RA9 and OA15. After the initial delay, the rate of accumulation of C2, factor B and C1-INH continued steadily throughout the remaining period of culture.

There was no delay in the onset of C3 and C4 synthesis which began to accumulate on the first day of culture. The rate of accumulation of these two proteins decreased towards the end of the culture period.

The concentrations of C1-INH and C3 in the culture supernatants were generally higher than the other complement components studied.

6. DETECTION OF FUNCTIONALLY ACTIVE COMPLEMENT COMPONENTS IN SYNOVIAL MEMBRANE CULTURE SUPERNATANTS

(a) Purification of D from human serum for use in the factor B haemolytic assay

In order to perform the factor B haemolytic assay, it was necessary to purify D from human serum. Biorex 70 cation-exchange chromatography was performed using 180ml of human serum and the resulting chromatograph is shown in figure 30. The exclusion peak (fractions 25-40) contained no D activity as shown by its failure to lyse rabbit erythrocytes. It was therefore used as an RD reagent (D depleted serum reagent) to screen the remainder of the column for D activity.

Fractions 184-195 were found to contain D activity and so they were pooled. A 1/10 dilution of this pool gave an OD_{280} reading of 0.424 which gave an approximate protein concentration of 4.24mg/ml in a total volume of 135ml. This pool was concentrated to 20.7mg/ml in a final volume of 20ml.

Before use in the factor B assay, it was necessary to titrate the purified D using a D assay and reconstitution of the RD reagent (figure 31). The concentration of D was calculated to be 19.3 units/ml. When D was titrated in the factor B assay, some lysis occurred in the samples containing D in the absence of factor B. This finding indicated trace contamination of D with factor B.

However, at a 1/40 dilution, the D alone sample produced no lysis. Thus a 1/40 dilution of D was used in all the factor B haemolytic assays.

Figure 30 - Biorex-70 cation exchange chromatograph of D purification from human serum. The OD_{280} (●—●) and conductivity at 4°C (△—△) were measured. Fractions 25-40 were pooled as the RD reagent while fractions 184-195 were found to contain the D activity.

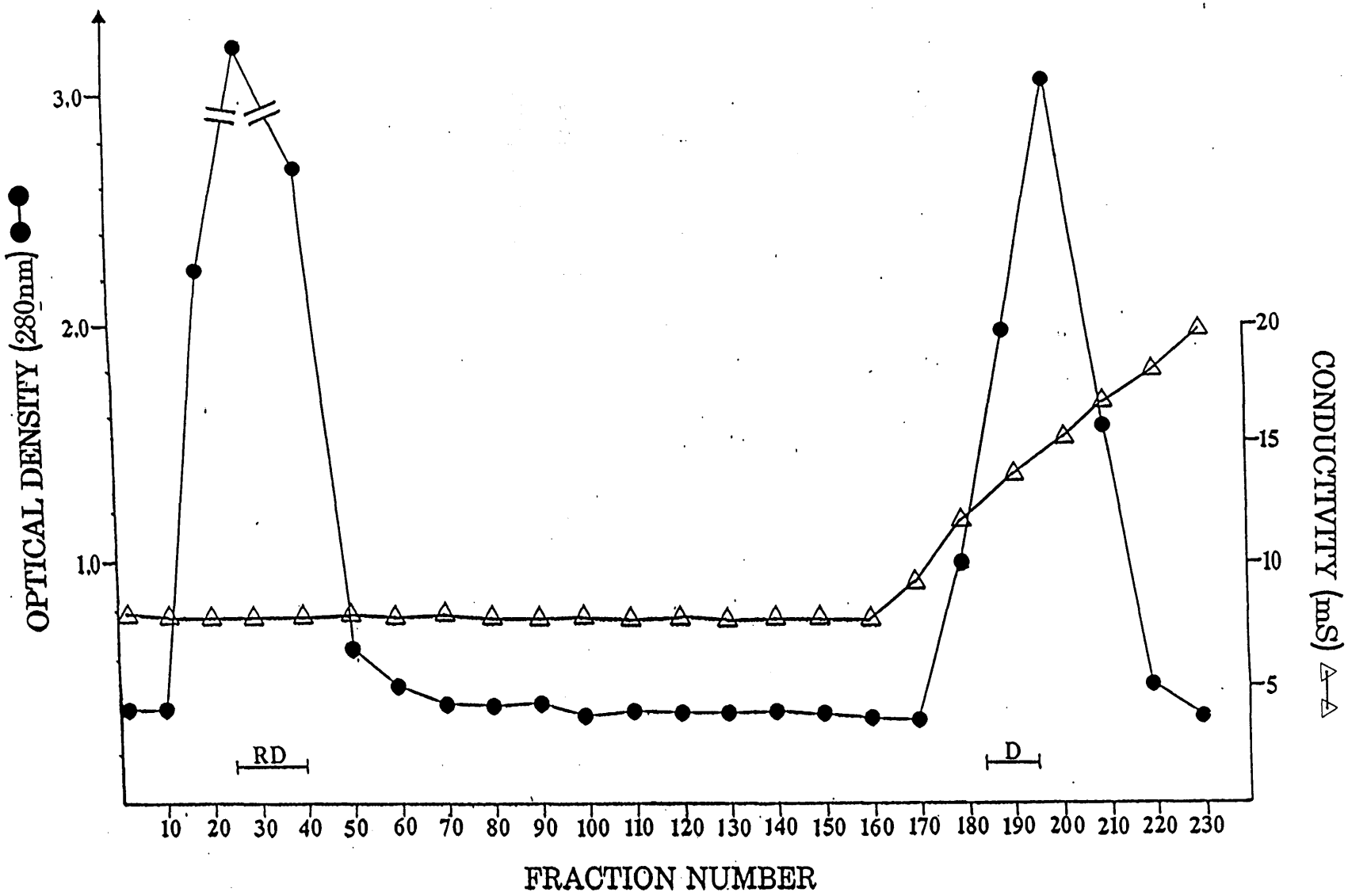
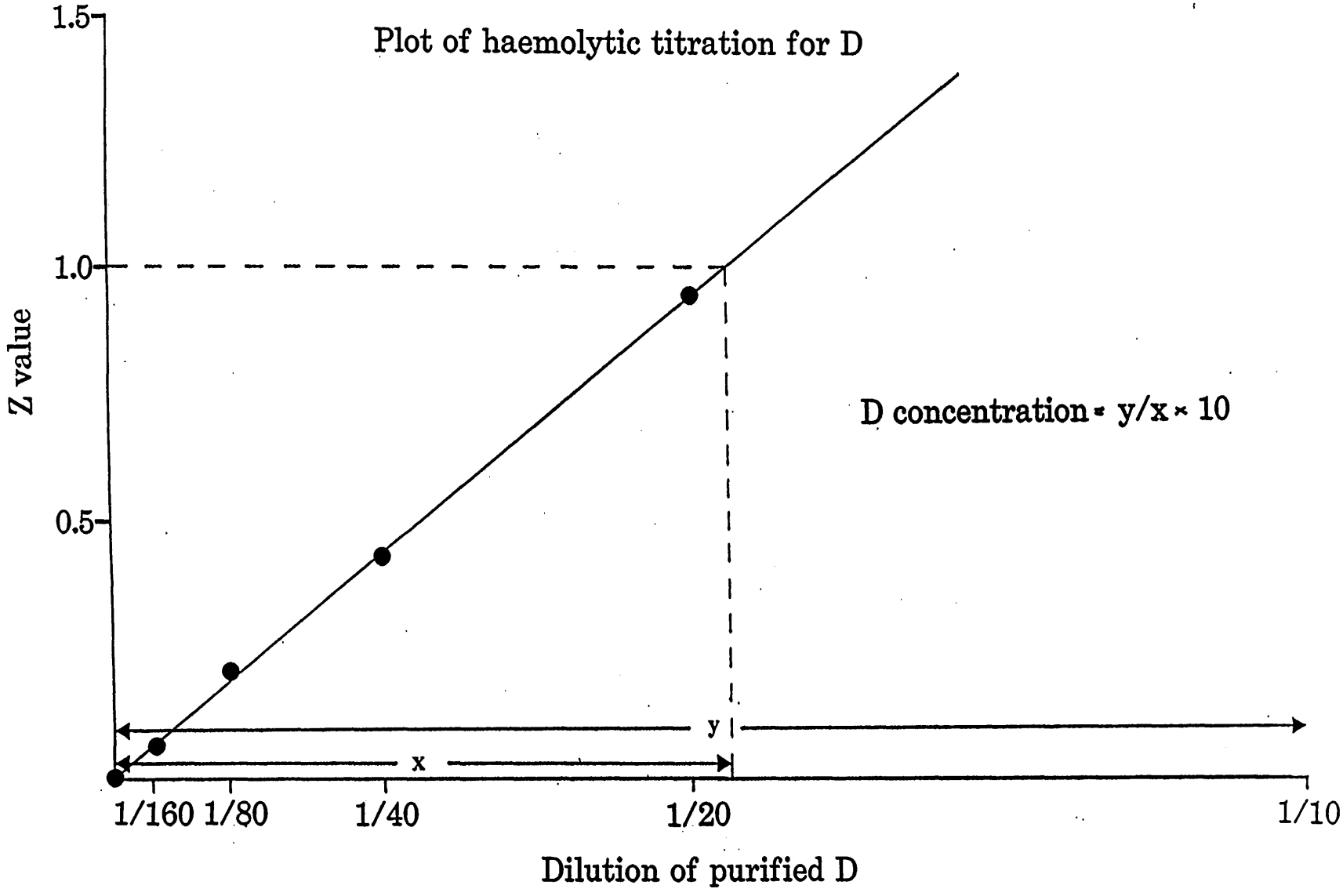

OPTICAL DENSITY (280nm)
3.0
2.0
1.0
CONDUCTIVITY (mS)
20
15
10
5
RD
D
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230
FRACTION NUMBER

Figure 31 - Titration of D purified from human serum, using reconstitution of the RD reagent.


Plot of haemolytic titration for D
1.5
1.0
0.5
Z value
D concentration = y/x × 10
y
x
1/160
1/80
1/40
1/20
1/10
Dilution of purified D

(b) T_{max} test

Before using a batch of EAC4 cells, a t_{max} test was performed. The reason for this test is explained below:

The natural decay of C2a from the C4b2a enzyme makes the titration of C2 somewhat more complicated than the titration of other components. When one studies the kinetics of formation of C142, it is noted that formation starts immediately, reaches its peak and then the enzyme decays. The time taken for the peak to be reached is called t_{max} and is dependent upon the concentration of C4b on EAC14, but is independent of the C2 concentration. The shorter the t_{max} the more functionally active C4b on EAC14 (Borsos et al., 1961).

The per cent lysis in each tube and the number of haemolytic sites (Z) were calculated. Z was then plotted against time and t_{max} determined by inspection (figure 32). The t_{max} of this batch of EAC4 cells was 3.5 min and in practice cells are accepted if they have a t_{max} of less than 6 min.

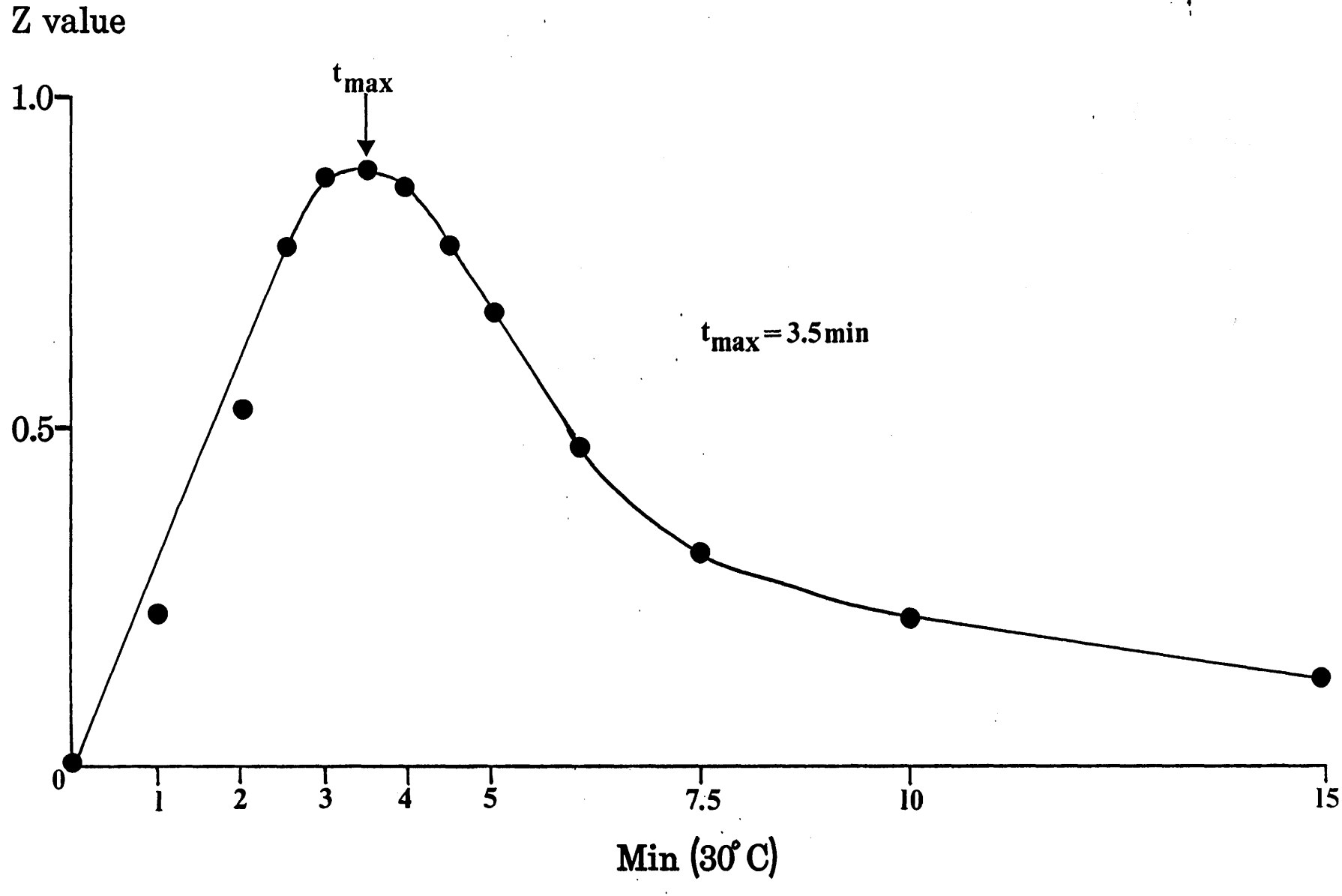
(b) Results of the haemolytic assays for C4, C2, C3, C5 and factor B

Figures 33 and 34 show the results of haemolytic assays performed on the synovial membrane culture supernatants from two patient samples (1 with osteoarthritis (OA15) and 1 patient (N1) who underwent patellectomy for recurrent patellar dislocation).

C4 assay

C4 haemolytic activity was detected in both samples on day 1: OA15 (12×10^7 e.m./100mg tissue) and N1 (9×10^7 e.m./100mg tissue). Thereafter no significant level of C4 haemolytic activity was detected in culture supernatants from either sample. In both samples, the C4

Figure 32 - Plot of t_{max} assay.


Z value
1.0
0.5
0
t_{max}
t_{max} = 3.5 min
1
2
3
4
5
7.5
10
15
Min (30° C)

haemolytic activity was not significantly higher than the C4 haemolytic activity detected in the supernatants of the cultures treated with cycloheximide.

C2 assay

Substantial C2 haemolytic activity was detected in both samples. At the end of the six day culture period, the accumulated C2 functional activity in the two patient samples was: OA15 (450 x 10^7 e.m./100mg tissue) and N1 (250 x 10^7 e.m./100mg tissue). N1 exhibited a steady increase in the rate of C2 activity increase throughout the period of culture while the rate increased from day 2 to 4 in OA15. In both samples, the rate of increase of C2 haemolytic activity had started to diminish slightly towards the end of the period of culture.

C3 assay

C3 haemolytic activity was detected in the culture supernatants of the two patient samples. After six days in culture, the cumulative total of C3 haemolytic activity had reached 12 x 10^7 e.m./100mg tissue and 15 x 10^7 e.m./100mg tissue for OA15 and N1 respectively. In both samples there was a slight increase in the rate of increase of C3 haemolytic activity towards the end of the period of culture, although on day 6, the rate in OA15 had started to decrease again.

C5 assay

C5 haemolytic activity (approximately 10 x 10^7 e.m./100mg tissue for both samples) was detected on day 1. However, there was no detectable C5 haemolytic activity during the remaining period of culture compared to the cycloheximide-treated cultures.

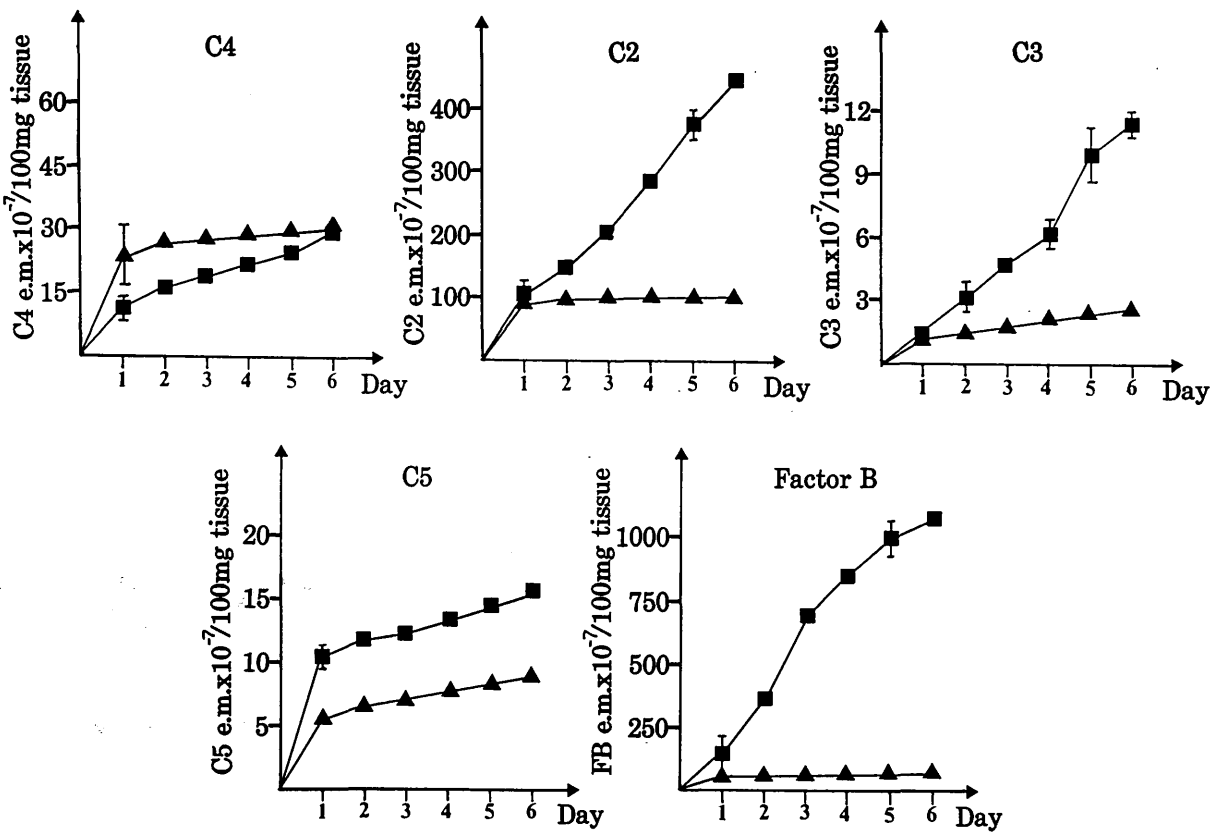


Figure 33 - Haemolytic assays of supernatants from cultured fragments of synovial membrane from osteoarthritis patient, OA15. Supernatants from the same cultures were analysed by both ELISA and haemolytic assay. Cumulative curves are plotted and haemolytic activity is expressed as effective molecules (e.m.) x 10^{-7} produced by 100mg tissue (mean±SD). Synovium fragment cultures were treated with 2.5µg/ml cycloheximide (▲—▲) or were left untreated and cultured in medium alone (■—■).

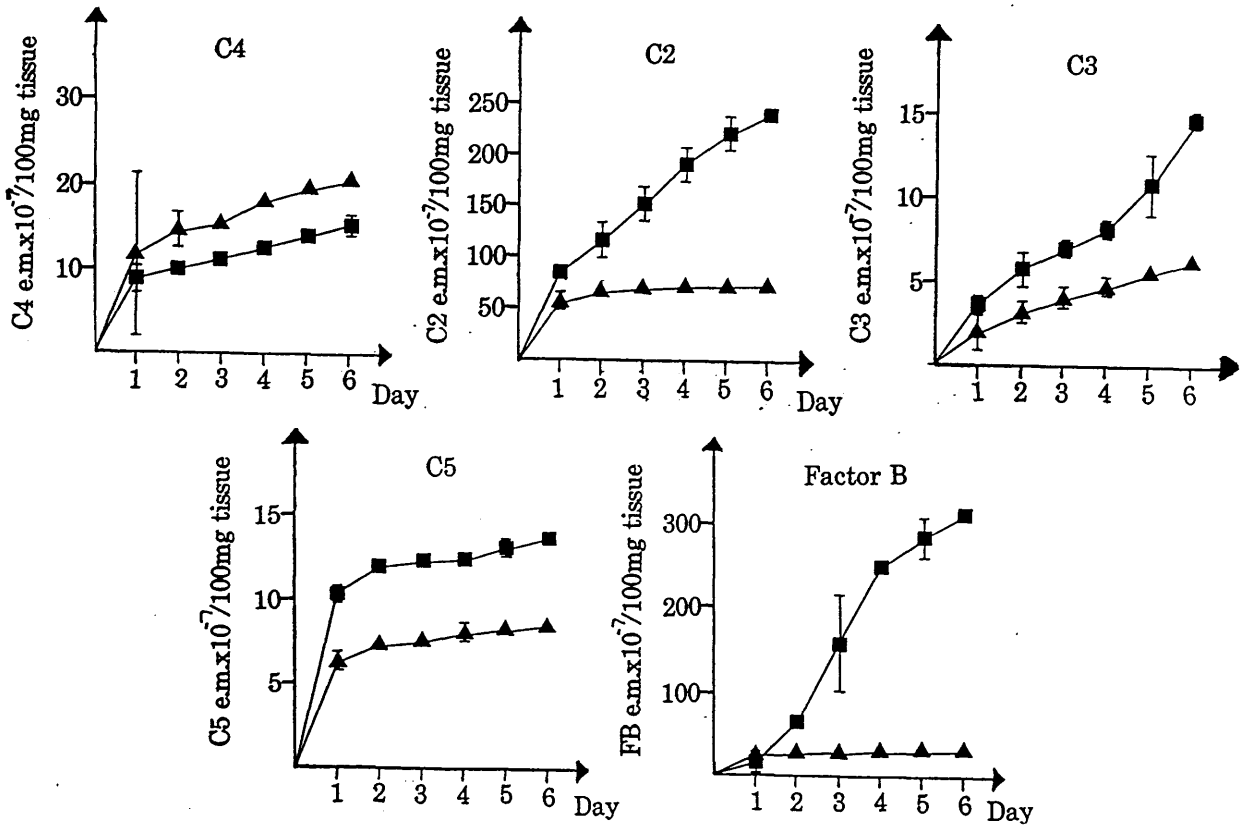


Figure 34 - Haemolytic assays of supernatants from cultured fragments of normal synovial membrane from a patient undergoing patellectomy, N1. Supernatants from the same cultures were analysed by both ELISA and haemolytic assay. Cumulative curves are plotted and haemolytic activity is expressed as effective molecules (e.m.) x 10^{-7} produced by 100mg tissue (mean±SD). Synovium fragment cultures were treated with 2.5µg/ml cycloheximide (▲—▲) or were left untreated and cultured in medium alone (■—■).

Factor B assay

Factor B haemolytic activity was detected at high levels in both samples. At the end of the six day culture period, the cumulative total of factor B haemolytic activity for the two patient samples was: OA15 (1000 x 10^7 e.m./100mg tissue) and N1 (300 x 10^7 e.m./100mg tissue). Culture supernatants from both OA15 and N1 showed an increasing rate of increase of factor B haemolytic activity during the first three days of culture after which time the rate gradually decreased.

In summary, haemolytic assays revealed that C2, C3 and factor B in synovial membrane culture supernatants were functionally active, while C4 and C5 haemolytic activity was not detected. C1-INH was not tested for functional activity as foetal calf serum contains high levels of C1-inhibitory activity (Muller et al., 1978).

7. INCORPORATION OF ^{35}S-methionine INTO COMPLEMENT PROTEINS

Incorporation of ^{35}S-methionine into the complement proteins synthesised by fragments of synovial membrane cultured *in vitro* was investigated. Synovial membrane from four patients (1 with rheumatoid arthritis (RA13) and 3 with osteoarthritis (OA16 - OA18)) was studied.

Table 13 shows the level of incorporation of ^{35}S-methionine into protein in each culture by measurement of the acid precipitable counts. On average, approximately 3% incorporation of ^{35}S-methionine was observed in each culture. Clearly, incubation of the synovial membrane cultures for 4 days (RA13*) had no detectable effect on the level of incorporation.

Immunoprecipitation of complement proteins in the synovial membrane culture supernatants was performed. These immunoprecipitates were washed thoroughly to remove any non-specific binding to the complexes. However, electrophoresis of the immunoprecipitates on SDS-PAGE and subsequent fluorography produced the same high background pattern which included an intense 60 kDa band in all samples. Attempts to remove this background using a variety of incubations with:

(i) *Staph. aureus* protein A

(ii) Sepharose protein A and protein G

or (iii) IgG-Sepharose,

resulted in only reducing or completely removing the entire signal previously detected by fluorography, including any bands considered to be specific.

Figures 35 and 36 show the SDS-PAGE results from the sample RA13 but the other 3 samples produced similar results:

PATIENT	PERCENTAGE INCORPORATION OF ^{35}S-methionine
RA13^{1}	2.02%
RA13^{2}	5.97%
RA13^{1}*	2.22%
RA13^{2}*	3.08%
OA16^{1}	2.82%
OA16^{2}	2.75%
OA17^{1}	0.94%
OA17^{2}	0.66%
OA18^{1}	7.07%
OA18^{2}	5.39%

Table 13 - **Incorporation of ^{35}S-methionine into acid precipitable protein. All samples were cultured in duplicate for 24 hours except for samples RA13^{1}* and RA13^{2}* which were cultured for 4 days.**

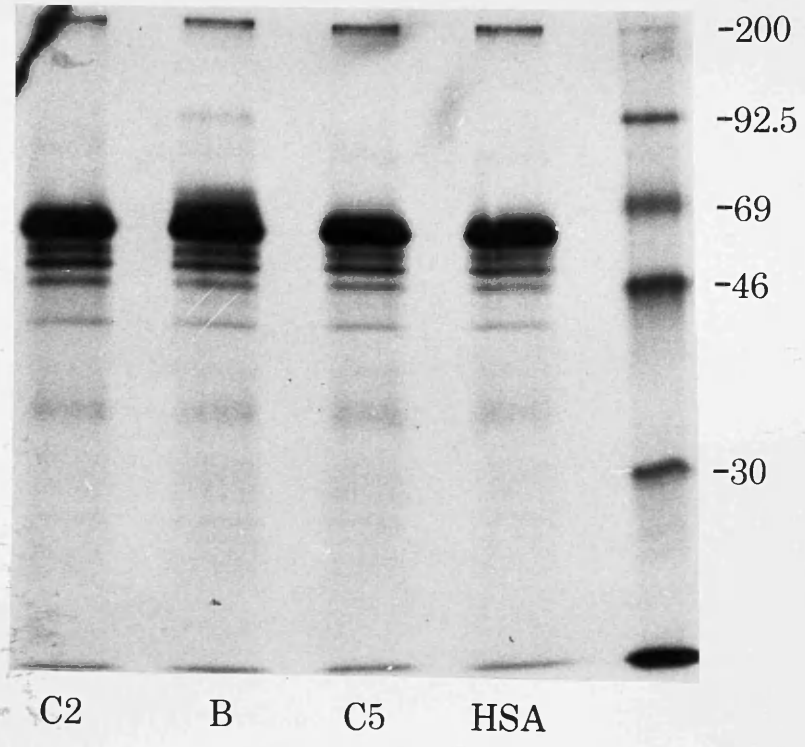


Figure 35 - SDS-polyacrylamide gel electrophoresis of ^{35}S-methionine labelled immunoprecipitates from the synovial membrane culture supernatants of rheumatoid arthritis patient, RA13. The synovium fragments were cultured for 24 hours. Immunoprecipitation was performed with the IgG fraction of the following goat anti-human antibodies: factor B, C2 and C5. Anti-human serum albumin was used as a negative control. Fluorography of the gel was performed at -70°C for 4 weeks.

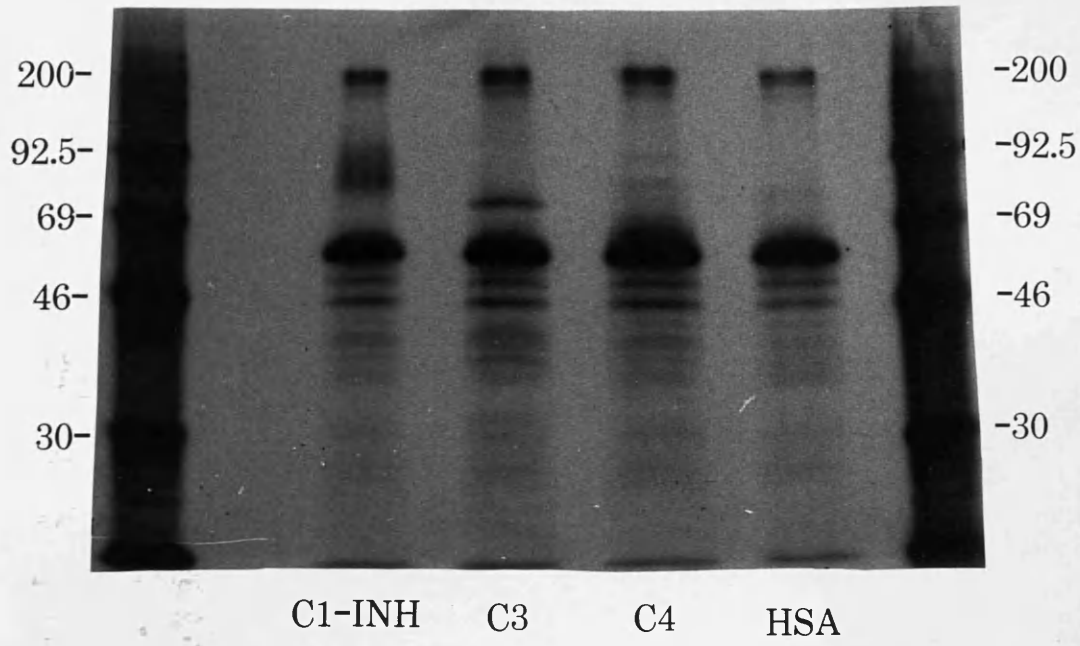


Figure 36 - SDS-polyacrylamide gel electrophoresis of ^{35}S-methionine labelled immunoprecipitates from the synovial membrane culture supernatants of rheumatoid arthritis patient, RA13. The synovium fragments were cultured for 24 hours. Immunoprecipitation was performed with the IgG fraction of the following goat anti-human antibodies: C1-INH, C3 and C4. Anti-human serum albumin was used as a negative control. Fluorography of the gel was performed at -70°C for 4 weeks.

(a) C1-INH - a smeared pattern at approximately 90 kDa was always detected for C1-INH. However this is significantly less than the correct size of C1-INH (110 kDa) and may represent degraded or unglycosylated C1-INH.

(b) C3 - a band at 75 kDa was detected for each sample. This band could represent the intact β-chain of the C3 molecule. However a band corresponding to the α-chain of C3 was not detected and most likely has been degraded.

(c) Factor B - this component produced the best results with a definite band at 92 kDa. This band corresponds exactly to the known molecular weight of the factor B protein.

Bands corresponding to the known molecular weights of the polypeptide chains of C4, C2 and C5 (or their cleavage products) were not detected in any patient sample.

8. *In vitro* COMPLEMENT BIOSYNTHESIS BY CELLS ISOLATED FROM HUMAN SYNOVIAL MEMBRANE

The ability of the cells isolated from human synovial membrane to synthesise complement components *in vitro* was measured by ELISA.

The synovial membrane from 4 patients was used in these studies (2 with rheumatoid arthritis, RA14 and RA15, and 2 with osteoarthritis, OA19 and OA20). The cells isolated from these tissue samples were plated at a cell density of 1 x 10^6cells/culture dish and then cultured for six days with the culture medium being changed daily. After 24 hours, those cells that had not adhered to the culture dish (usually 30-40% of the total number of cells) were separated from the adherent cells. The non-adherent cells were then cultured in a separate culture dish for the same length of time as the adherent cells. The stage at which the adherent and non-adherent cells were separated (ie. after the initial 24 hour culture period) was regarded as day zero in the biosynthesis experiments.

The concentrations of complement components in the culture supernatants from both the adherent and non-adherent cells were measured by ELISA in order to compare the rates of complement synthesis by these two cell populations. Figures 37-40 show the results of these ELISA analyses. In each case, complement biosynthesis was significantly reduced when cycloheximide (2.5μg/ml) was introduced into the medium.

C4 synthesis

After six days in culture, the non-adherent cells from samples RA14 and OA19 synthesised significantly more C4 (RA14, 0.2μg C4; OA19, 4.2μg C4) than the adherent cells (RA14, 0.05μg C4; OA19, 0.3μg C4). Indeed, the small amount of C4 synthesised by RA14 adherent cells was

not significantly more than that produced by the cycloheximide-treated cultures. However, samples RA15 and OA20 did not show such a marked difference between the different cell populations. After six days, the adherent and non-adherent cells of RA15 released 0.21μg C4 and 0.2μg C4 respectively into the culture supernatants while the adherent cells of OA20 released 1.1μg C4 as opposed to 0.6μg C4 by the non-adherent cells. Samples RA14 and RA15 showed similar patterns of C4 accumulation ie. the rate of increase in the concentration of C4 decreased towards the end of the period of culture. OA20 cells synthesised C4 at a steady rate throughout the six days while the rate of C4 accumulation by OA19 non-adherent cells increased daily.

C3 synthesis

The adherent cells of RA15 and OA20 synthesised significantly more C3 (2.9μg C3 and 1.2μg C3 respectively) than the non-adherent cells (0.9μg C3 and 0.62μg C3 respectively). Both cell populations of RA14 synthesised similar amounts of C3 (adherent, 1.45μg C3; non-adherent, 1.1μg C3), while OA19 non-adherent cells synthesised a lot more C3 (8.5μg C3) than the adherent cells (2μg C3). A steady rate of C3 synthesis throughout the culture period was shown for RA14 and RA15 while an increasing rate of C3 accumulation was observed for OA19 and also OA20.

Factor B synthesis

At the end of the culture period, the non-adherent cells of RA14 and OA19 had synthesised more factor B (1.4μg factor B and 3.2μg factor B respectively) than the adherent cells (RA14, 1.1μg factor B; OA19, 1μg factor B). The two cell populations of sample OA20 synthesised similar

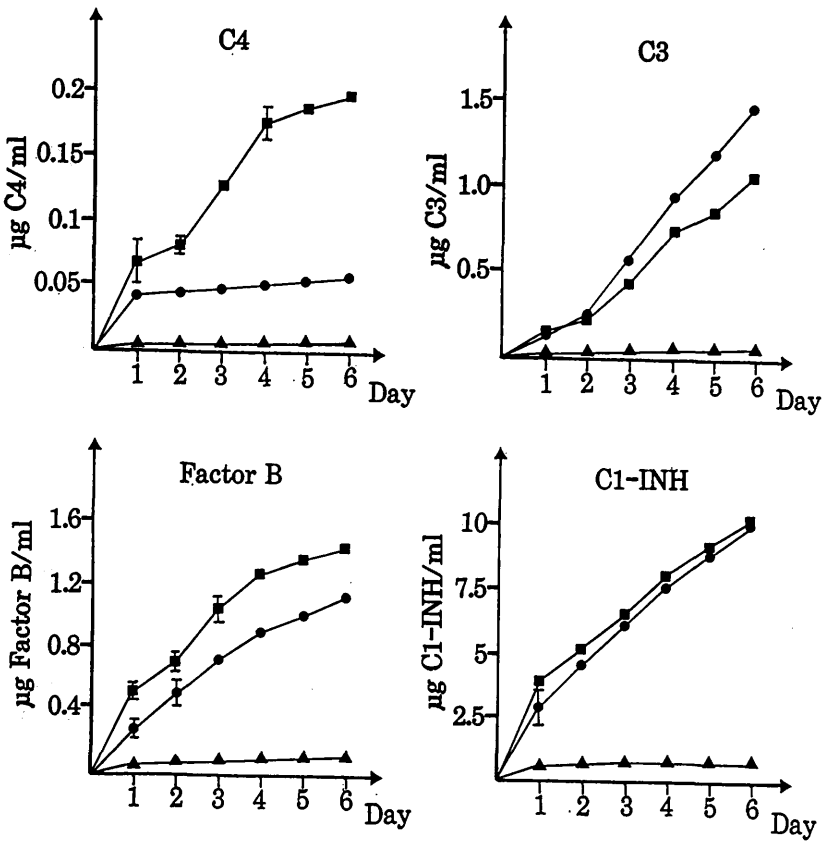


Figure 37 - Concentrations of complement components (measured by ELISA) in the culture supernatants from the adherent (●—●) and non-adherent (■—■) synovial cells of rheumatoid arthritis patient, RA14. The culture medium was changed completely every 24 hours and the amount of each complement component secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and complement component levels are expressed as µg complement component per 1ml of culture medium (mean±SD). One adherent and one non-adherent cell culture were treated with 2.5µg/ml cyclo-heximide (▲—▲).

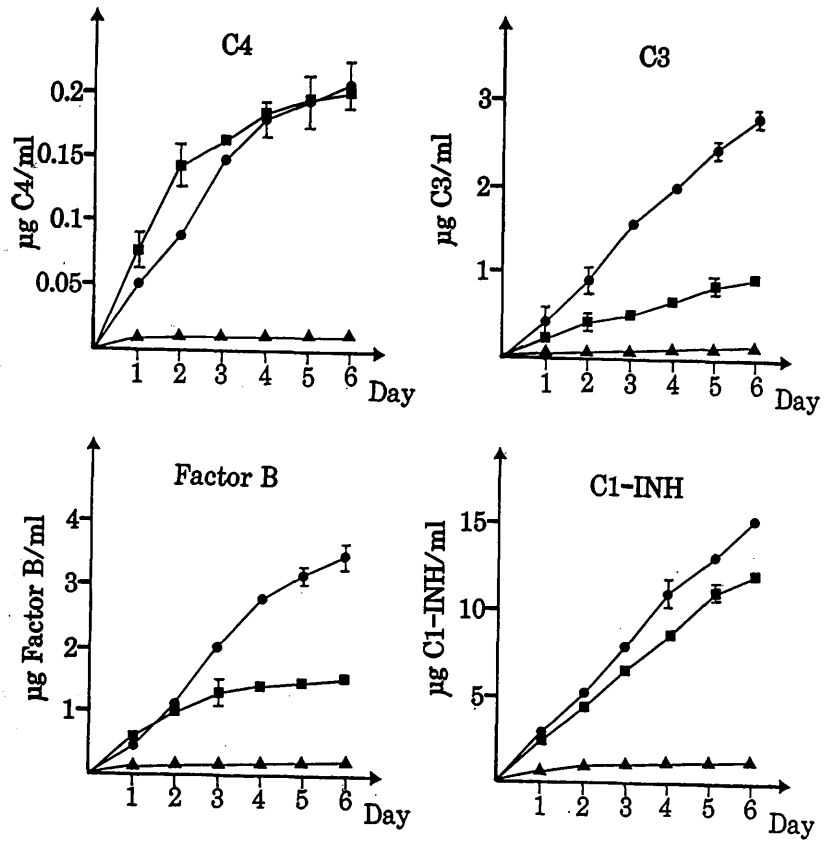


Figure 38 - Concentrations of complement components (measured by ELISA) in the culture supernatants from the adherent (●—●) and non-adherent (■—■) synovial cells of rheumatoid arthritis patient, RA15. The culture medium was changed completely every 24 hours and the amount of each complement component secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and complement component levels are expressed as µg complement component per 1ml of culture medium (mean±SD). One adherent and one non-adherent cell culture were treated with 2.5µg/ml cyclo-heximide (▲—▲).

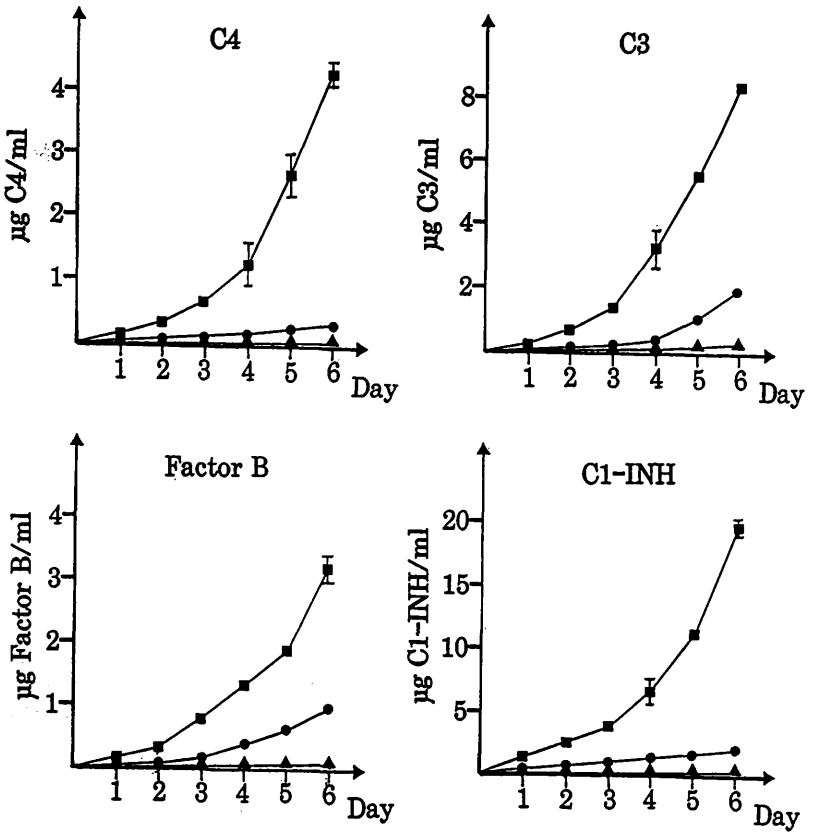


Figure 39 - Concentrations of complement components (measured by ELISA) in the culture supernatants from the adherent (●—●) and non-adherent (■—■) synovial cells of osteoarthritis patient, OA19. The culture medium was changed completely every 24 hours and the amount of each complement component secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and complement component levels are expressed as µg complement component per 1ml of culture medium (mean±SD). One adherent and one non-adherent cell culture were treated with 2.5µg/ml cyclo-heximide (▲—▲).

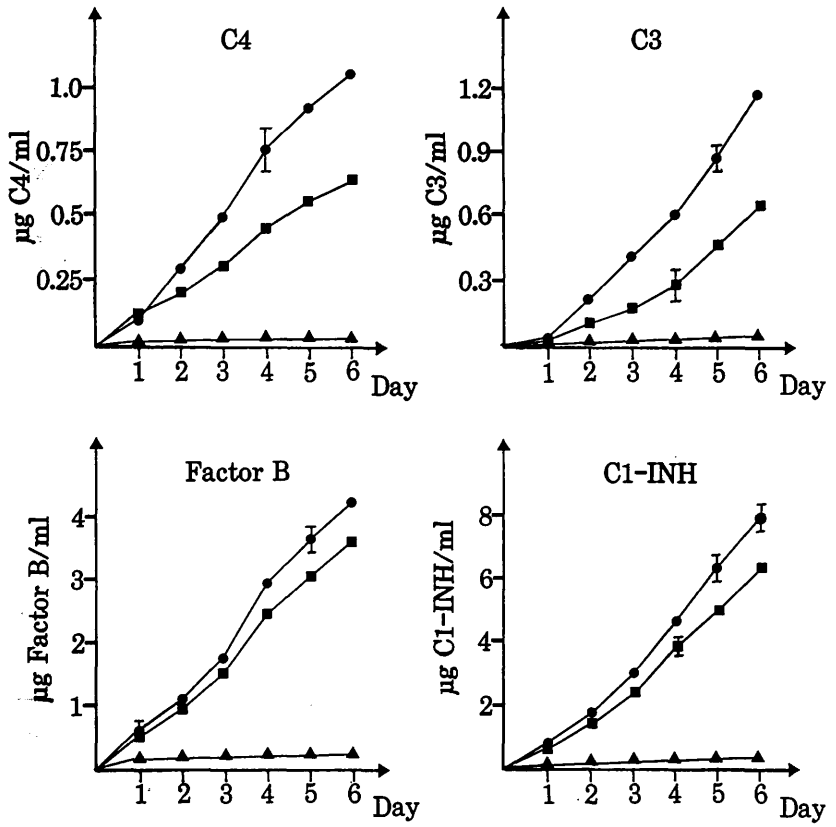


Figure 40 - Concentrations of complement components (measured by ELISA) in the culture supernatants from the adherent (●—●) and non-adherent (■—■) synovial cells of osteoarthritis patient, OA20. The culture medium was changed completely every 24 hours and the amount of each complement component secreted into the culture medium each day was measured. After six days in culture, the results were plotted as cumulative curves and complement component levels are expressed as µg complement component per 1ml of culture medium (mean±SD). One adherent and one non-adherent cell culture were treated with 2.5µg/ml cyclo-heximide (▲—▲).

amounts of factor B (adherent, 4.2μg factor B; non-adherent, 3.7μg factor B). However, the RA15 adherent cells synthesised substantially more factor B (3.5μg) than the non-adherent cells (1.3μg). RA14 and RA15 showed a steady decline in the rate of factor B synthesis while OA20 maintained a steady rate of factor B accumulation throughout the period of culture. OA19 showed a steady increase in the rate of factor B synthesis.

C1-INH synthesis

The amount of C1-INH synthesis by the adherent and non-adherent cells from each of RA14, RA15 and OA20 showed no significant difference (RA14 - adherent, 10μg; non-adherent, 10.1μg; RA15 - adherent, 15.5μg; non-adherent, 12μg; OA20 - adherent, 8μg; non-adherent, 6.5μg). The rate of C1-INH synthesis by cells isolated from these three synovium specimens, continued at a steady rate throughout the period of culture, although there was a slight decline in RA14 after one day. The non-adherent cells of OA19 synthesised substantially more C1-INH (20μg) than the adherent cells (2.5μg). Like the other complement components studied, a steady increase in the rate of C1-INH synthesis was observed throughout the culture period for OA19.

In summary, both adherent and non-adherent cells isolated from human synovial tissue synthesise C4, C3, factor B and C1-INH (although the adherent cells of one sample (OA19) synthesised only trace amounts of these four components). In most cases (except OA19), the adherent cells synthesised complement components at the same or higher levels than the non-adherent cells. As with studies on synovial tissue explant cultures, C5 synthesis was not detected.

9. IMMUNOHISTOCHEMICAL CHARACTERISATION OF CULTURED HUMAN SYNOVIAL CELLS

Immunohistochemistry was used to characterise the various cell types in both the adherent and non-adherent synovial cell cultures. This analysis was performed to characterise the cells responsible for the synthesis of complement components by the two cell populations.

After six days in culture, the cells were fixed in 10% neutral buffered formalin and characterised using the mouse monoclonal antibodies against vimentin (fibroblast marker) and LeuM5 (macrophage marker). Figure 41 shows that the vast majority (>95%) of the cells were fibroblasts while figure 42 shows that a small proportion of the cells were macrophages. These figures represent the results of immunohistochemistry performed on the adherent cells isolated from synovial tissue specimen RA15 but the non-adherent cells from this sample and the cells from all other samples showed similar results.

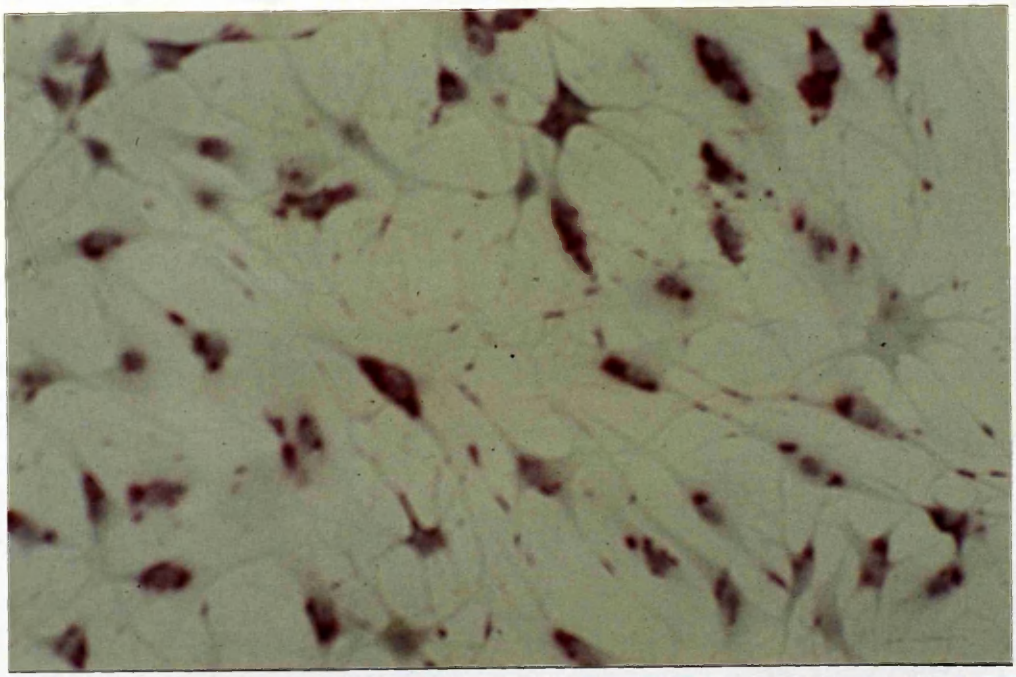
In all these studies normal mouse serum was used as a negative control and no positive staining was observed for any sample (figure 43).

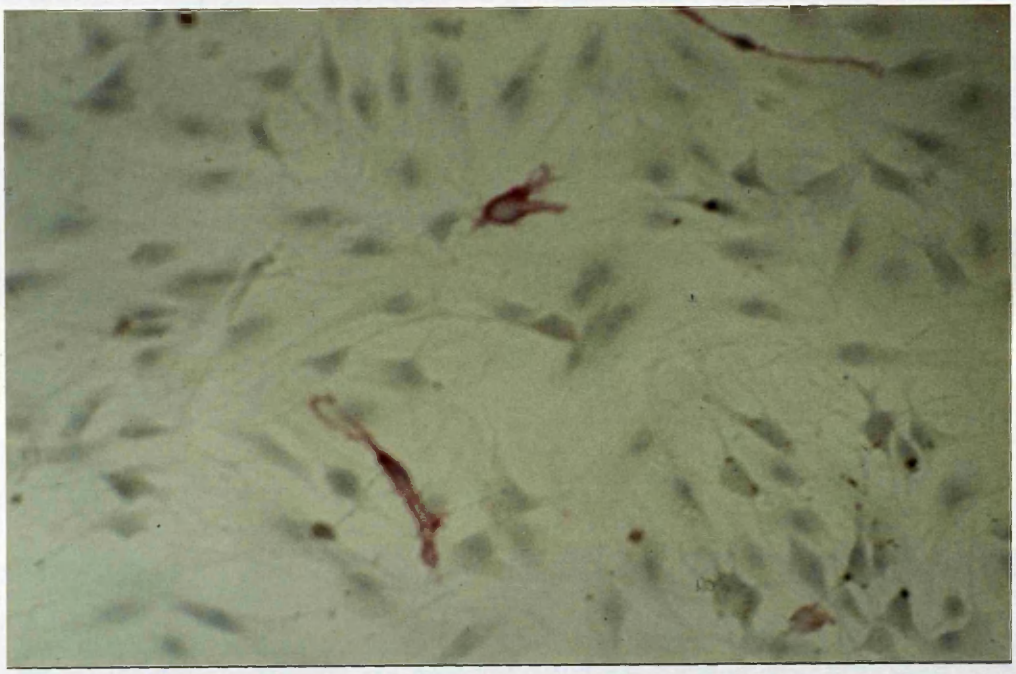
When the non-adherent cells from RA15 were replated onto a separate culture dish each day, it was found that ≈70% of the cells adhered to the culture dish every 24 hours so that by the fourth replating, virtually all the cells had adhered to the surface of the coverslip. After replating, all the cells were cultured until day 6 and then fixed in 10% neutral buffered formalin at the same time. Like the experiment described above, fibroblasts were shown to be the cell-type present in the greatest abundance in every culture of replated non-adherent cells, although a small number of cells were identified as macrophages (figures 44-49).

Figure 41 - Anti-vimentin (1/40) staining of RA15 adherent synovial cells (x250).

Figure 42 - Anti-LeuM5 (1/40) staining of RA15 adherent synovial cells (x250).

Figure 43 - Normal mouse serum (1/40) staining of RA15 adherent synovial cells (x250).





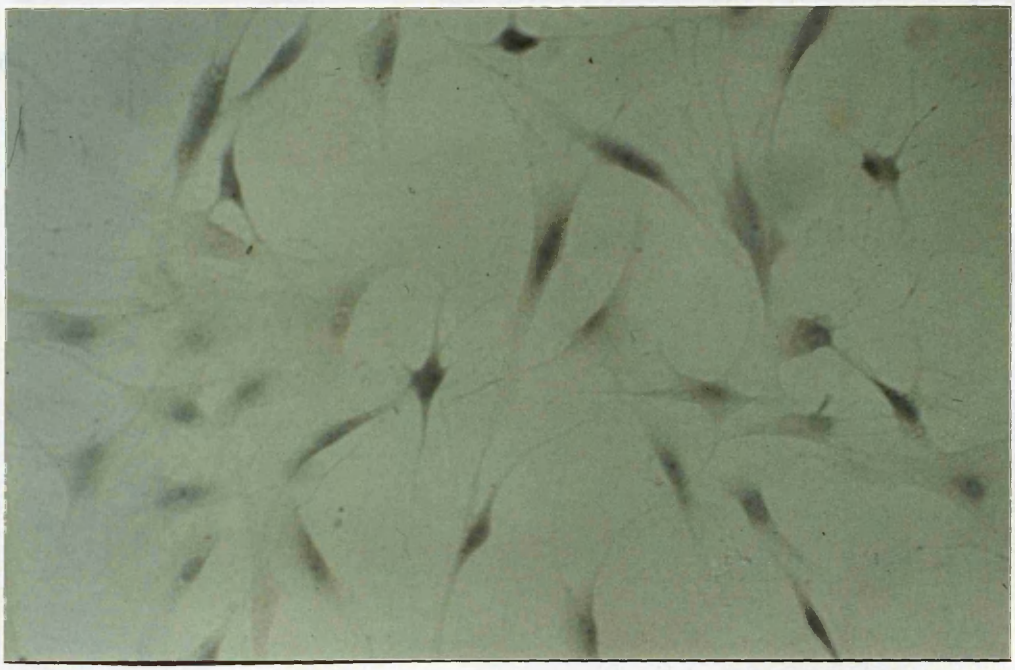
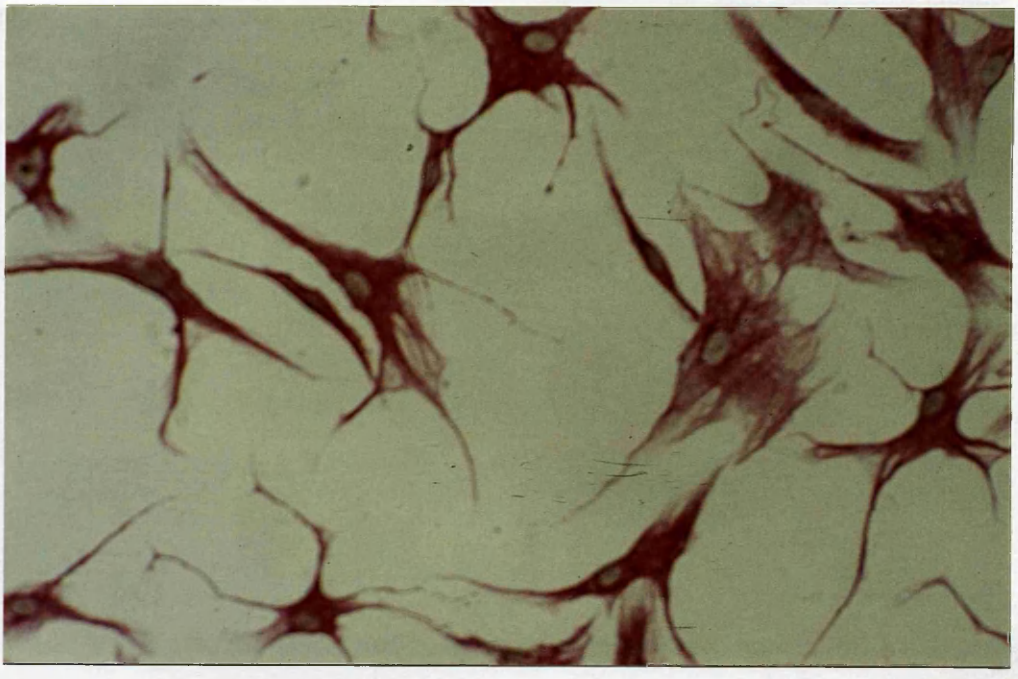


Figure 44 - Anti-vimentin (1/40) staining of RA15 non-adherent synovial cells replated on day 1 (x250).

Figure 45 - Anti-LeuM5 (1/40) staining of RA15 non-adherent synovial cells replated on day 1 (x250).



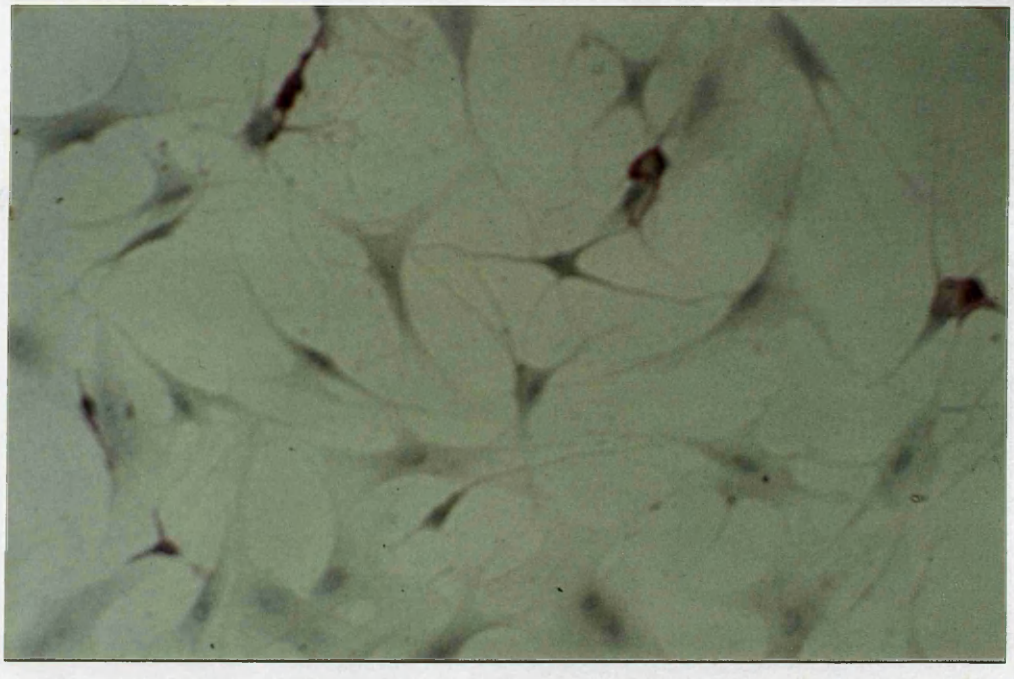
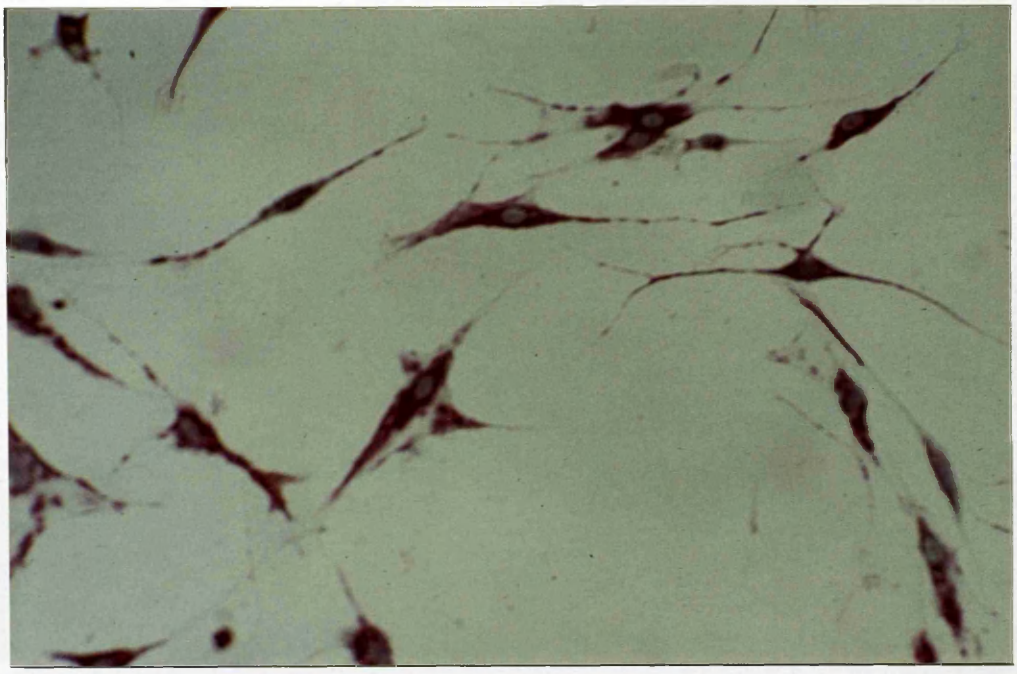


Figure 46 - Anti-vimentin (1/40) staining of RA15 non-adherent synovial cells replated on day 2 (x250).

Figure 47 - Anti-LeuM5 (1/40) staining of RA15 non-adherent synovial cells replated on day 2 (x250).



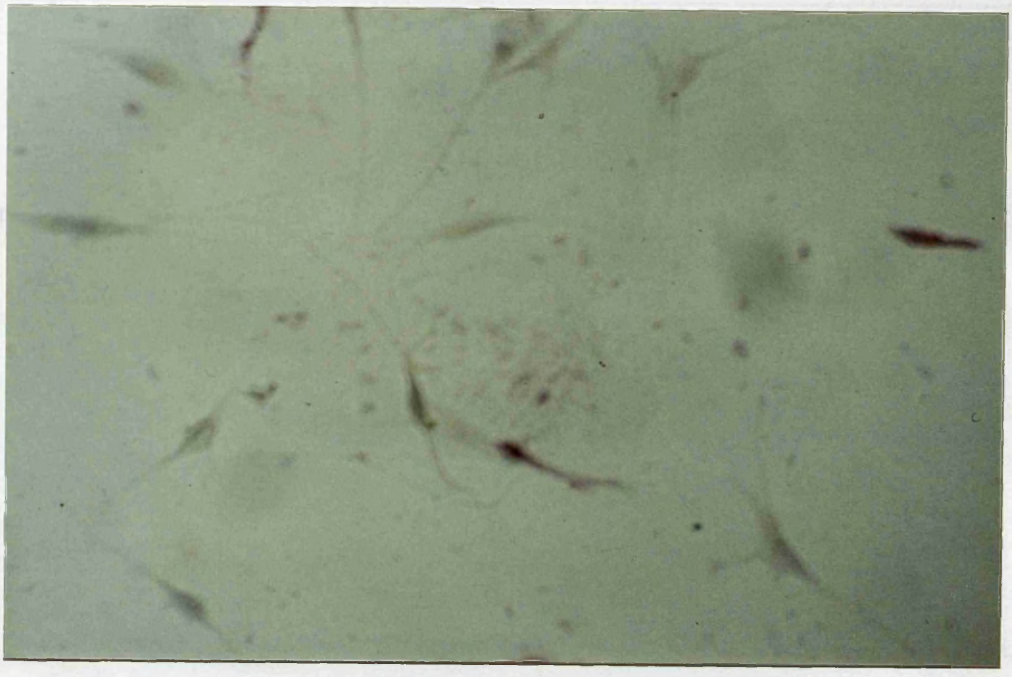
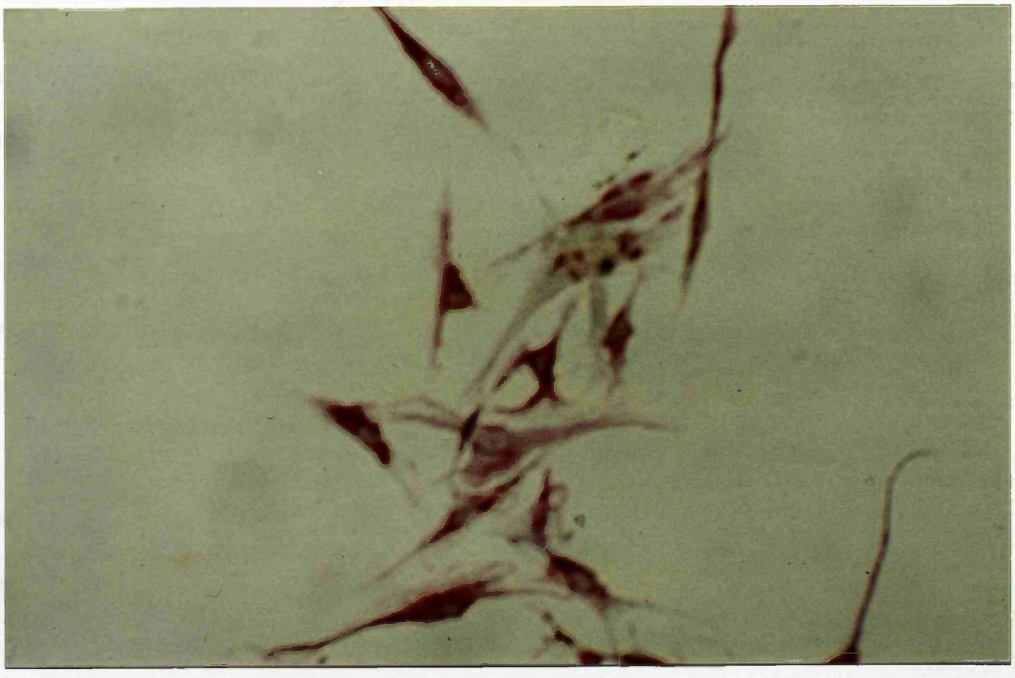
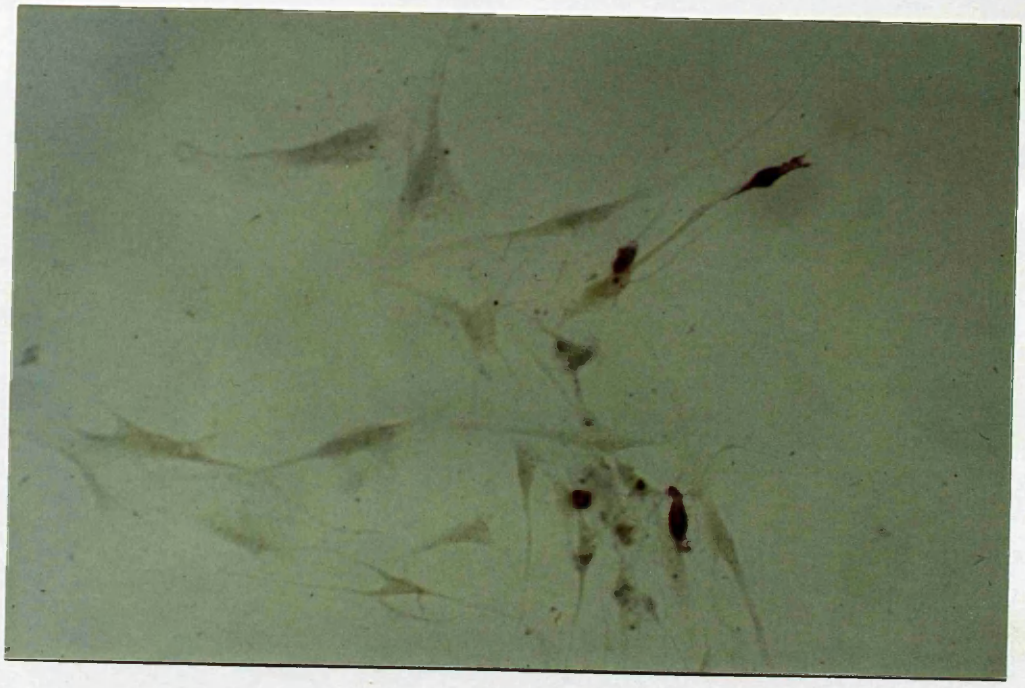


Figure 48 - Anti-vimentin (1/40) staining of RA15 non-adherent synovial cells replated on day 3 (x250).

Figure 49 - Anti-LeuM5 (1/40) staining of RA15 non-adherent synovial cells replated on day 3 (x250).





The concentrations of complement components in the culture supernatants were measured by ELISA to investigate the rates of complement synthesis by cells adhering at different stages of the culture period. Figure 50 shows the results of the ELISA analysis of the culture supernatants from sample RA15 but all other specimens showed similar results. The measurements first recorded on day 2 are for cells replated on day 1 (referred to as the second replating) while measurements first recorded on day 3 are for cells replated on day 2 (third replating), and so on.

Cells from the first replating showed a similar pattern of accumulation of C4, C3, factor B and C1-INH as the initial non-adherent cell cultures. Even after the second and third replating, the rate of C4 and C1-INH synthesis followed that of the initial non-adherent cultures, although the same levels were not reached presumably because of the fewer number of cells. However by the second replating the accumulation of C3 and factor B was not significantly higher than the cycloheximide-treated cultures.

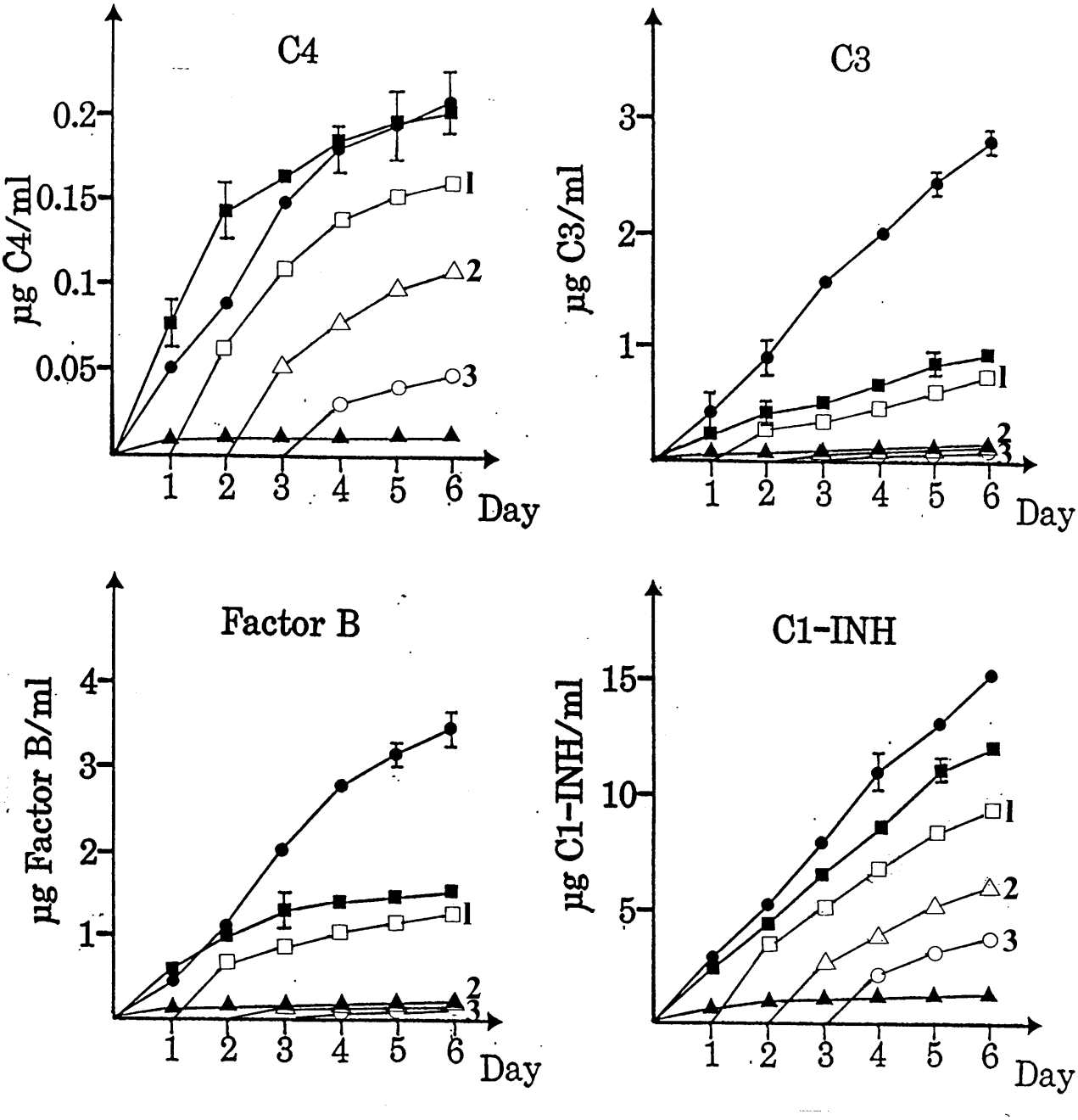


Figure 50 - Concentrations of complement components (measured by ELISA) in the culture supernatants from the adherent (●—●) and non-adherent (■—■) synovial cells of rheumatoid arthritis patient, RA15. Conditions were as described for fig. 38. In addition, for the first three days of culture the non-adherent cells were replated onto a separate culture dish each day. The amount of C4, C3, factor B and C1-INH synthesised by these cell populations was also measured, represented as follows: cells replated on day 1 (□—□, 1), cells replated on day 2 (△—△, 2) and cells replated on day 3 (○—○, 3).

10. IMMUNOHISTOCHEMICAL LOCALISATION OF COMPLEMENT COMPONENTS IN CULTURED HUMAN SYNOVIAL CELLS

Immunohistochemical analysis was used to localise C4, C3, C5, factor B and C1-INH within the adherent and non-adherent cells of cultured human synovial cells. Whole rabbit antisera were used for this analysis and normal rabbit serum was used as the negative control.

Figures 51-61 show the results of immunohistochemical localisation of C4, C3, C5, factor B and C1-INH in human synovial cells isolated from a patient suffering from osteoarthritis (OA21). The cells were fixed in 10% neutral buffered formalin after six days in culture. All other patient samples examined by immunohistochemical analysis (RA14, RA15, OA19 and OA20) showed similar results.

C1-INH and factor B were found in most cells in both the adherent and non-adherent cell cultures (figures 51-54). The positive pink staining was especially strong for factor B which was present in all cell types. C1-INH was also present in all cell types but the intensity of staining was not as strong as for factor B. However, the staining intensity may be due to different binding affinities of the antisera and not concentration of the complement component within cells.

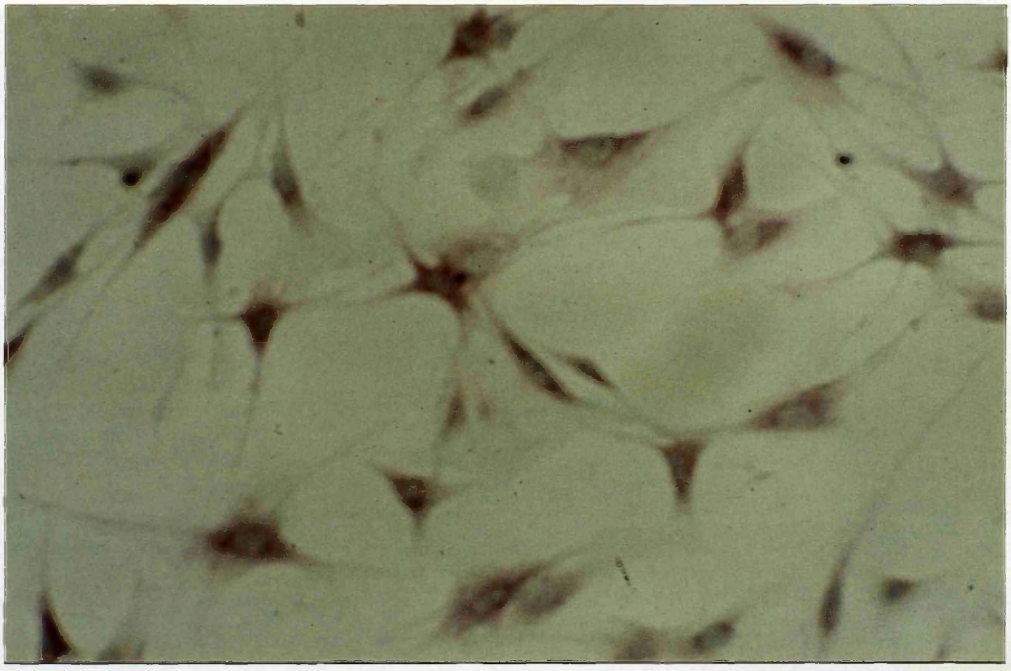
C3 and C4 were also present in most cells of both adherent and non-adherent cell cultures (figures 55-58). However, the intensity of signal was not as strong (particularly C4) as factor B and C1-INH.

As expected from the studies on synovial tissue described above, C5 was not detected in either the adherent or non-adherent cell cultures from any sample (figures 59 and 60).

Figure 61 shows the negative staining obtained with normal rabbit antiserum which acted as the negative control for these analyses.

Figure 51 - Anti-factor B (1/100) staining of OA21 adherent synovial cells (x250).

Figure 52 - Anti-factor B (1/100) staining of OA21 non-adherent synovial cells (x250).



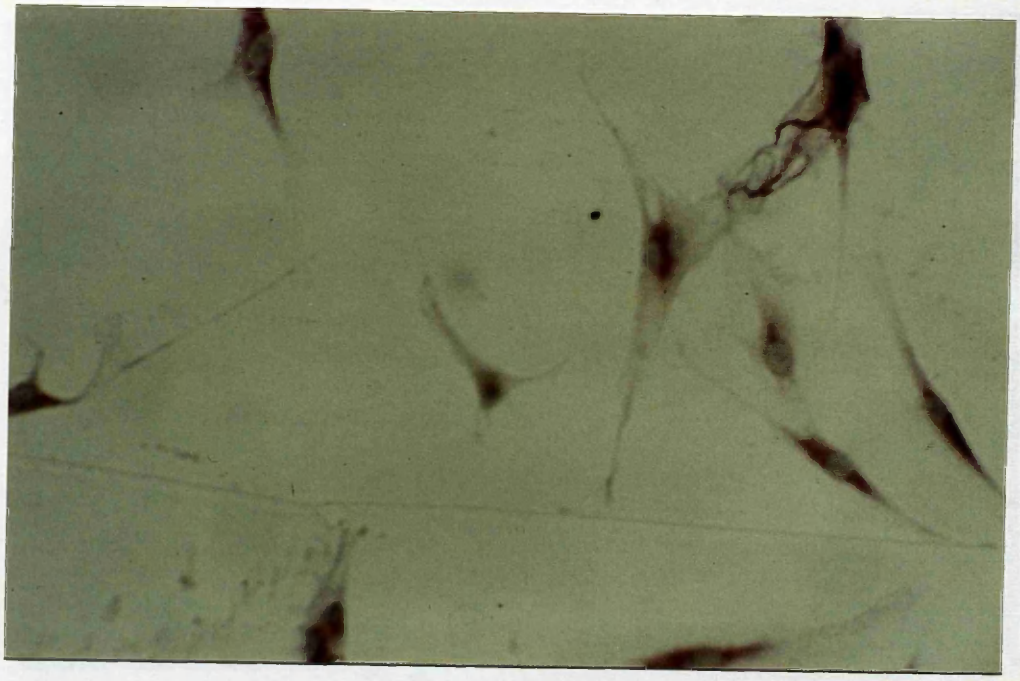
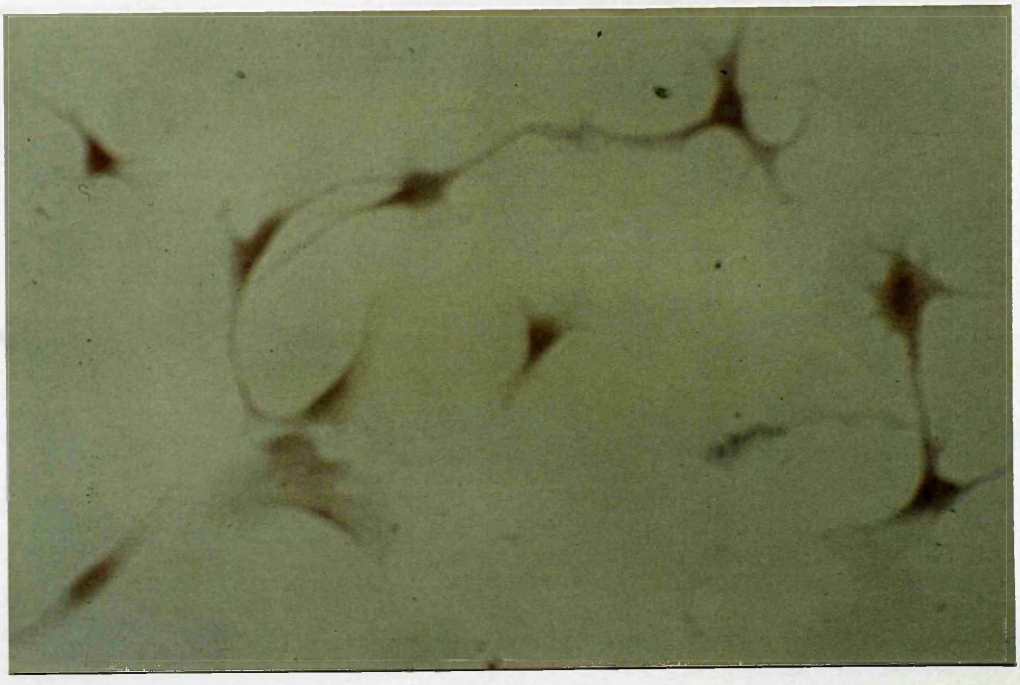


Figure 53 - Anti-C1-INH (1/100) staining of OA21 adherent synovial cells (x250).

Figure 54 - Anti-C1-INH (1/100) staining of OA21 non-adherent synovial cells (x250).



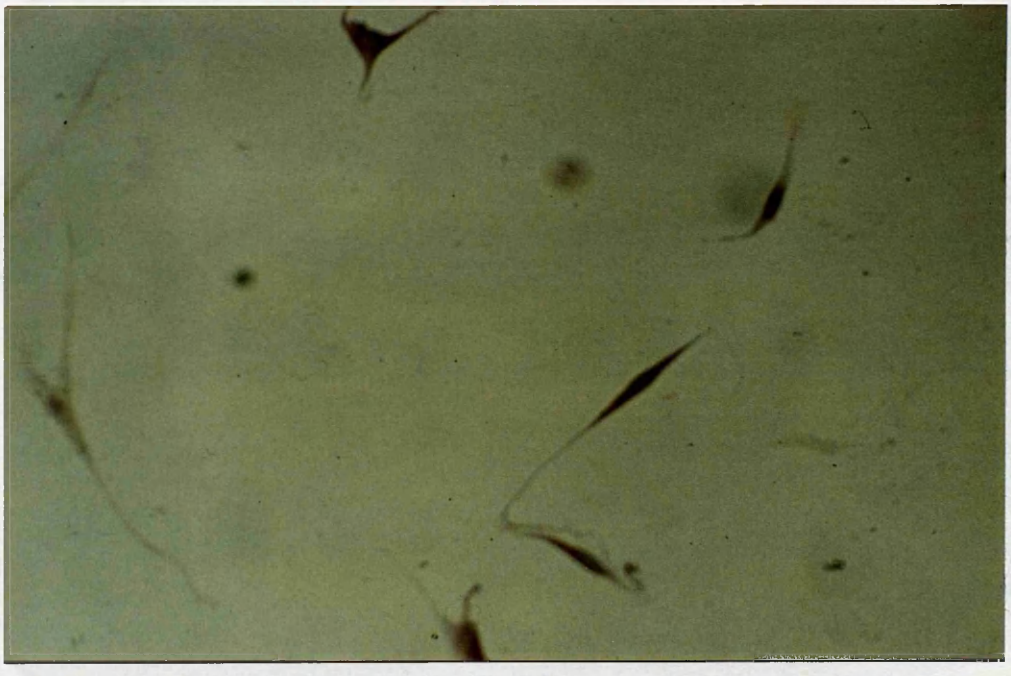
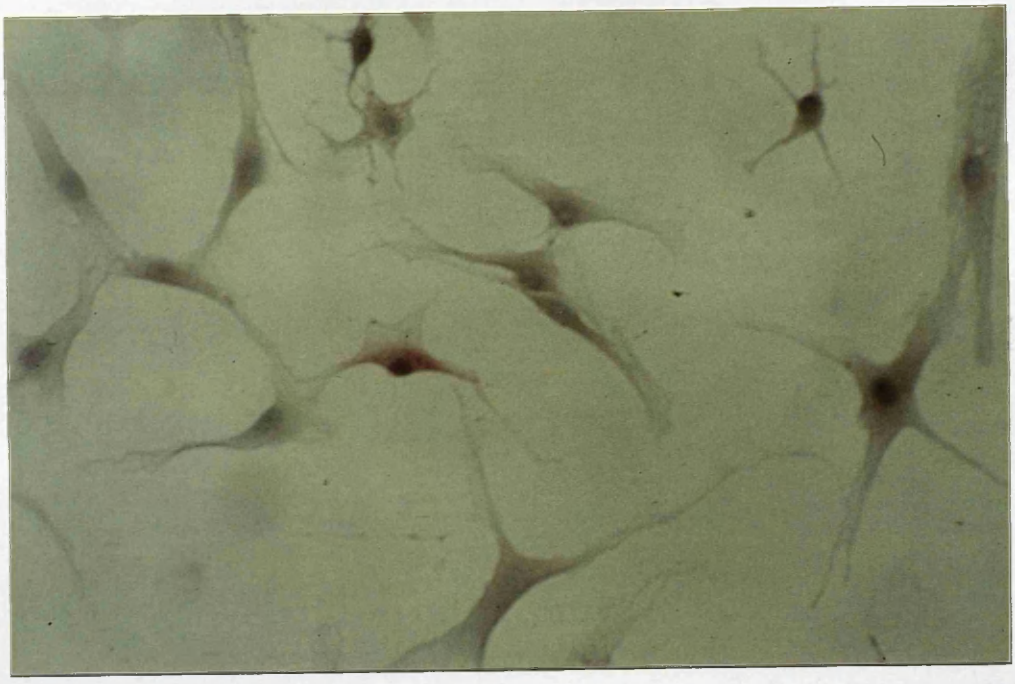


Figure 55 - Anti-C3 (1/100) staining of OA21 adherent synovial cells (x250).

Figure 56 - Anti-C3 (1/100) staining of OA21 non-adherent synovial cells (x250).



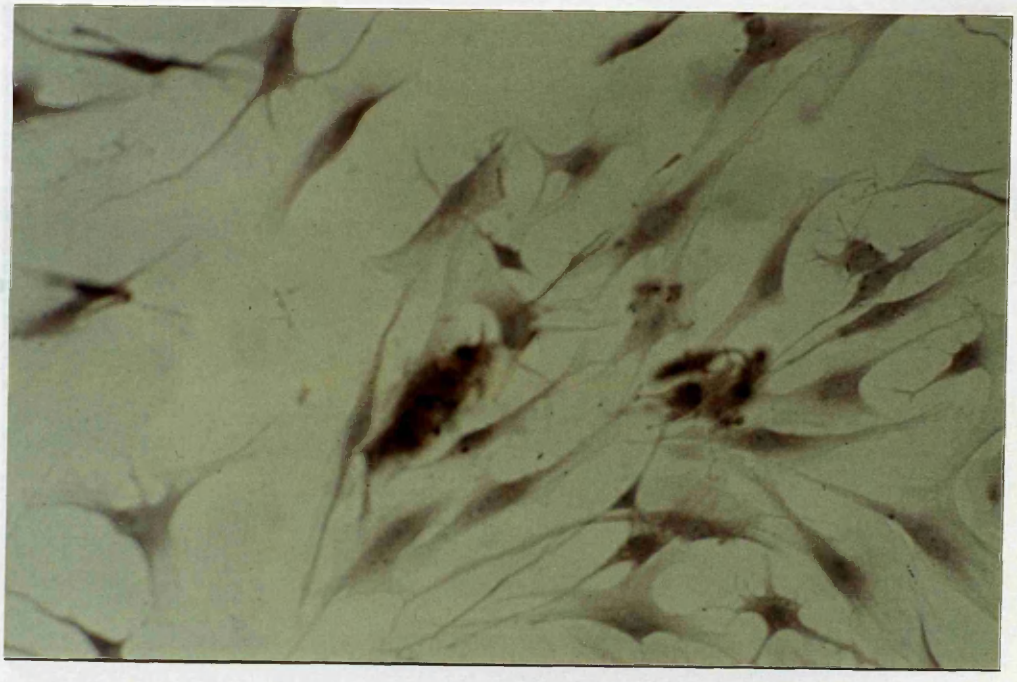


Figure 57 - Anti-C4 (1/100) staining of OA21 adherent synovial cells (x250).

Figure 58 - Anti-C4 (1/100) staining of OA21 non-adherent synovial cells (x250).

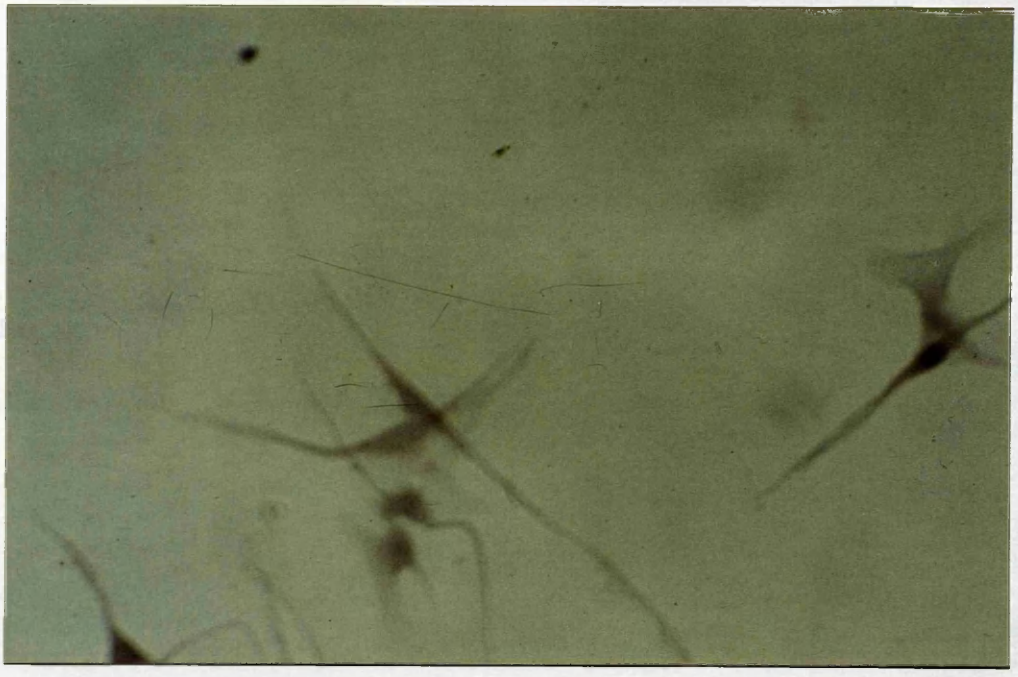
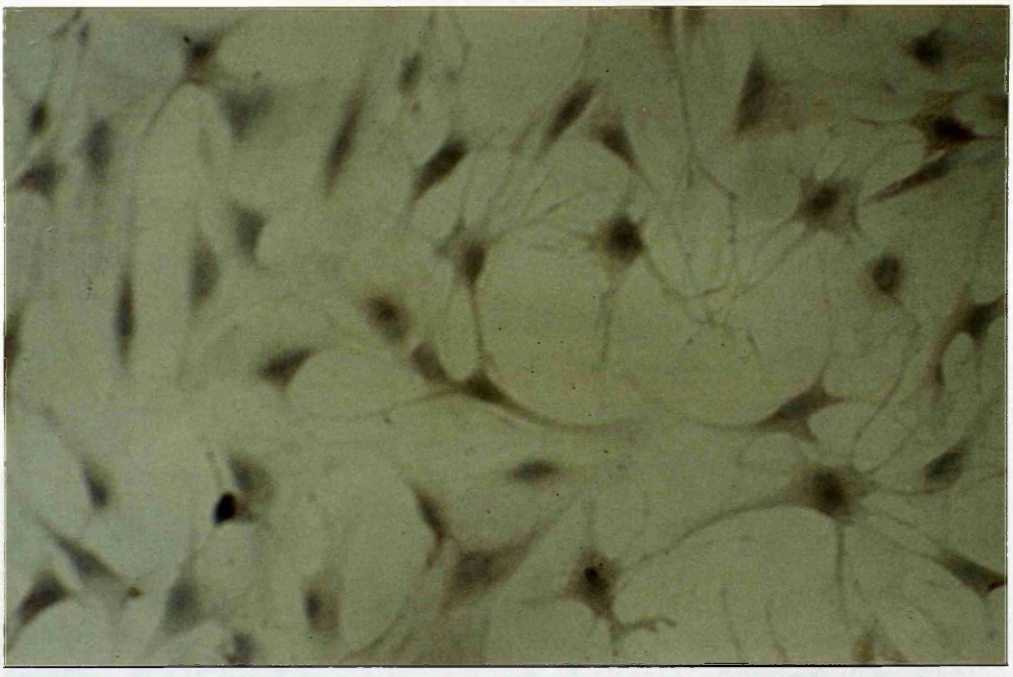


Figure 59 - Anti-C5 (1/100) staining of OA21 adherent synovial cells (x250).

Figure 60 - Anti-C5 (1/100) staining of OA21 non-adherent synovial cells (x250).



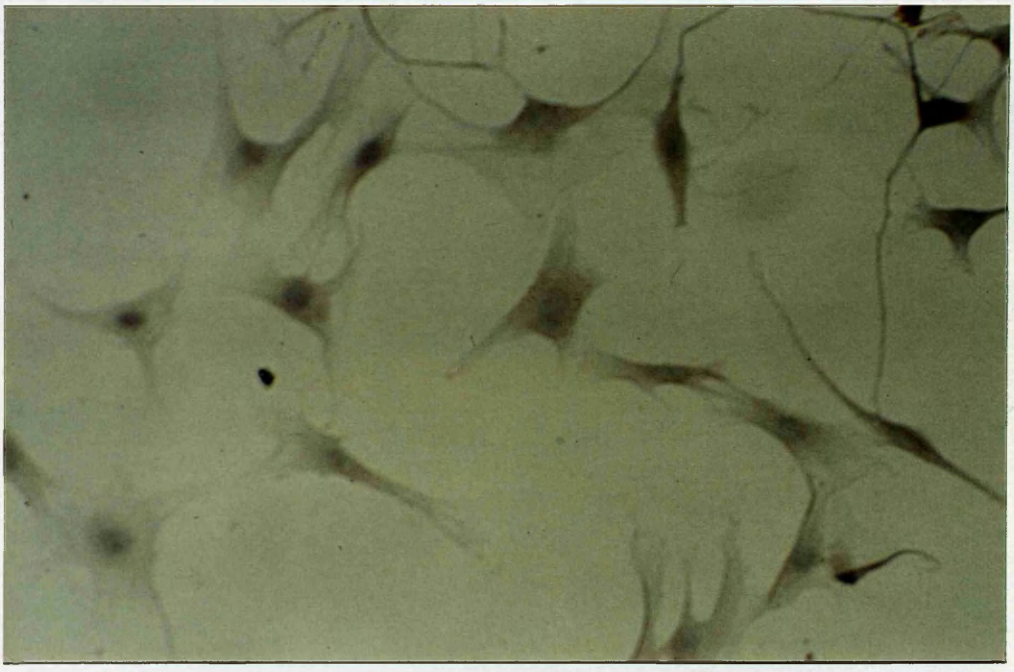
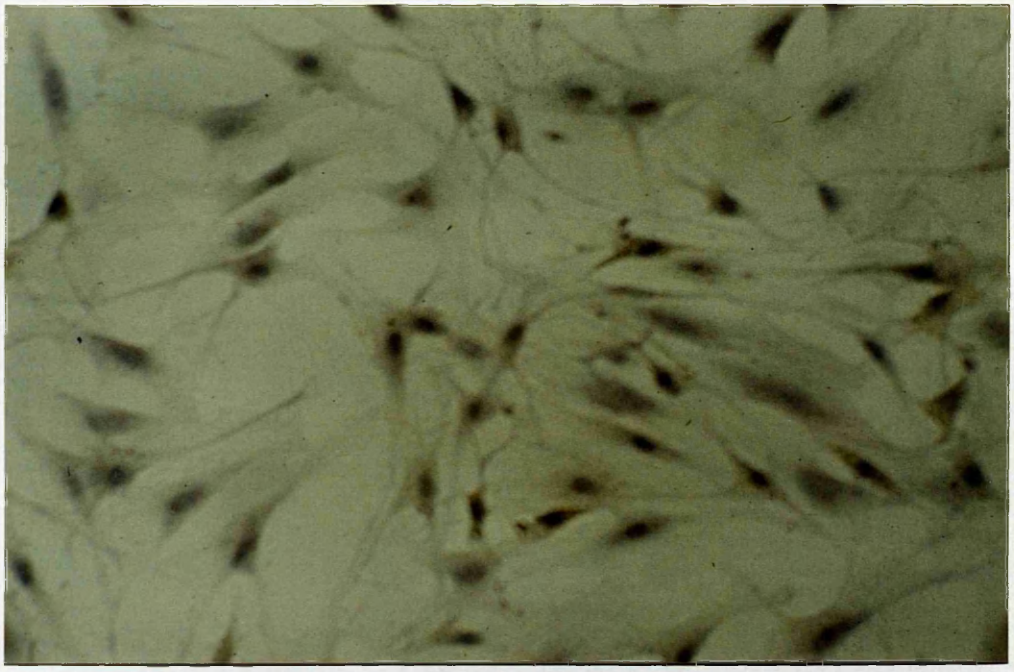


Figure 61 - Normal rabbit serum (1/100) staining of OA21 adherent synovial cells (x250).



DISCUSSION

AND

FINAL CONCLUSIONS

DISCUSSION

1. GENERAL APPROACH TO THE STUDY OF COMPLEMENT BIOSYNTHESIS IN HUMAN SYNOVIAL TISSUE

The principle aim of this study was to detect extrahepatic synthesis of complement components *in vivo* using inflamed synovium from patients with rheumatoid arthritis or osteoarthritis as a model system. In addition to being one of the first comprehensive studies of *in vivo* extrahepatic complement biosynthesis, it was hoped that, given the inflammatory effects of complement and the previous evidence for complement activation within the rheumatoid joint, the findings from this study would contribute to the understanding of the pathogenesis of rheumatoid arthritis.

Although plasma levels of most complement components are maintained by hepatic synthesis, the cell responsible being the hepatocyte (Colten, 1976), it is now well established that other cells including monocytes, macrophages, fibroblasts and the epithelial cells of the gastrointestinal and genitourinary tracts synthesise complement components when cultured *in vitro* (Colten, 1976). Such cells are potential sources of extrahepatic synthesis of complement components *in vivo*. Synovial fluid macrophages and adherent synovial membrane cells have been shown to synthesise complement components when cultured *in vitro* (De Ceulaer et al., 1980), as have cultured fragments of synovial membrane from patients with either rheumatoid arthritis or osteoarthritis (Ruddy & Colten, 1974). However, the only evidence for extrahepatic synthesis of complement occurring *in vivo* comes from metabolic studies with radiolabelled (^{125}[I]) C3, which indicated that up to 50% of C3 present in the rheumatoid joint was synthesised locally (Ruddy &

Colten, 1974).

The present studies, which have revealed the detection of the mRNAs coding for several complement components in human synovial tissue, as well as demonstrating complement component biosynthesis by cultured fragments of synovial tissue, have provided conclusive evidence that synthesis of a number of complement components including C4, C2, C3, factor B and C1-inhibitor, occurs locally within normal and inflamed synovium *in vivo*. The local synthesis of complement within normal synovial joints may be of importance in their defence against infection, whereas in inflamed joints it may contribute to the inflammatory response.

These conclusions are based upon the following observations:

(a) the detection of the mRNAs coding for C4, C2, C3, C1q B-chain, factor B and C1-INH by Northern blot and dot-blot analyses of total RNA isolated from the synovial membrane of rheumatoid arthritis and osteoarthritis patients and the detection of C1-INH mRNA in normal synovium. The expression of C5 mRNA was also investigated but was not detected by either Northern blot or dot-blot analyses.

(b) the demonstration of the accumulation of C4, C2, C3, factor B and C1-INH in the supernatants of cultured fragments of normal and inflamed synovial membrane. The observation that the presence of cycloheximide in the culture medium resulted in a reduction in the concentrations of these components in the supernatants shows that they were being synthesised *in vitro* rather than being released into the culture medium as a result of contamination of the synovial membrane by blood or inflammatory exudate.

(c) the C3, C2 and factor B produced by the cultured fragments of normal and inflamed synovial membrane were shown to be haemolytically active. C4 and C5 haemolytic activity, significantly higher than the cycloheximide-treated cultures were not detected.

(d) the incorporation of ^{35}S-methionine into a polypeptide corresponding to the correct molecular weight for factor B (92 kDa), released by cultured fragments of synovial membrane. A band corresponding to the molecular weight for the β chain of C3 (75 kDa) was visible but the α-chain was not detected. A smeared band below the correct molecular weight of C1-INH was detected while bands corresponding to C4, C2 and C5 were not detected.

2. AN APPRAISAL OF THE TECHNIQUES FOR ISOLATION OF RNA FROM SYNOVIAL TISSUE

The isolation of total RNA from human synovial tissue produced the greatest technical problem of this study. As stated in the Results chapter, the isolation of undegraded synovial tissue RNA was virtually impossible if there was a significant delay ie. more than 10 minutes, between removal of the tissue from the patient and the tissue being snap-frozen in liquid nitrogen.

The guanidinium thiocyanate method of RNA isolation was used to prepare RNA from synovial tissue. However, the major drawback of this method was the time involved to prepare RNA, which including the centrifugation steps, lasted 3 days. RNAzol, which has only recently become commercially available, was reported to be at least as effective as the guanidinium thiocyanate method but had the advantage that RNA could be prepared from either tissue or cells in less than one day. Testing the RNAzol solution showed that it could be used to isolate intact RNA from liver tissue as well as a wide range of cultured cells. However, when used on synovial tissue, the RNA was completely degraded while RNA prepared from the same tissue by the guanidinium thiocyanate method was intact. The degree of RNA degradation was tested by ethidium bromide staining of the Northern gel and analysis of the Northern blot for a relatively stable mRNA *in vivo* e.g. β_2-microglobulin.

Therefore, even although the guanidinium thiocyanate procedure is time-consuming, it would appear to be the better of the two methods for isolating RNA from human synovial tissue.

3. NORTHERN BLOT ANALYSIS OF SYNOVIAL TISSUE RNA

Northern blot analysis demonstrated the presence of mRNAs coding for C4, C2, C3, C1q B-chain, factor B and C1-INH in the synovial tissue from patients with rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and chondrocalcinosis.

Human cDNA probes were used throughout the Northern blot analysis and as such the filters could be washed in 2 x SSC, 0.5 x SSC and finally 0.1 x SSC each containing 0.1% SDS. Each wash was performed at 65°C for 30 min. Washing down to a stringency of 0.1 x SSC containing 0.1% SDS, ensured that all non-specific hybridisation was removed and therefore only specific DNA-RNA hybrids remained on the filter.

The Northern blot analysis demonstrated that mRNAs coding for C2, factor B, C1-INH and especially C3 and C4 in synovial tissue, show evidence of degradation. In order to exclude the possibility that these mRNAs had been degraded during the RNA isolation procedure the same Northern blots were reprobed with β_2-microglobulin cDNA, as the mRNA coding for this protein is known to be stable *in vivo*. As this mRNA was shown to be undegraded in the Northern blots, it was concluded that the mRNAs which code for the complement components being studied are unstable in synovial tissue *in vivo*.

The varying degrees of degradation of the different complement component mRNAs observed on Northern blot analysis probably occur because each mRNA species has a different rate of turnover in synovial tissue *in vivo*. The results would suggest that C3 and C4 mRNAs are more labile than C1-INH, C2, factor B and C1q B-chain mRNAs *in vivo*, while all are less stable than mRNA coding for β_2-microglobulin.

The reason for complement component mRNAs being relatively

unstable in human synovial tissue *in vivo* remains unclear as does the apparent differences in the *in vivo* stability of C2, factor B, C1-INH and C1q B-chain mRNAs compared with the mRNAs coding for C3 and C4.

The mRNAs coding for C3 and C4 are 5.2 kb and 5.5 kb long respectively, and are thus approximately twice as long as the mRNAs coding for C2 (2.9 kb), factor B (2.6 kb), C1-INH (2.1 kb) and C1q B-chain (1.4 kb). It is possible that because the mRNAs coding for C3 and C4 are longer, they are more susceptible to degradation. One possible cause may be that more of these RNA molecules could be exposed to ribonucleases resulting in a greater degree of degradation of C3 and C4 mRNA.

However, it is possible that mRNA instability constitutes a mechanism by which the expression of complement components is regulated. If this is the case, then the differences in mRNA stability may be related to the importance of individual proteins during complement activation.

As discussed previously, complement plays a major role in immunologically mediated inflammation. Thus, because of the inflammatory effects of the complement system, it is possible that complement component mRNAs, particularly C3 and C4 which play a central role in the activation of the complement system, have a short half-life *in vivo*, especially at extrahepatic sites such as synovial joints. This could be a possible mechanism for regulating the expression of the complement components, designed to prevent the complement proteins accumulating at high concentrations which may prove harmful to the local tissue environment.

(a) Possible mechanism for regulation of local C4 synthesis in synovial tissue

A mechanism for the regulation of C4 synthesis in guinea pig macrophages has been postulated, which if present in human synovial membrane could help explain the observations seen in Northern blot analysis of human synovial tissue RNA.

In guinea pigs, the proportion of macrophages producing C4 varied from one tissue site to another and at different stages of maturation of the mononuclear phagocyte series of cells. Auerbach et al (1983) showed that the binding of extracellular C4 protein to guinea pig peritoneal macrophages results in a specific inhibition of C4 biosynthesis and this correlates with a decrease in the steady state level of C4 mRNA. This feedback inhibition was reversible and was not accompanied by a change in C2 mRNA or factor B mRNA levels or total RNA content. These data suggest that feedback inhibiton of C4 biosynthesis is regulated at the level of transcription or post-transcriptional processing. In other words, C4 feedback inhibition may operate by preventing transcription of the C4 gene or by interrupting the post-transciptional processing of C4 mRNA. Alternatively, the feedback inhibition may decrease the stability of the C4 mRNA which would result in a reduction in the steady state level of C4 mRNA in addition to inhibiting translation into C4 protein

Coupled with the fact that human synovial membrane C4 mRNA appears to be labile *in vivo*, as suggested by Northern blot analysis, the above hypothesis of direct regulation of C4 would have important consequences in the evolution of an inflammatory response ie. would provide a mechanism for controlling constitutive secretion of C4 by

complement producing cells within the synovial tissue as well as a basis for replenishing C4 levels following complement consumption or diffusion from a site of inflammation.

Therefore local C4 synthesis within the synovial joint would not only be controlled by the inherent instability of C4 mRNA *in vivo*, but also by the feedback inhibition mechanism described above. The direct control of C4 synthesis would thus not depend on or necessarily correlate with the plasma concentrations of C4, but would reflect the local concentration of extracellular C4 in the synovial tissue environment.

(b) Possible mechanism for the regulation of local C3 synthesis in synovial tissue

Activation of C3 is the key step in the activation sequence of the complement system and as such it is possible that the regulation of C3 gene expression may be under even more stringent control than the other complement components.

It has been suggested that the local production of γ-interferon by activated T lymphocytes at sites of chronic inflammation, for example, the rheumatoid joint, plays a major role in the modulation of local complement synthesis (Lappin & Whaley, 1989). This suggestion was initially based on the observation that recombinant γ-interferon (rIFN-γ) increases C2, factor B and C1-INH synthesis but reduces synthesis of C3 and properdin by cultured human monocytes (Hamilton et al., 1987; Lappin & Whaley, 1987). Synovial membrane T lymphocytes, which release γ-interferon, have been shown to have the same effect on complement biosynthesis by human monocytes as rIFN-γ (Lappin & Whaley, 1989). The effects of both the T lymphocytes and rIFN-γ were abrogated in the

presence of a monoclonal antibody to γ-interferon. Thus it appears that γ-interferon may be the lymphocyte product which is responsible for the modulation of monocyte complement synthesis *in vivo*.

Monocytes and macrophages are present in large quantities in inflamed synovial tissue and from *in vitro* studies are suspected of playing a major role in the local synthesis of complement in the synovial joint *in vivo*. At sites of chronic inflammation e.g. in rheumatoid joints, γ-interferon is also present in abundance and as stated above, γ-interferon reduces monocyte C3 synthesis. The Northern blot analysis of human synovial tissue RNA showed that C3 mRNA was particularly labile *in vivo*, which may not only act as an inherent regulator of C3 synthesis but may also facilitate γ-interferon to exert its inhibitory effect on C3 synthesis.

4. DOT BLOT ANALYSIS OF SYNOVIAL TISSUE RNA

Both Northern and dot-blot analyses of synovial tissue RNA detected a wide range of steady state levels of each mRNA even within a group of patients suffering from the same joint disorder. Densitometry was performed on the dot-blot autoradiographs and quantitative analysis of the data by the students' t-test revealed no statistically significant difference between the steady state levels of C3, factor B or C1-INH mRNA between the two groups of patients, although there was a trend towards higher levels of C3, factor B and C1-INH mRNA in rheumatoid synovium compared with osteoarthritis synovial tissue. Although, the levels of C2 mRNA were significantly higher in rheumatoid synovium, and C4 mRNA levels were higher in osteoarthritis synovium, the P value was only marginally significant in both cases (<0.05).

This may appear rather surprising because rheumatoid arthritis is regarded as a classical example of chronic inflammation where one might expect local synthesis of complement components while osteo-arthritis is more of a 'wear and tear' disease as opposed to an inflammatory reaction.

Dot-blot analysis of RNA isolated from normal synovial membrane identified the expression of C1-INH mRNA by the normal tissue, although the small amount of RNA isolated from the small piece of synovium isolated from the normal patient, made it impossible to quantitatively compare the steady state level of C1-INH mRNA with that found in synovium from rheumatoid and osteoarthritis patients.

The suggestion that normal synovium may synthesise complement was first made by Katz and Strunk (1988b) who demonstrated that fibroblast-like cells isolated from normal synovium synthesised seven

complement components *in vitro.* However, apart from the fact that this evidence is based entirely on *in vitro* cell cultures, biosynthesis of complement components was only studied after these cells had been passaged several times.

Therefore, the demonstration of C1-INH mRNA in normal synovium provides the best evidence to date that local synthesis of complement in synovial tissue is constitutive. Such extrahepatic synthesis of complement may play a major role in host defence as well as contributing to the inflammation present in arthritic joints.

From the data presented here, it is difficult to interpret directly any of the results in relation to specific mechanisms for the regulation of gene expression. However, when considered alongside investigations performed by other workers, the dot-blot analysis of synovial tissue RNA and the subsequent statistical analysis of the data, supports the evidence for differential regulation of the closely linked C2, factor B and C4 genes.

The genes encoding complement components C2, factor B and C4 are closely linked in the class III region of the major histocompatibility complex on human chromosome 6 (Carroll et al., 1984). However several studies have previously shown that despite this close linkage, regulation of the expression of each of the three components is under independent control. Statistical analysis of the dot-blot data presented here, show that the steady state levels of factor B mRNA were not significantly different between rheumatoid arthritis and osteoarthritis patients. However, higher mean levels of C2 mRNA were detected in the rheumatoid synovium while the osteoarthritis synovium contained more C4 mRNA.

However, dividing the rheumatoid arthritis and osteoarthritis patients into two separate groups and studying the results of each patient individually, allowed a much closer examination of the densitometry data from the dot-blot analyses shown in Figure 21 and provides stronger evidence for differential regulation of C2, factor B and C4 expression in the synovial tissue of both rheumatoid arthritis and osteoarthritis patients. If one assumes the null hypothesis that expression of all three components is not independently regulated then following densitometry, the ratio of each patient's measurement to the mean value for the same component should equal the ratio obtained by the same calculation for the other two components ie. C2/mean C2 = FB/mean FB = C4/mean C4. However, Tables 11 and 12 show the results of such analysis on the densitometry data from the rheumatoid arthritis and osteoarthritis patients. For example, patient RA2 contains only 13% C2 mRNA of the mean value for C2 obtained from all 12 rheumatoid arthritis patients. This same patient contains 17% C4 mRNA compared to the mean C4 value and thus is quite similar to the C2 ratio. However, RA2 contains 104% factor B mRNA compared to the mean value obtained for factor B, which is almost seven times greater than the ratios calculated for C2 and C4 mRNAs.

Examination of the data reveals that virtually all patients show differences in such ratios between C2, factor B and C4 which supports the theory proposed from the results of several studies, that these closely linked genes are regulated independently.

For example, Strunk, Whitehead & Cole (1985) showed that the lipid A component of endotoxin increased the steady state level of factor B mRNA in cultured human monocytes which corresponded to a 2-3

fold increase in factor B protein. The steady state level of C2 mRNA was also increased but not to the same extent as factor B. However there was no net increase in the C2 protein production thus suggesting that perhaps a second signal is required to initiate a change in the translation rate of C2 mRNA.

Studies on a human hepatoma cell line (Ramadori et al., 1985) showed that interleukin-1 (IL-1) caused an increase in the steady state levels of factor B and C3 mRNAs but C2 gene expression was unaffected.

A cosmid bearing the factor B and C2 genes on a 30 kb segment of human DNA was introduced into mouse L-cells and both components were constitutively expressed (Perlmutter et al., 1985). These transfected cells synthesised and secreted functionally active C2 and factor B indistinguishable from the products released in primary human cell cultures. When the cells were incubated with IL-1, synthesis of human factor B and endogenous murine C3 expression were increased but as in the hepatoma cell line studies, human C2 synthesis was unaffected.

In the mouse, the C2, factor B and C4 genes are also closely linked in the MHC region. Similar studies to investigate the differential regulation of these three closely linked genes have been performed on resident peritoneal macrophages (Newell, Shreffler & Atkinson, 1982). In short term primary cultures of these cells, the rate of C4 secretion declined rapidly within the first few hours, although total protein synthesis remained constant while there was an apparent increase in factor B synthesis. During these experiments, there was an increase in steady state levels of factor B mRNA with a decrease in C4 mRNA levels. Hence the observed decrease cannot be simply due to a limitation of *in vitro* culture conditions. Instead it is most likely due

to a change in the rates of transcription of C4 and factor B or a change in post-transcriptional processing or a difference in the stability of the specific C4 and factor B mRNAs.

Factors regulating C2 and factor B synthesis have also been shown to depend on the stage of maturation of human mononuclear phagocytes (Cole et al., 1982, 1983). The secretion of active C2 exceeds factor B by 3.5 and 7 fold in milk and lung macrophages respectively whereas the ratio of C2 to factor B secreted by the peripheral blood monocytes is constant ie. the ratio is 1, for several weeks in culture.

Thus, currently available evidence points towards there being differential regulation of the closely linked C2, factor B and C4 genes in both murine and human cells.

5. DETECTION OF COMPLEMENT PROTEINS IN SYNOVIAL MEMBRANE CULTURE SUPERNATANTS

(a) Kinetics of synthesis of complement components

ELISA analysis of synovial membrane culture supernatants showed the patterns of accumulation of complement components could be divided into two groups:

(i) C3 and C4 - showed a steady increase in C3 and C4 concentration over the first two days of culture but this rate of accumulation decreased towards the end of the period of culture in all samples.

(ii) C2, factor B and C1-INH - either showed a steady increase in accumulation of these components throughout the period of culture or, in the case of RA8, RA9, OA15 and N1, a delay in the initiation of C2, factor B and C1-INH synthesis followed by an increasing rate of accumulation of these three components in the culture medium.

Therefore, ELISA analysis of most samples revealed a delay in the initiation of synthesis of C2, factor B and C1-INH while synthesis of C3 and C4 showed no such delay. Moreover, Northern blot analysis showed that the mRNAs coding for C2, factor B and C1-INH were substantially more stable *in vivo* than those coding for C3 or C4.

These observations could be explained if the mRNAs coding for C2, factor B and C1-INH were masked by proteins in stable ribonucleoprotein particles which protect them against degradation but also prevent translation. If this is the case then the *in vitro* culture conditions must allow the unmasking of the mRNAs. This 'masked message' hypothesis has previously been proposed to explain events during the fertilisation of sea urchin eggs (Grainger & Winkler, 1987) and the lack

of keratin synthesis by mouse squamous cell carcinomas that contain keratin mRNA (Winter & Schweizer, 1983).

As a corollary, the observation that the mRNAs coding for C3 and C4 are unstable in synovial tissue *in vivo* could be interpreted as evidence that they are not masked by ribonucleoprotein particles. This would permit translation of C3 and C4 mRNAs to proceed almost immediately in the synovial membrane fragment cultures.

As with all *in vitro* culture systems, these fragments of synovial membrane have been removed from their natural environment. Hence, it is unlikely that the stimulus or stimuli (which remain unknown) responsible for initiating complement synthesis and activation in synovial tissue, is being replenished and thus will probably be exhausted after a relatively short time, leading to an inhibition of complement synthesis. Alternatively, as the concentration of C3 and C4 increases in the culture supernatants, a feedback inhibition mechanism may operate switching off further synthesis of C3 and C4, as has been suggested for C4 synthesis by guinea pig peritoneal macrophages (Auerbach et al., 1983). It is possible that these inhibitory mechanisms may act at the level of transcription which would explain why synthesis of C3 and C4 decline towards the end of the culture period. The mRNAs coding for C3 and C4 have been shown to have a short half-life in synovial tissue. Hence, if after 2-3 days in culture, transcription of the C3 and C4 genes is terminated, then because of their short half-life the mRNAs coding for C3 and C4 will be rapidly degraded, and the rate of C3 and C4 protein synthesis will decrease markedly.

Such a mechanism, coupled with the 'masked message' theory, would also hold for the observed patterns of C2, factor B and C1-INH

accumulation. Even if transcription of these three genes was inhibited, translation of the RNA would continue as more of the ribonucleoprotein particles, which are protecting the RNA from degradation, were removed from the RNA molecule. If this hypothesis is correct, then once the ribonucleoprotein particles have been removed from the mRNAs coding for C2, factor B and C1-INH, degradation of these mRNAs would occur resulting in decreasing rates of C2, factor B and C1-INH synthesis. Indeed in some cases, the rate of synthesis of these three complement proteins did start to decrease even after six days in culture. Presumably, the patterns of C2, factor B and C1-INH accumulation would have reached a plateau (like C3 and C4 synthesis) if the cultures had been continued for a longer time.

One simple experiment which would help to answer whether control was being exerted at the transcriptional level, would be to introduce a transcription inhibitor, such as actinomycin D into the synovial membrane cultures and compare the results with the untreated cultures. Actinomycin D inhibits transcription by binding tightly to double-stranded DNA but not to single-stranded DNA or RNA, double-stranded RNA or RNA-DNA hybrids. At low concentrations, actinomycin D inhibits transcription without appreciably affecting DNA replication. Also, protein synthesis is not directly inhibited by actinomycin D. If the kinetics of complement component synthesis in the actinomycin D-treated cultures were similar to the untreated cultures, this would suggest that regulation of complement synthesis was possibly being exerted at the level of transcription.

(b) Different rates of complement synthesis by synovial membrane fragments from the same patient

In most cases, ELISA analysis showed that the four synovial membrane cultures from one patient synthesised similar amounts of complement as one another. However, there were examples when some cultures synthesised significantly higher or lower levels of complement components than other cultures from the same patient.

However, each culture contained fragments of tissue removed from different areas of the specimen. It is unlikely that the potential for complement biosynthesis was uniform throughout the entire specimen because some areas will contain higher concentrations of complement producing cells and hence will synthesise more complement proteins than others. Indeed, when studying the histology of synovial membrane the uneven distribution of various cell types is clearly visible (Athanasou et al., 1988). This uneven distribution of cell types would be the most likely explanation for the different amounts of complement synthesised by fragments of synovial membrane from the same patient.

6. DETECTION OF FUNCTIONALLY ACTIVE COMPLEMENT COMPONENTS IN SYNOVIAL MEMBRANE CULTURE SUPERNATANTS

Haemolytic assays were performed on the synovial membrane culture supernatants from one osteoarthritis patient (OA15) and one specimen of normal synovium from a pateint (N1) suffering from recurrent patellar dislocation. C2, C3 and factor B were shown to be functionally active in the culture supernatants from both patient samples, while C4 and C5 haemolytic activity, significantly higher than the cycloheximide-treated cultures, were not detected.

Discrepancy between the ELISA data and the haemolytic assay data concerning C2 synthesis

C2 synthesis data showed a discrepancy between the ELISA results, where a delay in the initiation of synthesis was detected, and the haemolytic assay where this delay was not apparent.

It has been shown that monocyte C2 behaves differently from serum C2 by having a higher specific functional activity (units/ng protein) and forming a C3 convertase (C4b2a) which is more stable ($T_{½}$ = 9.2 min at 30°C) than the C3 convertase formed with C2 purified from serum ($T_{½}$ = 3.5 min at 30°C) (Lappin & Whaley, 1989). Thus monocyte C2 behaves like oxidised serum C2 (Polley & Muller-Eberhard, 1967). It is likely that C2 which has been synthesised by monocytes has been oxidised by the secretory oxygen products of these cells. This conclusion was supported by the observation that purified C2 which had been incubated with monocyte cultures formed a more stable C3 convertase ($T_{½}$ = 7.2 min at 30°C) than that formed with the untreated C2. Furthermore, since the C3 convertase formed with monocyte C2 which had been oxidised with I_2/KI only showed a slight prolongation in its half-life, it is likely that

monocyte C2 has already been oxidised.

γ-interferon has been shown to stimulate the secretion of macrophage oxygen products (Nathan et al., 1983). In the above experiments, the specific functional activity of C2 and the stability of C4b2a of monocytes were increased by the presence of rIFN-γ, which is thought to be due to further oxidation. When a monoclonal antibody to rIFN-γ was introduced into monocyte cultures, which had been treated with peripheral blood lymphocytes or lymphocytes from rheumatoid synovial membrane, any increase in C2 synthesis was inhibited but neither the specific functional activity of C2 or the stability of C4b2a returned to the values seen in control monocyte cultures. This suggests that another lymphocyte product is responsible for much of their effects on C2 activity.

Therefore the discrepancy in the ELISA data and the results obtained from haemolytic assays for C2 synthesis by cultured fragments of synovial tissue may be explained if in the early stages of synovial membrane cultures, C2 is more oxidised and hence more functionally active than it is during the later period, resulting in C2 activity being detected when little C2 protein was present. The presence of 'oxidised' C2 at sites of inflammation will increase the efficiency of C3 activation by the classical pathway and may influence the outcome of the inflammatory response.

7. INCORPORATION OF ^{35}S-methionine INTO COMPLEMENT PROTEINS

This aspect of the study did not yield as clear results as was hoped. Indeed, the identification of a band corresponding to the correct molecular weight of factor B was the only positive result obtained from these experiments. A band corresponding to the molecular weight of the β-chain of C3 was also detected but a band corresponding to the α-chain was not visible. A smeared pattern was observed for C1-INH but it appeared below the correct molecular weight for C1-INH and hence its specificity must be questioned. Bands corresponding to the correct molecular weights of C2, C4 and C5 were not detected in any of the 4 patient samples studied (RA13, OA16, OA17 and OA18).

(a) Possible explanations for the lack of detection of C4, C2, and C3

The most likely explanation for the poor results in the incorporation of ^{35}S-methionine studies is that there was a high degree of degradation of C4, C2 and C3 within the synovial membrane cultures. However, within the same culture system, C2 and C3 haemolytic activity had been demonstrated which argues against excessive degradation of these two proteins.

One final possibility could be that insufficient cells were synthesising complement components for synthesis to be detected by this

type of analysis.

(b) Attempts to identify the 60 kDa band visible on all fluorographs

Although there was a lack of detection of C4, C2 and C3 in each patient sample, every track showed the presence of a very intense band at 60 kDa. Initially it was thought that this band may correspond to the heavy chain of IgG molecules which had been synthesised by the synovial membrane cultures and were immunoprecipitated along with the complement component immune complexes even although the culture supernatants had been preabsorbed with *Staph. aureus* protein A to remove any non-specific immune complexes. However, IgG heavy chains have a molecular weight of only 50 kDa which is smaller than the band detected. Indeed when incubations designed to remove immunoglobulin were performed e.g. *Staph. aureus* protein A, Sepharose protein G and protein A or IgG-Sepharose, they only succeeded in removing all the bands from the subsequent fluorograph, with no specific removal of the 60 kDa band. These experiments provided further evidence that this band did not correspond to the IgG heavy chain.

Another possibility considered was that this unidentified 60 kDa molecule was the recently characterised glycoprotein, gp60 (Ahmed & Whaley, 1988). This glycoprotein, which is found in normal sera, inhibits complement-mediated prevention of immune precipitation and the complement-dependent solubilisation of immune complexes. The sera of patients with rheumatoid arthritis contain increased concentrations of this protein. However, ELISA analysis of synovial membrane culture supernatants revealed no detectable synthesis of gp60 by cultured fragments of synovial tissue. Thus, it is unlikely that the 60kDa band represents gp60 and at present remains unidentified.

8. C5: SYNTHESIS OR RELEASE?

In contrast to a previous report (Ruddy & Colten, 1974), these studies have been unable to demonstrate synthesis of C5 by synovial membrane fragments cultured *in vitro*. C5 mRNA was undetectable in any sample by either Northern blot or dot-blot analyses, and the release of C5 into the culture supernatants was not significantly different from cycloheximide-treated cultures, after the first day of culture. The C5 detected by ELISA could represent C5 present in contaminating plasma or inflammatory exudate in the synovial tissue, or C5 pinocytosed *in vivo* and later secreted *in vitro*. These possibilities are supported by the observation that the release of albumin followed a similar pattern to that seen for C5 release, which suggests that the C5 detected by ELISA was not due to synthesis of the protein. Failure to detect C5 mRNA in any of these samples by Northern and dot-blot analyses supports this theory.

However, the serum concentration of albumin is approximately a thousand fold greater than C5, but albumin was found at a concentration only ten times higher than the C5 concentration in synovial membrane culture supernatants. This raises the possibility that the C5 detected in the culture supernatants may not be due to simple plasma contamination.

Another interesting finding from this study was that in every case, including C5 and albumin, the presence of cycloheximide reduced the amount of protein being released into the culture fluid. In light of the above studies, it is possible that in addition to its inhibitory effects on protein synthesis, cycloheximide may also inhibit the secretion of proteins.

I have been unable to explain the differences between my results and those of Ruddy and Colten (1974), but in view of the importance of the C5b-9 membrane attack complex, both as a cytolytic agent (Podack, 1986) and as an activator of inflammatory cells (Morgan et al, 1988), and of C5a as an anaphylatoxin and chemotactic agent (Chenoweth, 1987), it is important to resolve this issue.

9. IDENTIFYING THE CELLS RESPONSIBLE FOR COMPLEMENT BIOSYNTHESIS IN HUMAN SYNOVIAL TISSUE

In vitro cultures of cells isolated from human synovial tissue were studied as an initial step in determining the identification of the cells within synovial tissue that are responsible for complement biosynthesis *in vivo*.

Using ELISA analysis, primary cultures of cells isolated from synovial tissue were shown to synthesise C4, C3, factor B and C1-INH. The vast majority (>95%) of these cells are fibroblasts (as determined by performing immunohistochemistry on the cells with a mouse monoclonal antibody against human vimentin, which is a fibroblast marker), which is probably due to the rapid proliferation of these cells in culture compared with other cell types.

In the first case studied (OA19), the non-adherent cells synthesised more complement than those cells that readily adhered to the coverslip in the culture dish. It was thought likely that these non-adherent cells belonged to a different subset of cell types which were capable of synthesising higher levels of complement e.g. mononuclear phagocytes. However, investigation of these cells using an antibody against the fibroblast marker vimentin, identified them as predominantly (>95%) fibroblasts. This finding suggests that the rates of synthesis of complement components are reduced when fibroblasts are adherent to the surface of the culture dish. However subsequent studies of synovial membrane cells isolated from other patient samples showed that the adherent cells synthesised the same or higher levels of complement components as the non-adherent cells. In both cell populations, fibroblast-like cells comprised at least 95% of the total cell

population, and hence any differences in the rates of complement biosynthesis were unlikely to be due to the presence of different cell-types. Unlike sample OA19, (discussed above) the adhering of cells to the culture dish surface appeared to have little or no effect on the ability of the cells to synthesise complement components.

Although, these studies show that fibroblasts appear to be the cell most likely to synthesise complement components in synovial membrane, they are inconclusive, as the cells could have acquired the ability to synthesise the complement proteins during the isolation procedure or during the subsequent culture period. Similar immuno-histochemical analyses will have to be performed on synovial tissue sections to identify the sites of complement accumulation in the synovial tissue, as well as determining the morphology of the various cell types present in the synovial membrane. However, this type of analysis would still detect proteins that have been pinocytosed by cells. Thus, to identify the cells that actually synthesise the complement components, *in situ* hybridisation experiments will have to be performed on frozen sections of synovial tissue. *In situ* hybridisation will identify the cells in which the genes coding for the complement components are being expressed, by detecting the mRNAs encoding the complement proteins.

A number of cell-types that are present in synovial membrane have been shown to be capable of synthesising complement components *in vitro*. These include mononuclear phagocytes (Einstein et al, 1976; Whaley, 1980), and possibly the B-cells of the synovial membrane lining, fibroblasts (Katz et al, 1988) and endothelial cells (Ripoche et al, 1988b). In the human, none of these cells have been shown to synthesise

C4 or C5 so the synovial membrane cell which synthesises these components remains to be identified.

As discussed above, the finding of the synthesis of complement components in synovial tissue from patients with other forms of chronic inflammatory synovitis or with osteoarthritis, as well as in normal synovium, shows that this function of synovial tissue is not unique to rheumatoid arthritis.

The characteristic histopathological finding in osteoarthritis synovium is hyperplasia of the synovial lining cells, with only occasional foci of chronic inflammatory cells (Athanasou et al, 1988). In this disease, it is unlikely that these chronic inflammatory cells are the principle site of complement component synthesis, and raises the possibility that the synovial membrane lining cells or some other cell type may be involved. Indeed, in addition to the data presented here, Katz and Strunk (1988b) have shown that, after several passages, fibroblasts isolated from normal synovial membrane, synthesise a number of complement components during *in vitro* culture.

10. AREAS FOR FURTHER STUDY

I consider the following four areas to be sufficiently important to require further study:

(1) The definition of the range of complement components synthesised by the synovial membrane. As cDNA probes for nearly all the complement components are now available and can therefore be used in both Northern and dot-blot analyses of RNA isolated from human synovial membrane.

(2) The identification of and the distribution of cells which synthesise complement components within the intact synovial membrane should be undertaken. This study will involve the use of *in situ* hybridisation, histochemistry and immunohistochemistry on frozen sections of human synovial tissue.

(3) Studies of the regulation of the expression of each of the complement genes in each cell type.

(4) The interactions between the different cell types involved in complement synthesis and with other synovial membrane cells in the regulation of complement component synthesis.

11. FINAL CONCLUSIONS

Synovial membrane from patients with rheumatoid arthritis and with osteoarthritis have been shown to synthesise a number of complement components including C4, C2, C3, factor B and C1-INH. However, quantitative analysis of the dot-blots containing RNA isolated from the synovial membrane of these patients, showed no statistically significant difference in the steady state level of the mRNAs coding for these complement components (with the possible exception of C2 and C4) between the two disease groups. Furthermore, although the rheumatoid synovium did synthesise markedly greater quantities of C2 protein than the osteoarthritis synovial membrane fragments, no significant differences in the amount of the other complement proteins synthesised by cultured fragments of synovial membrane were observed between the rheumatoid arthritis and osteoarthritis patients.

Therefore, the significance of the contribution made by local complement biosynthesis to the inflammation seen in rheumatoid joints may not appear quite so apparent as was initially suspected. This statement arises because such severe inflammation of the synovium is not typical of osteoarthritis, but from these studies the ability of synovial tissue from osteoarthritis patients to synthesise complement does not appear to be significantly different from rheumatoid synovium. Moreover, normal synovium removed from a patient suffering from recurrent patellar dislocation was shown to synthesise C4, C2, C3, factor B and C1-INH *in vitro* while C1-INH mRNA was detected in a sample of this normal synovium that had been snap-frozen in liquid nitrogen immediately after removal from the patient. The demonstration of C1-INH mRNA in normal synovium provides the best evidence to date that

synthesis of complement components by synovial membrane is constitutive. Such extrahepatic synthesis of complement may play a major role in host defence.

It would have been advantageous to have examined more specimens of normal synovium. However, as with many studies of biological systems, the securing of physiologically normal tissue proved to be virtually impossible. The only synovium available was from patients undergoing a joint operation of some description and even in the above case of the recurrent patellar dislocation, it is likely that some degree of inflammatory response had occurred within the joint. Hence, this caveat has to be borne in mind whenever a comparative study such as this is undertaken.

However, even although complement biosynthesis has been detected in normal synovium, its role in the pathogenesis of chronic inflammatory synovitis in diseases such as rheumatoid arthritis cannot be discounted due to the biological effects of the complement system which are likely to contribute to the inflammatory response.

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