REGULATION OF COMPLEMENT ACTIVATION AT SITES OF CHRONIC INFLAMMATION

A thesis presented by
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for the degree of

DOCTOR OF PHILOSOPHY
OF THE
UNIVERSITY OF GLASGOW

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May 1993

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SUMMARY

Biosynthesis of the complement regulatory proteins C1-INH, C4-bp, factor H, factor I, S protein, SP-40,40, DAF, MCP, CD59 and CR1 has been investigated in synovial membrane tissue cultures obtained from normal individuals, and patients with osteoarthritis (OA) and rheumatoid arthritis (RA) by a combination of immunological and molecular biological techniques. Normal, OA and RA synovial membranes were capable of synthesizing this battery of regulatory complement proteins. The abundances of the mRNAs encoding the classical and alternative pathway secreted proteins were elevated in RA synovium as was that of CD59 mRNA. However, tissue samples were small and they may not reflect the whole situation in RA joints.

The cells present in synovial membrane, endothelial cells, monocytes, synovial fibroblasts and peripheral blood lymphocytes, were cultured and investigated in order to find which cell types provide which regulatory proteins detected in synovial membrane. Cell-specific differences were observed in the regulatory complement proteins synthesized. ELISA, immunohistochemical staining, Northern blot and doubling-dilution dot-blot analysis showed that both monocytes and fibroblasts expressed nine, endothelial cells expressed eight and lymphocytes expressed six of the eleven components under standard culture conditions. All of the cell types investigated synthesized factor H, DAF, MCP, CD59 and MIP. Endothelial cells and fibroblasts did not synthesize C4-bp and CR1. Also cell-specific differences observed in the amounts of secretion for some proteins.

Furthermore, cell specific differences were observed for the expression of different mRNA species of DAF, MCP and CD59 between endothelial cells and fibroblasts.

The effect of a cytokine, interferon–γ (IFNγ), on the synthesis of these complement regulatory proteins was also investigated. Treatment of endothelial cells with IFNγ caused an increase of transcription rate of DAF, MCP and CD59 but this did not correlate with the protein levels on the cell surface detected by flow cytometry.

This study indicated that complement regulatory protein synthesis is constitutive and varies between the different types of cells in synovial membrane. To some extent, the amounts of some complement regulatory proteins is regulated by IFNγ.

ACKNOWLEDGEMENTS

First and foremost my thanks must go to Prof. Keith Whaley for giving me the opportunity to start my PhD study and his friendly guidance throughout the writing of this thesis. I am also grateful to Dr. George D. Birnie for giving me the chance to join his team in Beatson Institute and for his constructive criticism and supervision from which I learned a lot. I like to thank my assessor Dr. John Pitts for his encouraging discussions.

I would also like to acknowledge the Scottish Home and Health Department for providing me the grant for this study.

I am indebted to Prof. MacSween for allowing me to use the facilities of the Department of Pathology and to Prof. John Wyke for allowing me to complete my study in the Beatson Institute.

I like to thank to Drs. Poonam Gulati, David Lappin, Elizabeth Holme, Grame Phimister and Gavin Sandilands, Mrs. Terry McShane and Sandra Howat for their help and friendship during my studies in the Western Infirmary.

I like to thank to Mrs. Elaine MacKenzie for her help in flow cytometry experiments and to all my friends in group R3 for their support. I appreciated the assistance of all the scientific, technical staff and the departmental secretaries in the Beatson Institute and in the Pathology department.

Thanks to my friends in Glasgow for making my stay so memorable and enjoyable.

I am grateful to my mother, my father and my sisters for all the love and constant support they have given me throughout my education in Turkey and in Glasgow.

Finally, I am grateful to my husband Oguz, the reason of my visit to Glasgow. His continual encouragement, love and help gave me strength and faith to carry on with this study. I feel proud and lucky having him as a life-partner.

PUBLICATIONS ARISING FROM THIS THESIS

FULL PAPERS

1. Lappin, D., **Guc, D.,** Hill, A., McShane, T. & Whaley, K. (1992). Effect of interferon-γ on complement gene expression in different cell types. *Biochemical Journal,* **281,** 437-442.

2. Whaley, K., **Guc, D.,** Gulati, P. & Lappin, D. (1992). Synthesis of complement components by synovial membrane. *Immunopharmacology* **24,** 83-89.

3. Gulati, P., **Guc, D.,** Lemercier, C., Lappin, D. & Whaley, K. (1993). Expression of the components and regulatory proteins of the classical pathway of complement in normal and diseased synovium. *Clinical Experimental Immunology.* (submitted for publication).

4. **Guc, D.,** Gulati, P., Lemercier, C., Lappin, D., Birnie, G.D. & Whaley, K. (1993). Expression of the components and regulatory proteins of the alternative complement pathway and the membrane attack complex in normal and diseased synovium. *Clinical Experimental Immunology* (submitted for publication).

ABSTRACTS

1. Lappin, D., **Guc, D.,** Hill, A., McShane, T. & Whaley, K. (1991). Complement gene expression of C2, B, C3, C1-inh, C4-bp and H(I). *Complement and Inflammation,* **8,** 180 (Abst. No:148).

2. Lappin, D., **Guc, D.,** Hill, A., McShane, T. & Whaley, K. (1991). Cell specific regulation of stability of mRNAs encoding C2, B, C3, C1-inh, C4-bp and H. *Complement and Inflammation,* **8,** 180 (Abst. No:149).

BOOK CHAPTER

1. Gulati, P., Lemercier, C., **Guc, D.,** Lappin, D. & Whaley, K. (1993). Regulation of the synthesis of C1 subcomponents and C1-inhibitor. In: *Behring Institute Research Communications,* Behring Institute Publications, Mitteilungen, Germany, (in press).

OTHER

1. **Guc, D.,** Gulati, P., Birnie, G.D. & Whaley, K. (1992). CD59 gene expression in human endothelial cells and fibroblasts. *Proceedings of Biochemical Society-Scottish Area Predoctoral Students Meeting, Poster Programme* (September 14), Glasgow, U.Kingdom.

2. Gulati, P., **Guc, D.,** Lemercier, C., Lappin, D. & Whaley, K. (1992). Cell-specific expression of C1 subcomponents. *Proceedings of III. International Workshop on C1* (November 6-9), Mainz, Germany.

3. **Guc, D.,** Gulati, P., Birnie, G.D. & Whaley, K. (1992). Expression of CD59, DAF, MCP mRNAs in endothelial cells and fibroblasts. *Proceedings of British Society for Immunology Autumn Meeting* (November 18-20), London, U.Kingdom.

ABBREVIATIONS USED IN THIS THESIS

C1	First component of complement
C1q, C1r, C1s	Sub-components of C1
C1-INH	Inhibitor of activated C1
C2	Second component of complement
C2a, C2b	Fragments of C2
C3	Third component of complement
C3a	Anaphylatoxin fragment of C3
C3b	Largest fragment of C3
C3bi	Inactivated C3b
C3c, C3d, C3e	Fragments of C3
C3dg	C3bi fragment produced by control proteins
C4	Fourth component of complement
C4a, C4b	Fragments of C4
C4bp	C4 binding protein
C5	Fifth component of complement
C5a	Anaphylatoxin fragment of C5
$C5_{des\ Arg}$	Desarginyl derivative of C5a
C5b	Largest fragment of C5
C6	Sixth component of complement
C7	Seventh component of complement
C8	Eight component of complement
C9	Ninth com. ponent of complement
DAF	Decay accelerating factor
MCP	Membrane cofactor protein
HRF	Homologous restriction factor
MIP	Membrane inhibitory 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)
CR4	Complement receptor type 4
CR5	Complement receptor type 5
H	Factor H
I	Factor I

PRE	Phorbol 12-myristate 13 acetate response element
CRE	cAMP response element
Mr	Molecular rate
C4A	Isotype of C4
C4B	Isotype of C4
B	Factor B
N	Normal

P	Properdin
D	Adipsin
S	S Protein or vitronectin
SP-40,40	Clustrin
IgA	Immunoglobulin class A
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	Regulators of complement activation
kb	Kilobases
kDa	Kilodalton
HSA	Human serum albumin
PMN	Polymorphonuclear leucocyte
RA	Rheumatoid arthritis
OA	Osteoarthritis
IFNγ	Interferon gamma
IFNα	Interferon alpha
IL-1	Interleukin one
IL-6	Interleukin six
MOPS	Sodium morpholino propanesulphonic acid
Tris	Tris hydroxymethyl-methylamine
w/v	Weight per volume
v/v	Volume per volume
FCS	Foetal calf serum
HUVEC	Human umbilical vein endothelial cells
PHA	Phytohaemagglutinin
PWM	Pokeweed mitogen
Con A	Concanavalin A
CHX	Cycloheximide
PCR	Polymerase chain reaction

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CHAPTER 1. INTRODUCTION

1.1 INFLAMMATION

1.1.1. Definition of Inflammation

Inflammation is defined as a localized protective event, elicited by injury, which serves to destroy, dilute or wall off both injurious agent and the injured tissues. It involves a complex series of events, including dilatation of arteries, capillaries and venules, with increased blood flow and vascular permeability and exudation of body fluids and plasma proteins. These processes are often rapidly followed by the adhesion of leucocytes to the vascular endothelium with a subsequent influx of the cells into the surrounding tissues.

The various mediators which regulate local blood flow and the cells of the inflammatory response are discussed below.

1.1.2. Inflammatory Mediators

The components which mediate inflammatory reactions are derived from the complement system, lymphocytes, granulocytes, mast cells and platelets, causing an increase in blood flow and vascular permeability.

The immune system can initiate acute inflammatory reactions either *via* the classical complement pathway which is activated by antigen-antibody complexes containing IgG or IgM antibodies, or by recruiting mast cells by sensitizing them with IgE so that they are triggered following contact with antigen. The anaphylatoxins C3a, C4a and C5a also trigger mast cells and basophils. C5a is chemotactic for, and activates, neutrophils and macrophages. Eicosanoids released from the mast cells affect the local vasculature, and leukotriene B4 (LTB4) is itself chemotactic. Additional eicosanoids are produced by the macrophages

themselves, as well as by the local endothelium. Once present at the site of inflammation, inflammatory cells (granulocytes, macrophages, lymphocytes) come under the additional controls of cytokines including interferon γ (IFNγ), tumor necrosis factor (TNF) and interleukin-1 (IL1). Macrophages and lymphocytes are of particular importance in chronic inflammatory reactions. The mediators of inflammation are summarized in Table 1.1.

According to its duration, inflammation is classified as acute or chronic.

1.1.3. Acute Inflammation

There are three main events in the acute inflammatory process.

1) Vasodilatation and changes in the blood flow: a transient vasoconstriction of the arterioles occurs immediately after the injury and is followed by vascular dilatation affecting arterioles mainly but also to a lesser extent the venules and the capillaries. Blood flow through the injured tissue is increased immediately after the injury, but is followed by slowing of blood flow and stasis. Decreased blood flow is thought to be due to increased blood viscosity.

2) Exudation of plasma: contraction of the endothelial cells increases vascular permeability. This permits large molecules to traverse the endothelium and allows the soluble plasma mediators of immunity (*e.g.* complement, antibody) to reach the damaged tissue.

3) Emigration of neutrophil leucocytes: increased migration of cells from the blood vessels into the affected area occurs following their adhesion to endothelial cells, their emigration out of the vessel by insinuating themselves between the endothelial cells and finally, their movement through the tissues to the site of inflammation along a

Table 1.1. **MEDIATORS OF INFLAMMATION**

Mediator	Sources	Activators	Principle effects
Bradykinin	High mol weight kininogen	Factor XIIa kallikrein proteases	vasodilatation increased vascular permeability,pain
Histamine	mast cell, basophil, granules	IgE,antigen anaphylatoxin	vasodilatation increased vascular permeability, spasmogenic chemokinesis
C3a,C5a	complement system	C3 and C5 convertases proteases	mast cell degranulation chemotaxis increased vascular permeability.
Platelet Activating Factor (PAF)	basophils neutrophils macrophages	depends on cell type	platelet factor release,spasmogen neutrophil activation increased vascular permeability
Eicosanoids (see below)	macrophages monocytes endothelialcell mast cell basophils.	many inducers	modulate effects of other mediator vasoactive
PGE_2	cyclo-oxygenase pathway	many inducers	vasodilatation potentiates LTB4, C5a, bradykinin, 5-HT effects
PGI2	cyclo-oxygenase pathway	many inducers	vasodilatation blocks platelet aggregation broncho-constriction

Table 1.1. **MEDIATORS OF INFLAMMATION (continued)**

Mediator	Sources	Activators	Principle effects
TxA2	cyclo-oxygenase pathway	many inducers	vasoconstriction platelet aggregation broncho-constriction
LTB4	lipo-oxygenase pathway	many inducers	chemotaxis modulates increased vascular permeability
LTD4	lipo-oxygenase pathway	many inducers	spasmogen increase vascular permeability bronchospasm
Interleukin1 (IL-1)	macrophages	macrophage activators (e.g. C5a)	pyrogen, PG production induce acute phase proteins activation of T cells
Interferonγ (IFNγ)	T cells	antigen	macrophage and T cell activation modulates inflammatory reaction
Tumour Necrosis Factor (TNFα,β)	macrophages lymphocytes	bacteria protozoa some tumour cells	activation of granulocytes, macrophages and cytotoxic cells increased leucocyte-endothelial cell adhesion,cachexia

chemical gradient, a process called chemotaxis. Activation of the neutrophils facilitates phagocytosis, bacterial killing and the degradation of phagocytosed material.

Acute inflammatory foci contain mostly polymorphonuclear leucocytes, whereas regions of chronic inflammation contain a high proportion of lymphocytes and macrophages, and possibly epithelioid and giant cells.

1.1.4. Chronic Inflammation

Inflammation is said to be chronic when its duration is relatively prolonged for months or years. The tissues are infiltrated by several types of cells such as mononuclear phagocytes, lymphoid cells, fibroblasts, and endothelial cells. In addition there may be granulocytic leucocytes, particularly eosinophils. Mononuclear phagocytes are present as macrophages, epithelioid cells, or multinucleated giant cells. Lymphoid cells are present as lymphocytes, plasma cells or immunoblasts. Fibroblasts produce collagen and ground substance and proliferate to form more fibroblasts. Endothelial cells migrate and proliferate forming a rich neovascular network necessary for the maintenance of the inflammatory response. One of the most important components of chronic inflammation is the macrophage and its derivatives. They recognize material to be phagocytosed by means of their surface receptors which include the complement receptors C3b and iC3b and the Fc portion of IgG. By facilitating recognition and phagocytosis, complement acts as an opsonin. Macrophages also present antigen to lymphocytes. Finally, they have potential microbial effector mechanisms such as reactive oxygen intermediates (.OH, $O^{\cdot}$, O_2^{-} and H_2O_2), toxic oxidants produced by interaction of H_2O_2 on halides in the presence of peroxidase catalase,

phagosome acidification, lysosomal enzymes and growth inhibitors, including lactoferrin and arginase. Necrosis is a common feature of chronic inflammation which may be associated with the formation of granulation tissue and fibrous tissue. A proliferative type of lesion may result and is called "granuloma".

Rheumatoid arthritis is an example of a chronic inflammatory disease and it is discussed in more detail below.

1.1.5. Rheumatoid Arthritis

Rheumatoid arthritis is a chronic progressive inflammatory arthritis of unknown aetiology which affects principally the joints and is characterized by the tendency to spontaneous remissions and subsequent relapses. It is two to three times more frequent in females and usually involves the small joints of the hands and feet.

1.1.5.1. *Pathologic anatomy*

The basic tissue changes of rheumatoid disease are not specific but in combination they give a fairly typical and diagnostically suggestive picture. Several features which characterize the rheumatoid lesion are as follows.

1. Diffuse or focal infiltration of the tissue by lymphocytes or plasma cells or both, with the development of lymphoid follicles.

2. Vasculitis with endothelial proliferation, narrowing or occlusion of the lumen, fibrinoid change or necrosis of the walls and perivascular aggregation of lymphocytes and plasma cells.

3. The rheumatoid granuloma, a focal lesion with an amorphous center composed of necrotic tissues.

1.1.5.2. *Immunogenetics in rheumatoid arthritis*

Susceptibility to rheumatoid arthritis (RA) is linked to genes within the major histocompatibility complex (Payami et al., 1986). Patients with classical or definite disease, 70% exhibit either HLA-DR4 or DR1 (Nepom et al., 1989; Wordsworth et al., 1989).The high association with RA appears to reside in the specific sequences in the third hypervariable region of the HLA-DR β1 chain (Wordsworth et al., 1989). However this association is not absolute since there are some patients who lack DR4 or DR1. Wordsworth et al. (1991) have shown a significant association with DRw10 in the DR4/DR1 negative RA group.

Evidence that immune processes are important in the development of the rheumatoid lesion can be summarized as follows.

1) The presence of IgG, IgA, IgM in synovial fluid, in synovial lining cells and in the articular cartilage.

2) The presence of antigen-antibody complexes in synovial fluid and synovial tissue.

3) The presence of rheumatoid factor (RF), IgM antibody reactive to the Fc portion of IgG, in the plasma cells in synovial tissue.

4) The presence of activated complement components in synovial fluid, in synovial tissue, in macrophages, in the interstitial connective tissue and synovial lining cells. This is asssociated with decreased levels of complement components in the synovial fluid.

In addition, the presence of other autoantibodies in the sera of patients (*e.g.* antinuclear antibodies and antibodies to type II collagen) is further evidence that autoimmune processes are involved in the pathogenesis of RA.

1.1.6. Osteoarthritis

Osteoarthritis (OA) is a non-inflammatory disorder of the synovial joints. OA characterized by the degenerative changes in articular cartilage and by overgrowth of subchondral and juxta-articular bone and intra-articular soft tissues.

The aetiology of osteoarthritis is multifactorial. But primary and secondary osteoarthritis must be distinguished although morphologically, the two types are indistinguishable. Secondary osteoarthritis is often post-traumatic in origin due to malalignment of a joint due to congenital malformations, fractures or the deposition of extraneous injurious material within the articular tissues. Genetic (increased familial incidence), hormonal (increased severity of OA associated with acromegaly and diabetes) and nutritional factors (frequent coexistence of OA and obesity) and age have been shown to determine the susceptibility of the articular cartilage to becoming arthritic (Altmann et al., 1986).

1.2. THE COMPLEMENT SYSTEM

The initial discoveries leading to the understanding of complement were made about a century ago. Grohman (reviewed by Colten, 1976) reported that cell-free blood was capable of destroying bacteria *in vitro*. This lytic activity was shown to be lost when the blood was stored at room temperature, incubated at 55 ^{o}C, or dialysed against distilled water. At the same time, progress was made leading to the discovery of serum antibodies and the subsequent division of bactericidal activity of serum into two factors: 1) a heat-stable factor confined to immune sera and 2) a heat-labile factor constituting the bactericidal entity itself (quoted by

Morgan, 1990). The sequential nature of the two factors was elucidated by Erlich and Morgenroth (Bouldan, 1910), whose studies led to the concept that a specific antibody reacted with a target acceptor and than activated the lytic activity of the second factor. They first termed the second factor "alexin" but later introduced the term "complement" as it complemented antibody function. Over the next few years an intense debate developed concerning the nature of complement and its interaction with antibodies, the main protagonists being Bordet and Erlich (reviewed in Morgan, 1990).

The complement system has evolved as the major humoral defense mechanism against infection, and complement activation products mediate many of the processes of inflammation. These include 1) the binding of fragments of the third component (C3) to the surfaces of the microorganisms or other antigens targets them to cells expressing C3 receptors (*e.g.* phagocytes); 2) chemotactic factors (C5a and $C5a_{des\ Arg}$) recruit phagocytic cells to the area where complement activation takes place; 3) cell killing is mediated through the formation of the C5b-9 membrane attack complex.

An essential requirement for such a destructive capacity to exist *in vivo* is the existence of stringent control mechanisms. Multiple inhibitory and control proteins influence virtually every stage of the complement system, preventing excessive activation and damage to host cells.

1.2.1. Nomenclature

The proteins of the classical activation pathway and the membrane attack pathway are called "components" and are symbolized by the letter "C" followed by a number, *e.g.* C1, C4, C2, C3, C5, C6, C7, C8, C9.

The proteins of the alternative activation pathway are termed "factors" and are symbolized by letters, *e.g.* B, D, P.

The control proteins are referred to with contraction of their trivial names *e.g.* C1 inhibitor (C1-INH), C4-binding protein (C4-bp).

The enzymically active form of components are denoted by a bar over the number *i.e.* $\overline{C1}$. Cleavage fragments are denoted with suffixed lower case letters *e.g.* C3a, C3b, C3c, C3d. Polypeptide subunit chains are suffixed with Greek letters starting with the largest chain, *e.g.* C3α and C3β.

1.3. THE ACTIVATION OF COMPLEMENT SYSTEM

The complement system consists three interacting pathways:

1) the classical pathway;

2) the alternative pathway;

3) the membrane attack sequence.

1.3.1. The Classical Pathway

Immune complexes containing antibodies of either IgG (subclasses IgG1, IgG2 and IgG3) or IgM initiate classical pathway activation (Figure 1.1). The classical pathway consists of four plasma proteins, C1, C4, C2, C3 (Table 1.2).

The first component of complement is a large Ca^{2+}-dependent complex composed of one molecule of C1q and two molecules each of C1r and C1s (Lepow et al., 1963).

C1q, the recognition unit of the classical pathway, is a multichain molecule consisting of a central stem which separates into six fibrillar

Table 1.2. CLASSICAL PATHWAY PROTEINS

Proteins	Serum Concentration (mg/L)	Mr (kDa)	Subunit Structure	Chromosomal Assignment
C1q	80	460	6 X (A,B,C) chains (26 kDa)	1p34.1-36.3
C1r	50	83	Homodimer	12p13
C1s	50	83	Single but Ca^{2+}dependent dimer	12p13
C4	600	200	1α chain (97 kDa), 1β chain (75 kDa), 1γ chain (33 kDa)	6p21.3
C2	20	100	Single chain	6p21.3
C3	1300	190	1α chain (110 kDa), 1β chain (75 kDa)	19q

stalks each of which terminates in a globular head, giving it the appearance of a bunch of tulips (Knobel et al., 1975). The globular head region of C1q binds to specific sites in the Fc region of the IgG and IgM. IgG3 is the IgG subclass which binds C1q most efficiently, followed by IgG1 and IgG2 (Lucisano-Valim and Lachmann, 1991). IgG4 does not appear to bind C1q. Complexes of polycations with polyanions such as protamine with heparin, or C polysaccharide with C-reactive protein (CRP), bacterial lipopolysaccharide and myelin are also capable of binding C1q and activating C1 (Claus et al., 1977).

Binding of C1q to immunoglobulins or other activators of classical pathway initiates conformational changes within both C1q and C1r, resulting in the expression of neoantigens on C1q and of proteolytic activity by C1r. Activated C1r ($\overline{C1r}$) activates C1s by producing a single proteolytic cleavage (Arlaud et al., 1987) (Figure 1.1).

Activated C1s ($\overline{C1s}$) cleaves C4 at a single site in the α chain, thereby releasing a 77 amino acid (8 kDa) N-terminal fragment, C4a, (Gorski et al., 1979) which is a weak anaphylatoxin. The remaining fragment, C4b, contains an internal thiolester group in the α chain which is exposed during C1s cleavage. This thiolester provides a binding site permitting the covalent binding of C4b to surfaces. The thiolester is extremely labile, being active for only a few microseconds and decays as a result of hydrolysis if C4b does not encounter a suitable surface. Only about 5% of C4b actually becomes bound to the activating surface, and the distribution is limited to a small area around the activating C1 complex.

C4 occurs in two structurally and functionally distinct isotypes, C4A and C4B, each of which are encoded by distinct genes (Carroll et al., 1984). C4A reacts preferentially with amino groups, C4B with hydroxyl groups.

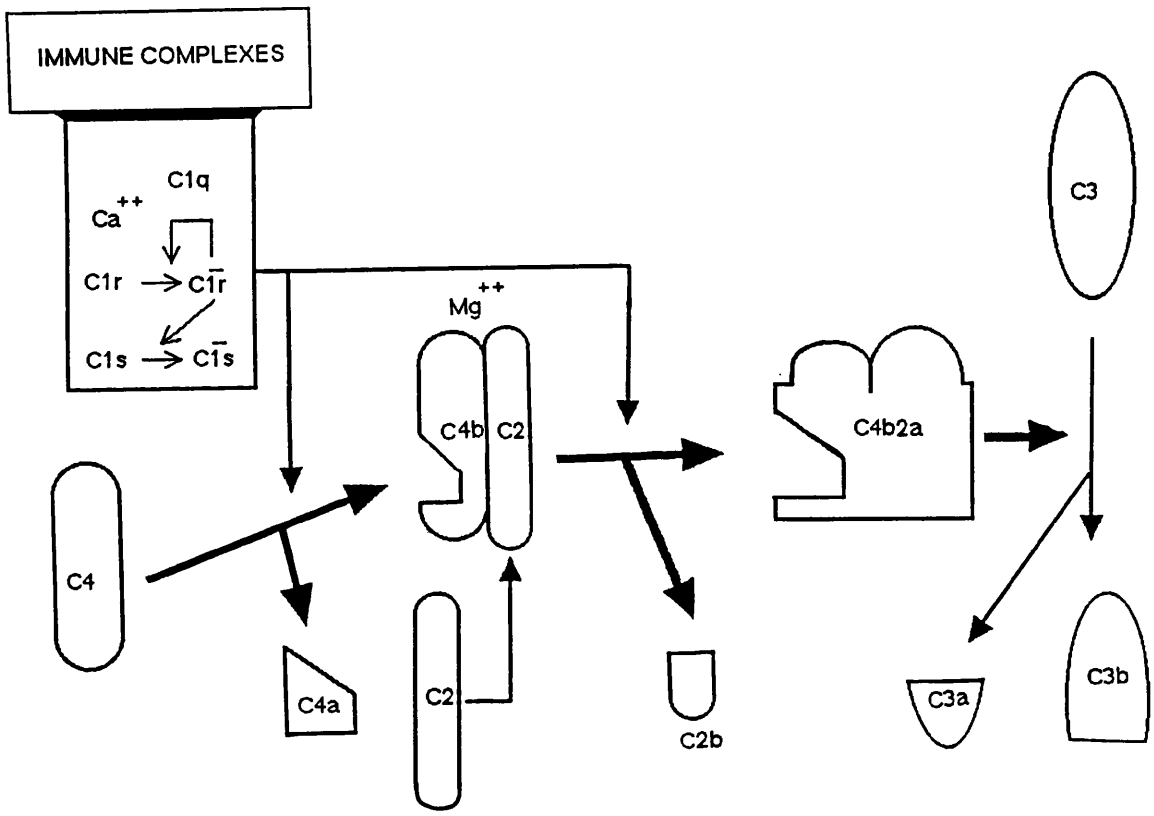


Figure 1.1: Summary of the classical pathway. Binding of C1q to immune complexes activates C1r and C1s. $\overline{C1s}$ cleaves C4a from C4 which binds C2 in the presence of Mg^{++}. C1s cleaves C2b from this complex to leave a classical pathway C3 convertase (C4b2a) (Adapted from Roitt et al., 1990).

C2 binds to C4b and subsequently becomes a substrate for C1s. It is cleaved into two fragments C2a (85 kDa) and C2b (35 kDa) (Polley and Muller-Eberhard, 1968). Although C1 can cleave free C2, the efficiency of cleavage is greatly enhanced when C2 is bound to an adjacent C4b molecule. C2a associates with C4b to form the C4b2a complex, the classical pathway C3 convertase. C2b may be released into fluid phase or it may remain loosely attached to the C4b2a complex (Ziccardi and Cooper, 1980; Gigli and Austen, 1969) (Figure 1.1).

C3 is the complement component which is present in highest concentration in plasma (1.5 mg/l) and is of central importance. It is involved in both classical and alternative pathways. Both the classical and the alternative pathway C3 convertases (C4b2a and C3bBbP) respectively cleave C3 in the fluid-phase. This cleavage releases a small N-terminal fragment, C3a (9kDa), which is an important anaphylatoxin, and a large fragment, C3b, which has a thiolester group which can bind the hydroxyl or amino groups on surfaces, forming ester or amide bonds respectively (Law and Levine, 1977) (Figure 1.1). The thiolester has an extremely short life (approximately 60 ms) and if it does not bind to a surface by that time, the thiolester bond undergoes hydrolysis and C3b becomes inactive fluid-phase C3b. Thus, although each C4b2a complex can activate several hundred C3 molecules, only approximately 10% of C3b molecules bind to surfaces. C3b which binds to C4b in C4b2a alters the specificity of the enzyme to that of a C5 convertase (C4b2a3b). This occurs because C3b in C4b2a3b act as a receptor for C5 and presents C5 to C2a for activation (Medicus et al., 1976).

1.3.2. The Alternative Pathway

The addition of polysaccharides such as the yeast cell wall extract, zymosan or Gram-negative bacterial endotoxin to the serum of animals congenitally deficient of either C4 or C2 leads to cleavage of C3 and to the assembly of the membrane attack complex by a mechanism that is independent of the classical activation pathway. IgA is also thought to be the activator of the alternative pathway. IgG2 is the best IgG subclass for alternative pathway activation but requires high epitope density and equivalence or antibody excess (Lucisano-Valim and Lachmann, 1991). This pathway comprises six plasma proteins (Table 1.3), three of which form the C3 and C5 convertases (C3, factor B and factor D) while properdin has a positive regulatory property (Gotze and Muller Eberhard, 1974) and factor H and I are negative regulators (Whaley and Ruddy, 1976; Pangburn et al., 1977). Phylogenetically the alternative pathway is older than the classical pathway.

The alternative pathway continuously undergoes low-grade spontaneous non-enzymatic activation (Lachmann and Hughes-Jones, 1984). This activation occurs because of spontaneous hydrolysis of the internal thiolester of C3 to form a C3 derivative molecule, $C3(H_2O)$. Apart from its inability to bind covalently to surfaces, $C3(H_2O)$ is functionally equivalent to C3b. In the presence of magnesium ions, $C3(H_2O)$ binds to B in the fluid-phase, rendering it susceptible to cleavage activation by D and thereby forming a low efficiency fluid phase C3 convertase $C3(H_2O)$, Bb (Fearon et al., 1974; Pangburn and Muller-Eberhard, 1984) (Figure 1.2). Unlike C3b, C3(H2O) is relatively resistant to the control proteins, H and I.

Factor B is the alternative pathway homologue of C2. Upon binding to activated C3, it is rendered susceptible to cleavage by factor D,

Table 1.3. ALTERNATIVE PATHWAY PROTEINS

Proteins	Serum Concentration (mg/L)	Mr (kDa)	Subunit Structure	Chromosomal Assigment
Factor B	210	93	Single chain	6p21.3
Factor D	2	24	Single chain	N.D.
Properdin	26	220	Exists as polymers of identical 60kD subunits usually trimers or tetramers	Xp 11.23-21.1
C3	1300	190	1α chain (115 kDa) 1β chain (75 kDa)	19q

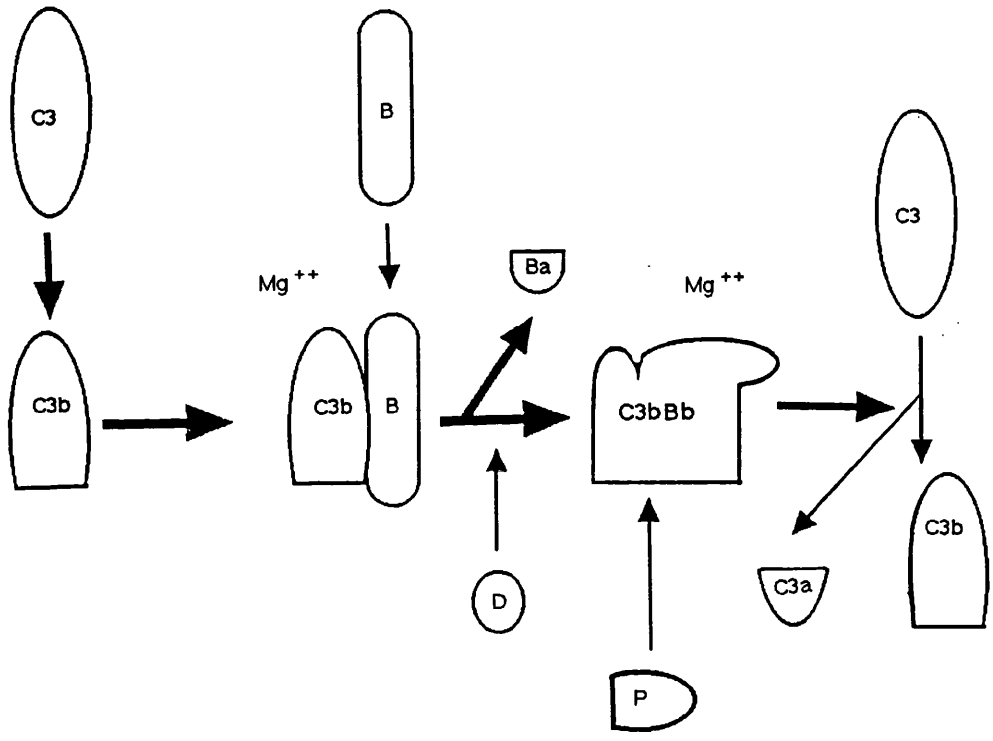


Figure 1.2: Alternative pathway. In the presence of Mg^{++} ions C3b binds to B, rendering it susceptible to cleavage by factor D and thereby forming an alternative pathway C3 convertase (C3bBb). Properdin binds to C3bBb and stabilizes the C3 convertase (Adapted from Roitt et al., 1990).

exist in plasma as an active serine protease present in its active state in the fluid phase. Cleavage of B releases a 33 kDa fragment, Ba, which may have mitogenic activity. The Bb, 60 kDa fragment has serine protease activity and like C2 is capable of cleaving C3 and C5 (Lambris and Muller-Eberhard, 1984).

The spontaneously generated fluid-phase convertase $C3(H_2O)Bb$ cleaves C3 in precisely the same way as the C4b2a complex, releasing the anaphylatoxin C3a and exposing the thiolester binding site on C3b. C3b formed in this way binds to any surface it encounters during the lifetime of its binding site. Thus, C3b is deposited continuously in a random and nonspecific way on the surfaces of host cells and pathogenic organisms alike. C3b which is bound to nonactivating surface (*e.g.* host cells) is rapidly inactivated by the control proteins H and I (Figure 1.3), whereas C3b bound to an activating surface is protected from these factors (H and I) and therefore persists and binds B to form a membrane bound C3bBb (Fearon and Austen, 1977; Pangburn and Muller-Eberhard, 1978). The bound C3bBb complex can then cleave more C3, initiating a positive feedback cycle which rapidly amplifies the amount of C3 bound to the activator and the amount of anaphylatoxin generated. The control of such a potentially explosive cycle is achieved by the spontaneous decay of C3bBb complex (half-life of seconds at 37 ^{o}C) and by the action of control proteins H and I.

Although C3b is already present in the C3 convertase C3bBb a second C3b must bind to the original C3b to form a C5 convertase. This second C3b in "C3bBbC3b" acts as the C5 receptor and presents C5 for the activation by Bb (Medicus et al., 1976; Schreiber et al., 1975). C3bBbC3b cleaves C5 at exactly the same site as does C4b2a3b.

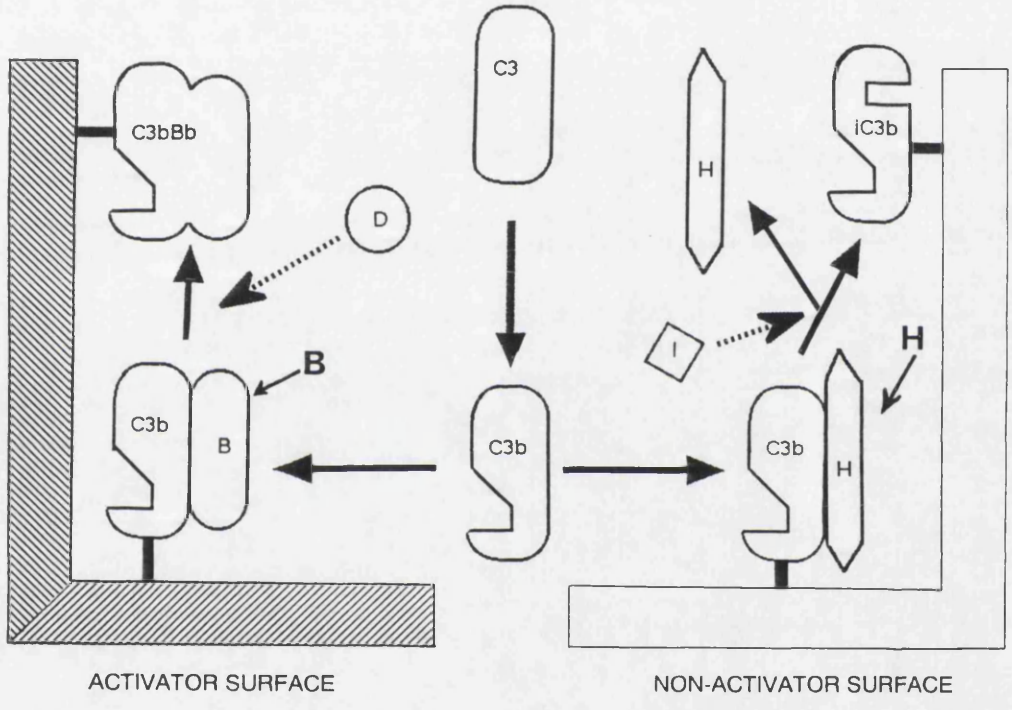


Figure 1.3: Regulation of the amplification loop. C3b which has bound to an activator surface binds factor B and an alternative pathway C3 convertase (C3bBb) is generated. C3b which has bound to self surfaces promote the binding of factor H rather than factor B, thus C3b becomes inactivated by factor I (Adapted from Roitt et al., 1990).

Properdin binds to C3bBb and stabilizes the alternative pathway C3 and C5 convertases by slowing the rate of decay of Bb from the complex (Fearon et al., 1975; Czop et al.,1978).

1.3.3. The Membrane Attack Sequence

The membrane attack sequence compromises of five complement components C5, C6, C7, C8 and C9 (Table 1.4). Cleavage of C5 by the convertases of either pathway is the final enzymatic step in the complement activation cascade.

C5 consists of two disulphide-linked polypeptide chains. C2a in the C4b2a3b complex, Bb in the C3BbC3b complex cleave C5 at a single site in its α-chain, releasing a 74 amino acid N-terminal fragment, C5a (12kDa), which has important anaphylactic and chemotactic activities. The major cleavage product C5b expresses a labile membrane binding site which is not present on native C5. This allows C5b to bind weakly and reversibly to cell membranes. Another binding site recognizes C6 which, if it binds within a few minutes, stabilizes the low affinity membrane binding site. However, as the membrane binding site is of low affinity, the C5b6 complex may be released into the fluid-phase and binds to cell membranes distal to the site of complement activation. As the haemolytic potential of complex is preserved, damage to cells outside the area of the original injury may occur (reviewed by Muller, 1986).

Binding of C6 creates a stable complex with weak membrane binding properties and the capacity to bind C7, a single chain protein of 110 kDa. Binding of C7 causes a major conformational change in the complex, which effects its release from the C5 convertase and exposes a hydrophobic membrane binding site. If the C5b67 does not encounter a membrane rapidly, it self-aggregates and interacts with inhibitory

Table 1.4. MEMBRANE ATTACK PATHWAY PROTEINS

Proteins	Serum Concentration (mg/L)	Mr (kDa)	Subunit Structure	Chromosomal Assigment
C5	70	190	1α chain (115 kDa) 1β chain (75 kDa)	9q 32-24
C6	65	110	Single chain	5
C7	55	110	Single chain	5
C8	55	150	1α chain (64 kDa) 1β chain (64 kDa) 1γ chain (22 kDa)	α,β 1p32
C9	60	69	Single chain	9q

proteins (S protein and SP-40,40) in the fluid-phase so that membrane insertion cannot occur (Podack et al., 1978, 1984; Kirszbaum et al., 1992) (Figure 1.4).

As mentioned earlier, C5b6 can dissociate from the target cells and bind to host cells. The subsequent binding of C7, C8 and C9 leads to "bystander lysis". Alternatively, C7 may bind to C5b6 in the fluid-phase and then bind to cells in the immediate vicinity. The subsequent binding of C8 and C9 leads to "reactive lysis" (Lachmann and Thompson, 1970). Once inserted into the membrane, the C5b-7 complex becomes a stable membrane protein although cytolysis does not occur until C8 and C9 have been incorporated (Figure 1.4).

C8 comprises three polypeptide chains α, β, γ. The α and γ chains are disulphide-linked, while the β chain is noncovalently bound. C8 binds to C5b67 complex *via* a binding site in the C8 β chain. The incorporation of the C8 to the C5b67 complex causes the complex to become more deeply embedded in the membrane making it slightly leaky. The leak may be sufficient to cause slow lysis of metabolically inert targets like aged erythrocytes but actively metabolizing cells are resistant by virtue of their ion pumps (reviewed in Morgan, 1990).

C9 consists of a single polypeptide chain. Binding of C9 to the α-chain of C8 initiates a major conformational change in C9 allowing it to insert deeply into the membrane (Stewart and Sodetz, 1985). Once the first molecule of C9 has bound to C5b-8, a second molecule binds and undergoes the same conformational change. This process continues until a complete cylinder of poly-C9 (containing between 12 and 18 molecules) has been formed (Stanley, 1988) (Figure 1.5). This cylinder traverses the cell membrane and has an everted lip at its outer aspect. In anuclear cells, the formation of transmembrane channels occurs through the centre of the cylinder and the passage of water and electrolytes ensures that cell

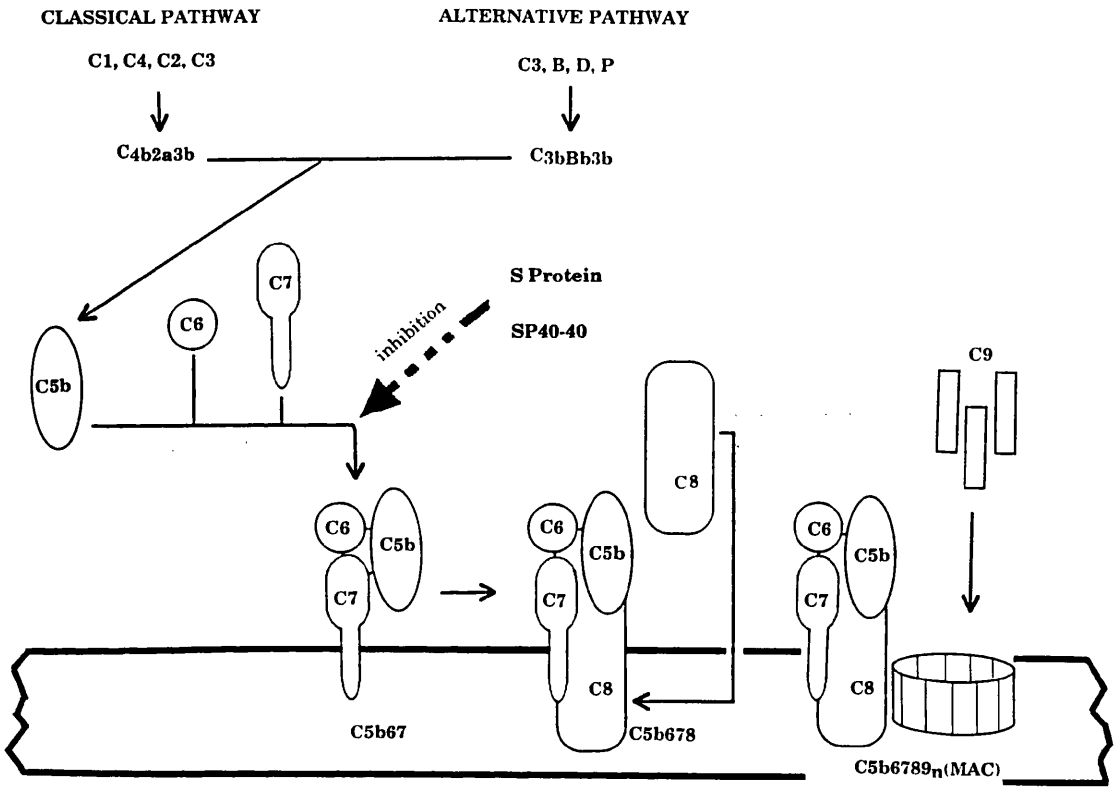


Figure 1.4: Generation of membrane attack complex. The major cleavage product of classical and alternative pathway C5 convertases (C4b2aC3b and C3bBbC3b respectively) is C5b. Binding of C5b to C6 than C7 exposes a hydrophobic membrane binding site on C5b67 complex. The subsequent binding of C8 and C9 leads to formation of transmembrane channels. If the C5b67 does not encounter a membrane rapidly, it interacts with inhibitory proteins S protein and SP-40,40 in the fluid phase so that membrane insertion cannot occur (Adapted from Roitt et al., 1990).

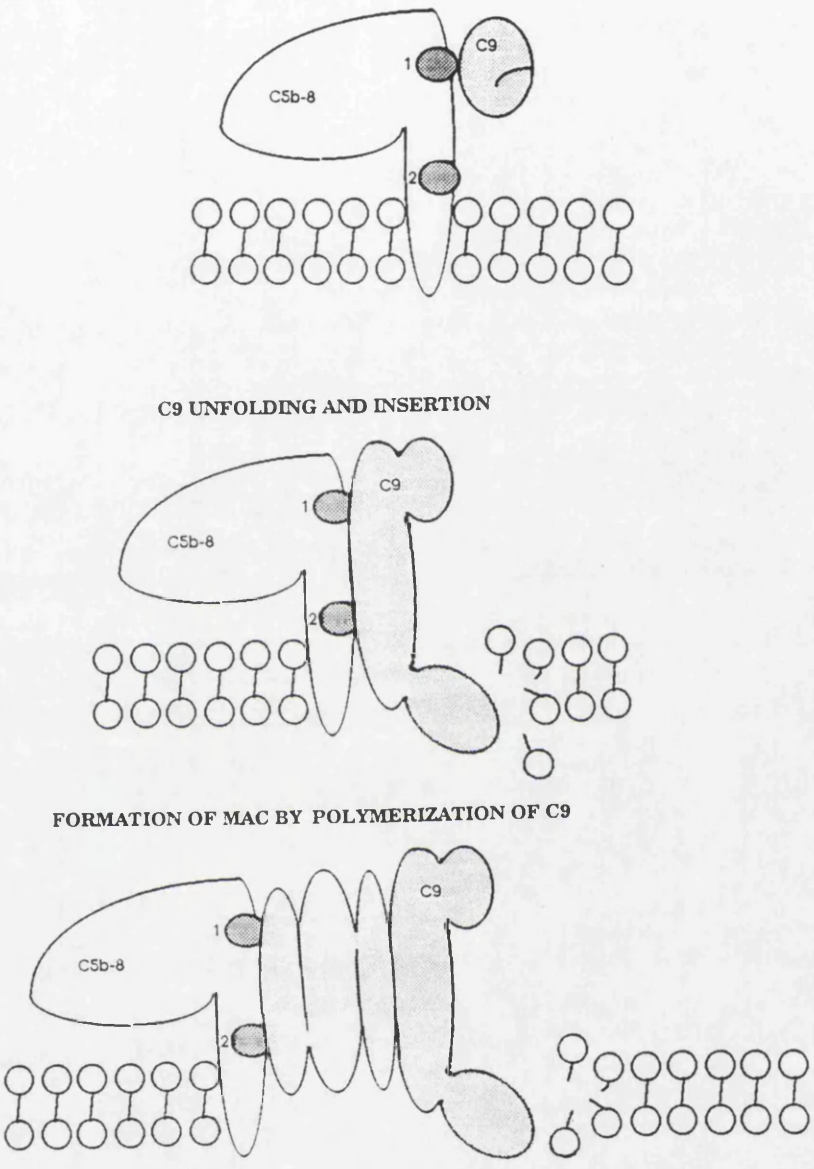


Figure 1.5: Polymerization of C9. Binding of C9 to the α-chain of C8 initiates a major conformational change in C9 allowing it to insert deeply into the membrane. Once the first molecule of C9 has bound to C5b-8, a second molecule binds and this process continues until a complete cylinder of poly C9 has been formed (Adapted from Meri et al., 1990)

death occurs by osmotic lysis. In nucleated cells the mechanism of lysis is less well understood. Multiple molecules of poly-C9 damage the membrane directly, but in addition influx of Ca^{2+} and ATP depletion occur, suggesting that cell death may be a metabolically active process (Morgan, 1989).

1.4. THE BIOLOGICAL ACTIVITIES OF COMPLEMENT SYSTEM

1.4.1. Cytolysis

Membrane attack complex polymerized C9 forms cylindrical structures. The insertion of large number of membrane attack complexes into cell membranes disrupts the membrane and eventually uncontrolled electrolytes and water flux results in osmotic swelling and subsequent lysis.

1.4.2. Increased Vascular Permeability

The anaphylatoxins C4a, C3a and C5a increase vascular permeability, C5a being the most potent and C4a the least. C3a and C5a act on mast cells to release histamine (Johnson et al., 1975). However, as C5a induces smooth muscle contraction independently of histamine release (Regal et al., 1980), it is possible that it also has a direct effect on the endothelial cells of the microcirculation. Additionally, the contraction of the endothelial cells opens up the intercellular gaps which increases plasma exudation.

1.4.3. Neutrophil Adhesiveness

In order to emigrate through blood vessel walls to the sites of inflammation, neutrophils must first adhere to the vascular endothelium. It has been shown that C5a and desarginyl dervative of C5a, $C5a_{des\ Arg}$, act on neutrophils and promote their adherence to endothelial cells. It is possible that the diffusion of C5a and or $C5a_{des\ Arg}$ products through the walls of the microcirculation during vascular stasis results in the aggregation of neutrophils and their adherence to the vascular endothelium which is a prerequisite for their emigration (Tonnesen et al., 1984)

1.4.4. Chemotaxis

The loss of C-terminal residue of C5 following cleavage by carboxypeptidase B produces $C5a_{des\ Arg}$ which posess relative stability and it can diffuse long distances from the inflammatory site recruiting inflammatory cells from a wide area. Thus C5a and $C5a_{des\ Arg}$ are potent chemotactic agents which attract neutrophils, eosinophils and monocytes at site of inflammation (Damerau et al., 1978; Fernandez et al., 1978).

1.4.5. Leucocytosis

A peptide derived from the α chain of C3 causes an initial leucopenia followed by leucocytosis. This peptide, termed C3e, appears to be produced as a result of proteolytic cleavage of C3c by an unidentified plasma protease (Ghebrehiwet and Muller-Eberhard, 1979). Another fragment of C3 (C3d-K) which is produced by the proteolysis of iC3b by

kallikrein also releases leucocytes from the bone marrow and inhibits T-cell proliferative responses. Although it is almost identical to C3dg, its N-terminal sequence of nine amino acids are not a part of the C3dg molecule. The C3e fragment probably contains the same sequence, which explains why they are both able to induce leucocytosis (Hoeprich et al., 1985).

1.4.6. Phagocytosis

Microorganisms or antigen-antibody complexes coated with C3b are able to bind to the cells which possess receptors for this ligand. The C3b receptors on phagocytic cells promote phagocytosis and killing of microorganisms and the internalization and the degradation of immune complexes (Gigli and Nelson, 1968). CR1 is a receptor which is present on many blood cells and binds C3b. Erythrocyte CR1 may be involved in the removal of complement-coated antigen-antibody complexes from the circulation.

1.4.7. Lymphocyte Function

The presence of complement receptors on lymphocytes suggests that C3 cleavage products might influence immune responses. Studies on mice which had been complement depleted by the administration of cobra venom factor revealed that thymus-dependent antibody responses and the generation of B memory cells were impaired (Pepys, 1974; Klaus and Humphrey, 1976).

1.5. THE REGULATION OF THE COMPLEMENT SYSTEM

The control of complement activation is achieved by a combination of regulatory proteins and the spontaneous decay of active factors.

Regulatory proteins may act in the fluid-phase (Table 1.5) to prevent spontaneous or abortive activation; or on cell membranes (Table 1.6) to protect host cells from the destructive action of complement. Some of the membrane proteins with regulatory activities may also act as receptors for complement activation products enabling the cell to respond to complement-coated particles (CR1) (Table 1.6).

Within the last decade, the amino acid sequences of the majority of the complement components and control proteins have been determined. This has provided new insights into the way the proteins interact with one another and, moreover, has revealed that homology exists within the system, which enables the components to be categorized according to their functional and structural similarities. In addition, the chromosomal localization of many of the genes has shown that genes encoding proteins with similar functions occur in groups. For example, pulse-field gel electrophoresis has recently revealed that MCP, CR1, CR2, and DAF as well as α and β subunits of C4-bp are encoded by a 900 kb segment in order of MCP-CR1-CR2-DAF-C4-bp α-C4-bp β while the factor H gene is located at a distance of more than 500 kb from this group (Rey-Campos et al., 1988; Carrol et al., 1988; Bora et al., 1989; Pardo-Manuel et al., 1990). All of the members of this group are called "Regulators of Complement Activation" (RCA). They share short consensus repeats(SCRs) of 60 amino acids, characterized by a framework of four very highly-conserved cysteines and several other residues (reviewed by Klickstein et al., 1987). The number of the SCRs in each component varies: 4 for DAF and MCP, 8 for C4-bp, 16 for CR2, 20 for factor H and 30 for CR1 (Figure 1.6).

Table 1.5. FLUID PHASE REGULATORY PROTEINS

Proteins	Serum Concentration (mg/L)	Mr (kDa)	Subunit Structure	Chromosomal Assignment
C1-INH	200	105	Single chain	11q11-13.1
C4-bp	250	550	7 identical subunits (70kDa) 1 β subunit (45kDa)	1q32
Factor I	50	90	1α chain (50 kDa) 1β chain (40 kDa)	4q25
Factor H	300	150	Single chain	1q28
S Protein	150	80	Single chain	17q11.1
SP40-40	65	80	Two subunits (40 kDa)	8

Table 1.6. MEMBRANE REGULATORY PROTEINS

Proteins	Ligand	Mr (kDa)	Subunit Structure	Chromosomal Assigment
DAF	Clasical and alternative pathway C3, C5 convertases	75	Single chain	1q32
MCP	C3b/C4b	45-70	Single chain	1q32
CD59/HRF20	C5b-8	18	Single chain	11
MIP/HRF/C8BP	C5b-8	65	Single chain	?
CR1	C3b, C4b, iC3b	160, 190 220, 250	Single chain	1q32
CR2	iC3b, C3d, C3dg	140	Single chain	1q32

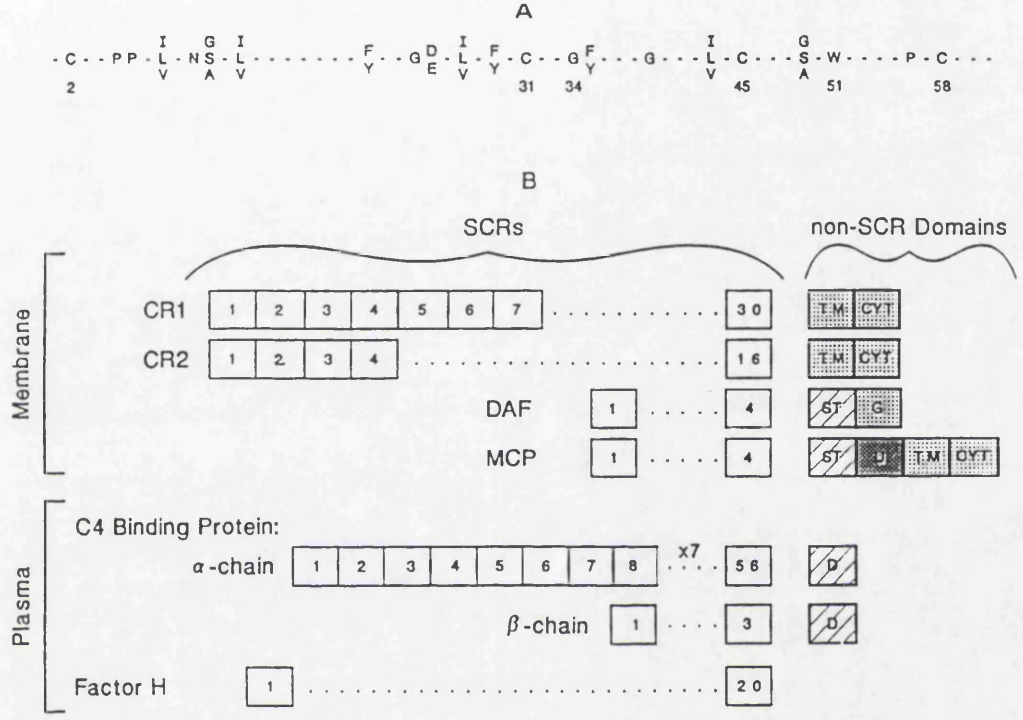


Figure 1.6: Regulators of complement activation. All the members of this group share short consensus repeat (SCRs) of 60 amino acids characterized by a frame-work of four very highly conserved cystein and proline, glycin, phenylalanine and tryptophan residues. The number of the SCRs in each component varies: 4 for decay accelerating factor (DAF) and membrane cofactor protein (MCP), 8 for C4 binding protein (C4-bp), 16 for CR2, 20 for factor H and 30 for CR1. TM, transmembrane domain; CYT, cytoplasmic domain; ST, serine/threonine-enriched area; G, glycolipid anchor; U, domain of unknown functional significance (Adapted from Hourcade et al., 1989).

Although many of the members of the RCA group share the function of binding to C3, the SCRs are not directly homologous with this function. They are also found in other complement proteins, including C2 and B (three each) and C1r and C1s (two each), as well as in β-2 glycoprotein I, a protein of unknown function, the IL-2 receptor, the leucocyte common antigen and factor XIII of the clotting system.

The functional, biochemical and genetic properties of the regulatory fluid-phase and the regulatory membrane proteins are discussed below (sections 1.5.1 and 1.5.2.).

1.5.1. Functional and Genetic Features of the Fluid-phase Regulatory Proteins

1.5.1.1. *C1-inhibitor*

C1-INH is a member of the SERPIN superfamily of serine protease inhibitors (Table 1.5). It is able to inactivate several plasma serine proteases, including C1r and C1s. C1-INH also inhibits certain enzymes in the coagulation, kallikrein and plasmin systems (Levy and Lepow, 1959; Ratnoff et al., 1969).

C1-INH binds covalently to the enzymatic site of C1r and C1s. The resultant C1r:C1s:C1-INH complex (ratio 1:1:2) dissociates from the C1 molecule, leaving C1q attached to the immune complex. This restricts the half-life of activated C1 to only about 20 seconds at physiological concentrations. C1-INH also prevents spontaneous activation of fluid-phase C1 probably by combining loosely with the molecule in plasma (Ziccardi, 1985).

Genomic clones for C1-INH have been isolated and characterized. The gene is 17 kb long with eight exons and seven introns and localized on chromosome 11 (Davis et al., 1986; Carter et al., 1988).

Northern blot analyses of mRNA demonstrate a major 2.1 kb band (Bock et al., 1986).

1.5.1.2. *C4-binding protein*

C4-bp is a glycoprotein (Table 1.5) composed of seven identical disulphide-linked 70 kD subunits (α chains) (Dahlback and Stenflo, 1981; Gigli et al., 1979). Each 70 kD unit of C4-bp which contains 48 kD chymotryptic fragment possesses the C4b binding and factor I cofactor activity (Nagasava et al., 1982; Fujita et al., 1985). C4-bp also contains a unique 45 kD β chain. It is disulphide-linked to the central core and contains a binding site for the vitamin K-dependent protein S (Hillarp and Dahlbach, 1988)

C4-bp regulates the classical pathway by acting as a cofactor to factor I in the degradation of C4b and by accelerating the decay of the C3 convertase C4b2a (Fujita et al., 1978; Gigli et al., 1979). The major physiological role of C4-bp and factor H appears to be the inhibition of C3 and C5 convertases in the fluid-phase, although these proteins undoubtedly contribute to the inhibition of C3b and C4b which are attached to the cell surfaces (Cooper, 1975).

The cDNA sequence studies demonstrated that the α chain of C4-bp contains 549 amino acid residues (Chung et al., 1985). The N-terminal 491 amino acids of C4-bp can be divided at into eight internal homologous regions each approximately 60 amino acids long (SCRs) (Figure 1.6). Mouse C4-bp is 51% homologous to human counterpart although it lacks the equivalents of the human SCRs 5 and 6. Since the mouse protein

binds to human C4b as well as to mouse C4b and as it is an active cofactor in the cleavage of human C4-bp mediated by mouse I (Hourcade et al., 1989), it is unlikely that human SCR 5 and 6 are required to the expression of these activities.

Northern blot analysis indicated that the mRNA for C4-bp is approximately 2.5 kb long (Chung et al., 1985).

Recently Hillarp and Dahlback (1990) isolated and characterized full length cDNA clones for the β chain. The β chain contains five potential N-linked glycosylation sites. Northern blot analysis of human liver mRNA using β chain cDNA as a probe demonstrates a major mRNA species of approximately 1.0 kb (Hillarp and Dahlback, 1990). From the N-terminus, the β chain contains three tandem repeat units (60 amino acids long) which are homologous to those present in the α chain. The C-terminal region which is unrelated to the tandem repeats demonstrates sequence similarity with the corresponding region of the α chain. In both α and β chains these regions contain two cysteine residues which probably form the interchain disulphide bridges (Hillarp and Dahlback, 1990).

1.5.1.3. *Factor H*

Factor H (formerly called β1H) is a single-chain plasma and membrane-bound glycoprotein (Whaley and Ruddy, 1976) (Table 1.5). On limited digestion with trypsin, H is cleaved in a specific manner to give 35 kDa (N-terminal) and 120 kDa (C-terminal) fragments (Alsenz et al., 1984). The N-terminal 35 kDa fragment shows co-factor activity in the factor I-mediated conversion of C3b to iC3b while the remainder of the molecule is inactive in this respect (Alsenz et al., 1984). Factor H binds to C3b and prevents the binding of B and C5. As the affinity for C3b is

higher than that of B, the formation of the alternative pathway C3 and C5 convertases is restricted (Figure 1.3). In addition, H will bind to C3b which is present in the C3 and C5 convertases of the alternative pathway and accelerate their decay by displacing Bb (Whaley and Ruddy, 1976).

The signal sequence of human factor H is similar to that of mouse factor H (Kristensen and Tack, 1986), having 11 out of 18 identities and three conservative replacements. The factor H amino acid sequence is arranged in 20 homologous repeating units based on a frame work of four invariant cysteine residues and highly-conserved acidic proline, asparagine, glycine, tyrosine, phenylalanine and tryptophan residues (Ripoche et al., 1988).

Three different species of human factor H mRNA (4.3 kb; 1.8 kb; 1.4 kb) are expressed abundantly in the liver. The 4.3 kb mRNA which encodes the major factor H serum protein of 150 kDa was sequenced completely by Ripoche et al. (1988). The sequence of 1.8 kb mRNA shows that this species is largely identical to the 5' portion of the 4.3 kb mRNA and it appears that these two mRNAs arise by alternative splicing from a single gene (Schwaeble et al., 1991). Schwaeble et al. (1991) have also shown that the 1.4 kb factor H mRNA is the product of a distinct gene.

1.5.1.4. *Factor I*

Factor I is a serine proteinase, although it is not inhibited by di-isopropylfluorophosphonate, a general inhibitor of this class of enzyme (Table 1.5). Unlike the majority of plasma serine proteinases, factor I does not appear to have a circulating proenzyme form.

Human factor I is responsible for the cleavage of the α chains of C4b and C3b in the presence of the cofactors such as H, C4-bp, CR1, CR2 and MCP.

The α chain of C4b is cleaved by factor I at two sites resulting in the formation of C4d, an α chain fragment which is covalently linked to the activating surface because it contains the thiolester and C4c, the fluid-phase fragment of α chain, and the intact β and γ chains (Pangburn et al., 1977). The first two cleavages of C3b by factor I liberates a 2 kDa C3f fragment and yields iC3b (Figure 1.7). Further cleavage of iC3b by factor I occurs, releasing a large fragment C3c, while a smaller piece, C3dg remains attached to the membrane (Lachmann et al., 1982).

Northern blot analysis of human liver mRNA probed with factor I cDNA indicated that the mRNA is about 2.4 kb (Catteral et al., 1987).

1.5.1.5. *S Protein*

S Protein (vitronectin) (Table 1.5) is a plasma protein functioning as a regulator of the membrane attack complex (Podack et al., 1978).

Vitronectin binds to the exposed hydrophobic region of the newly formed C5b-7 complex to form the water-soluble SC5b-7 complex (Figure 1.4). The SC5b-9 complex is incapable of membrane insertion and is therefore not cytolytic. C9 polymerization does not occur in SC5b-9 (Podack et al., 1978; 1984; Podack and Muller-Eberhard, 1979; Bhakdi and Tranum-Jensen, 1982).

Recent studies strongly suggested that ionic interactions between the negatively charged "class A" cysteine-rich domain of terminal complement proteins and the heparin-binding region of S protein trigger the binding of S protein to nascent terminal complement complexes (Tschopp et al., 1988).

S protein may also have a regulatory role in the coagulation system (Podack and Muller-Eberhard, 1979). S Protein can be observed in a

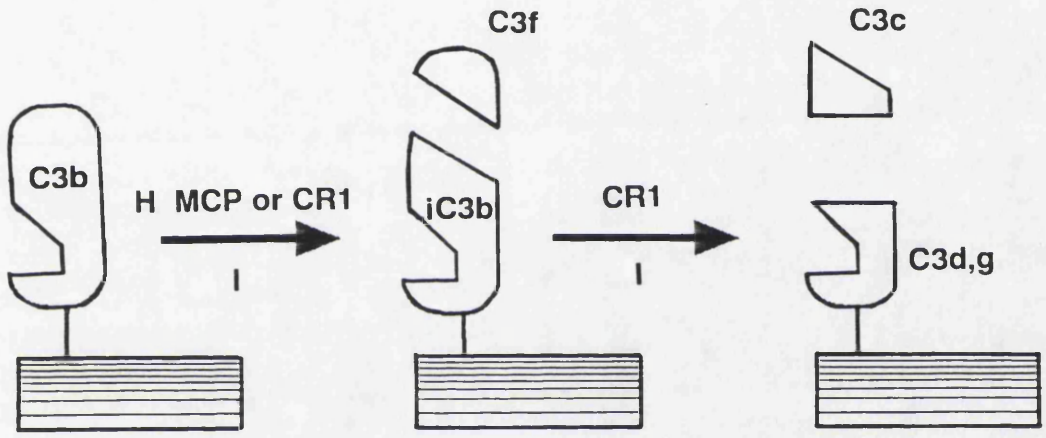


Figure 1.7: Degradation of C3b by factor I. Factor H, membrane cofactor protein (MCP) and complement receptor 1 (CR1) are cofactors for factor I mediated cleavage of C3b to iC3b. CR1 involves further cleavage of iC3b to C3d,g and C3c (Adapted from Roitt et al., 1990).

complex with thrombin in serum after coagulation but not in plasma. This complex contains antithrombin III (Jenne et al., 1985).

S protein and the serum-spreading factor, vitronectin, are identical. The human vitronectin gene (4.5-5 kb) consists of eight exons and seven introns (reviewed in Preissner, 1991). The S protein amino acid sequence can be divided into five regions with different functional significance (Suzuki et al., 1985) as follows.

(1) The first 44 residues of S protein (somatomedin B) contains four disulphide bonds and have the appearance of an independently-folding cysteine-rich domain. No homology was observed between this region and high cysteine region of complement component C9.

(2) Adjacent to the somatomedin B domain, there is a three amino acid (Arg-Gly-Asp) motif which is shared by several molecules exibiting cell attachment activity (Hayman et al., 1985).

(3) This is followed by a proline-rich region (residues 48-347) which contains three possible sites for attachment of N-linked oligosaccarides, but shows no amino acid homology with collagen or fibronectin.

(4) The following 32 residues of the molecule contain 14 positive charges, no negative charges and only two hydrophobic residues. This region has been identified as a heparin-binding site (Suzuki et al., 1984). This site could also be involved in the binding of S protein to the terminal components of complement pathway since it has been observed that polylysine can block the inhibition of C5b-7 by serum factors.

(5) Following the heparin-binding region is the C-terminal region which is found to be partially cleaved in plasma and which is cleaved rapidly when purified S protein is treated with trypsin (Podack and Muller-Eberhard, 1979). Endogenous proteolytic cleavage of this carboxy-terminal fragment accounts for the two molecular weight forms of both S protein and vitronectin.

Vitronectin gene has been mapped to the centromeric region of the long arm of chromosome 17 at 17q11 (Fink et al., 1992).

1.5.1.6. *SP-40,40*

SP-40,40 (clusterin) is a heterodimeric structure (Table 1.5). It has two chains which are of a similar size (40 kD). The amino terminal sequences of the chains are unrelated to one another (Murphy et al., 1988).

SP-40,40 acts as a control protein of the membrane attack complex of complement. Recent studies have shown that SP-40,40 potentially inhibits C5b-6 initiated lysis, by preventing the hydrophilic-amphilic transition of C5b-C7 complex during the assembly of the lytic pore C5b-9 complex (Kirszbaum et al., 1992) (Figure 1.4).

The cDNA sequence encoding the SP-40,40 was reported by Kirszbaum et al. (1989). The two chains of SP-40,40 are coded in a single open reading frame on the same mRNA molecule indicating the existence of a biosynthetic precursor protein which matures postsynthetically by proteolysis (Kirszbaum et al., 1990). The coding order of SP-40,40 chains upon the mRNA is β, α. The precursor contains six N-linked glycosylation sites which are distributed equally between the two chains. The sequence of the SP40,40 bears a 77% identity to a rat sulphated glycoprotein-2 (SGP-2, clusterin) which is the major secreted product of Sertoli cells (Jenne and Tschopp, 1989). SP-40,40 and SGP-2 are the serum and seminal forms of the same protein. A sequence of 23 amino acids within the β chain of SP-40,40 exhibits significant homology to corresponding segments located within complement components C7, C8 and C9. This short, cysteine-containing motif represents the only evidence of a possible

vestigial relationship between SP-40,40 and other complement components.

Northern blot analysis showed that a single species of mRNA (approximately 2 kb) is normally present in various tissues (Collard and Griswold, 1987; Bettuzi et al., 1989).

1.5.2. Functional and Genetic Features of the Membrane Regulatory Proteins

1.5.2.1. *Decay Accelerating Factor (DAF)*

DAF is a protein (Table 1.6) anchored in the cell membrane by a covalently-linked glycolipid (Medof et al., 1987; Davitz et al., 1986) rather than by a transmembrane anchor. This non-amino acid structure consists of a phospholipid-containing fatty acid, glycerol and inositol linked to an oligosaccaride-containing non-acetylated glucosamine and ethanolamine (Medof et al., 1987). The presence of glycophospholipid anchor in DAF increases its mobility. This increased mobility could enhance the ability of a limited number of DAF molecules to contact a large number of C3b and C4b fragments on the cell surface (Thomas et al., 1987). Other possible roles for the glycophospholipid anchor include serving as a means to release the protein from the cell membrane and transducing an intercellular signal (Lublin and Atkinson, 1989). Although DAF is present in plasma and in other body fluids, it is not known whether it arises from the membrane by endogenous phospholipases.

The initial purification of DAF was based on its ability to accelerate the spontaneous decay of preformed classical C3 convertase C4b2a (Nicolson-Weller et al., 1982). It has also been shown that DAF prevents the assembly of C3 and C5 convertases of the classical and

alternative pathways (Figure 1.8). But Fujita et al. (1987) showed that DAF does not prevent the initial binding of C2 or B to the cells containing C4b or C3b respectively, but rather, it rapidly dissociates C2a or Bb from their binding sites, thus preventing the assembly of the C3 convertase. It has no effect on the structures of C3b and C4b (Medof et al., 1985) and thus its effect is reversible. DAF lacks cofactor activity for factor I-mediated cleavage of C3b and C4b (Pangburn et al., 1983).

DAF cDNA has been fully sequenced and the data show that DAF mRNA encodes a protein of 347 amino acids preceded by a hydrophobic rich stretch of amino acids characteristic of an NH_2 terminal leader sequence. It has a single N-glycosylation site at position 61 and multiple potential O-glycosylation sites in the serine-threonine-rich region. There are four contiguous SCR repeats of internal homology of approximately 61 amino acids (Figure 1.6). DAF SCRs 1, 2 and 4 are encoded by a single exon, while SCR 3 is encoded by two exons (Rodrigez de Cordoba et al., 1985).

DAF cDNA hybridises to two major mRNA species of ~2.7 kb and ~2 kb and a minor mRNA species of ~3.1 kb.

Recently the DAF gene promoter region has been characterized (Ewulonu et al., 1991). The DAF gene promoter possesses a region with the negative regulatory activity and responds to PMA and cAMP induction presumably PRE and CRE like elements (Ewulonu et al., 1991). The physiological function of the negative regulatory sequence in DAF's promoter could maintain the markedly divergent levels of DAF expression found in different cell types (Cheung et al., 1988).

The 35 kb long DAF gene is flanked upstream by the genes for complement receptors CR1 and CR2 and downstream by the genes for C4-bp other C3/C4 regulatory proteins (Lublin et al., 1987).

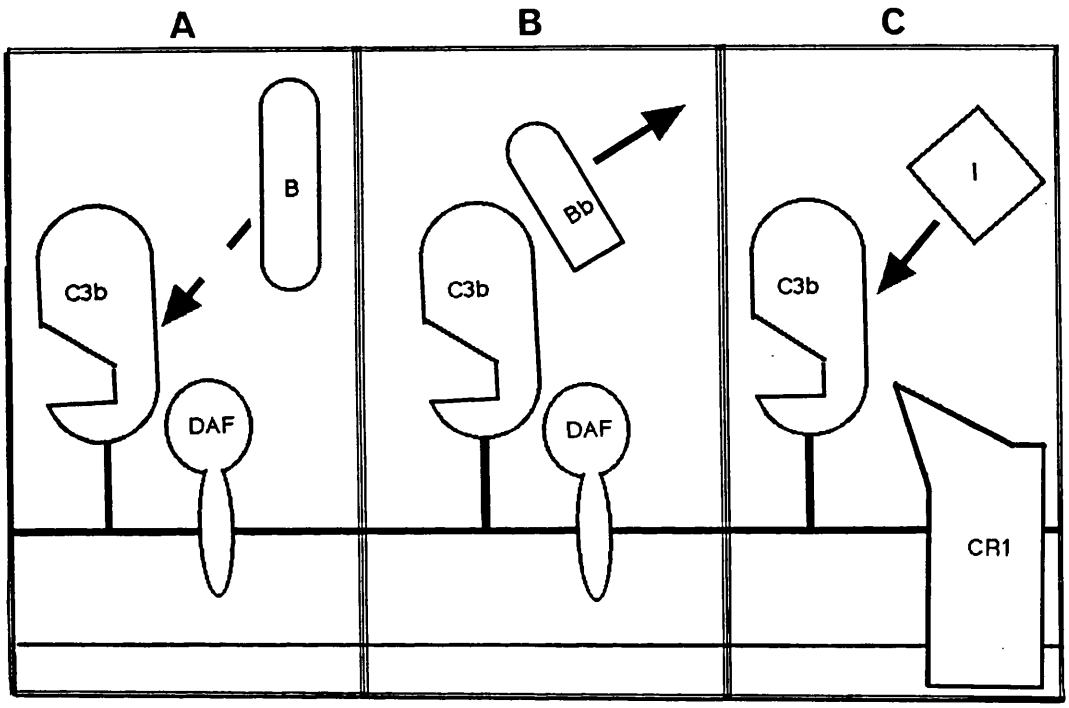


Figure 1.8: Regulation of C3 convertases. Decay acceleration factor (DAF) and CR1 inhibit the association between C3b and B and promote dissociation of the C3bBb complex. CR1 also promotes the factor I mediated cleavage of C3b (Adapted from Roitt et al., 1990).

1.5.2.2. *Membrane cofactor protein (MCP)*

MCP was first identified as an iC3/C3b-binding membrane protein (Cole et al., 1985) (Table 1.6). It was subsequently purified and shown to be a potent cofactor for factor I mediated cleavage of fluid-phase C3b (Seya et al., 1986). This protein does not accelerate the decay of the C3 convertases, therefore its functional profile is complementary to that of DAF. Like DAF, MCP is widely distributed and it is found on epithelial, endothelial cells, fibroblasts (McNearny et al., 1989) and all peripheral blood cells except for erythrocytes (Seya et al., 1988). Moreover, the structures of MCP and DAF are similar, consisting of four short consensus repeat (SCRs), altough MCP is a transmembrane protein wheras DAF possesses a glycolipid anchor (Figure 1.6) (Lublin et al., 1988).

The cDNA sequence encoding the MCP has been reported by Lublin et al. (1988). The 1.5 kb cDNA encodes a protein of 384 amino acids with three sites for N-linked glycosylation. Following the SCR domains there is a segment of 29 amino acids enriched in serine, threonine and proline residues which are likely to be a site of heavy O-linked glycosylation.

On Northern blot analysis, the cDNA for MCP detects a major and a minor mRNA of 4.2 and 4.8 kb (Lublin et al., 1988). Analysis of alternative splicing sites of MCP mRNA demonstrates the existence of 14 transcripts generated by the alternative splicing of five exons (Figure 1.9). Splicing of one exon is controlled by sequences within or near the MCP gene and is thus genetically determined. Splicing of other exons is regulated by tissue-specific factors or activation events (Russell et al., 1992).

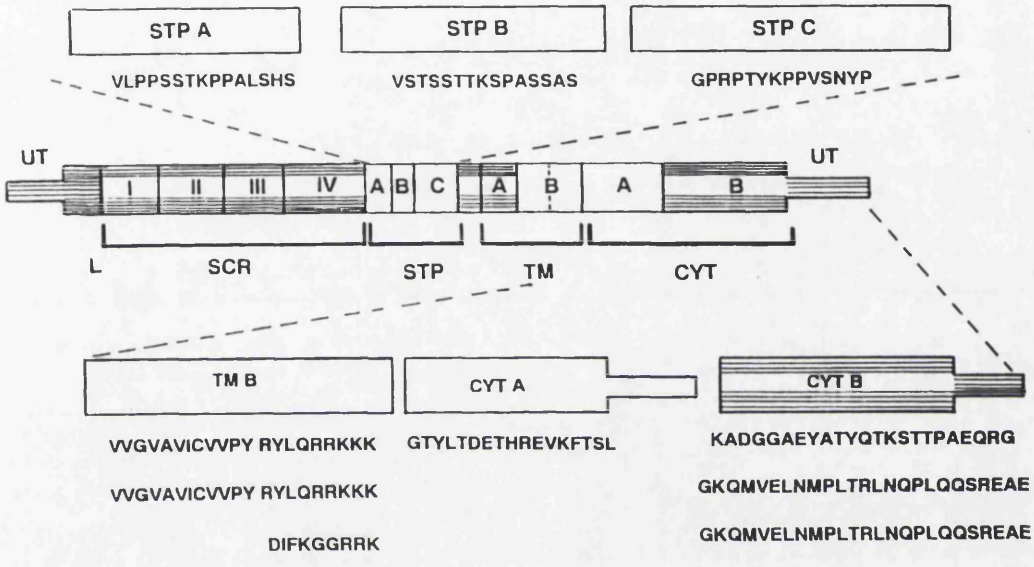


Figure 1.9: Exon structure of membrane cofactor protein. White boxes represent exons that undergo alternative splicing. Letters under enlarged boxes indicate amino acid sequence of isoforms from alternatively spliced transcripts; spaces in amino acid sequences indicate deletion of exons (Adapted from Russel et al., 1992).

A yeast artificial chromosome (YAC) clone identified an MCP-like genetic element which is 93% and 84% homologous to MCP, at the nucleotide and amino acid levels, respectively. It is not known if this homologue is transcribed or is a pseudogene (Liszewski et al., 1991).

1.5.2.3. *CD59*

CD59 [also known as protectin, membrane inhibitor of reactive lysis (MIRL) or homologous restriction factor 20 (HRF20)] is a membrane protein (Table 1.6) (Zalman et al., 1986). CD59 is a powerful inhibitor of complement cytolysis and exerts its activity by limiting the polymerisation of C9 in membrane C5b-9 complex (Meri et al., 1990) (Figure 1.10).

Phosphatidylinositol-specific phospholipase C digestion of the protein abolished 50% of its C5b-9 inhibitory activity and removed approximately 15% of the protein from the human erythrocytes (Rollins and Sims, 1990). This suggests that CD59 is linked to the cell surface by means of a glycolipid anchor. Thus CD59 antigen is absent from the surface of the affected erythrocytes of patients suffering from paroxysmal nocturnal haemoglobinuria (PNH) as such cells lack proteins that use a glycolipid anchor like DAF and HRF (Okada et al., 1987; 1989).

The cDNA sequence of CD59 is 1154 bp, and encodes a precursor polypeptide of 128 amino acids. The sequence shows that CD59 does not resemble any other complement component or regulatory protein, but shows 26% amino acid identity with that of murine LY-6 antigen (Davies et al., 1989).

Northern blot analysis revealed four species of CD59 mRNA, approximately 0.6 kb, 1.2 kb, 1.9 kb and 2.2 kb, which are present at varying amounts in different cells (Davies et al., 1989).

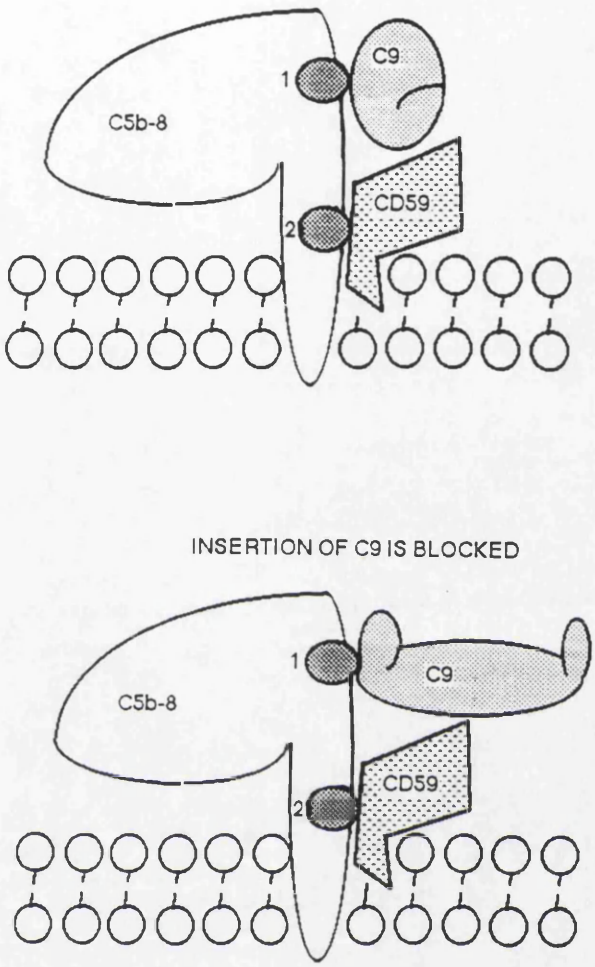


Figure 1.10: Inhibition of C9 polymerization by CD59. In the presence of CD59 the initial binding of C9 to C5b-8 occurs but the subsequent insertion of C9 is prevented (Adapted from Meri et al., 1990)

1.5.2.4. *Homologous restriction factor (HRF)*

HRF [also known as C8-binding protein (C8-bp) or Membrane Inhibitory Protein (MIP)] is a membrane protein which is highly effective in inhibiting complement-mediated cytolysis (Table 1.6). Like CD59, it recognizes the C5b-8 membrane attack complex, and prevents C9 polymerization (Zalman et al., 1986; Schonermark et al., 1986). HRF possesses a glycophosphatidylinositol anchor and paroxysmal nocturnal haemoglobinuria (PNH) erythrocytes are lacking in this protein (Zalman et al., 1987). Amino acid and cDNA sequences of MIP are not available.

1.5.2.5. *Complement receptor 1 (CR1)*

Human CR1 is a glycoprotein that exibits genetically regulated structural and quantitative polymorphism (Table 1.6).

CR1 is mainly expressed on cells in the blood, whereas DAF and MCP are more widely distributed. Like DAF, CR1 accelerates the decay of the classical and alternative pathway C3 and C5 convertases. Moreover, like MCP, CR1 act as a co-factor for factor I to mediate inactivation and degradation of C3b and C4b (Figure 1.6). Thus CR1 possesses the properties of both DAF and MCP (Ahearn and Fearon, 1989). CR1 on erythrocytes can bind immune complexes bearing C3b/iC3b and may be important in the clearance of such complexes from the blood (Wong and Fearon, 1985).

Wong et al. (1985) identified a cDNA for CR1. This cDNA hybridized with mRNA species of 9 and 11 kb.

1.5.2.6. *Complement receptor 2 (CR2)*

CR2 is a complement protein that serves as a receptor (Table 1.6) for C3dg (Ross et al., 1982) and for the Ebstein-Barr virus (Fingeroth et al., 1984; Nemerow et al., 1985). CR2 is a cofactor for factor I in the conversion of C3b to iC3b (Mitomo et al., 1987).

Human lymphocyte CR2 is known to act as an alternative pathway activator on which homologous C3 is deposited. This CR2 activity can not be suppressed by the DAF and MCP on the cell membrane.

1.6. RECEPTORS OF THE COMPLEMENT SYSTEM

Complement receptors and their ligands, in addition to CR1 and CR2, are summarized in Table 1.7.

1.7. BIOSYNTHESIS OF COMPLEMENT PROTEINS

1.7.1. In Liver

In 1911, Muller (reviewed by Colten, 1976) found that perfusion of liver with serum led to an increase in complement activity in the effluent, and concluded that complement synthesis occured in the liver. Alper and coworkers (1969) also confirmed this observation and showed that the liver was the major site of serum complement synthesis. The liver is the main site of synthesis of almost all of the serum complement proteins except C1q, D and properdin and hepatocytes are the main cell type

Table 1.7. COMPLEMENT RECEPTORS

Proteins	Mr (kDa)	Subunit Structure	Specificity	Cell Distribution
CR3 (iC3bR)	260	α chain (165) β chain (95)	iC3b, C3dg C3d	Monocyte/macrophage neutrophils, T cells NK cell
CR4 (p150/95)	245	α chain (150) β chain (95)	iC3b, C3dg C3d	Granulocytes,T cells B cells, mast cells
C1qR	65		C1q	B lymphocytes neutrophils monocytes
C3a/C4aR	?		C3a/C4a	Mast cells, T cells granulocytes
C5aR	50		C5a	Mast cells, granulocytes Monocyte/macrophage
C3eR	?		C3e,C3dk	Monocytes, Neutrophils

responsible. Kupffer cells are probably a rich source of C4 and C2. Table 1.8 shows the complement proteins secreted by the liver.

1.7.2. By Extrahepatic Sources

Complement proteins of the classical, alternative and membrane attack pathways are synthesized in several cell types in various tissues (Table 1.8).

Mononuclear phagocytes are widely distributed in tissues and their circulating precursors, monocytes, are able to be recruited rapidly into inflamed tissue. Endothelial cells are present throughout the body since they line the blood vessels. Fibroblasts are widespread in the connective tissues. In culture *in vitro* all three cell types synthesize complement proteins under basal conditions. Other cells which synthesize complement include astrocytes (Gasque et al., 1992), alveolar type II epithelial cells (Strunk et al., 1988) and intestinal epithelial cells (Colten, 1976).

Stimulation of these cells by mediators of inflammation (*i.e.* cytokines) changes the pattern of the secretion rates of the complement proteins. For example, IL-1, IL-6 and TNF all stimulate alternative pathway activating components (Katz & Strunk, 1989b); IFNγ increases synthesis of C1-INH, factor H, C2, factor B and C4-bp by monocytes, endothelial cells and skin fibroblasts (Lappin et al., 1992).

1.7.2.1. *Mononuclear phagocytes*

Monocytes mature to macrophages once they leave the blood stream. Together with the neutrophils, macrophages are the main phagocytes in the mammalian organism. Mononuclear phagocytes are the single richest source of complement proteins with synthesis of all the

Table 1.8. BIOSYNTHESIS OF COMPLEMENT COMPONENTS

Cell types	Classical Pathway Proteins	Alternative Pathway Proteins	Terminal Sequence	Secretory Regulatory Proteins	Membrane Regulatory Proteins
Liver (hepatocyte)	C1r, C1s, C4, C2, C3	C3, B,	C5, C6, C7, C8, C9	C4bp, C1-INH, H, I, SP, SP40-40	
Monocyte/ Macrophage	C1q, C1s, C4, C2, C3	C3, B, P, D	C5, C6, C7 C8, C9	C4bp, C1-INH, H SP40-40	DAF, MCP, CR1
Fibroblast	C1r,C1s, C4 C2, C3	C3, B, D	C5, C6, C7 C8, C9	C1-INH, H, I, SP, SP40-40	DAF, MCP CD59
Endothelial	C1q, C3	C3, B		C1-INH, H, I SP, SP40-40	DAF, MCP CD59
Epithelial	C1q, C1r, C1s, C3	C3, B		SP40-40	DAF, MCP
Alveolar type epithelial	C4, C2, C3	C3, B	C5		

activator and inhibitor proteins in both activation pathways (Littman and Ruddy, 1977; Whaley, 1980; Hetland et al., 1986; Lappin et al., 1990). Complement proteins synthesized by mononuclear phagocytes are listed in Table 1.8.

1.7.2.2. *Fibroblasts*

Tissues are not solely composed of cells. A substantial amount of their volume is the extracellular space, which is largely filled by an intricate network of macromolecules constituting the extracellular matrix. In most connective tissues, these macromolecules such as collagen, elastin, fibronectin and laminin are secreted by fibroblasts. Recent studies demonstrated that fibroblasts also synthesize some complement proteins and cytokines. Thus, they actively contribute to local inflammatory response. The pattern of complement protein synthesis (Table 1.8) under resting conditions favours inhibition of both pathways. Rates of synthesis of inhibitors (C1-INH, factor H) are greater than those of activators in fibroblasts (Katz and Strunk, 1988a; 1989a). Under stimulatory conditions by mediators of inflammation, this pattern changes with an increase in the rates of synthesis of activators being greater than those of inhibitors (Katz and Strunk, 1988b).

1.7.2.3. *Endothelial cells*

All tissues depend on blood supply and blood supply depends on the endothelial cells. They create an adaptable life support system extending into every region of the body. If there were no endothelial cells extending and remodeling the network of the blood vessels, tissue growth and repair would be impossible. Endothelial cells play a vital role by

allowing white cells to migrate into the inflamed area and also allowing the exudation of plasma into the inflamed region. They also synthesise some complement proteins (Colten, 1976; Ripoche et al, 1988) (Table 1.8).

1.7.2.4. *Other cell types*

Epithelial cells, alveolar type II epithelial cells (Strunk et al., 1988), astrocytes (Gasque et al., 1992), intestinal epithelial cells (Colten, 1976), are also capable of synthesizing some complement proteins (Table 1.8).

1.8. COMPLEMENT DEFICIENCIES

Congenital and/or acquired deficiencies of the complement proteins have been described in man. With the exception of C1-INH deficiency (autosomal dominant) and properdin deficiency (X-linked recessive), deficiencies of all complement components are autosomal recessive.

C1-INH deficiency may result from the failure to secrete protein due to an abnormal gene or because the transcript of the abnormal gene encodes a functionally inactive protein. Hereditary angioedema (HAE) is a disease which results from inherited C1-INH deficiency and is characterized by recurrent episodes of subcutaneous and submucosal oedema (Davies, 1988).

Complete deficiencies of either factors H or I result in uncontrolled turnover of the alternative pathway with secondary deficiencies of C3 and B due to depletion. These patients also have a high incidence of immune complex diseases and severe systemic bacterial infections (reviewed in Morgan and Walport, 1991).

Deficiency of C4-bp has been reported in association with Behçet's syndrome and angioedema (Trapp et al., 1987).

Deficiency of DAF (Nicholson-Weller et al., 1983), HRF (Zalman et al., 1987) and CD59 (Holguin et al., 1989) occurs in patients with paroxysmal nocturnal haemoglobinuria. In this acquired clonal disorder of bone marrow cells, GPI-anchored proteins cannot be inserted in the cell membrane so that they are susceptible to complement lysis.

The features of deficiencies of the other complement components are listed in Table 1.9.

Table 1.9. SUMMARY OF COMPLEMENT DEFICIENCIES*

Component	Number of cases	Chromosomal location of gene	Disease associations
C1q	>40	A, B chains 1,p C chain ?	SLE in majority; pyogenic infections including meningitis
C1r/C1s	10	Closely linked on 12,p13	As above
C4	17	Two genes C4A and C4B in MHC on chromosome 6	As above
C2	>100	MHC locus on chromosome 6 adjacent to factor B	Pyogenic infections; SLE;
Factor D	1	?	Neisserial infections
Properdin	>50	X	Neisserial infections; rarely other pyogenic infections
C3	16	19	Pyogenic infections
Factor I	15	4	As above
Factor H	12	1 in RCA cluster	As above; haemolytic uraemic syndrome
C5	19	9	Neisserial infections; rarely SLE
C6	>50	5 closely linked to C7	As above
C7	26	5	As above
C8	32	α, β closely linked on 1 γ on chromosome 9	As above
C9 Caucasoid	5	5	Neisserial infections
C9 Japanese	many		Weak association with Neisserial infections

* Adapted from Morgan and Walport (1991).

1.9. THE AIM OF THE PRESENT STUDY

Rheumatoid arthritis (RA) is a disease characterized by chronic inflammation of the synovium which leads to the destruction of cartilage and bone. The formation of immune complexes within the articular and peri-articular tissues is thought to play a major role in its pathogenesis. Complement system is activated by immune complexes. Detection of decreased levels of complement components and the presence of activation products (anaphylatoxins, SC5b-9, MAC, C3dg) in the synovial fluid provide strong evidence for intra-articular complement activation in RA. Activated complement components can become anchored to host cells as well as to the target organisms. Various types of cells such as fibroblasts, endothelial cells, macrophages, adipocytes, and synoviocytes (type A and type B, macrophages-like and fibroblast-like, respectively) are all found in the synovial membrane. In this potentially cytotoxic environment, the host cells should be protected from the destructive capacity of complement by fluid-phase and membrane-bound regulatory proteins.

Therefore, the aim of this project was firstly, to investigate which fluid-phase and membrane-bound proteins are synthesized in the synovial membrane from patients with RA and OA, and from normal individuals who did not have the diseases.

It was secondly, to examine the expression of the genes encoding the complement regulatory proteins in some of the cell types which are present in the synovial membrane (*i.e.*, endothelial cells, monocytes, lymphocytes and fibroblasts from RA, OA patients and from normal individuals).

And finally, it was to investigate the effects of cytokines (*e.g.* IFNγ) on the expression of some of these complement components.

CHAPTER 2. MATERIALS AND METHODS

2.1. REAGENTS

The following reagents were purchased from the sources shown.

Flow Laboratories, Rickmansworth, Herefordshire, England

Linbro multiwell tissue culture dishes, Linbro 75 cm^2 tissue culture flasks, trypsin EDTA solution in Pack's saline (trypsin/EDTA), Dulbecco's modified Eagle's medium (DMEM), RPMI 1640.

GIBCO BRL, Paisley, Renfrewshire, Scotland

Foetal calf serum (FCS), penicilline-streptomycin, amphotericin solution, L-glutamine, Hank's balanced salt solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM), trypsin-EDTA, nonessential amino acids, Nunclon tissue culture flasks 25 cm^2; 75 cm^2; 175 cm^2, Taq DNA polymerase, sodium bicarbonate, fungizone, DNA ladder (1 kb), RNA ladder (0.24-9.5 kb), low melting temperature agarose.

Boehringer Mannheim, Mannheim, FRG

Random primed DNA labelling kit, DNAase I (RNAase free), RNAase inhibitor, restriction endonucleases (*Eco*RI, *Hind*III, *Bam*H I).

SIGMA Chemical Co., Poole, Dorset, England

Diethylpyrocarbonate, gelatin 2% solution, endothelial cell growth supplement, collagenase type II, collagenase type I, trypsin type II, deoxyribonuclease 1, Concanavalin A, phytolacca Americana (pokeweed mitogen), cycloheximide, extra avidin biotin staining kit mouse, polaroid photography film type 57, lysozyme, 3-N-morpholino propane sulfonic acid sodium salt (MOPS), hexadecyl trimethyl ammonium bromide

(CTAB), spermidine free base, anti-mouse IgG FITC conjugate, Triton X 100, Tween X 20 .

Biogenesis, Bournemouth, England

RNAzol, purified human alpha interferon (lymphoblastoid), purified human beta interferon (fibroblast), recombinant human interferon gamma.

British Biotechnology Ltd., Cowley, Oxford

Human interleukin 1β (recombinant), human interleukin 6, human TNF α (recombinant), human TNF β (recombinant).

Amersham International, Bucks, England

Hybond-N nylon membranes, hyperfilm-MP (10x12") ZRPN 1680, α^{32}P-dCTP, γ^{32}P-ATP, α^{32}P-dUTP

Serotec, Oxford, England

Mouse monoclonal antibodies to human CR1 receptor, human membrane co-factor protein and human decay accelerating factor.

Atlantic Antibodies Incstar Ltd, Workingham, Berkshire

Goat anti-human C1-INH, goat anti-human factor I, sheep anti-human C4-bp, goat anti-human factor H

Bioquotec Limited, 3 Mount Plaisant Court, West Yorkshire

Human vitronectin cDNA probe (1 kb)

BGRL Research Products BPL Commercial Department, Herts

Mouse monoclonal antibody to CD59.

DIFCO Labs, 146 Central Ave, East Molesley, Surrey

Bactotryptone, yeast extract,

Fluka Chemica, Peakodale Road, Glossop, DerbyShire

Formamide.

Welcome Diagnostic, Temple Hill, Dartford

Phytohaemagglutinin.

Mc Quilkin Co., 21 Palmadif St., Glasgow, Scotland

Chloroform, propan-2 ol

Plasmids

The plasmid pCI (C1-INH cDNA) was a gift from Dr Philip Carter, (Department of Biochemistry, University of Aberdeen; Carter et al., 1988). The plasmid BP-8 C4bp (C4 binding protein cDNA) was a gift from Dr. K.B.M. Reid, (Immunochemistry Unit, Department of Biochemistry, University of Oxford; Chung et al., 1985). The plasmid B38-1 FH (factor H cDNA) and the plasmid pSp 64 (factor I cDNA) were gifts from Dr. R.B. Sim, (MRC Immonochemistry Unit, Department of Biochemistry, University of Oxford; Ripoche et al., 1988, Catteral et al., 1987). S protein (vitronectin) cDNA was purchased from Bioquote Ltd., (Ilkley, Yorkshire, England; Suzuki et al., 1985). The plasmid LK 107 (SP-40,40 cDNA) was a gift from Dr. Brendan Murphy, (St. Vincent's Hospital, Melbourne, Australia; Kirszbaum et al., 1989). The plasmid YTH 53.1/1 (CD59 cDNA) was a gift from Professor H Waldmann, (Department of Pathology,

University of Cambridge, Cambridge, England; Davies et al., 1989). The plasmid pGEM DAF 2.1 (DAF cDNA) was a gift from Dr. E Medof, (Department of Pathology Casewestern Reserve University, Cleveland, Ohio, USA; Medof et al., 1987). The plasmid MCP-9 (MCP cDNA) was a gift from Dr. D.M. Lublin, (Washington University School of Medicine, St. Louis, Missouri,USA; Lublin et al., 1988). The plasmid pCR1.1 (CR1 cDNA) was a gift from Dr.D.T. Fearson, (Department of Medicine, Jonhs Hopkins University, Baltimore, Maryland, USA; Wong et al., 1985)

2.2. BUFFERS

2.2.1. Preparation of Culture Media

2.2.1.1. *Cell and tissue culture media*

Endothelial cell culture medium

Seventy mililitres RPMI 1640, 15 ml fetal calf serum (heat inactivated 2 hours at 56 ^{o}C), 10 ml human serum (heat inactivated 2 hours at 56 ^{o}C), 1 ml glutamine (0.2 M) and 2 ml penicillin/streptomycin (5,000 units/ml; 5000 µg/ml), 1 ml endothelial cell growth supplement (3 mg/ml), 1 ml heparin (1000 u/ml) were mixed together and stored at 4 ^{o}C up to two weeks.

Monocyte culture medium

Eighty-seven mililitres RPMI 1640, 10 ml human serum (heat inactivated 2 hours at 56 ^{o}C), 1 ml glutamine (0.2 M) and 2 ml penicillin/streptomycin (5,000 units/ml; 5000 µg/ml) were mixed and stored at 4 ^{o}C. After the third day, instead of human serum, fetal calf serum (heat inactivated 2 hours at 56 ^{o}C) was added to the medium.

Synovial tissue and fibroblast cell culture medium

Twenty mililitres 10 x Dulbecco's Modified Eagle Medium (DMEM), 20 ml fetal calf serum (FCS, heat inactivated for 2 hours at 56 ^{o}C), 8 ml sodium bicarbonate (7.5 %), 2 ml glutamine (0.2 M) and 2 ml penicillin/streptomycin (5,000 units/ml; 5000 µg/ml) were mixed together and the volume made up to 200 ml with sterile water and stored at 4 ^{o}C for up to 2 weeks. The same medium was used for skin fibroblast cell culture.

Transport medium

Sodium bicarbonate (70 mg), 20 ml 10 x Hank's solution, 4 ml FCS (heat inactivated for 2 hours at 56 ^{o}C), 10 ml penicillin/streptomycin were mixed and the volume made up to 200 ml with sterile water. The final solution was stored at -20 ^{o}C in aliquots of 10 ml.

Collagenase/DNase 1

Collagenase (100 mg), deoxyribonuclease 1 (10 mg) and sodium bicarbonate (35 mg) were dissolved in 10 ml 10 x HBSS and 2 ml penicillin/streptomycin. The volume was made up to 100 ml with sterile water. The final solution was divided into 25 ml aliquots and stored at -20^{o}C.

Trypsin/EDTA

Trypsin (100 mg) and EDTA (40 mg) were dissolved in 20 ml 10 x phosphate-buffered saline and the volume was made up to 200 ml with sterile water. The final solution was stored at -20 ^{o}C as 25 ml aliquots.

2.2.1.2. *Bacteria culture media*

L-Broth

Bactotryptone (10 g), NaCl (10 g), yeast extract (5 g), were dissolved in water and the volume was made to 1 litre. The solution was sterilized by autoclaving and stored at room temperature.

L-Broth Agar

Bactotryptone (10 g), NaCl (10 g), yeast extract (5 g), and bactoagar (20 g) were dissolved in water and the volume was made up to 1 litre. The solution was sterilized by autoclaving and stored at room temperature.

Lysosyme mix

Lysosyme was dissolved to a final concentration of 2 mg/ml in Tris.HCl (25 mM, pH 8.0) containing glucose (50 mM) and EDTA (10 mM). The mixture was stored at 4 ^{o}C.

GTET

Glucose (8 g), 10 ml EDTA (0.5 M, pH 8.0), 10 ml Tris.HCl (0.5 M, pH 8.0) was added to 80 ml water. The solution was filtered through 0.2 μm filter units, 100 ml Triton X 100 was added.

1 x Tris Borate EDTA (TBE)

Tris (10.8 g) and boric acid (5.5 g) were dissolved in 500 ml water after which 4 ml of EDTA (0.45 M) was added. The pH was adjusted to 8.0 before making the volume up 1 litre with water. The solution was stored at room temperature.

20 x Phosphate-Buffered Saline (PBS)

Sodium chloride (320 g), dipotassium hydrogen phosphate (48.4 g) and potassium dihydrogen phosphate (13.6 g) were dissolved in water and the volume was made up to 2 litres and stored at room temperature.

2.2.2. Buffers for Nucleic Acid Manipulation

10 x MOPS

Sodium morpholino-propanesulphonic acid (42 g), sodium acetate (6.8 g) and EDTA (3.7 g) 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 room temperature.

20 x SSPE

Sodium chloride (3.6 M), sodium dihydrogen orthophosphate (0.2 M) and EDTA (0.02 M), the pH was adjusted to 7.7 and the volume was made up to 1 litre and stored at room temperature.

20 x SSC

Sodium chloride (3 M) and trisodium citrate (0.3 M), the pH adjusted to 7.2 and the volume made up to 1 litre. The solution was stored at room temperature.

100 x Denhart's solution

Ficoll (2 g), polyvinyl pyrolline (2 g) and bovine serum albumin (2 g) were dissolved in water, the volume was made up to 100 ml and the final solution was filtered through 0.2 μm filter units before dividing into 1.25 ml aliquots which were stored at -20 ^{o}C.

Salmon sperm DNA (1 mg/ml)

Salmon sperm DNA (0.1 g) was added to 100 ml of water. The DNA was dissolved by autoclaving and the final solution was divided into aliquots and stored at -20 ^{o}C.

Sample buffer (Northern blotting)

Formamide (250 µl), formaldehyde (80 µl) and 10 x MOPS (50 µl) were mixed in a microfuge tube and stored at -20°C.

Sample buffer (Slot blotting)

Formamide (250 µl), formaldehyde (80 µl) and 20 x SSC (50 µl) were mixed in a microfuge tube and stored at -20 °C

Dye-mix

Two hundred and fifty microlitres of ethidium bromide (1 mg/ml), 50 µl bromophenol blue (10 mg/ml) and 250 µl glycerol were mixed in a microfuge tube and stored at -20 °C.

TE buffer

Two mililitres of Tris.HCl (0.5 M, pH 8.0) and 20 ml EDTA (0.5 M, pH 8.0) were mixed and the volume was made up to 100 ml with water. The solution was filtered through 0.2 µm filter units and stored at room temperature.

Neutralising solution

Sodium chloride (1.5 M), Tris.HCl (0.5 M, pH 7.2) and EDTA (0.001 M) were dissolved in one litre of water.

Lysis buffer

Tris.HCl (10 mM, pH 7.4), sodium chloride (10 mM), $MgCl_2$ (3 mM) and Nonidet P-40 (5 %; v/v) were dissolved in water. The solution was stored at room temperature.

Storage buffer

Tris.HCl (50 mM, pH 8.3), $MgCl_2$ (5 mM), EDTA (0.1 M) and glycerol (40 %) were dissolved in one litre of water and filtered through 0.2 μm filter.

2.3. EUKARYOTIC CELLS AND TISSUE CULTURE

2.3.1. Endothelial Cells

Primary cultures of human umbilical vein endothelial cells (HUVEC) were prepared from freshly obtained umbilical cords from normal deliveries at the Royal Maternity Hospital, Glasgow, U.K. Umbilical cords were transferred to the laboratory in sterile 1 x HBSS. After catheterization, the vein was washed twice with 1 x HBSS. One end of the cord was tied with sterile clamp and then 10 ml collagenase solution (10 ml of 0.1 % w/v) was injected into the lumen of the vein. The cord was then incubated at 37 ^{o}C for 10 minutes. The collagenase was flushed into a sterile Falcon culture flask and the vein was further washed with 1 x HBSS in order to collect the remaining endothelial cells. The pooled cell suspension was centrifuged for 5 minutes at 1000 rpm at room temperature, the cell pellet was resuspended in RPMI 1640 medium (see section 2.2.1) and cultured at 37 ^{o}C, in a humidified atmosphere consisting of 5 % of CO_2 in air (Jaffe et al., 1973). Cells were grown to confluence in 25 cm^2 flasks coated with 1 % gelatin

Phase-contrast microscopy proved that at confluence, the cells formed a monolayer which had a uniform cobblestone appearance typical of endothelial cells (Figure 3.1).

Weibel-Palade bodies, rod-shaped organelles in endothelial cells, were detected by electron microscopy (Figure 3.2).

Clotting factor VIII antigen, which is considered to be the most reliable marker for endothelial cells, was detected when the cultured cells were stained with a monoclonal factor VIII antibody.

For all experimental work, the cells were used during their fourth passage.

2.3.2. Monocytes

Human monocyte monolayers were prepared from the buffy coat of blood donations (Scottish Blood Transfusion Service, Law Hospital, Carluke, Scotland, U.K.). The buffy coat was centrifuged at 2,000 rpm for 10 minutes which formed three phases.

The lower phase contained erythrocytes, the upper phase was plasma and the middle phase leukocytes. The leukocytes were aspirated and transferred to a fresh falcon tube, diluted with an equal volume of PBS and then layered onto a Ficoll-Hypaque gradient which was centrifuged at room temperature at 1,500 rpm for 30 minutes. The mononuclear cells at the interphase were transferred to a falcon tube, diluted with an equal volume of PBS and recentrifuged at 2000 rpm for 10 minutes at room temperature. The supernatant was removed and the cells were resuspended (1 x 10^6 cells per ml) in RPMI 1640 containing 10% ABS (heat-inactivated 2 hours at 56 ^oC). The cells were placed into 24 well Linbro dishes (1 ml per well), and cultured at 37 ^oC in a humidified 5% CO_2/air atmosphere. The non-adherent cells were removed from the dishes following the five hours incubation. After 3 days, cells were washed extensively and the medium changed to RPMI 1640 containing 20% FCS (heat-inactivated). The cells were incubated under the same conditions for another 24 hours before any experiments were performed.

The cells were found to consist of 95 % monocytes when assessed by phase-contrast and light microscopy (Figure 3.5), staining for non-specific esterase and phagocytosis of latex particles.

2.3.3. Peripheral Blood Lymphocytes

Human Peripheral Blood Lymphocytes were prepared from the buffy coat of blood donations (Scottish Blood Transfusion Service, Law Hospital, Carluke, Scotland, U.K.). The buffy coat was centrifuged at 2,000 rpm for 10 minutes and formed three phases.

The lower phase contained erythrocytes, the upper phase was plasma and the middle phase leukocytes. The leukocytes were aspirated and transferred to a fresh falcon tube, diluted with an equal volume of PBS and then layered onto a Ficoll-Hypaque gradient which was centrifuged at room temperature at 1,500 rpm for 30 minutes. The lymphocytes at the interphase were transferred to a falcon tube, diluted with an equal volume of PBS and recentrifuged at 2000 rpm for 10 minutes at room temperature. The supernatant was removed and the cells were resuspended (1×10^6 cells per ml) in RPMI 1640 containing 15 % FCS (heat-inactivated 2 hours at 56 ^{o}C). The cells were placed into 75 cm^2 flask, and cultured at 37 ^{o}C in a humidified 5 % CO_2/air atmosphere. The adherent cells, monocytes, were depleted from the culture by removing the non-adherent cells, lymphocytes, to a fresh flask following the three and five hours incubations.

2.3.4. Synovial Tissue Fibroblasts

Specimens of synovium were collected from the knees of 9 patients undergoing orthopaedic surgery in Gartnavel General Hospital, Glasgow, U.K.. Three patients were suffering from rheumatoid arthritis (RA), three from osteoarthritis (OA), and three patients from menisceal injuries. None of this last group had evidence of inflammatory joint disease or OA. Synovial tissue specimens were transported in Hank's transport medium.

The tissues obtained were used for fibroblast cell cultures and for tissue cultures (see section 2.3.6).

Synovial fibroblasts were prepared according to the method of Dayer et al. (1976) except that the concentration of clostriopeptidase-A (crude bacterial collagenase type I) was 0.1 % (w/v).

The specimen was washed twice with Dulbecco's calcium and magnesium-free phosphate buffered saline (PBS), and cut into 1-2 mm^3 fragments in a large Petri dish containing Hank's transport medium. The tissue fragments were transferred to a 100 ml flask and allowed to settle for a few minutes before the supernatant was discarded. The tissue was then washed twice with PBS and the supernatant was discarded. The tissue was digested for 3.5 hours at 37 ^oC in a shaking water bath with 25 ml Hank's balanced salt solution containing 0.1 % (w/v) collagenase and 0.01 % (w/v) deoxyribonuclease I. Cells and debris were removed by transferring the sample to a universal container which was centrifuged at 400 g for 10 min at room temperature. After the supernatant was discarded, the cells and the tissue were washed into a conical flask used before with 50 ml Dulbecco's PBS containing 0.05 % trypsin and 0.02 % EDTA, and further incubated at 37 ^oC in a shaking water bath for 1 hour. The cells were separated from the undigested residue by allowing the residue to settle for 5 minutes, and then by pipetting off the supernatant containing the cells. The residue was then washed with 10 ml PBS, allowed to resettle and the supernatant was collected and pooled with the first supernatants. The cells were harvested from the supernatants by centrifugation at 400 g for 10 minutes at room temperature and the cell pellet washed three times with DMEM containing 10 % FCS (v/v), resuspeded in DMEM containing 10 % FCS and a cell count was performed. The cell suspension was adjusted to 2 x 10^6 cell/5 ml and 5 ml portions transferred to 25 cm^2 flasks. The flasks were incubated at 37 ^oC,

in a humid 5 % CO_2/air atmosphere. Nonadherent cells were removed after 48 hours and culture of the adherent cells was continued after the addition of fresh medium (DMEM/FCS).

Fibroblasts were identified by their characteristic fusiform morphology under light microscopy (Figure 3.3) and their specific staining with anti-vimentin antibody (Figure 3.4). See figure 3.18A for the control staining

2.3.5. Harvesting Cells

Once the cells reached confluence in the 25 cm^2 flasks, they were washed twice with PBS and harvested by trypsinisation with trypsin/EDTA solution. They were resuspended in 10 ml of fresh medium and transferred into 75 cm^2 flasks. When the cells reached confluence in 75 cm^2 flasks they were trypsinised and cultured in 175 cm^2 flasks until confluence was achieved. They were then divided into three 175 cm^2 flasks and cultured until confluence when experiments were performed.

2.3.6. Synovial Membrane Tissue Culture

Specimens of synovium were collected at the time of surgery from the knee joints of three patients with rheumatoid arthritis (RA), three with osteoarthritis (OA) and three from individuals undergoing meniscectomy who did not have OA or any chronic inflammatory joint disease.

To allow optimal gas and nutrient exchange, gas-liquid interface technique was used. Medium (containing DMEM supplemented with 10 % FCS) enough to keep wet filter was added to the dishes. The tissue was washed 3 times with Dulbecco's calcium and magnesium-free PBS. Tissue fragments (40-80 mg) were placed onto stainless steel grids with sterile

filters in a humidified 5 % CO_2 atmosphere. The culture medium was changed every second day.

2.3.7. Treatment of Cell Cultures

2.3.7.1. *Cycloheximide*

Three sets of HUVEC cultures, human synovial fibroblast cultures, and synovial membrane tissue cultures were treated with 2.5 μg/ml of cycloheximide. Culture supernatants were collected at days 1, 3, 5 and 7 in order to examine *de novo* synthesis of the complement components by ELISA, and total cellular RNA was isolated from the cells (section 2.8.).

2.3.7.2. *Actinomycin D*

Three sets of HUVEC cultures were exposed to actinomycin D (5 μg/ml) in order to measure the stabilities of DAF, MCP and CD59 mRNA. The total cellular RNA was isolated as described in section 2.8.1, at the time intervals of 0, 1, 2, 4, 6, 8 and 24 hours. The isolated RNA was immobilized onto membranes as described in section 2.9.2 for further analysis.

2.3.7.3. *Cytokines*

Three sets of human endothelial cell cultures were incubated with two different concentrations of (0.1, and 10 ng/ml) IFNγ, INFα, IL-1 and 1L-6 for 24, 48 and 72 hours. The supernatants were collected to examine the synthesis of regulatory complement components by ELISA.

After 72 h stimulation by cytokines, the total cellular RNA was isolated as described in section 2.8.1. and Northern blot analysis was carried out (see section 2.9.1).

Two sets of endothelial cell cultures stimulated with 10 ng/ml IFNγ for 24 h were further treated with actinomycin-D as described in section 2.3.7.2. to examine the mRNA stability in IFNγ stimulated cell cultures.

2.4.7.4. *Mitogens*

Three sets of peripheral blood lymphocytes and three sets of monocyte depleted peripheral blood lymphocytes were exposed to Phytohaemagglutinin (120 μg/ml), Pokeweed mitogen (120 μg/ml) and Concanavalin A (100 μg/ml) separately for 72 hours in order to activate the lymphocytes. The supernatants were collected to examine the synthesis of regulatory complement components by ELISA.

2.4. GENERAL PROCEDURES INVOLVING NUCLEIC ACIDS

2.4.1. Preparation of the Plasmid DNA

2.4.1.1. *Preparation of competent cells*

20 µl of glycerol stock of *E.coli* HB101 were inoculated into 10 ml of L-broth and incubated at 37 °C overnight in an orbital incubator. 100 µl of this culture was added to a tube containing 10 ml of L-broth and incubated at 37 °C for further 3 to 4 hours, until the optical density at 520 nm was approximately 0.6. The culture tube was cooled on ice for 10 minutes. The cells were pelletted by centrifugation at 2000 rpm, at 4 °C for 5 minutes. The supernatant was discarded, the pellet was resuspended in 5 ml of ice cold $MgCl_2$ (100 mM). It was incubated on ice for 30 minutes and then centrifuged at 2,000 rpm, at 4 °C for 5 minutes. The pellet was resuspended in 1 ml of $CaCl_2$ (100 mM), and incubated on ice for 40 minutes after which the cells were ready for use in the transformation protocol (see below).

2.4.1.2. *Transformation of bacterial cells*

Three hundred microlitres of competent cells (section 2.4.1.1.) in ice-cold $CaCl_2$ (100 mM) were transferred to microcap tubes and approximately 0.5 µg of plasmid DNA was added. The cells were incubated on ice for 30 minutes, 42 °C for 2 minutes (heat-shock) and then 5 minutes at 4 °C to recover from the heat-shock. One ml of L-broth at 37 °C was then added and the mixture was incubated with shaking for 1 hour. The cells were centrifuged at 6500 rpm (microfuge) and all but 100 µl of the medium was removed. The cell pellet was resuspended in

this residual medium and spread on agar plates containing 100 μg/ml ampicillin. The culture was allowed to dry on the agar and was incubated overnight at 37°C. Ampicillin-agar plates were selected for those clones containing closed circular plasmid DNA with an intact ampicillin resistance gene. The analysis of recombinant colonies was performed by restriction analysis as described in section 2.6.1.

The negative control consisted of an agar plate on which competent cells alone were spread.

2.4.2. DNA Purification

2.4.2.1. *Small scale isolation of plasmid DNA*

The glycerol stock of *E.coli* that had been transformed with the appropriate plasmid [C1-INH (pC1), C4-bp (BP-8), factor I (pSp 64), factor H (B38-1), Sp-40,40 (LK107), DAF (pGEM DAF2.1), MCP (MCP-9), CD59 (YTH 53.1/1), CR1 (pCR1.1)] was plated onto the agar containing antibiotic (ampicillin 50 μg/ml or tetracycline 12.5 μg/ml) using a flame-sterilized platinum loop. The agar plate was incubated at 37 °C overnight. A single colony was picked from the plate and was inoculated into a universal container containing 5 ml of L-broth with the appropriate antibiotic, and incubated for 4 to 5 hours at 37 °C in an orbital incubator.

An aliquot (1.5 ml) of L-broth culture was transferred to a microcap tube and centrifuged at 8000 rpm for 1 minute at room temperature. The supernatant was discarded and another 1.5 ml of the culture was added and centrifuged as above. After centrifugation, the pellet was resuspended in 200 μl lysis buffer and incubated for 5 minutes at room temperature, before the addition of 400 μl of freshly prepared alkaline solution (NaOH 0.2 M, SDS 1 %, w/v). The mixture was mixed by

inverting 5 or 6 times and then incubated on ice for 5 minutes. Three hundred microliters of 7.5 M ammonium acetate solution (pH 7.8) were added and the contents were mixed gently for a few seconds. The suspension was kept at 4 ^{o}C for 10 minutes to allow the precipitation of most of the protein, high molecular weight RNA and chromosomal DNA. The tube was centrifuged for 3 minutes at 13,000 rpm in a microfuge and the clear supernatant was transferred to the fresh microcap tube. Isopropanol (0.6 volume) was added and the tube was incubated at room temperature for 10 minutes, prior to centrifugation at 15,000 rpm for 10 minutes. The supernatant was discarded and the DNA pellet was washed with 70 % (v/v) ethanol and air dried, before being dissolved in 100 µl Tris.HCl/EDTA (TE). The purified plasmid DNA was digested with appropriate restriction enzymes and examined by gel electrophoresis (see sections 2.5.1. and 2.5.2).

2.4.2.2. *Large scale isolation of plasmid* DNA

One mililitre of an overnight culture of *E.coli* which had been transformed with the appropriate plasmid (see small scale plasmid DNA preparation in section 2.4.2.1) was added to 500 ml L-broth containing requisite antibiotic and incubated overnight at 37 ^{o}C in an orbital incubator. The culture was transferred to a sterile polycarbonate bottle and centrifuged at 5000 rpm for 5 minutes at 4 ^{o}C. The supernatant was decanted, the pellet was drained and the inside of the bottle was dried with a tissue. The pellet was resuspended in 30 ml of GTET solution (glucose 8 %, w/v; Tris-HCl 50 mM; EDTA 50 mM; Triton X 100 0.1 %, v/v) and it was transferred to a sterile 50 ml centrifuge tube. Lysozyme (1.2 ml of 50 mg/ml solution) was then added and the solution was mixed by gently inverting the tube 5 to 10 times. The solution was

centrifuged at 12,000 rpm for 15 min and the supernatant was transferred to a fresh tube. One hundred microlitres of RNase (10 mg/ml) were added and the mixture was incubated at 68 ^{o}C for 20 minutes. After centrifugation at 10,000 rpm for 15 min at room temperature, the supernatant was transferred to a fresh tube and 1.5 ml of CTAB (5 %, w/v) was added. After mixing, the solution was centrifuged at 10,000 rpm for 15 minutes at 4 ^{o}C . The pellet was dissolved in 15 ml NaCl (1.2 M) and after the addition of an equal volume of isopropanol and incubation at -20 ^{o}C for 1 hour, the tube was centrifuged at 10,000 rpm for 10 minutes at 4 ^{o}C. The supernatant was discarded and the pellet was washed twice in ice-cold ethanol (10 ml; 75 %, v/v). The pellet was air dried and dissolved in TE buffer.

2.5. GEL ELECTROPHORESIS OF NUCLEIC ACID

2.5.1. Agarose Gel Electrophoresis of DNA

One gram of agarose was dissolved in 100 ml of 1 x TBE buffer in a microwave oven for 2 minutes. The solution was removed from the oven and allowed to cool to 50 ^{o}C. Ethidium bromide (10 µl of a 5 mg/ml solution) was added and the mixture was swirled gently, poured into the gel mould where it was left to set for 30 minutes at room temperature, the comb was removed carefully and then the electrophoresis buffer (1 x TBE with 0.5 µg/ml ethidium bromide) was poured into the gel tank.

The DNA (5 µg) sample was mixed with loading buffer (0.25 %, w/v bromophenol blue; 50 %, v/v glycerol; 15 % w/v Ficoll in H_2O) and was heated at 65 ^{o}C for 5 min and cooled on ice. DNA samples were loaded into the slots of the gel and run at 50 V, 50 mA for 1 hour at room temperature. A set of standard markers (1 kb ladder) was also run in the same gel, in order to determine the size of DNA being analysed.

2.5.2. Agarose Gel Electrophoresis of RNA

One gram of agarose was dissolved in 85 ml of water by heating in a microwave oven for 2 minutes. The solution was then cooled to 50 ^{o}C by standing at room temperature, after which 10 ml of 10 x MOPS buffer and 5 ml of formaldehyde (37 %, v/v) were added, mixed and immediately poured into the gel mould. The gel was left to set for 1 hour at room temperature in a fume cupboard.

10 µg of RNA sample was mixed with sample buffer and denatured at 65 ^{o}C for 15 minutes then cooled on ice for 10 minutes. 3 ml of dye-mix was added and the RNA sample was loaded into the slots. The gel was set

to run at 100 V and at 50 mA for 2 hours. A set of standard markers (0.24-9.5 kb) was also run in the same gel, in order to determined the size of mRNA being analysed (Figure 2.1).

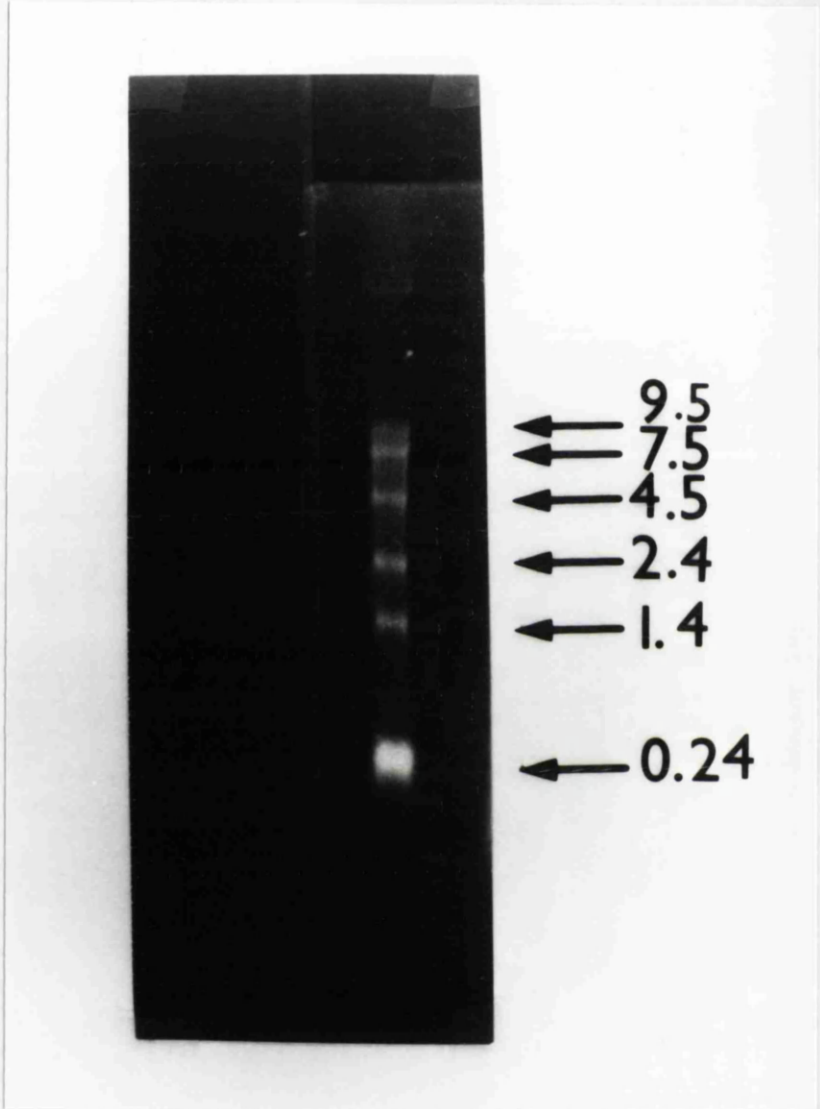


Figure 2.1: The RNA size marker.

2.6. PURIFICATION OF DNA RESTRICTION FRAGMENTS

2.6.1. Low Melting Point Agarose Gel Electrophoresis of DNA

The restriction endonucleases used to excise the cDNA inserts from their plasmid vectors were as follows: *Bam*HI and *Hind*III for C1-INH, C4-bp, factor H and DAF, *Eco*RI for factor I, MCP, CR1 and CR2.

The volume of the reaction mixture which contained 20 U of suitable restriction enzyme(s), 10 µg of plasmid DNA, 5 µl of spermidine (20 mM) and 10 µl of enzyme buffer was adjusted to 100 ml by the addition of water. The solution was incubated at 37 ^{o}C overnight. Next day the solution was heated to 65 ^{o}C for 5 min and cooled on ice. 1/10 volume of dye mix was added.

0.9 % of low melting point (LMP) agarose gel was dissolved in 50 ml of 1 x TBE by heating in microwave oven for 2 min, 5 µl of ethidium bromide (10 mg/ml) was added and left to set in the cold room (4 ^{o}C) for 1 hour.

The DNA samples were loaded in wells and the electrophoresis was performed at 4 ^{o}C, at 50 V and 50 mA for 2 to 3 hours. The insert was purified as described in section 2.6.1

2.6.2. Purification of the DNA from Agarose Gels

Gel electrophoresis was applied to the plasmid DNA as described in section 2.6.1. The band of interest was located by using UV light, the slice of agarose containing the band excised with a scalpel blade and transferred to a microcap tube. Five volumes of sterile water were added and the tube was incubated at 65 ^{o}C for 5 minutes to melt the agarose.

An equal volume of phenol was added. The solution was mixed in a vortex mixer for 15 seconds. Following 5 minutes of centrifugation at 13,000 rpm at room temperature the aqueous phase was removed. Then it was re-extracted once with phenol-chloroform and once with chloroform.

The aqueous phase was transferred to a fresh tube and 1/10 volume of 3 M sodium acetate (pH 6.0) and 2 volume of ice cold absolute ethanol was added prior to incubation overnight at -70 ^{o}C.

The tube was centrifuged at 13,000 rpm for 15 min at 4 ^{o}C, the supernatant was discarded and the pellet was washed twice with ice cold 75 % (v/v) ethanol. The pellet was then dried under vacuum and dissolved in 50 µl of TE buffer.

2.6.3. cDNA Amplification by Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used to amplify the cDNA of C4-bp, factor H, S Protein and DAF. The reaction mixture was prepared in 0.5 ml microcap tubes. The Gene Amp PCR Reagent Kit (Pekin-Elmer Cetus) was used. The composition of the solution is given in Table 2.1.

The mixture (100 µl) was added to a microcap tube and overlaid with 70 µl of paraffin oil to prevent evaporation. The tube was then placed on DNA thermal cycler (Pekin-Elmer Cetus). A negative control mixture, which included all the components of the PCR solution except the template DNA, and a positive control, which included two control primers and a control template DNA, were also used.

The DNA was denatured by heating at 90 ^{o}C for 1 minute (denaturation), the reaction mixture was then cooled to 42 ^{o}C for 2 minutes to allow the oligonucleotide primers to anneal to their target sequence (annealing), then the temperature of the mixture was raised to

SUBSTANCE	VOLUME	FINAL CONCENTRATION
sterile water	69.5 µl	
10x reaction buffer	10 µl	1x
dATP	2 µl	200 mM
dCTP	2 µl	200 mM
dGTP	2 µl	200 mM
dTTP	2 µl	200 mM
Taq DNA polymerase	0.5 µl	2.5 U/100 ml
primer 1	5 µl	1 mM
primer 2	5 µl	1 mM
cDNA	1 µl	20 ng
Total volume	**100 µl**	

Table 2.1. Composition of reaction mixture for polymerase chain reaction.

72 ^{o}C for 1 min to allow DNA synthesis (extension) to occur. This reaction sequence was repeated for 30 cycles. The oligonucleotides used were as follows.

For factor H and C4-bp (pAT 153 primers)

5' -CTC ATG TTT GAC AGC TTA TC- 3'

5' -CAC GAT GCG TCC GGC GTA GA- 3'

For S protein (cDNA)

5' -GCG TCG ACA GAT GGC CAG GA- 3'

5' -GCG AAT TCA CCG ACT CAA GAA C- 3'

For DAF (pGEM primers)

5' -CTC ACT ATA GGG AGA CC- 3'

5' -ACC TTA TGT ATC ATA CAC AT- 3'

2.7. PREPARATION OF RADIOLABELLED DNA/RNA PROBES

2.7.1. Random Prime Labelling of cDNA

Radioactive labelling of the cDNA probes was performed using the Random Primed DNA Labelling Kit. A typical reaction contained 50 ng of DNA, 20 nmol of each of unlabelled dATP, dGTP, dTTP, 5µl [α-^{32}P] dCTP (specific activity > 3000 Ci/mmol; 10 µCi/µl) and 2 µl Klenow fragment of *E.coli* DNA polymerase (1 U/µl) in a total reaction volume of 20 µl. The mixture was incubated at 37 ^{o}C for 30 minutes after which the reaction was stopped by adding 20 µl of dye-mix. The mixture was applied to a small Bio-gel column as described in section 2.7.3. An aliquot (5 µl) of the probe was transferred to a tube which contained 5 ml of liquid scintillation solution (Ecoscint A) and the radioactivity was counted by liquid scintillation spectrometry (1217 Rack Beta). For hybridization reactions, the radioactivity level was adjusted to 10^6 cpm/ml in hybridization buffer.

2.7.2. Kinase 5'-end Labelling of the Oligonucleotide

Synthetic oligonucleotides (28 S ribosomal RNA) were end-labelled with (γ-^{32}P)ATP. The reaction mixture contained 10 pmol of oligonucleotide, 2 µl 10 X bacteriophage T4 polynucleotide kinase buffer [0.5 M Tris-HCl (pH 7.6), 1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine-HCl, 1 mM EDTA (pH 8.0)], 2 µl (γ-^{32}P)ATP (specific activity 5000 Ci/mmol; 10 pmol) and 1 µl of T4 kinase in a total volume of 10 µl. This mixture was incubated at 37 ^{o}C for 30 minutes. Ammonium acetate (7.5 M; 1/10 volume of the mixture) and 2 volumes of ice-cold absolute ethanol was added prior to incubation at -70 ^{o}C for 30 minutes. The mixture was

centrifuged at 13,000 rpm for 15 minutes at 4 ^{o}C. The supernatant was discarded and the pellet was washed twice with ice-cold absolute ethanol. The pellet was air dried and dissolved in 100 µl of TE. An aliquot (5 µl) of the probe was transferred to a tube which contained 5 µl of liquid scintillation solution (Ecoscint A) and the radioactivity was counted by liquid scintillation spectrometry (1217 Rack Beta). For hybridization reactions, the radioactivity level was adjusted to 10^6 cpm/ml in hybridization buffer.

2.7.3. Column Chromatography

Agarose beads (Bio-gel 1.5, 100-200 mesh) were used for gel filtration column chromatography. The lower part of the barrel of a glass Pasteur pipette was plugged with a piece of glass wool (about 1 cm long) and then filled with Bio-gel 1.5 and washed with 0.1 X (w/v) SSC, 0.1 % (w/v) SDS. The labelled probe (20 µl) (see section 2.7.1) was diluted in equal amounts of dye mix and layered onto the surface of the gel and washed through the column with 0.1 X (w/v) SSC, 0.1 % (w/v) SDS to separate the radiolabelled probe from the free radioactivity. The radiolabelled probe was collected in a fresh microcap tube and used for hybridization (see section 2.10.3).

2.7.4. Nuclear Run-on Assay

Approximately 1 x 10^7 cells were pelletted at 2000 rpm in a Beckman J-6B centrifuge for 5 minutes at 4 ^{o}C. They were washed in ice-cold PBS and resuspended in 1 ml of ice-cold lysis buffer and then left on ice for 5 minutes. The nuclei were then washed twice in lysis buffer for 5 minutes at 2000 rpm at 4 ^{o}C. The supernatant was removed. The nuclear

pellet was resuspended in 100 µl ice-cold storage buffer and stored at -70°C. Nuclei were thawed and incubated at 30 °C for 30 minutes in 100 µl reaction mixture [10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, 200 µCi of [α-^{32}P]UTP (>800 Ci/mmol), 5 mM dithiothreitol]. The labelled RNA was then isolated from the nuclei using the RNAzol method.

RNAzol (1.3 ml) and 130 µl of chloroform were added to the reaction mixture,which was then vortexed for 15 seconds and incubated on ice for 15 minutes. The lysate was centrifuged for 15 minutes and the aqueous phase was transferred to a fresh tube. An equal volume of isopropanol was added and incubated at -20 °C for 45 minutes. After centrifugation at 13,000 rpm for 15 minutes at 4 °C, the RNA pellet was washed in ice-cold ethanol 75 % (v/v), air dried and dissolved in 200 µl of water. An aliquot (2 µl) was added in 5 ml of scintillation solution in order to determine the amount of radioactivity incorporated into the mRNA transcripts. The concentration of radioactivity was adjusted to 10^7 cpm/ml in hybridization buffer. The labelled transcripts were then hybridized to filters coated with the appropriate single-stranded cDNA at 65 °C for 72 hours (see section 2.10.3).

5 µg of plasmid DNA was denatured for 5 minutes at 95 °C in 100 ml water. An equal volume of 20 x SSC was added to each tube and the DNA and then it was blotted directly onto a Hybond-N^+ membrane. The membrane was placed for 1 hour on a filter paper pad which was soaked in denaturation solution and then placed (DNA side up) on a filter paper pad which was soaked in neutralizing solution for a further 1 minute. The membrane was then briefly rinsed in 5 x SSPE and hybridization was carried out as described in section 2.10.3.

2.8. RNA PURIFICATION

2.8.1. Isolation of the Total Cellular RNA

The RNAzol method was used to isolate cellular RNA (Chomczynski and Sacchi, 1979). Monolayers of cells were washed twice in ice-cold PBS prior to lysis by the addition of RNAzol (0.2 ml per 10^6 cells) to each flask. The RNA was solubilized by repeated pipetting of the lysate which was then transferred to a microcap tube. Chloroform (1/10 volume of lysate) was added and the mixture was vortexed for 15 seconds and then left on ice for 15 minutes. Prior to centrifugation (13,000 rpm for 5 minutes at room temperature in a microfuge) the colourless upper aqueous phase containing the RNA was transferred to a fresh tube and an equal volume of isopropanol was added, after which the tube was incubated at -20 ^{o}C for 1 hour. After the sample was centrifuged at 13,000 rpm, at 4 ^{o}C, for 15 minute in a microfuge, the supernatant was removed and the pellet washed twice with ethanol (75 %, v/v). Then the pellet was dried briefly under vacuum for 10-15 minutes. Finally the RNA pellet was dissolved in TE (15-50 µl) and the amount of RNA was measured by spectrophotometry (see section 2.8.3).

Specimens of synovium were collected at the time of surgery from the knee joint of three patients with RA, three with OA and from three individuals undergoing meniscectomy who did not have 0A or chronic inflammatory joint disease. Immediately after collection the tissue specimens were snap-frozen in liquid nitrogen and stored at -70 ^{o}C until required. Finely ground frozen tissue fragments were homogenised in RNAzol (2 ml/100 mg), before adding 1/10 volume of chloroform and mixing vigorously for 15 seconds and standing on ice for 15 minutes. The suspension was centrifuged (12,000 rpm for 15 minutes at room

temperature) and the aqueous phase was transferred to a fresh tube to which an equal volume of ice-cold isopropanol was added. After standing on ice for 15 minutes the tube was centrifuged (13,000 rpm for 15 minutes at room temperature), the supernatant was removed and the pellet washed twice with ice cold 75 % (v/v) ethanol before being dissolved in water (100 ml). RNA samples which were contaminated with genomic DNA were incubated (5 minutes at 37 ^{o}C) with RNase inhibitor (10 units/mg RNA) prior to incubation (10 minutes at 25 ^{o}C) with RNase-free DNase I (5 units/100 µl). The samples were then re-extracted with RNAzol and chloroform, and precipitated with isopropanol as described above.

2.9. QUANTITATION OF DNA/RNA

To quantify the amount of DNA or RNA, readings was taken by spectrophotometry (Du-64 spectrophotometer, Beckman) at wavelengths of 260 nm and 280 nm. An optimal density (OD) of 1.0 corresponds to approximately 50 µg/ml for double-stranded DNA, 40µg/ml for single-stranded DNA or RNA at 260 nm wavelengths.

1 µl of DNA or RNA was diluted in 1 ml of sterile water and read at a wavelengths of 260 nm.

The ratio between the readings at 260 nm and 280 nm was used to estimate of the purity of the nucleic acid. A ratio of 1.9 or greater indicated a satisfactory degree of purity.

2.10. ANALYSIS OF mRNA FROM EUKARYOTIC CELLS

2.10.1. Northern Blot Analysis of RNA

For Northern blot analyses, total cellular RNA (10 μg/track) was fractionated by electrophoresis in 1 % agarose-formaldehyde gels as described in section 2.5.2. After electrophoresis, the gel was examined under UV light and photographed. Transcript sizes were determined by comparing their migrations relative to a RNA ladder which was used as a size marker (Figure 2.1).

Transfer of the RNA to the Hybond-N^+ membranes was performed by using the alkaline transfer protocol outlined by Amersham International. The transfer buffer was 0.05 M NaOH and the transfer was allowed to continue for 2 to 3 hours after which the blot was rinsed in 2 x SSC and kept at 4 ^oC until used.

2.10.2. Dot-blot Analysis of RNA

10, 5, 2.5, 1.25, 0.625 and 0.312 μg of total RNA in 5 μl of sample buffer were denatured at 65 ^oC for 15 minutes and cooled on ice. A filtration manifold was used to apply the RNA samples onto the Hybond-N^+ membranes. The membranes were air dried, fixed in 0.05 M NaOH for 10 to 20 minutes and then rinsed in 2 x SSC for 5 minutes. Hybridization was performed as described in section 2.10.3.

2.10.3. Hybridization Conditions

The protocol applied were set out by Amersham International (membrane transfer and detection methods). The hybridization buffer

consisted of 12.5 ml of 20 x SSPE, 5 ml of 50 x Denhardt's solution, 2.5 ml of 10 % (w/v) SDS, 5 ml water, 25 ml of formamide (37 %), 500 µl (10 mg/ml) of salmon sperm DNA. All prehybridization and hybridization reactions were performed overnight at 42 ^{o}C in a hybridization oven, except those for run-on assay which was prehybridized for 18 hours at 65 ^{o}C, and hybridized for 48-72 hours at 65 ^{o}C.

The labelled probe was boiled for 5 minutes and then cooled on ice before being added to the hybridization buffer. The labeled probe was added at a concentration of $1x10^{6}$ cpm/ml.

The filters were washed sequentially in 2 x (w/v) SSC , 0.5 x (w/v) SSC, 0.1 x (w/v) SSC each containing 0.1 % (w/v) SDS. The first wash was performed at room temperature for 30 minutes, followed by three washes of 30 minutes each at 65 ^{o}C.

The membranes were then covered with Saran-Wrap and Kodak XAR-5 film exposed to [illegible] intensifying screens at -70^{o}C. Exposure times varied from 1 to 15 days.

2.10.4. Stripping Probes from Nylon Membranes

Filters were washed in 0.1 % (w/v) SDS at 98 ^{o}C, allowed to cool to 20 ^{o}C and air dried. To ensure total removal of probe, the stripped membranes were subjected to autoradiography. The membranes were stored at 4 ^{o}C in a sealed plastic envelope until hybridized with another probe.

2.10.5. Scanning of the Autoradiographs

The relative abundance of mRNA species was determined by scanning densitometry using a Joyce-Loebl Chromoscan-3 (Joyce-Loebl, Gateshead, Tyne and Wear, U.K.). Further analysis was performed by

using a Molecular Dynamics laser scanner with PDI Quest system software running on a Sun-Sparc computer.

An arbitrary value of 1.00 was assigned to the control level of expression. The abundances of 28S ribosomal RNA was assumed as constant. Thus variation in the 28S RNA hybridization signals were taken to represent the unequal loading of RNA samples, so the results for the expression of other genes were adjusted accordingly.

2.11. ANALYSIS OF THE SECRETED PROTEINS

2.11.1. Preparation of the Reagents and Buffers

Coating buffer

Sodium bicarbonate (Na_2CO_3, 0.79 g) and 1.46 g of sodium hydrogen bicarbonate ($NaHCO_3$) were dissolved in water and the pH was adjusted to 9.0. The volume was made up to 500 ml with water and stored at 4 ^oC for up to one week.

PBS-Tween (PBST)

PBS containing 0.05 % (v/v) Tween was prepared by adding 1 ml Tween 20 to 100 ml 20 x PBS and making the volume up to 2 litres with water.

Blocking buffer

Bovine serum albumin (0.5 g) was dissolved in 500 ml 1 x PBS and the final solution was stored at 4 ^oC for up to 1 week.

Peroxide substrate

Disodium hydrogen phosphate (28 ml, 0.2 M) was added to 44 ml of citric acid (0.1 M) and the final pH was adjusted to 5.6 by the dropwise addition of one of these solutions. The volume was made up to 100 ml with water and the solution was protected from the light. O-phenylenediamine (34 mg) was dissolved in the solution which was then kept in the dark. Hydrogen peroxide (40 µl) was added to the solution immediately before its use as the substrate.

2.11.2. Enzyme-linked Immunoadsorbent Assay (ELISA)

A double antibody sandwich ELISA method was used to measure the synthesis of complement components by cultured human endothelial cells, monocytes, skin fibroblasts, synovial fibroblasts and synovial membrane.

ELISA plates were coated with the IgG fraction of goat anti-human C1-INH (10 μg/ml), factor H (5 μg/ml), factor I (10 μg/ml), and the sheep anti-human C4-bp (2.5 μg/ml) were used.

The antibody (100 μl) diluted in coating buffer was added to the wells of an ELISA plate [Dynatech Immunolon 4 (C1-INH, H), Corning (C4-bp, I)] which were then covered with Saran-Wrap and incubated at 4 ^{o}C overnight.

The plates were washed five times with PBS containing 0.05 % (v/v) Tween, using a Dynatech plate washer; 250 μl of blocking buffer was added to each well. The plates were incubated in a moist atmosphere at room temperature for 1 hour.

The plates were washed five times with PBS and then 100 μl of culture supernatant was added to each well. In the case of synovial membrane culture supernatants, multiple dilutions were performed. The samples were assayed in duplicate.

A pool of human serum which contained known concentrations of complement components was used as a standard. This standard serum pool was diluted sequentially in PBS-Tween and 100 μl of each dilution was added to a series of wells. All standards were assayed in duplicate. The concentrations of complement component covered by each standard curve were as follows:

C1-INH : (60 ng/ml to 0.06 ng/ml)

C4-bp : (12.5 ng/ml to 0.01 ng/ml)

factor H: (30 ng/ml to 0.03 ng/ml)

factor I: (200 ng/ml to 0.19 ng/ml)

The negative controls were culture medium/FCS. The plates were incubated in a moist atmosphere at room temperature for 2 hours, before being washed 5 times with PBS-Tween. Biotin-conjugated antibody (100 µl) was added to each well. The concentrations of the conjugated antibodies were:

C1-INH : 1/5000

C4-bp : 1/3000

factor H: 1/4000

factor I: 1/500

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

The plates were washed again five times with PBS-Tween and 100 µl of avidin-linked horse radish peroxidase (HRP) were added to each well. The concentration of the avidin-HRP was 1/5000 for C1-INH, factor H, factor I and was 1/3000 for C4-bp. The plates were incubated in a moist atmosphere at room temperature for 1 h.

The plates were washed five times with PBS-Tween, 100 µl of peroxidase substrate (OPD-HCl 2 mg/ml in 0.1 M citrate phosphate pH 5.6) were added and the plates were left in the dark until the colour reaction had developed sufficiently. The reaction was stopped by the addition of 25 µl 4 N H_2SO_4 to each well. The intensity of the colour in each well was then measured on a microplate reader (Dynatech MR700).

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 that standard. The concentration of each complement component in the culture supernatants was determined by interpolation from the standard curve. Secretion rates for each component were determined from the difference in concentration between days seven and one.

2.12. IMMUNOHISTOCHEMICAL DETECTION OF MEMBRANE PROTEINS

2.12.1. Fixation of the Cells

Human peripheral blood monocytes, and human synovial fibroblasts were grown on glass slides in a petri dish under standard culture conditions (see sections 2.3.2. and 2.3.3.). The culture medium was removed and the slides were washed twice with PBS. Human peripheral lymphocytes slides were prepared by cytospin. The glass slides containing monocytes, lymphocytes, fibroblasts and the frozen section of synovial membrane were placed in a Coplin jar and fixed in acetone for 5 minutes. Following a further two washes with PBS, immunostaining was carried out as described in section 2.11.2.

2.12.2. Immunohistochemical Staining

2.12.2.1. *Immunoalkaline phosphatase method*

Normal swine serum (500 µl) diluted 1:5 in PBS was spread on slides and left at room temperature for 5 minutes. The excess serum was removed with paper tissue. The primary antibodies against DAF (IA10H6 monoclonal), MCP (E4.3 monoclonal), CD59 (YTH53.1 monoclonal), CR1 (E11 monoclonal) were added at 1:10, 1:50, 1:100, 1:1000 dilutions to the slides. They were incubated at room temperature for one hour in a moist atmosphere. The slides were again placed in a Coplin jar and washed with PBS at least 10 times over 30 minutes. Then they were placed in a staining rack and dried with tissue paper. Later alkaline phosphatase conjugated secondary antibodies were applied at a dilution of 1:15. The

slides were incubated at room temperature for an hour. They were washed in PBS 10 times over 30 minutes. The substrate [20 ml veronal acetate buffer (pH 9.2), 10 mg fast violet, 5 μg α-naphtol (dissolved in dimethyl formamide), 2.5 mg levamisol] was flooded over the slides and incubated for 5 minutes at room temperature. The slides were washed sequentially using three Coplin jars which contained 1) PBS, 2) tap water, and 3) buffered formalin before being rinsed in tap water. The slides were placed in a metal slide rack and dipped in haematoxylin for 15 seconds, rinsed with tap water and then rinsed in PBS as described above. The slides were mounted in aquamount and examined by light microscopy.

2.12.2.2. *Immunoperoxidase method*

The technique used was similar to that described for immunoalkaline phosphatase except that, as a secondary antibody, peroxidase-conjugated swine IgG was used at a dilution of 1:50. The substrate applied was diaminobenzine [450 ml Tris buffer (pH 7.4), 50 ml imidazole 0.1 M, 250 ml H_2O_2, 2 ml DAB solution (5 g DAB, 50 ml Tris buffer 0.05 M pH 7.4] and the slides were mounted in Histomount.

2.13. FLOW CYTOMETRIC ANALYSIS

1×10^6 cells were detached from the flasks by the cell scraper and washed twice in ice cold PBS/BSA (0.2 %) at 2000 rpm, at 4 ^{o}C. The pellet was resuspended in 1 ml of PBS/BSA and 100 µl of suspension was used for each experiment. The appropriate monoclonal antibody (5 µl) against DAF (CD55, BRIC110, IgG1 mouse), MCP (CD46, J4-48, IgG1, mouse), CD59 (BRIC 229, IgG2b mouse) and CR1 (CD35, E11, IgG1 mouse) was added to the each 100 µl of cell suspension and the mixture incubated on ice for 40 minutes. The mixture was washed twice with ice-cold PBS/BSA at 2000 rpm for 5 minutes at 4 ^{o}C. Anti-mouse IgG (100 µl FITC conjugated at 1/50 dilution) was added to the resuspended cell pellet which was incubated on ice for 30 minutes. Following two further washes with PBS/BSA, the pellet was resuspended in 500 µl PBS. The sample was then analysed by flow cytometry (Beckman FACS Analyser) which was connected to a CONSORT 30 computer with software designed to acquire, store and analyse the data.

CHAPTER 3. RESULTS

3.1. CHARACTERIZATION OF CELLS

Endothelial cells were isolated from human umbilical cord. Cells were cultured until fourth passage and then characterized. In culture, the cells formed a monolayer which had a typical cobblestone appearance (Figure 3.1). Endothelial cells were also examined by electron microscopy and Weibel-Palade bodies were detected (Figure 3.2). Weibel-Palade bodies are the rod-shaped organelles in endothelial cells. They containe granule membrane proteins-140 (GMP-140). They are important in the acute inflammatory response and coagulation. They facilitate the adhesion of leucocytes or platelets respectively (Lackie and More, 1992). They are an excellent marker for endothelial cells (Gimbrone et al., 1974; Zetter, 1981).

Fibroblasts were isolated from human synovial tissue and identified by their characteristic fusiform morphology under light microscopy (Figure 3.3.). Fibroblasts were also examined by immunohistochemistry. They were positively stained with anti-vimentin antibody (Figure 3.4) which is expressed in fibroblasts (Freshney, 1987).

Monocytes, isolated from the buffy coats of human blood donations, were examined by phase-contrast and light microscopy and were identified by their characteristic morphology (Figure 3.5) and by staining for non-specific esterase and phagocytosis of latex particles.

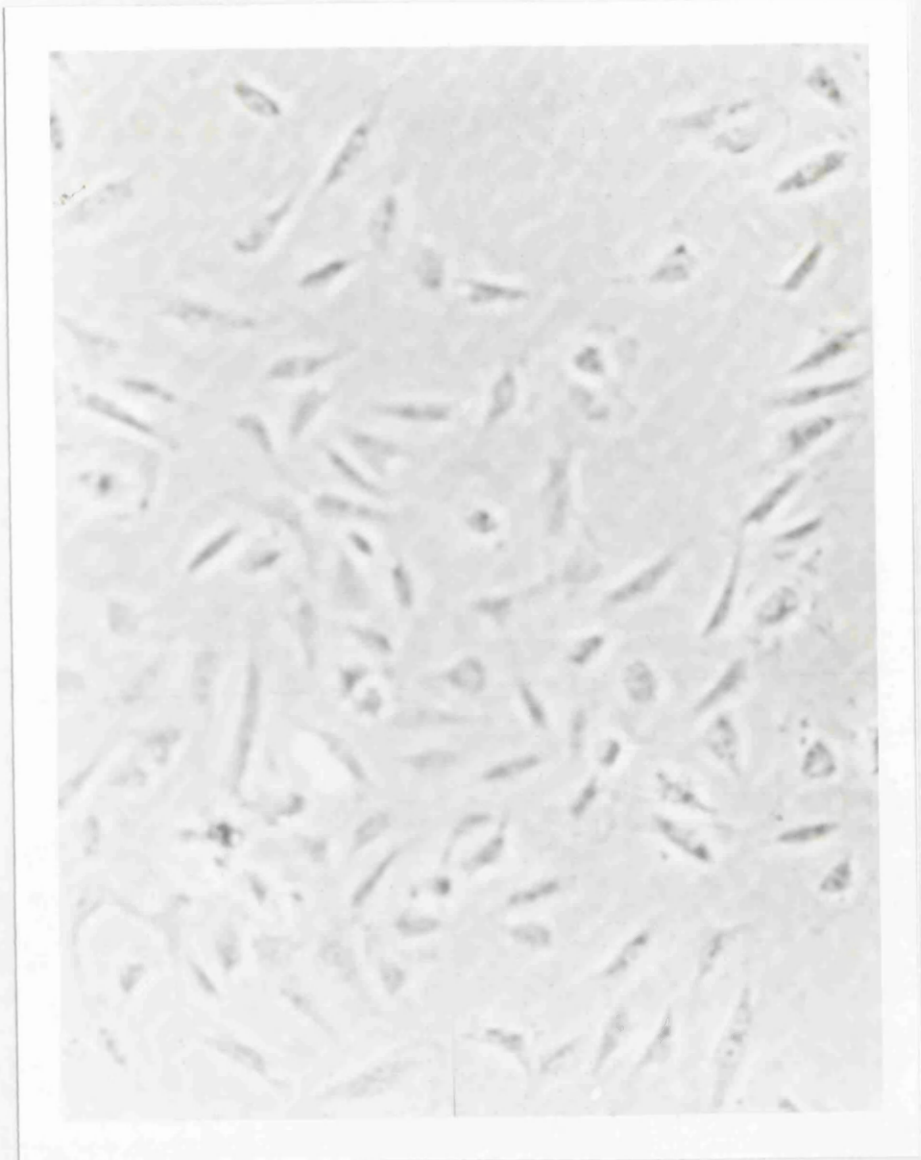


Figure 3.1: Characteristic cobblestone appearance of endothelial cells in culture by light microscopy (x 250).

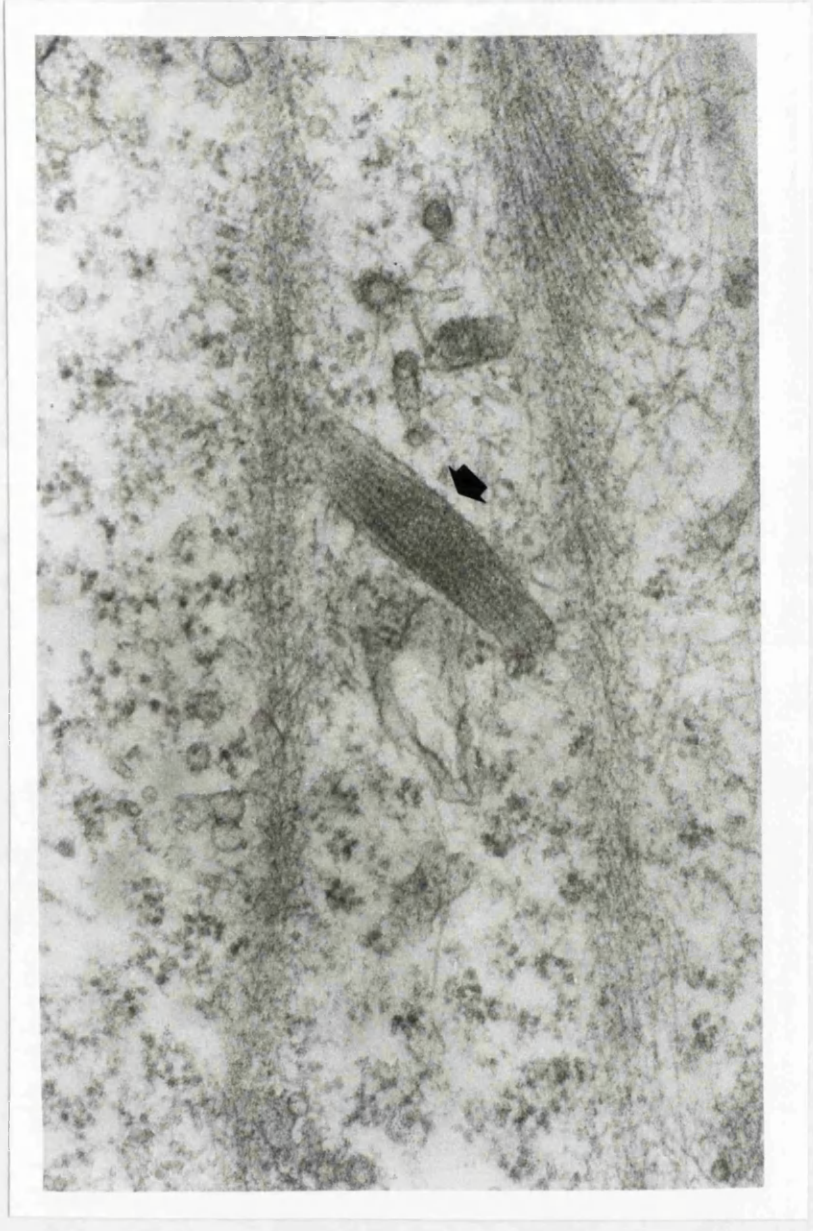


Figure 3.2: Endothelial cell analysis by electron microscopy (x 28080) Weibel-Palade body indicated with an arrow.

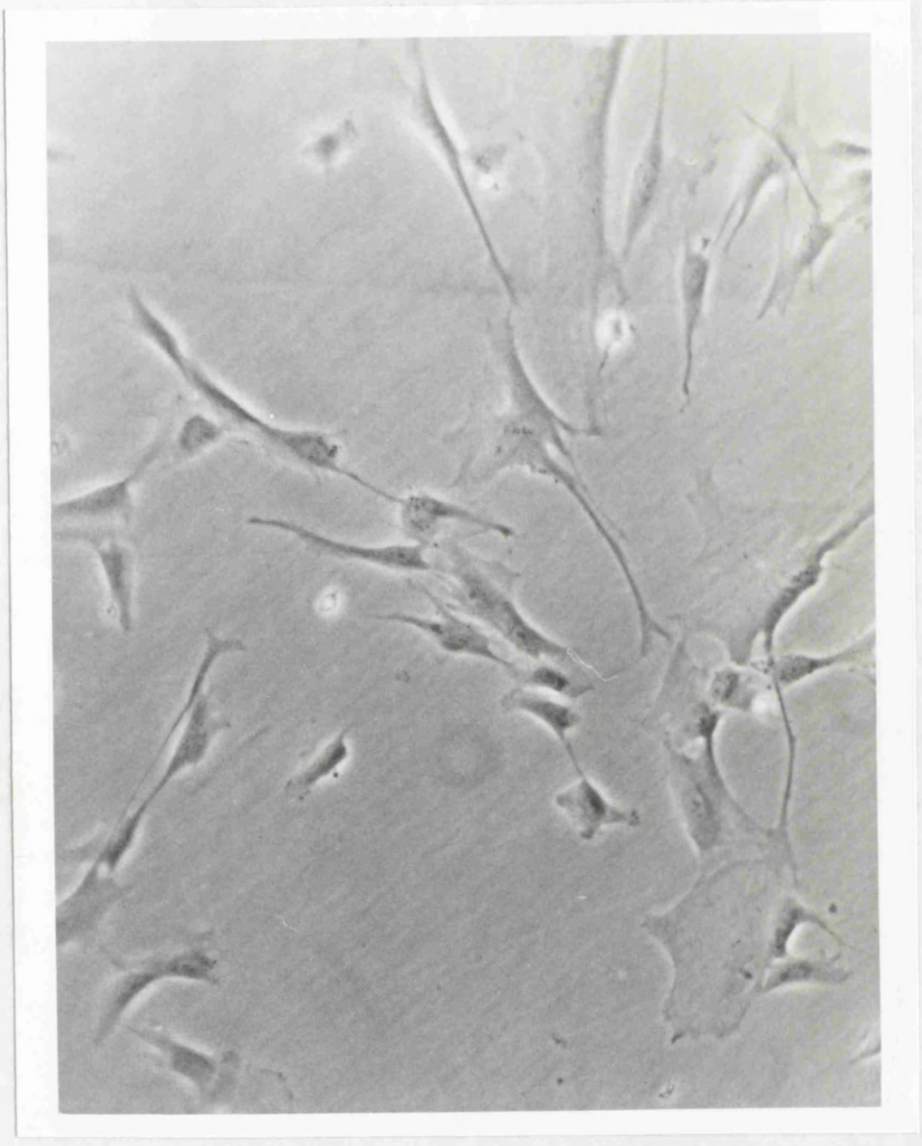


Figure 3.3: Characteristic fusiform morphology of fibroblasts in culture by light microscopy (x 250).

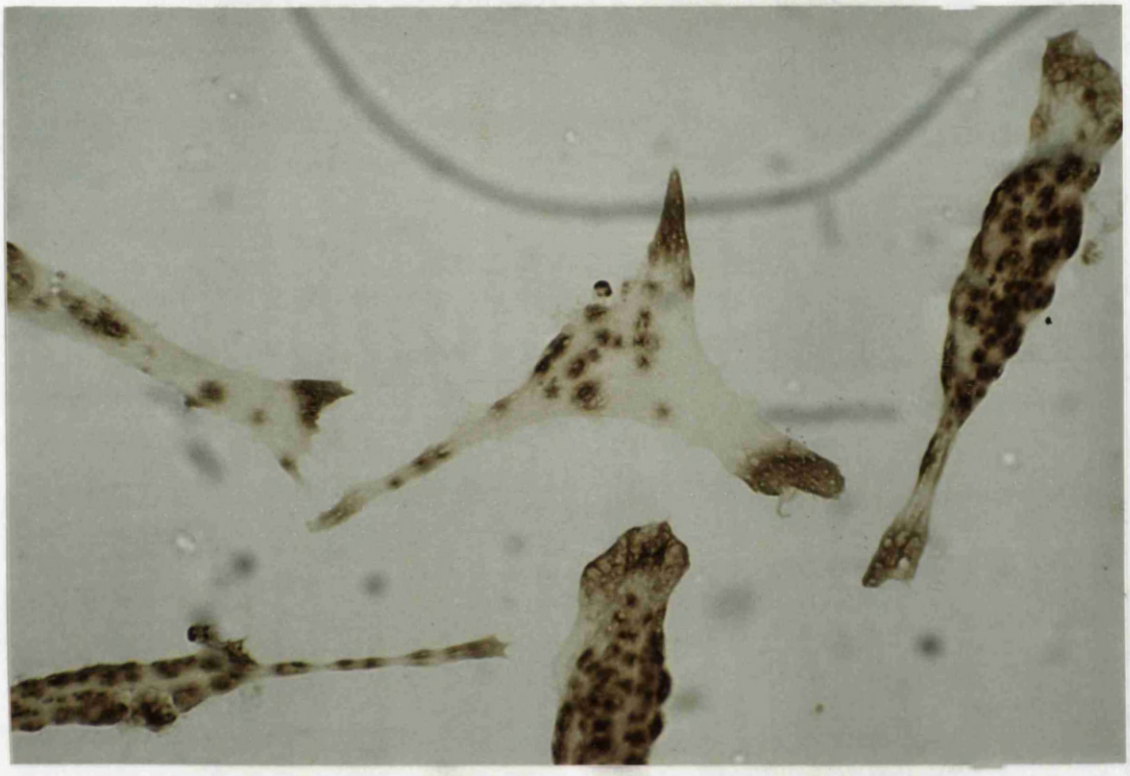


Figure 3.4: Immunohistochemical characterization of fibroblasts. Cells were stained by immunoperoxidase method. Monoclonal antibody to human vimentin was used at 1/50 dilution. Vimentin localized on fibroblasts (brown colour) (x 250).

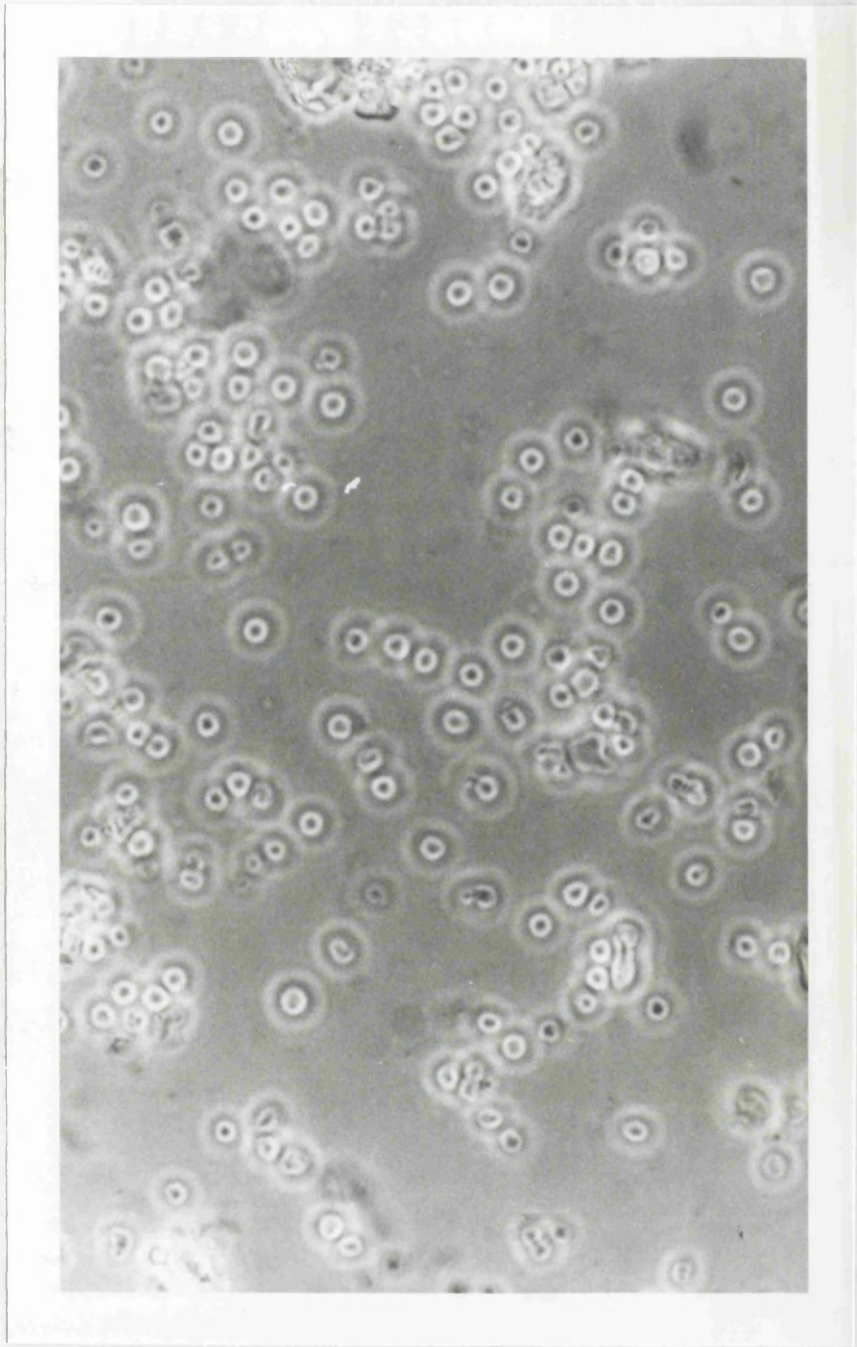


Figure 3.5: Peripheral blood monocytes in culture under light microscopy (x 250).

3.2. PREPARATION OF CLONED HYBRIDIZATION PROBES

A human CI-INH cDNA had been cloned into the *Puv*II site of plasmid pAT153/*Puv*II/8 by Bock et al. (1986). Digestion of the recombinant plasmid with *Bam*HI and *Hind*III excised a 1.25 kb fragment (Figure 3.6 a, lanes 3 and 4) containing 1250 bp of CI-INH cDNA corresponding to the 3' end of the C1-INH gene.

A human C4-bp cDNA had been cloned into the *Pvu*II site of the vector pAT153/*Puv*II/8 by Chung et al. (1985). The cDNA of C4-bp was amplified by PCR (see section 2.6.2.). The size of this cDNA was 1.6 kb (Figure 3.6 b). Clone pBP8 contains the cDNA between the codon for the 60^{th} amino acids of C4-bp and the 85^{th} nucleotide in the 3' untranslated region of the C4-bp gene.

A human factor H cDNA had been cloned into the *Pvu*II site of the vector pAT153/*Puv*II/8 by Ripoche et al. (1988). The cDNA of factor H was amplified by PCR (see section 2.6.2.). The size of this cDNA was 1.6 kb (Figure 3.6 b) containing 1644 bp of factor H cDNA corresponding to the N-terminus one third of factor H.

A human factor I cDNA had been cloned into the *Puv*II site of plasmid pAT153/*Puv*II/8 by Catteral et al. (1987). Digestion of the recombinant plasmid with *Eco*RI excised a 2.0 kb fragment (Figure 3.6 d) containing 2000 bp of factor I cDNA which corresponded to the full-length of factor I.

The cDNA of S protein was purchased from Bioquote Ltd. S protein cDNA was amplified by PCR (see section 2.6.2.). The size of the cDNA was 1.0 kb which corresponded to the 951 bp of the coding region plus a portion of the 3' untranslated region S protein (Figure 3.7 a).

A human SP-40,40 cDNA had been cloned into the multiple cloning site of plasmid pUC19 by Kirszbaum et al. (1989). Digestion of the

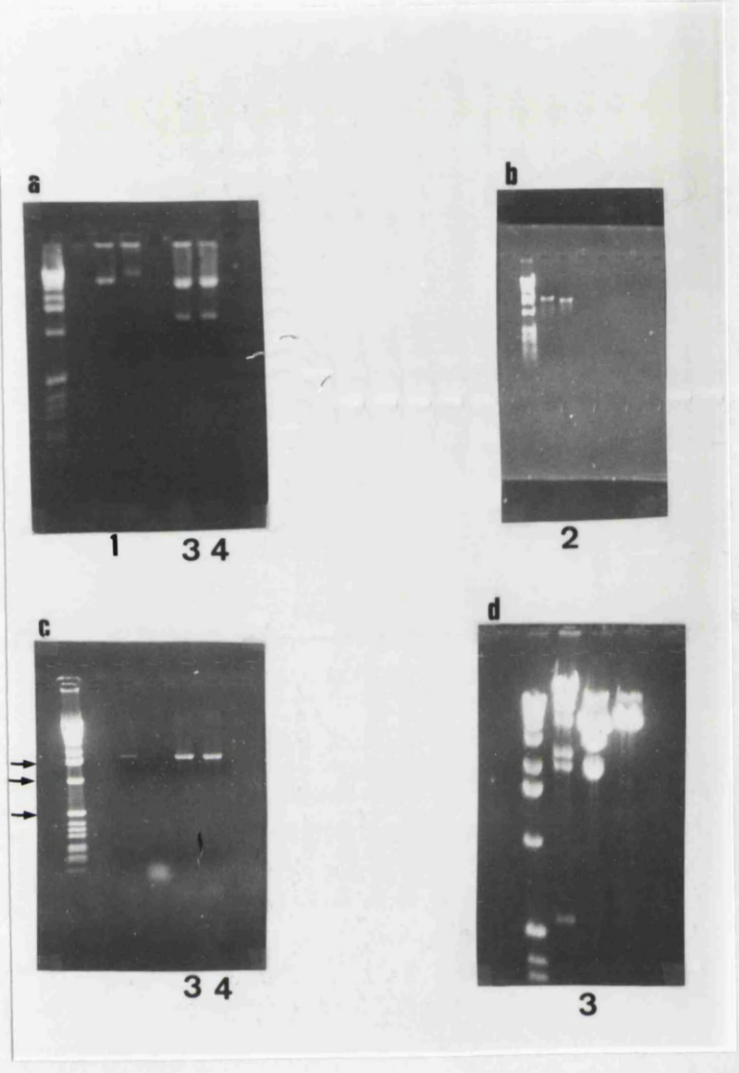


Figure 3.6: Analyses of cloned cDNAs.

a) Restriction digests of C1-INH by *Bam*HI + *Hind*III and of CR1 by Eco RI resulted in inserts of 1.25 kb (lanes 3 and 4), and 0.8 kb (lane 1) respectively. Remaining lanes are irrelevant in this experiment.

b) C4-bp was amplified by PCR; the size of the cDNA was 1.6 kb (lane 2).

c) Factor H was amplified by PCR; the size of the cDNA was 1.6 kb (lanes 3 and 4).

d) Restriction digest of factor I by *Eco*RI resulted an insert of 2.0 kb (lane 3).

recombinant plasmid with *Eco*RI excised a 0.78 kb fragment (Figure 3.7b) containing 780 bp of SP-40,40 cDNA corresponding to the 3' end of the SP-40,40 gene.

A human decay accelerating factor (DAF) cDNA had been cloned into the *Eco*RI site of the plasmid pGEM1 by Medof et al. (1987). The cDNA of DAF was amplified by PCR (see section 2.6.2.). The size of the cDNA was 1.9 kb (Figure 3.7 c) containing 1900 bp of DAF cDNA corresponding to the coding region (except the first 3 amino acid in N-terminal of DAF) and 3' untranslated region of DAF gene.

A human membrane cofactor protein (MCP) cDNA had been cloned into the multiple cloning site of plasmid pUC19 by Lublin et al. (1988). Digestion of the recombinant plasmid with *Eco*RI excised a 1.6 kb fragment (Figure 3.7 d, lane 4) containing 1546 bp of MCP cDNA corresponding to the full-length of MCP.

A human homologous restriction factor 20 (CD59) cDNA had been ligated into plasmid CDM8 by Davies et al. (1989). Digestion of the recombinant plasmid with *Xba*I excised a 1.2 kb fragment (Figure 3.7 e, all lanes) containing 1154 bp of CD59 cDNA corresponding to 1.4 kb mRNA species of CD59.

A human complement receptor 1 (CR1) cDNA had been cloned into the *Eco*RI site of plasmid pBR 327 by Wong et al. (1985). Digestion of the recombinant plasmid with *Eco*RI excised a 0.8 kb fragment (Figure 3.6 a, lane 1) containing 800 bp of CR1 cDNA corresponding to the 5' end of CR1 gene.

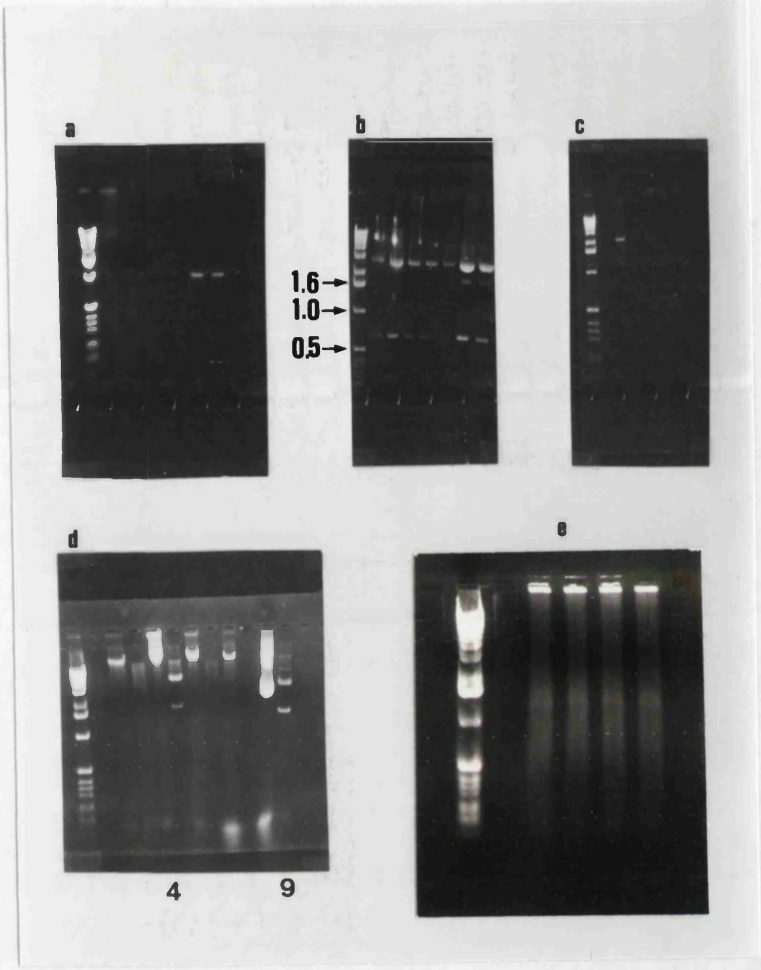


Figure 3.7: Analyses of cloned cDNAs.

a) S protein was amplified by PCR; the size of the cDNA was 1.0 kb.

b) Restriction digest of SP-40,40 by *Eco*RI resulted an inserts of 789 bp (all lanes).

c) DAF was amplified by PCR; the size of the cDNA was 1.9 kb.

d) Restriction digest of MCP by *Eco*RI resulted an inserts of 1.6 kb (lane 9).

e) Restriction digest of CD59 by *Xba*I resulted an insert of 1.2 kb (all lanes).

3.3. SYNTHESIS OF COMPLEMENT REGULATORY PROTEINS BY SYNOVIAL MEMBRANE

3.3.1. Analysis of Secreted Regulatory Proteins by ELISA.

Fragments of synovial membranes from RA and OA patients and from normal individuals were cultured for seven days as described in section 2.2.6. Culture supernatants were collected at days 1, 3, 5 and 7. The samples were assayed for the complement components C1-INH, C4-bp, factor H and factor I by ELISA. The results from three separate experiments are summarized in Figures 3.8 and 3.9, and the secretion rates (calculated as molecule/10 mg tissue/min) in Table 3.1. They show that, there was great variation between experiments and, that all the components measured accumulated rapidly in the culture medium during the first and often the second day of culture. Despite washing the synovial membrane pieces five times prior to culture, this pattern of component accumulation persisted. Because I was unable to inhibit totally or consistantly the accumulation of these components by the addition of cycloheximide (2.5 μg/ml) to the culture media, one must conclude that part of each component measured was derived from plasma contamination of the synovial membrane fragments. However, the partial inhibition observed in some cultures implies that a proportion of the components measured in the supernatants had been synthesized locally within the synovial membrane.

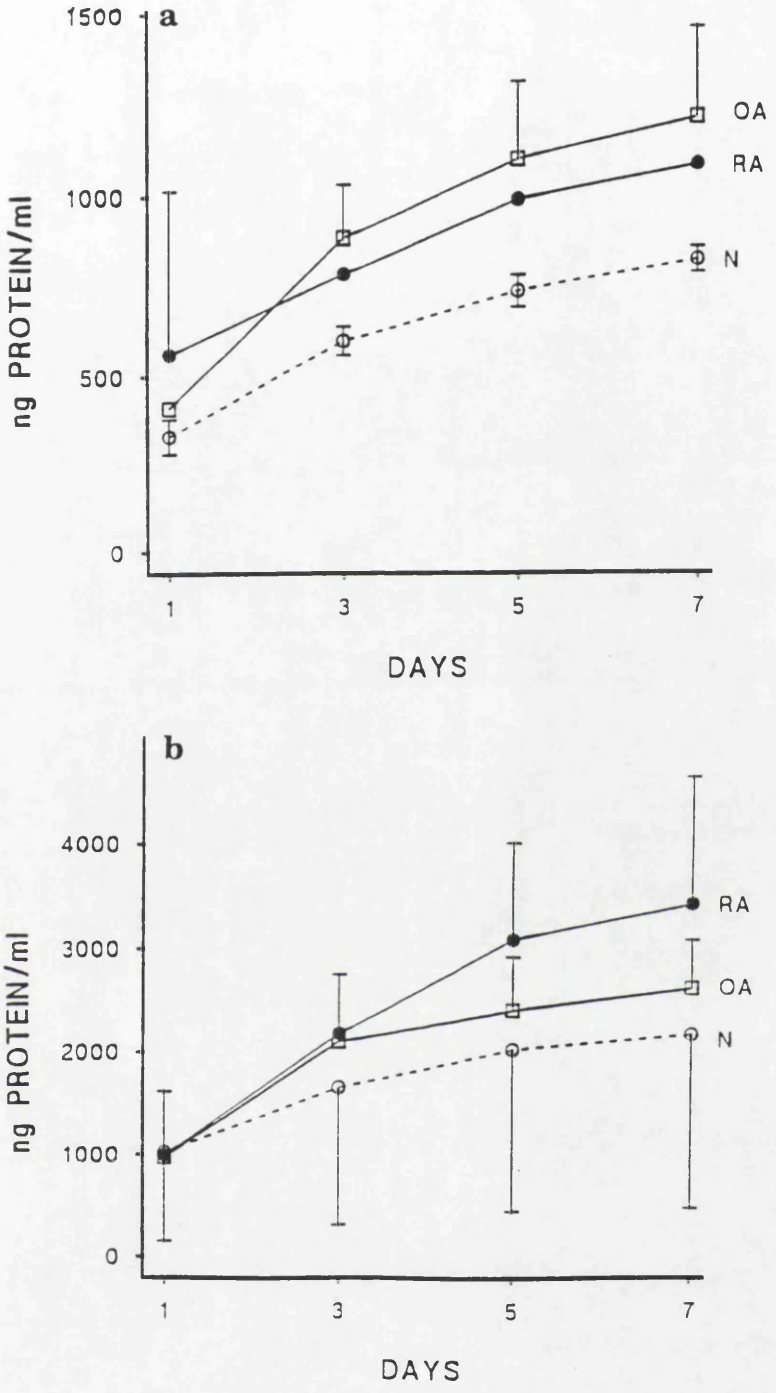


Figure 3.8: ELISA analyses for **a)** C1-INH and **b)** C4-bp secreted by synovial membrane in tissue culture from normal (N) individuals, osteoarthritis (OA) and rheumatoid arthritis (RA) patients. The culture medium was removed completely at day 1, 3, 5 and 7. The amounts of C1-INH and C4-bp secreted into the culture medium at the indicated days was measured. After seven days in culture, the results were plotted as cumulative synthesis. Each point represents the mean with the standard deviation of three experiments.

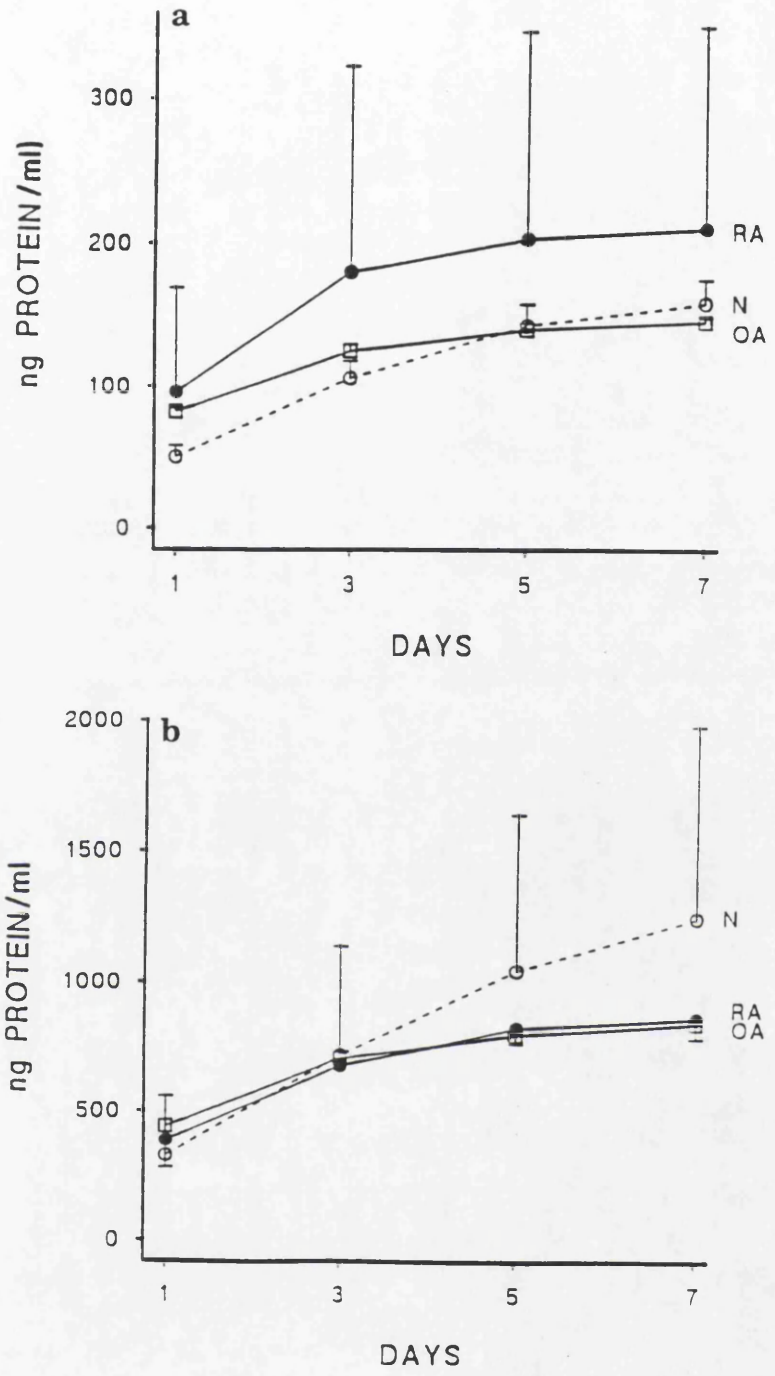


Figure 3.9: ELISA analyses for **a)** factor I and **b)** factor H secreted by synovial membrane in tissue culture from normal (N), osteoarthritis (OA) and rheumatoid arthritis (RA) patients. The culture medium was removed completely at day 1, 3, 5 and 7. The amounts of factors H and I secreted into the culture medium at the indicated days was measured. After seven days in culture, the results were plotted as cumulative synthesis. Each point represents the mean with the standard deviation of three experiments.

TISSUE	C1-INH	H	I	C4-bp
N	1654 ± 1239	1252 ± 390	398 ± 275	1119 ± 1065
OA	1427 ± 2	554 ± 198	130 ± 2	351 ± 249
RA	1517 ± 989	842 ± 405	435 ± 286	1251 ± 639

Table 3.1: Secretion rates of the regulatory fluid-phase complement components C1-INH, factor H (H), factor I (I) and C4-bp expressed as molecule/10 mg tissue/min in normal (N), osteoarthritis (OA) and rheumatoid arthritis (RA) synovial membrane. Figures are the mean ± SD of three separate experiments.

3.3.2. Immunohistochemical Analysis of Membrane Regulatory Proteins.

The presence of membrane regulatory proteins of the complement system are investigated by immunohistochemistry. Frozen sections of synovial membrane obtained from the operation of rheumatoid arthritis patients were stained with monoclonal antibodies against DAF (IA10H6) (Fig 3.10 b), MCP (E4.3) and CD59 (YTH53.1) (Figure 3.11 a and b), CR1 (E11) and MIP (anti-MIP) (Figure 3.12 a and b). DAF, MIP and CD59 were expressed on most cells, particularly on cells lining the synovium, endothelial cells and lymphocytes. MCP was expressed very weakly compared to other components examined. CR1 was only expressed on macrophages and lymphocytes.

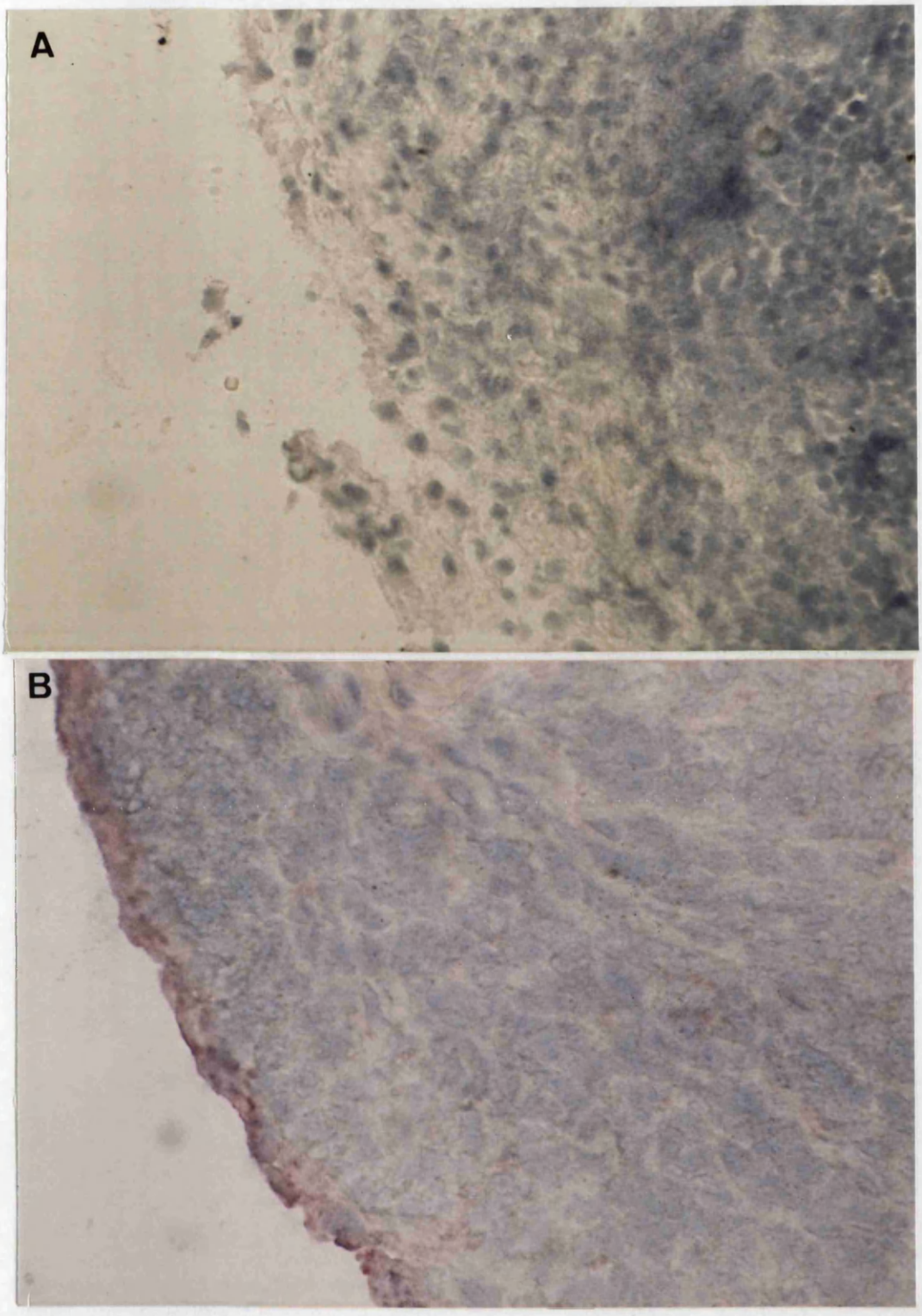


Figure 3.10: Immunohistochemical analyses of RA synovial membrane. Frozen sections were stained by the alkaline phosphatase method. **a)** Mouse IgG was used at 1/80 dilution as control. **b)** Monoclonal antibody to human DAF (IA10H6) was used at 1/80 dilution. DAF localized on cells lining the synovium (red colour) (x 250).

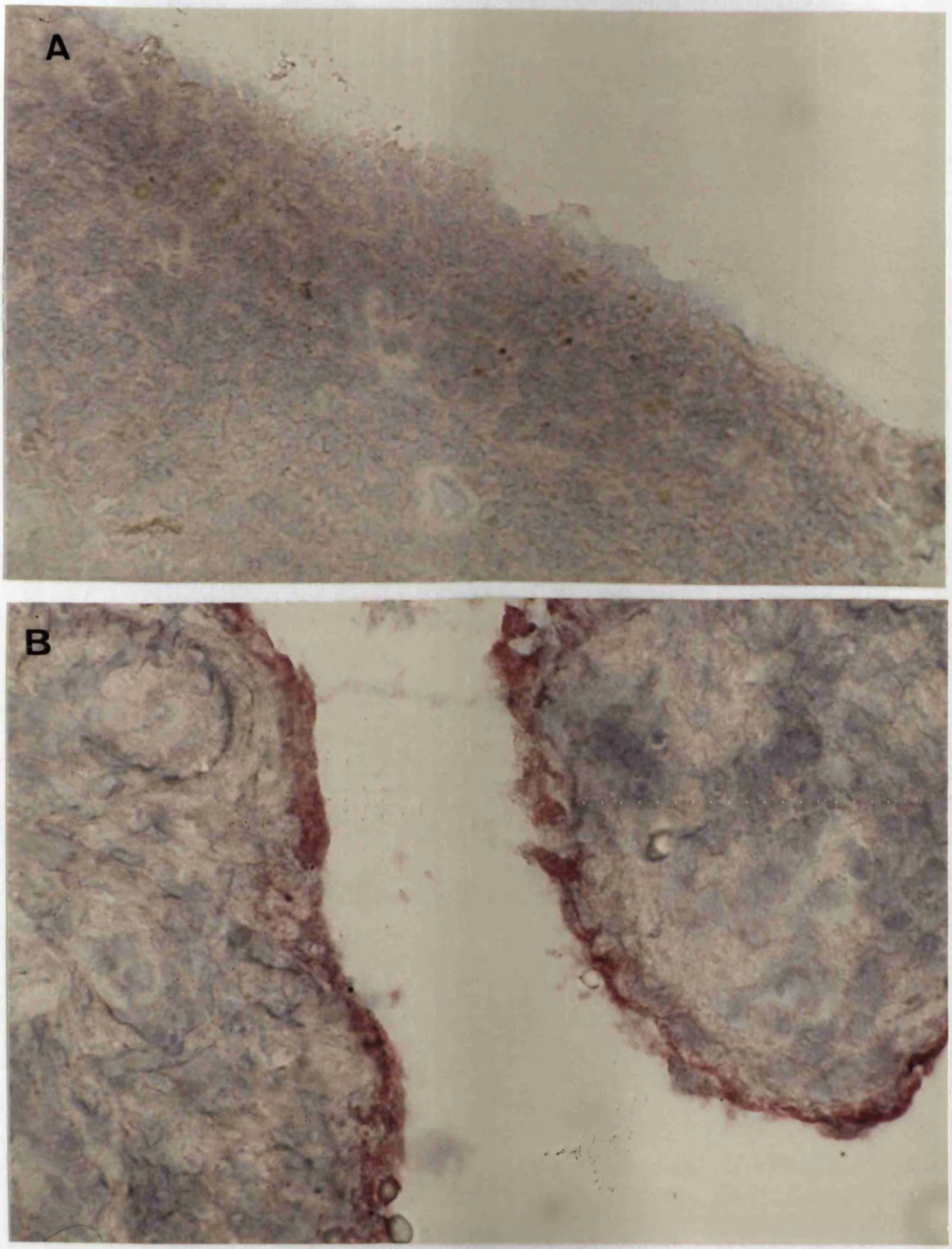


Figure 3.11: Immunohistochemical analyses of RA synovial membrane. Frozen sections were stained by the alkaline phosphatase method. **a)** Monoclonal antibody to human MCP (E4.3) was used at 1/80 dilution. MCP staining was very weak compared to the other antibodies used (pink colour). **b)** Monoclonal antibody to human CD59 (YTH53.1) was used at 1/80 dilution. CD59 localized on cells lining the synovium (red colour) (x 250).

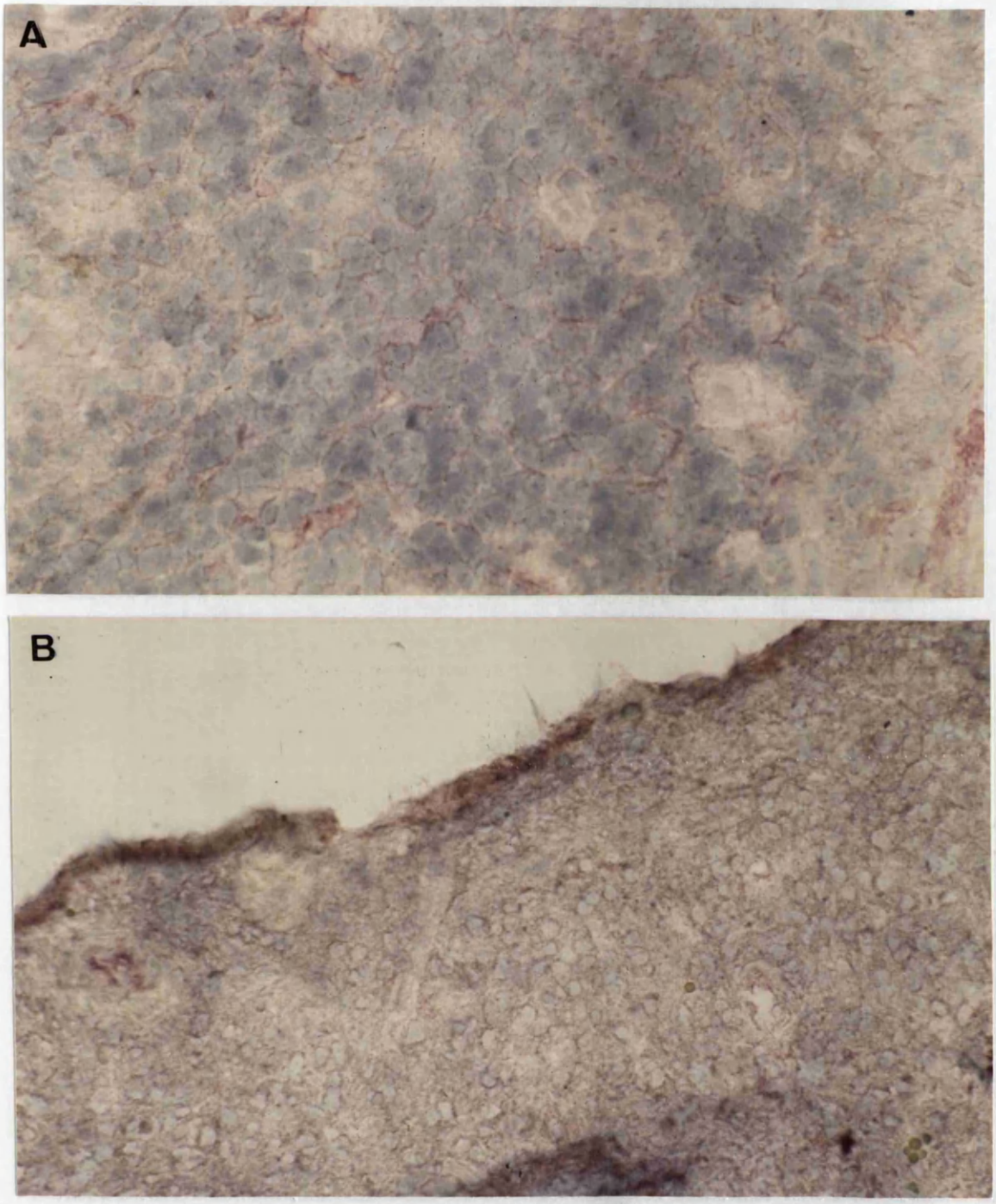


Figure 3.12: Immunohistochemical analyses of RA synovial nembrane. Frozen sections were stained by the alkaline phosphatase nethod. **a)** Monoclonal antibody to human CR1 (E11) was used at 1/80 diution (red colour). **b)** Monoclonal antibody to human MIP (anti-MIP) wis used at 1/80 dilution. MIP localized on cells lining the synovium (red colour) (x 250).

3.3.3. Abundance of mRNAs Encoding Regulatory Complement Proteins.

Since a large proportion of the complement regulatory proteins detected by ELISA (C1-INH, C4-bp, factor H and factor I) or by immunohistochemical analysis (DAF, MCP, CD59, CR1 and MIP) could have been the result of contamination of the synovial membrane fragments with plasma proteins, the tissues were analyzed for the presence of the mRNAs encoding each of these proteins. Total RNA, extracted from synovial membranes from RA and OA patients and normal individuals was analyzed by doubling-dilution dot blots hybridized with ^{32}P-labelled probes for each of the mRNAs (C1-INH, C4-bp, factor H, factor I, S Protein, SP-40,40, DAF, MCP, CD59, and CR1). The dot-blots were stripped and rehybridized with a probe for 28S rRNA to allow corrections for unequal loading to be made. The results are summarized in Figures 3.13, 3.14 and 3.15; the abundances of the mRNAs relative to those in normal synovium and corrected for variations in loading are summarized in Table 3.2. The data suggest that the abundance of the mRNAs encoding the secreted proteins is elevated in RA synovium, as is that of CD59 mRNA. Only S protein mRNA is elevated in OA synovium, whereas DAF and MCP may be reduced in abundance in both RA and OA synovium. However, these data must be interpreted with caution because the tissue samples available were small and they may not reflect the mRNA abundance in whole synovial membrane. Also, severe limitations in the supply of tissue samples precluded confirmation of the differences noted by repeated assays.

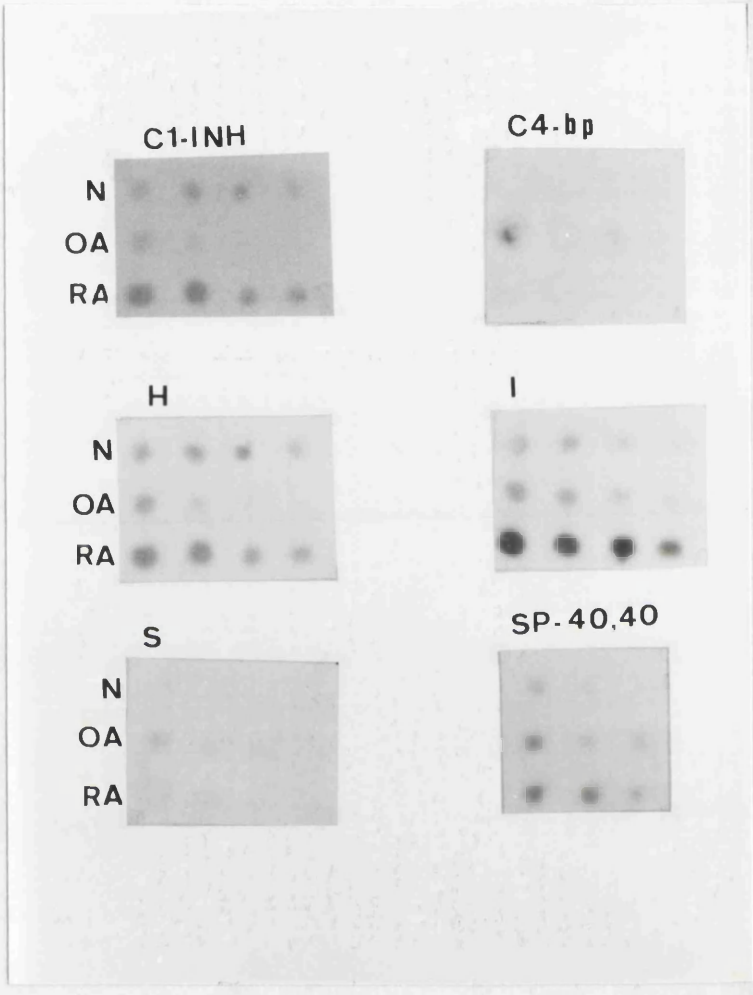


Figure 3.13: Dot blot analyses of RNA obtained from synovial membranes from normal (N) individuals, osteoarthritis (OA) and rheumatoid arthritis (RA) patients. The dot blots (5, 2.5, 1.25 and 0.62 µg) were hybridized with probes for C1-INH, C4-bp, factor H (H), factor I (I), S protein (S) and SP-40,40 mRNAs.

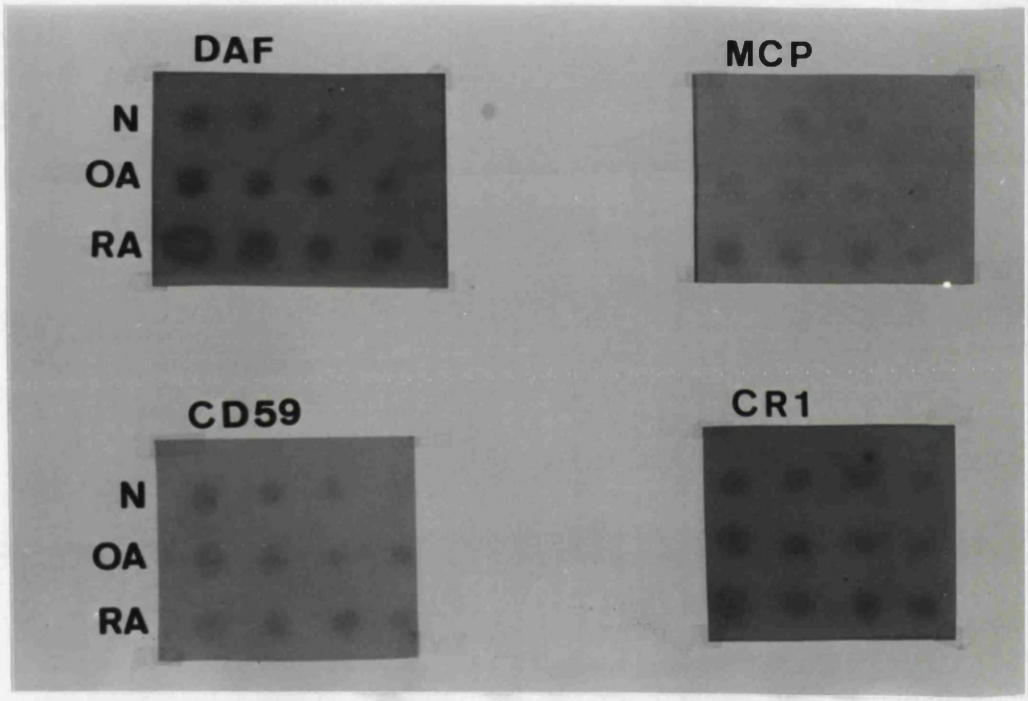


Figure 3.14: Dot blot analyses of RNA obtained from synovial membranes form normal (N) individuals, osteoarthritis (OA) and rheumatoid arthritis (RA) patients. The dot blots (5, 2.5, 1.25 and 0.62 μg) were hybridized with probes for DAF, MCP, CD59 and CR1 mRNAs.

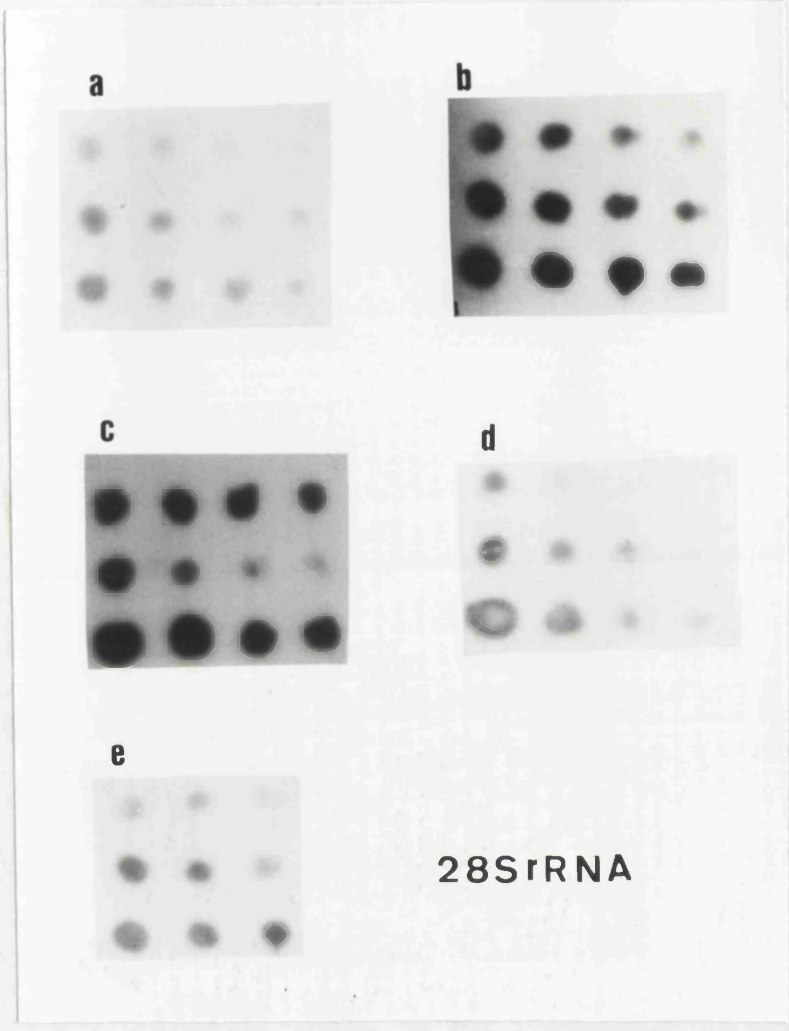


Figure 3.15: Rehybridization of the dot-blots shown in figures 3.13 and 3.14 with a probe for 28S rRNA. **a)** Dot blot used for MCP, CD59 and CR2, **b)** factor I, **c)** C1-INH and factor H, **d)** C4-bp, S protein and DAF, **e)** SP-40,40.

COMPONENTS	N	OA	RA
C1-INH*	1.0	1.8	4.4
Factor H*	1.0	1.0	2.0
Factor I*	1.0	0.7	4.3
C4-bp*	1.0	1.1	3.0
S Protein*	1.0	3.2	1.5
SP-40,40*	1.0	0.3	0.9
CD59*	1.0	0.5	2.4
DAF	1.0 ± 1.0	0.4 ± 0.3	0.6 ± 0.004
MCP	1.0 ± 0.03	0.8 ± 0.1	0.8 ± 0.2
CR1	1.0 ± 0.5	1.0 ± 0.2	0.9 ± 0.2

Table 3.2. Relative abundances of mRNAs for regulatory complement components in normal (N), osteoarthritis (OA) and rheumatoid arthritis (RA) synovial membranes. The abundance of each mRNA in normal synovial membrane was given an arbitrary value of 1 unit. Data for DAF, MCP and CR1 are the mean ± SD of three experiments.

* Data from one set of observation.

Various types of cells such as fibroblasts, endothelial cells, macrophages, adipocytes, macrophage-like (type A) and fibroblast-like (type B) synoviocytes are present in the synovial membrane. Detection of the mRNAs encoding the whole battery of regulatory complement proteins in synovial membrane raised the question of which types of cells are expressing which complement regulatory proteins in synovial membrane. Human synovial fibroblasts, endothelial cells, monocytes and lymphocytes were investigated to find an answer to this question.

3.4. SYNTHESIS OF COMPLEMENT REGULATORY PROTEINS BY SYNOVIAL FIBROBLASTS

3.4.1. Analysis of Secreted Regulatory Proteins by ELISA.

Fibroblasts isolated from synovial membranes from RA and OA patients and from normal individuals were cultured for seven days as described in section 2.3.4. Culture supernatants were collected at days 1, 3, 5 and 7. The samples were assayed for the complement components C1-INH, C4-bp, factor H and factor I by ELISA. The results from three separate experiments are summarized in Figures 3.16 and 3.17, and the secretion rates (calculated as molecule/cell/min) in Table 3.3.

C1-INH and factor H were detected in the culture supernatants from synovial fibroblasts isolated from all RA and OA patients and normal individuals. Although factor I was detectable in all normal fibroblast cell culture supernatants, only one RA and one OA fibroblast culture showed detectable levels of factor I. C4-bp was not detectable in any type of fibroblast cell culture supernatants. The secretion of proteins was inhibited approximately 80 % by the addition of cycloheximide (2.5 μg/ml) in the culture medium (data not shown). This result shows that the accumulation of complement regulatory proteins in the culture supernatants was due to *de novo* synthesis and not to secretion of preformed protein.

The ELISA data indicate that fibroblasts from the synovial membrane of RA and OA patients, and from normal individuals, are capable of synthesizing and secreting C1-INH, factor H and factor I *in vitro*. The amounts of C1-INH, factor I and factor H secreted increased linearly throughout the seven day culture period. Statistical analysis by the Mann-Whitney ranking procedure showed significant differences in

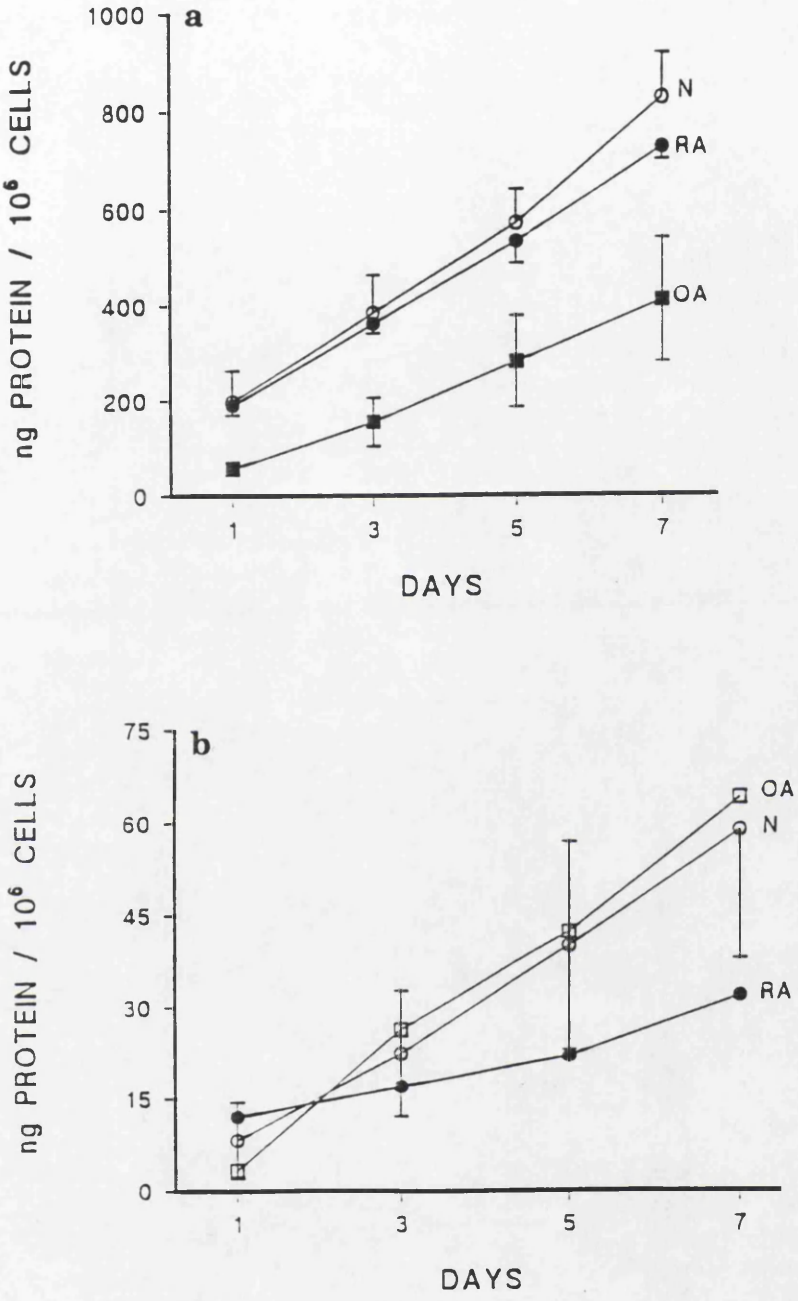


Figure 3.16: ELISA analyses for **a)** C1-INH and **b)** factor I secreted by synovial fibroblasts in culture from normal (N), osteoarthritis (OA) and rheumatoid arthritis (RA) patients. The culture medium was removed completely at day 1, 3, 5 and 7. The amount of C1-INH and factor I secreted into the culture medium at the indicated days was measured. After seven days in culture, the results were plotted as cumulative synthesis (ng/10^6 cells). Each point represents the mean with the standard deviation of three experiments.

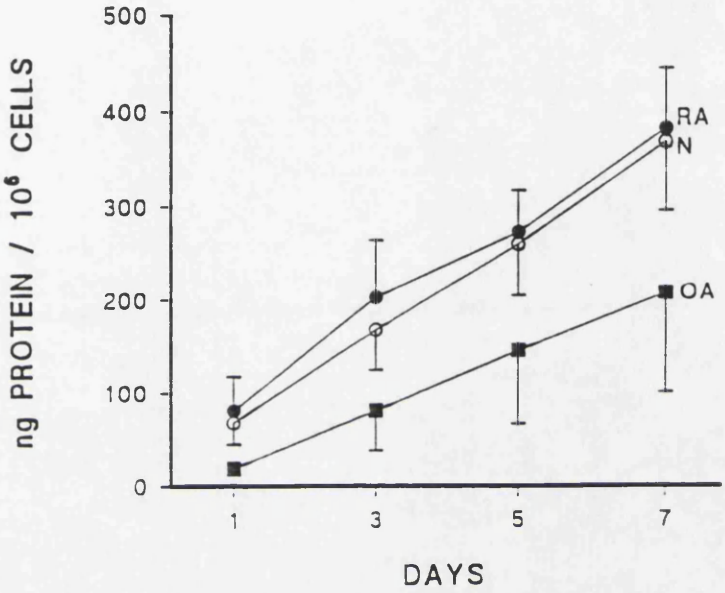


Figure 3.17: ELISA analyses for factor H secreted by synovial membrane fibroblast in culture from normal (N), osteoarthritis (OA) and rheumatoid arthritis (RA) patients. The culture medium was removed completely at day 1, 3, 5 and 7. The amount of factor H secreted into the culture medium at the indicated days was measured. After seven days in culture, the results were plotted as cumulative synthesis (ng/10^6 cells). Each point represents the mean with the standard deviation of three experiments.

CELL TYPE	C1-INH	H	I	C4-bp
HUVEC	0	74 ± 12	40 ± 12	0
MONOCYTE	109 ± 18	65 ± 6	0	23 ± 0.3
N (F)	418 ± 80	140 ± 22	39 ± 10	0
OA (F)	233 ± 80 *	79 ± 50	16 ± 27	0
RA (F)	357 ± 14	143 ± 19	6 ± 10 *	0

Table 3.3: Secretion rates of regulatory fluid-phase complement components C1-INH, factor H (H), factor I (I) and C4-bp expressed as molecule/cell/min in human umbilical cord endothelial cells (HUVEC), monocytes, normal (N), osteoarthritis (OA) and rheumatoid arthritis (RA) synovial fibroblasts (F). Each point represents the mean ± SD of three experiments. * indicates significant difference ($P < 0.05$) when compared to corresponding normal (F) values.

secretion rates of factor I between normal and RA fibroblasts ($p<0.01$), and of C1-INH between normal and OA fibroblasts ($p<0.04$). The other regulatory complement components was not significantly different in RA, OA and normal fibroblasts.

3.4.2. Immunohistochemical Analysis of Membrane Regulatory Proteins.

The presence of membrane regulatory proteins was investigated by immunohistochemical staining. Fibroblasts isolated from synovium from normal individuals were grown on glass slides under standard culture conditions as described in section 2.3.4. Cells were fixed by acetone (section 2.11.1.) and stained with monoclonal antibodies against DAF (IA10H6) (Figure 3.18. b), MCP (E4.3) and CD59 (YTH53.1) (Figure 3.19. a and b), MIP (anti-MIP) and CR1 (E11) (Figure 3.20. a and b) by the immunoperoxidase method. The staining for DAF and CD59 was strong while staining for MIP and MCP was weak. CR1 was not detected on fibroblasts.

3.4.3. Abundances of mRNAs Encoding Regulatory Complement Proteins.

A large proportion of regulatory complement proteins (except C4-bp and CR1) were detected in synovial fibroblasts. Fibroblasts were analyzed for the presence of the mRNAs encoding each of these proteins. Total RNA extracted from synovial fibroblasts from RA and OA patients and normal individuals was analyzed by Northern blots hybridized with ^{32}P-labelled probes for each of the mRNAs (C1-INH, C4-bp, factor H, factor I, S Protein, SP-40,40, DAF, MCP, CD59, and CR1). As single

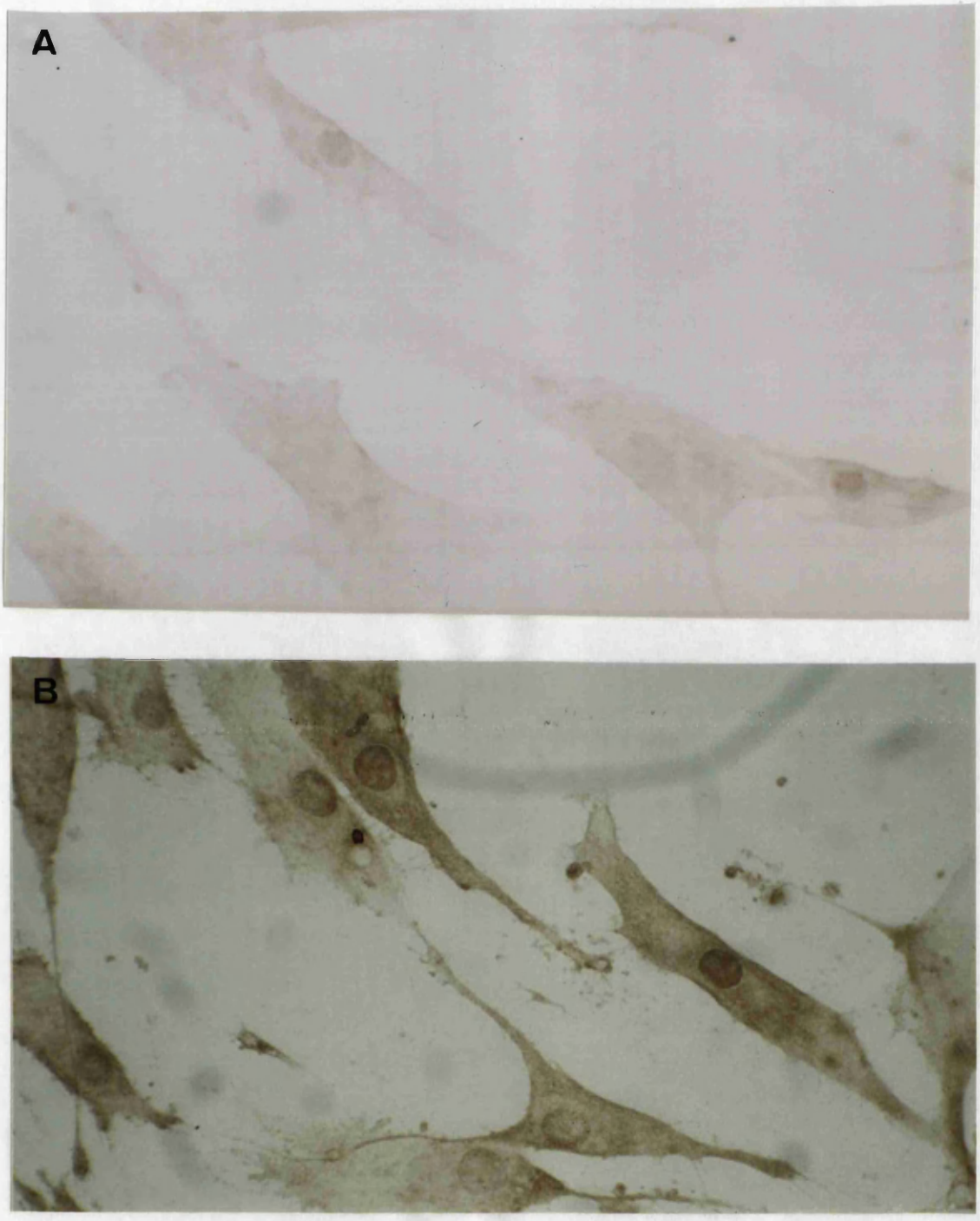


Figure 3.18: Immunohistochemical analyses of normal synovial fibroblasts. Cells grown on slides were stained by the immunoperoxidase method. **a)** Mouse IgG was used at 1/100 dilution as control. **b)** Monoclonal antibody to human DAF (IA10H6) was used at 1/100 dilution. DAF localized on synovial fibroblasts (brown colour) (x 250).

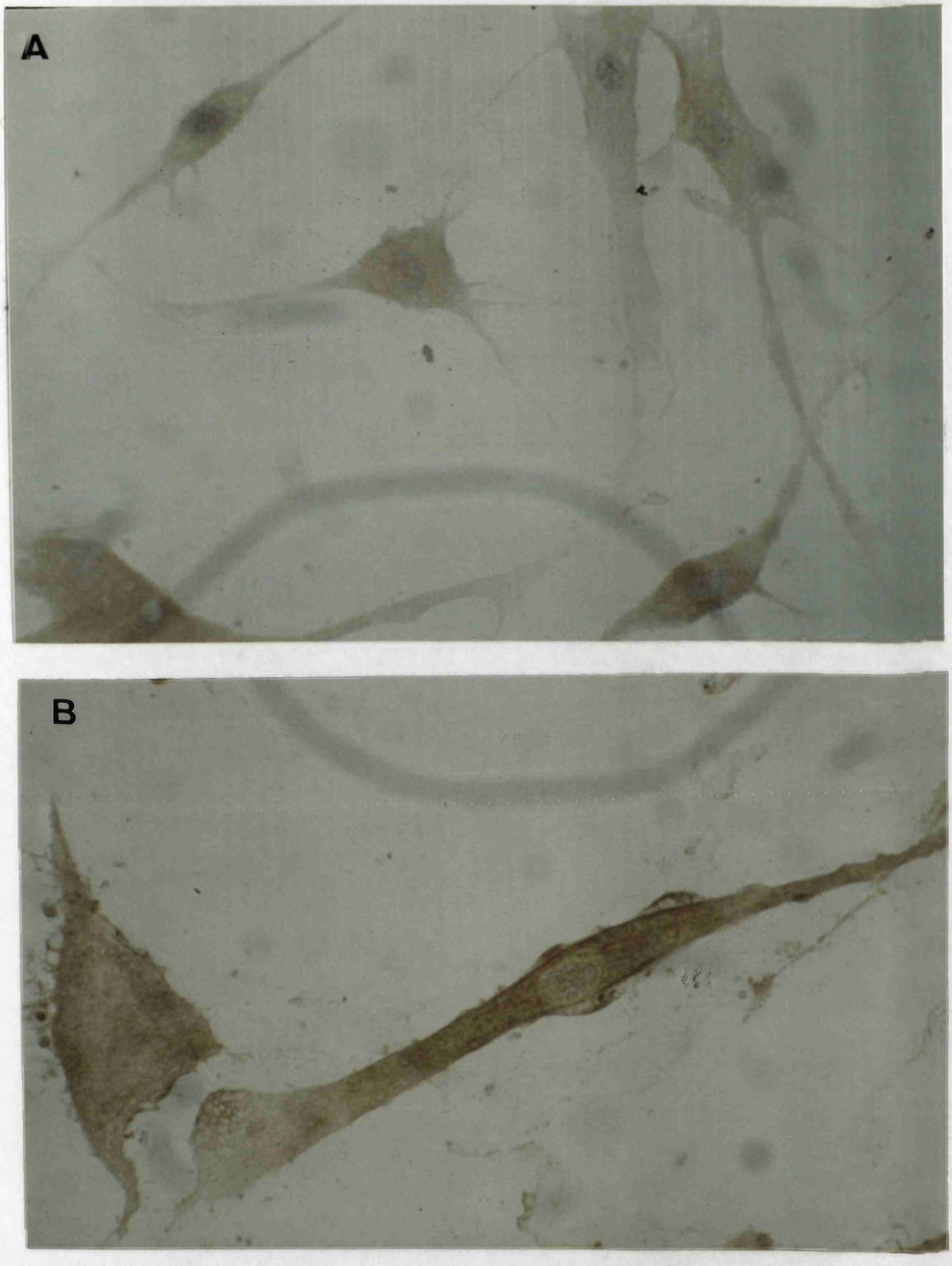


Figure 3.19: Immunohistochemical analyses of normal synovial fibroblasts. Cells grown on slides were stained by the immunoperoxidase method. **a)** Monoclonal antibody to human MCP (E4.3) was used at 1/100 dilution. MCP staining was very weak compared to the other antibodies used (brown colour). **b)** Monoclonal antibody to human CD59 (YTI53.1) was used at 1/100 dilution. CD59 localized on synovial fibroblasts (brown colour) (x 250).

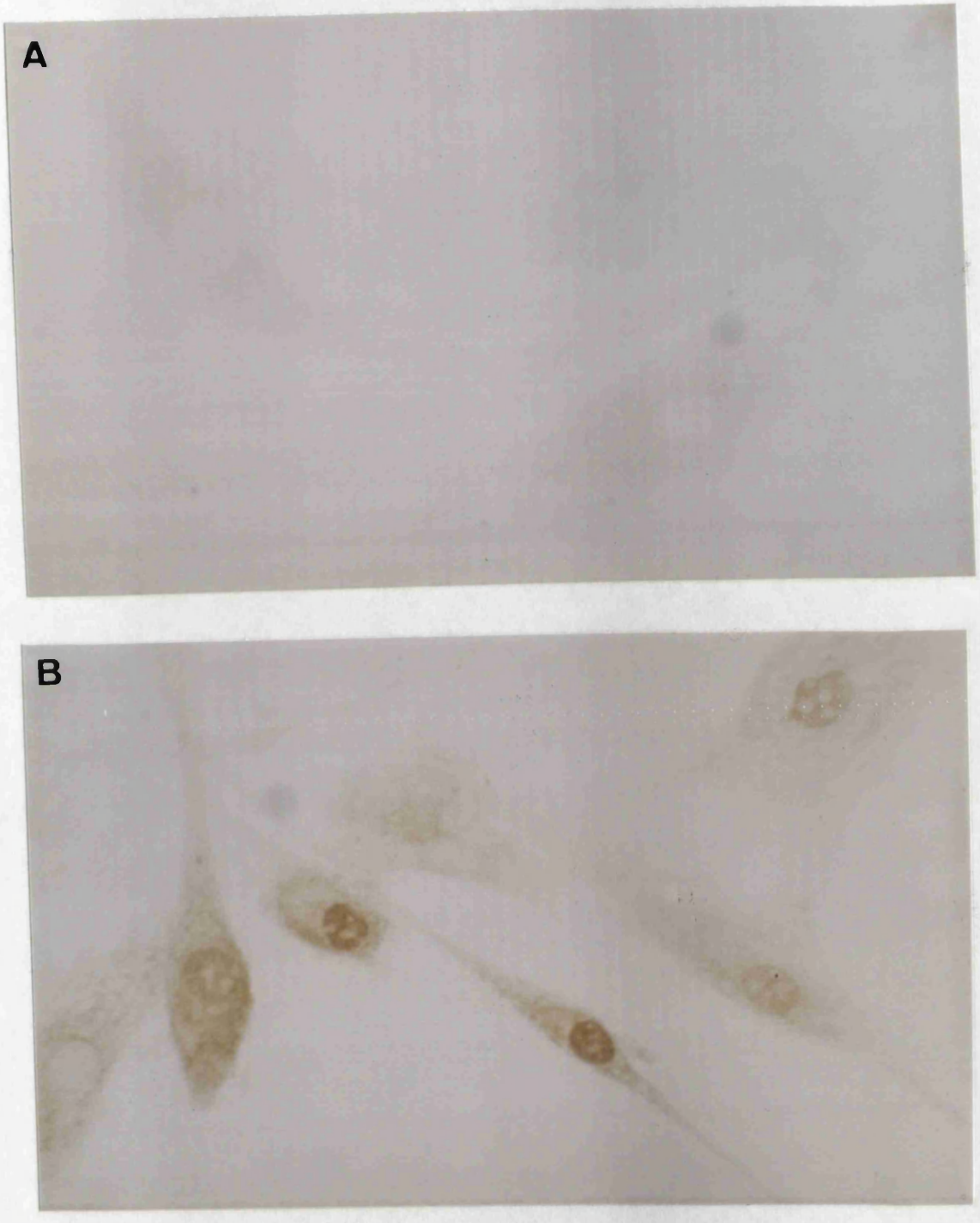


Figure 3.20: Immunohistochemical analyses of normal synovial fibroblasts. Cells grown on slides were stained by the immunoperoxidase method. **a)** Monoclonal antibody to human CR1 (E11) was used at 1/100 dilution. Staining was negative for CR1. **b)** Monoclonal antibody to human MIP (anti-MIP) was used at 1/100 dilution. MIP localized on synovial fibroblasts (brown colour) (x 250).

species of mRNA were observed for C1-INH (2.1 kb), factor I (2.4 kb), S Protein (2.8 kb), SP-40,40 (1.8 kb), MCP (4.2 kb) whereas multiple bands were observed for factor H (4.3 kb, 1.8 kb), DAF (2.7 kb, 1.5 kb) and CD59 (2.0 kb, 1.4 kb, 0.8 kb). Although Northern blots were also probed for C4-bp and CR1 hybridisation signals were not detected. The Northern blots were stripped and rehybridized with a probe for 28S rRNA to allow corrections for unequal loading to be made. The results are summarized in Figures 3.21, 3.22 3.23 and 3.24; the abundances of the mRNAs relative to those in normal fibroblast, and corrected for differences in loading, are summarized in Table 3.4.

The relative abundances of the mRNAs for factor I ($p<0.006$), factor H (4.3 kb species; $p<0.04$), S protein ($p<0.01$) and SP-40,40 ($p<0.02$) were significantly different in normal and RA patient fibroblasts. The relative abundances were greater for factor H and SP-40,40 but smaller for factor I and S protein in RA fibrolasts than in normal fibroblasts. Furthermore, the 2.0 kb species of CD59 was greater ($p<0.001$) in normal fibroblasts than OA fibroblasts. Moreover, the relative abundances of the mRNAs for factor I ($p<0.002$), SP-40,40 ($p<0.04$), CD59 (2.0 kb species, $p<0.01$) and DAF (2.7 kb species, $p<0.001$) were significantly different in RA and OA fibroblasts. Except for factor I the relative abundances were always greater in RA than OA fibroblasts. No significant differences were observed in the abundances of C1-INH and MCP mRNAs in normal, OA and RA fibroblasts. The results of the Northern blot analyses showed that the only mRNAs present were those for which the protein had been detected by ELISA or immunohistochemistry.

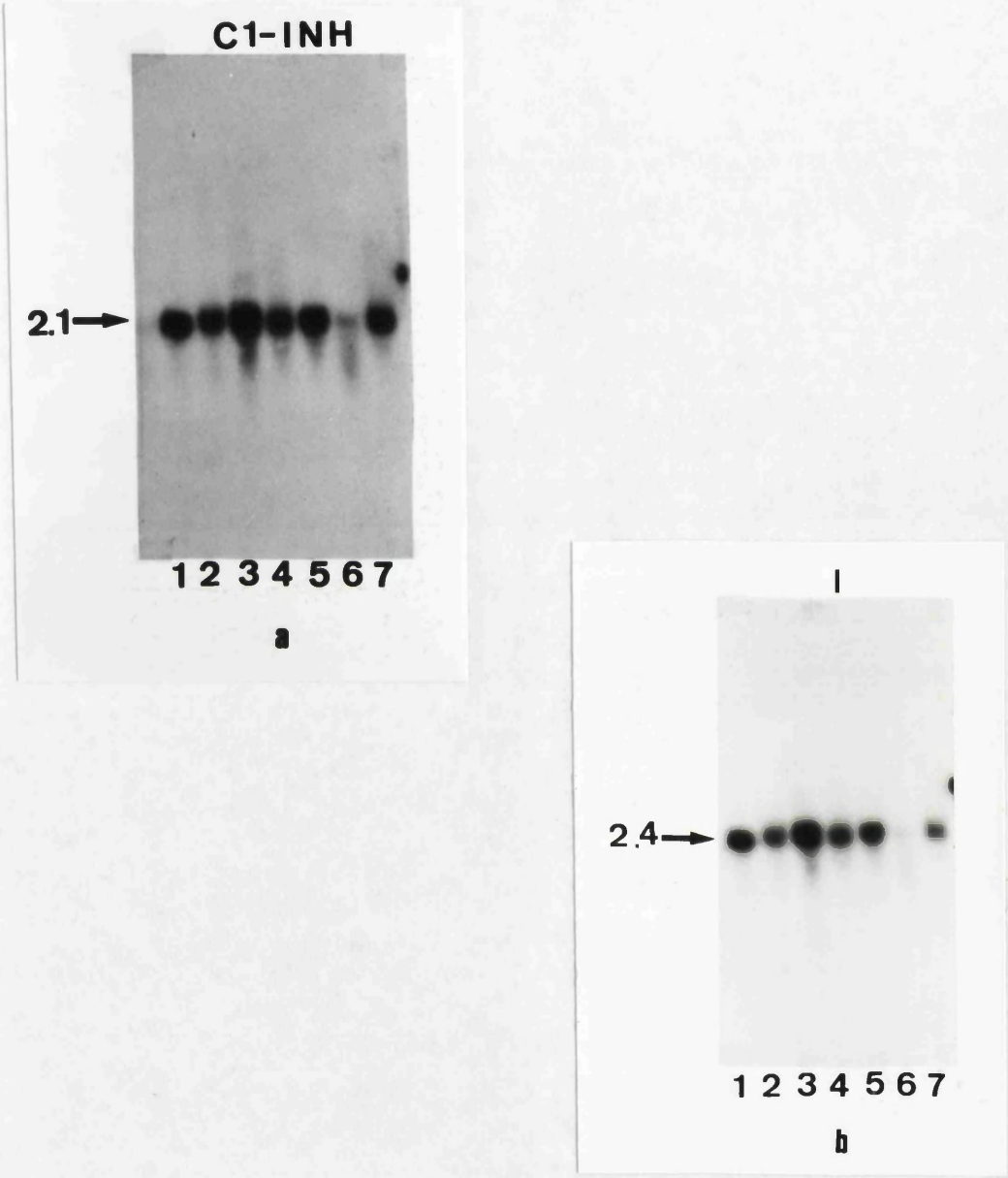


Figure 3.21: Autoradiographs of Northern blot analyses of RNA derived from cultured synovial fibroblasts from normal individuals (Lanes 1, 2, 3), osteoarthritis (Lanes 4, 5) and rheumatoid arthritis (Lanes 6 and 7) patients. ^{32}P-labelled **a)** C1-INH and **b)** factor I probes were used for hybridisation. The sizes of the mRNAs were: C1-INH (2.1 kb) and factor I (2.4 kb).

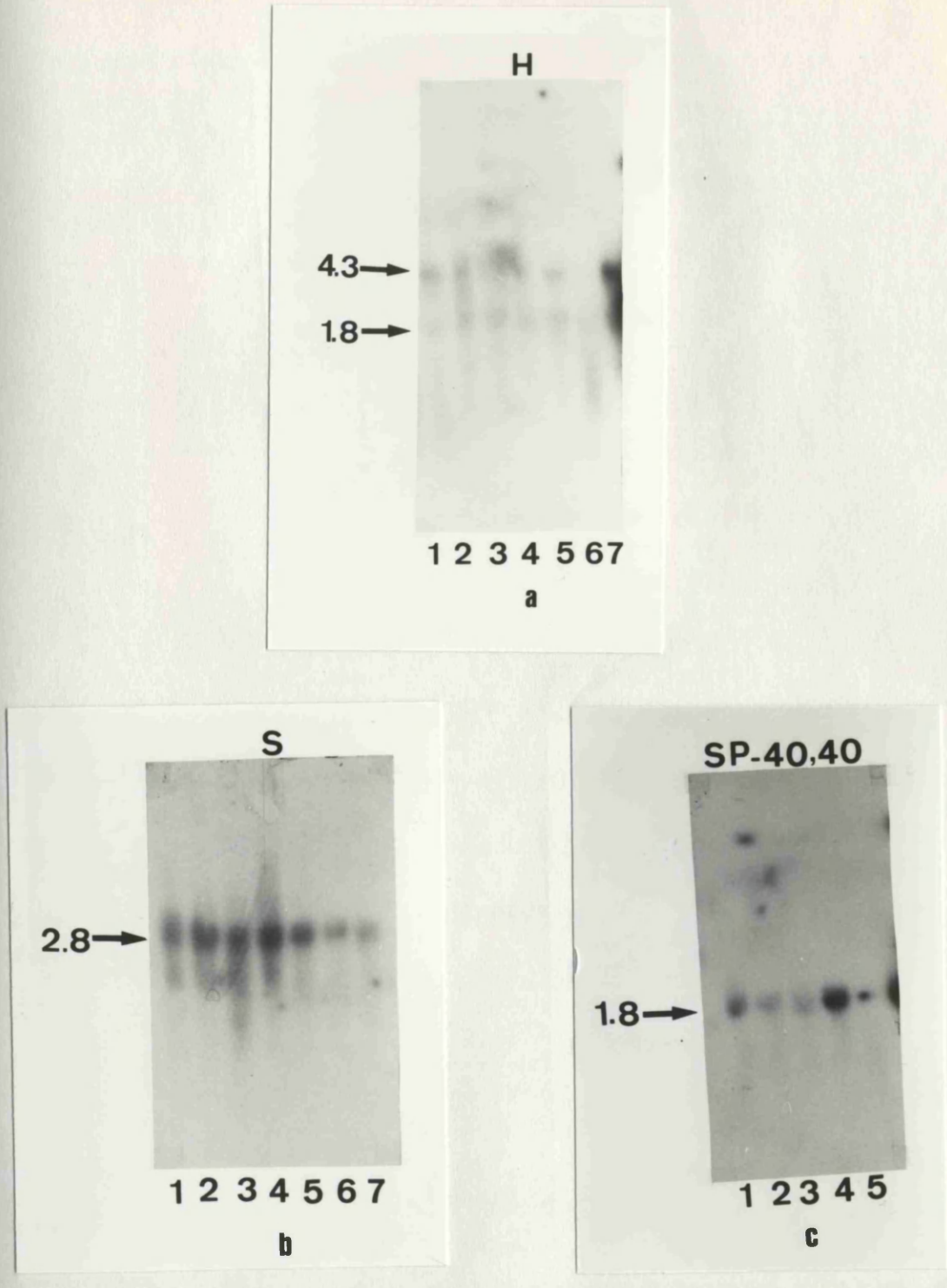


Figure 3.22: Autoradiographs of Northern blot analyses of RNA derived from cultured synovial fibroblasts from normal individuals (Lanes 1, 2, 3 in a and b; Lanes 1, 2 in c), osteoarthritis (Lanes 4, 5 in a and b; Lanes 3, 4 in c) and rheumatoid arthritis (Lanes 6, 7 in a and b; Lanes 5, 6 in c) patients. ^{32}P-labelled **a)** factor H, **b)** S protein and **c)** SP-40,40 probes were used for hybridisation. The sizes of the mRNAs were: factor H (4.3 kb, 1.8 kb), S protein (2.8 kb) and SP-40,40 (1.8 kb).

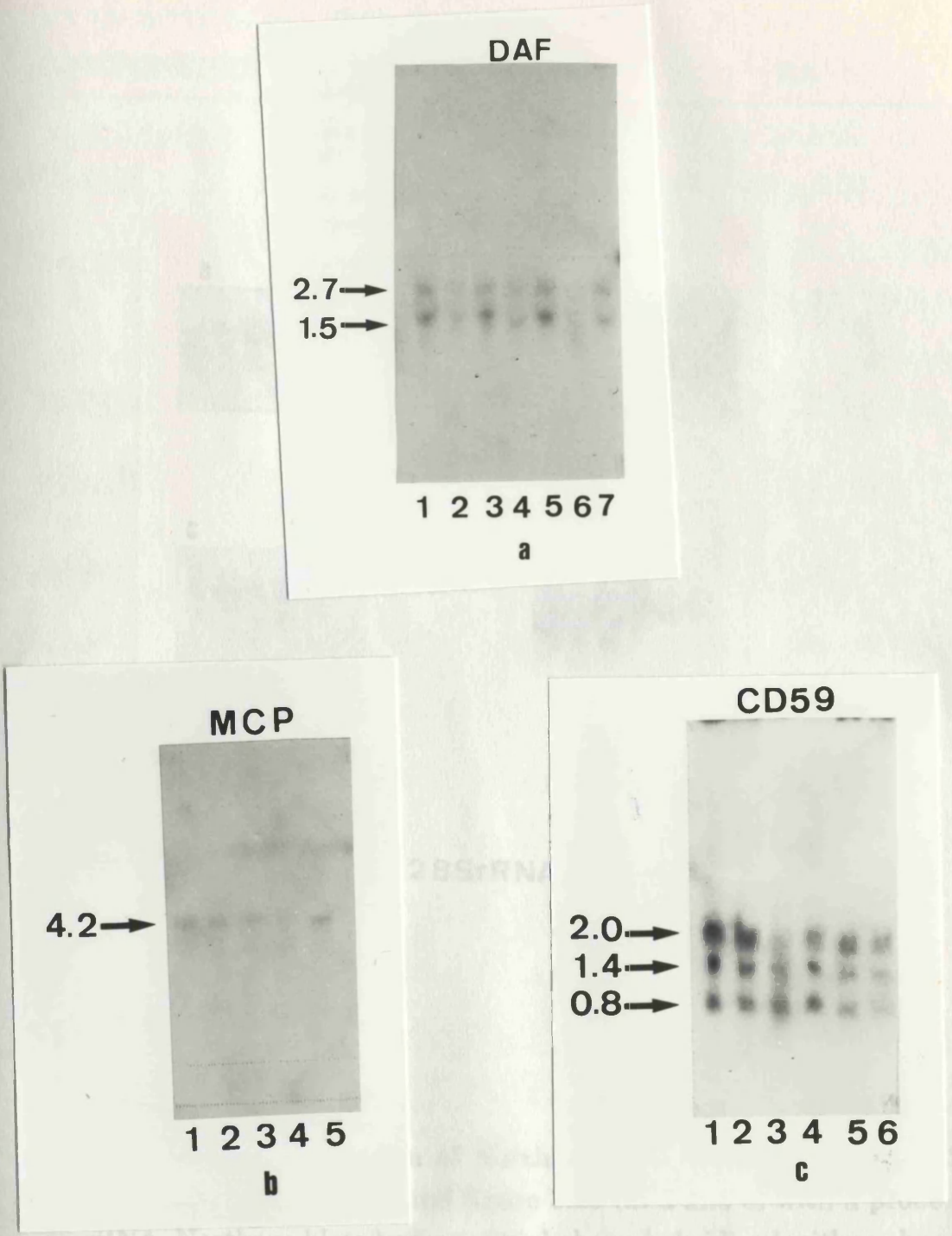


Figure 3.23: Autoradiographs of Northern blot analyses of RNA derived from cultured synovial fibroblasts from normal individuals (Lanes 1, 2, 3 in a, Lanes 1, 2 in b and c), osteoarthritis (Lanes 4, 5 in a; Lanes 3, 4 in b and c) and rheumatoid arthritis (Lanes 6, 7 in a; Lane 5 in b; Lanes 5, 6 in c) patients. ^{32}P-labelled **a)** DAF **b)** MCP and **c)** CD59 probes were used for hybridisation. The sizes of mRNA were; DAF (2.7 kb, 1.5 kb), MCP (4.2 kb) and CD59 (2.0 kb, 1.4 kb and 0.8 kb).

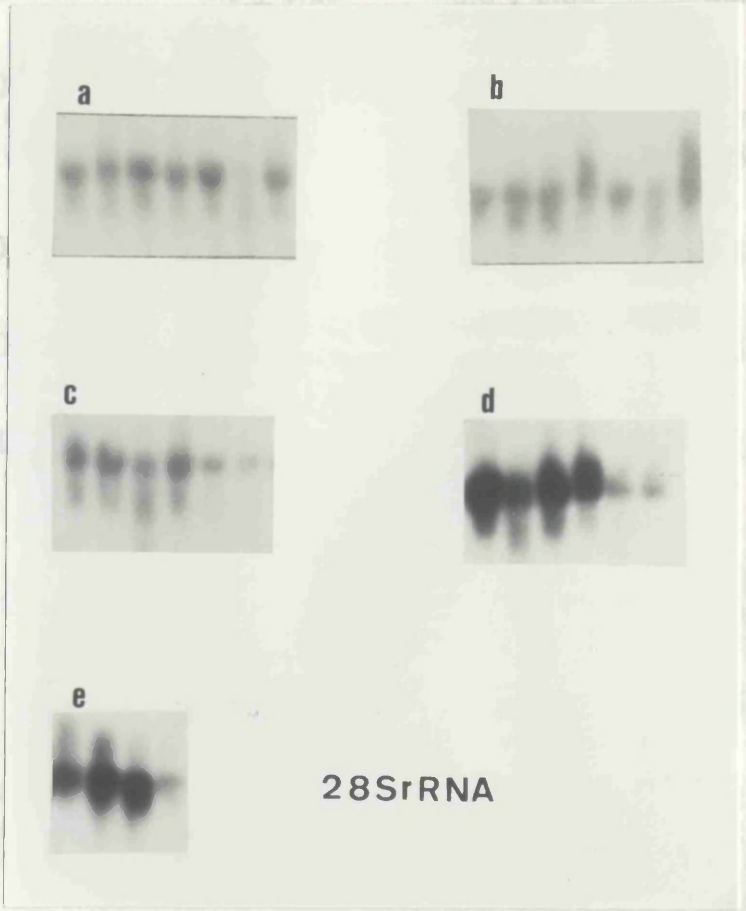


Figure 3.24: Rehybridization of Northern blots shown in figures 3.21, 3.22 and 3.23 (in a, b and c) and figure 3.29 (in d and e) with a probe for 28S rRNA. Northern blots had previously been hybridized with probes for **a)** C1-INH, factor I, DAF, MCP and SP-40,40, **b)** factor H, S protein, **c)** CD59 **d)** CD59 **e)** DAF.

COMPONENTS		N	OA	RA
C1-INH		1.0 ± 0.18	0.8 ± 0.06	0.9 ± 0.03
FACTOR H				
	(4.3kb)	1.0 ± 0.18	1.0 ± 0.43	2.7 ± 1.0 * (+)
	(1.8kb)	1.0 ± 0.42	1.0 ± 0.001	1.8 ± 1.75
FACTOR I		1.0 ± 0.20	0.7 ± 0.08	0.4 ± 0.005 * (+)
S PROTEIN		1.0 ± 0..20	1.5 ± 0.79	0.4 ± 0.125 *
SP-40,40		1.0 ± 0.75	1.3 ± 0.78	3.1 ± 0.70 * (+)
DAF				
	(2.7kb)	1.0 ± 0.28	0.8 ± 0.05	1.1 ± 0.05 (+)
	(1.5kb)	1.0 ± 0.40	0.8 ± 0.03	1.4 ± 1.1
MCP		1.0 ± 0.47	0.6 ± 0.07	0.5 ± 0.43
CD59	(2.0kb)	1.0 ± 0.10	0.5 ± 0.06 *	1.3 ± 0.30 (+)
	(1.4kb)	1.0 ± 0.10	0.9 ± 0.57	1.2 ± 0.41
	(0.8kb)	1.0 ± 0.07	1.3 ± 0.86	1.1 ± 0.06

Table 3.4: Relative abundances of mRNAs for regulatory complement components in normal (N), osteoarthritis (OA) and rheumatoid arthritis (RA) synovial fibroblasts. Results represent the mean ± SD of three experiments. The abundance of each species of mRNA in normal synovial membane was assigned an arbitrary value of 1 unit. * significantly different ($P < 0.05$) from corresponding normal values, (+) sigificantly different ($P < 0.05$) from corresponding OA values.

3.5. SYNTHESIS OF REGULATORY COMPLEMENT PROTEINS BY ENDOTHELIAL CELLS

3.5.1. Analysis of Secreted Regulatory Proteins by ELISA.

Endothelial cells are also present in synovial membrane. Endothelial cells were isolated from human umbilical cord and cultured for seven days as described in section 2.3.1. Culture supernatants were collected at days 1, 3, 5 and 7. The samples were assayed for the complement components C1-INH, C4-bp, factor H and factor I by ELISA. Factor H and factor I were detected. Although secretion of C1-INH by endothelial cells has been reported by Schmaier et al. (1989), I could not detect it under the unstimulated culture conditions employed. C4-bp was not detected in any set of cultures. The addition of cycloheximide (2.5 μg/ml) inhibited secretion of proteins approximately 90% by endothelial cells (data not shown). This result shows that the accumulation of factors H and I in the culture supernatants was due to *de novo* synthesis and not to secretion of preformed protein. The results from three separate experiments are summarized in Figure 3.25. and the secretion rates (calculated as molecule/cell/min) in Table 3.3. These data indicate that endothelial cells, like fibroblasts, are capable of synthesizing and secreting some of the regulatory complement proteins *in vitro*.

3.5.2. Flow-cytometric Analysis of Membrane Regulatory Proteins.

The presence of regulatory complement proteins on endothelial cells was investigated by flow cytometry. Cells were treated with monoclonal antibodies against DAF (BRIC 110), MCP (J4-48), CD59

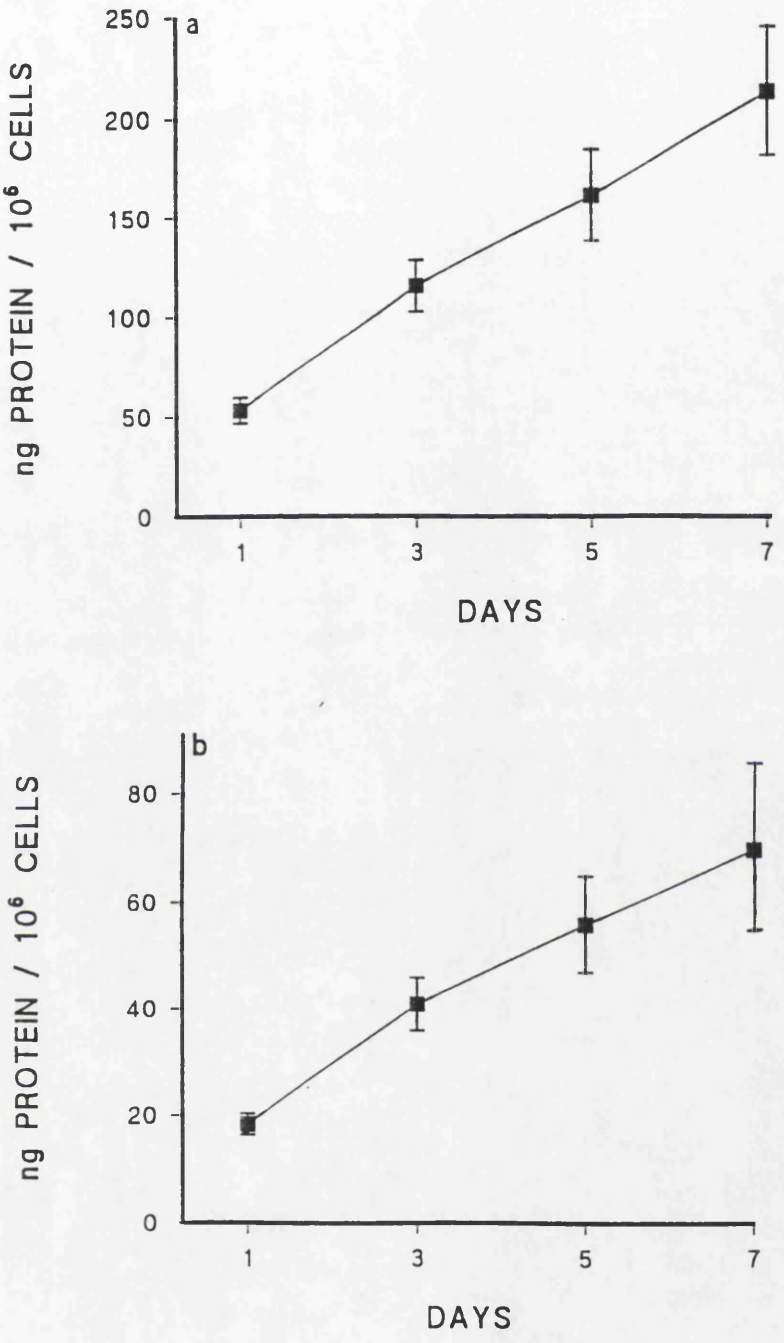


Figure 3.25: ELISA analyses for **a)** factor H and **b)** factor I secreted by endothelial cells. The culture medium was removed completely at day 1, 3, 5 and 7. The amount of factor H and factor I secreted into the culture medium at the indicated days was measured. After seven days in culture, the results were plotted as cumulative synthesis (ng/10^6 cells). Each point represents the mean with the standard deviation of three experiments.

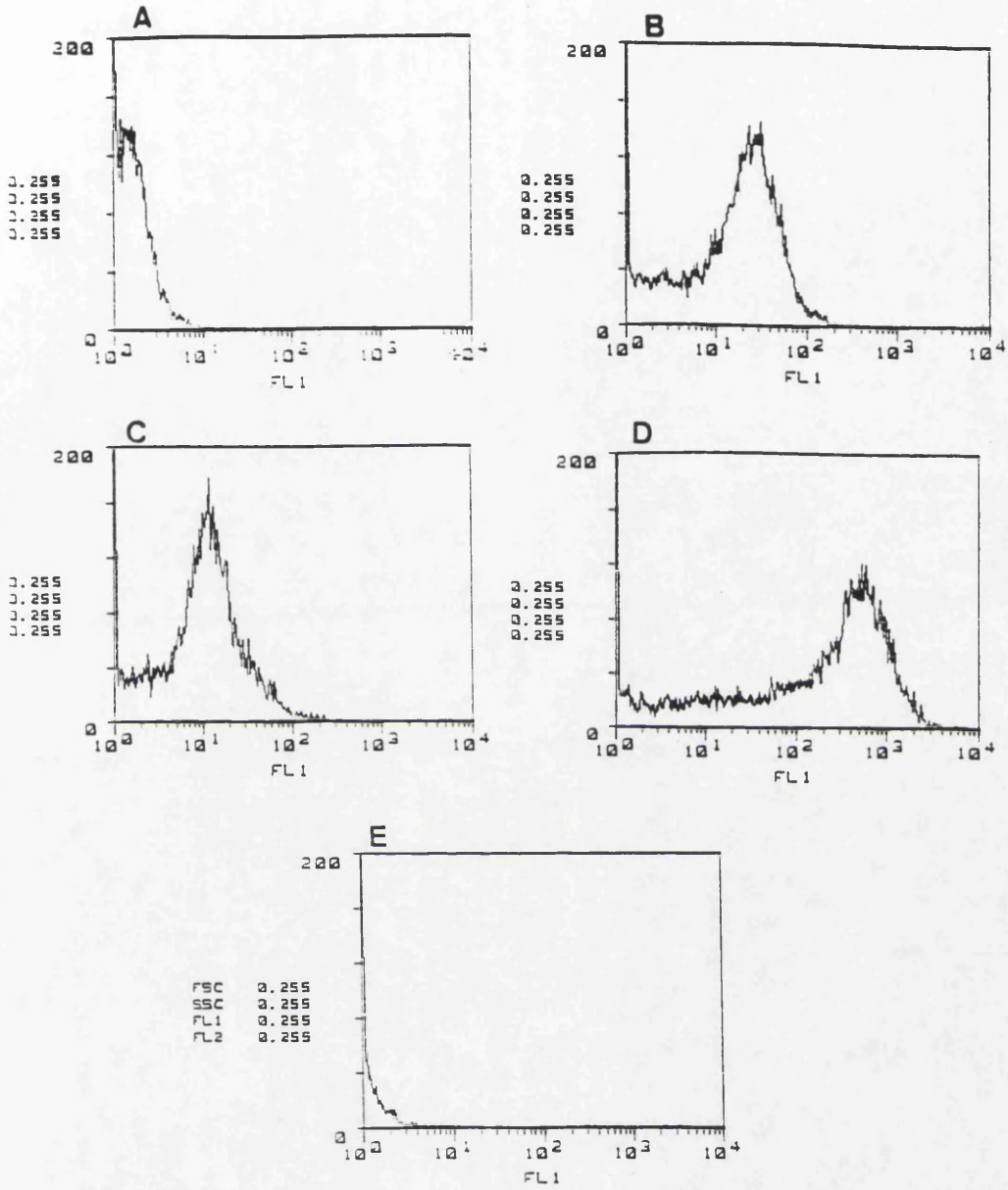


Figure 3.26: Flow-cytometric analyses of endothelial cells during the fourth passage. Cells were treated with **a)** BSA (0.2%, w/v) in PBS as a background control **b)** anti-DAF (BRIC110, IgG1 mouse), **c)** anti-MCP (J4-48, IgG1 mouse), **d)** anti-CD59 (BRIC229, IgG2b mouse) and **e)** anti-CR1 (E11, IgG1 mouse) and FITC-conjugated anti-mouse Ig. Fluorescence intensity is plotted on a logarithmic scale (x-axis), and cell number on a linear scale (y-axis).

(BRIC 229) and CR1 (E11) as described in section 2.12. The results indicated that DAF, MCP and CD59, but not CR1, were present on endothelial cells (Figure 3.26). The fluorescence intensity for CD59 was high while that for DAF was relatively low.

3.5.3. Abundances of mRNAs Encoding the Regulatory Complement Proteins.

ELISA and flow cytometry analysis showed that endothelial cells are capable of secreting factor H and I, and expressing DAF, MCP and CD59 on their surfaces. Thus, these cells synthesize the majority of regulatory complement proteins. Total cellular RNA was extracted from endothelial cells to investigate the mRNAs encoding each of these detected proteins, and also those that were not detected by ELISA or flow cytometry. Northern blot analyses were performed as described in section 2.9.1. and hybridized with ^{32}P-labelled probes for each of the mRNAs (C1-INH, C4-bp, factor H, factor I, S Protein, SP-40,40, DAF, MCP, CD59 and CR1). The Northern blots were stripped and rehybridized with a probe for 28S rRNA to allow corrections for unequal loading to be made. The results are summarized in Figures 3.24, 3.27, 3.28, and 3.29. Northern blots hybridized with probes for C1-INH, C4-bp and CR1 did not show any signal. A single species of mRNA was observed for factor I (2.4 kb), S Protein (2.8 kb), SP-40,40 (1.8 kb), but multiple bands were observed for factor H (4.3 kb, 1.8 kb), DAF (2.7 kb, 1.5 kb), MCP (4.8 kb, 4.2 kb) and CD59 (2.0 kb, 1.4 kb, 0.8 kb). Densitometric analysis enabled the ratios of the different species of mRNA to be calculated. The ratios of the abundances of the two species of DAF mRNA differ between endothelial cells and fibroblasts (Table 3.5) but not between normal, OA and RA fibroblasts. The ratios of the abundances of the CD59 mRNAs are

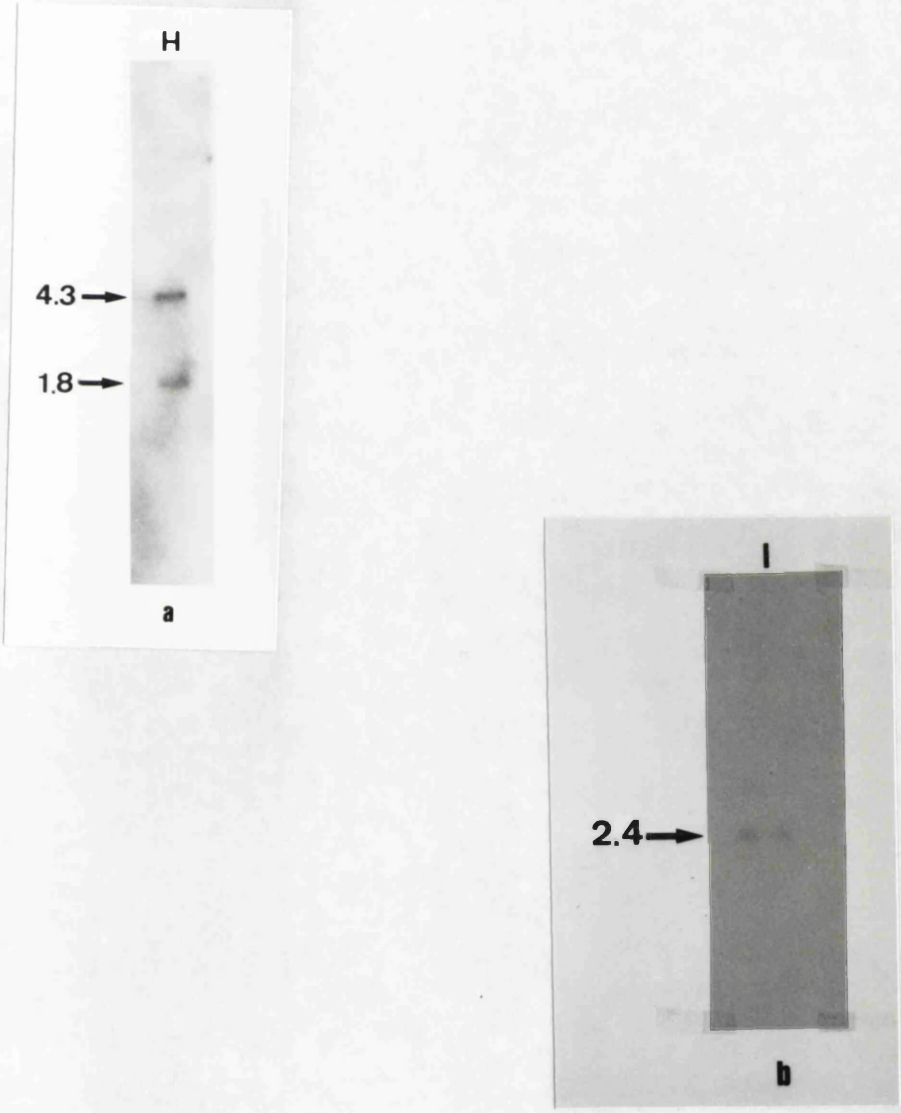


Figure 3.27: Autoradiographs of Northern blot analyses of RNA derived from cultured endothelial cells. The blots were hybridized with probes for **a)** factor H and **b)** factor I. The sizes of the mRNAs were: factor H (4.3 kb, 1.8 kb) and factor I (2.4 kb).

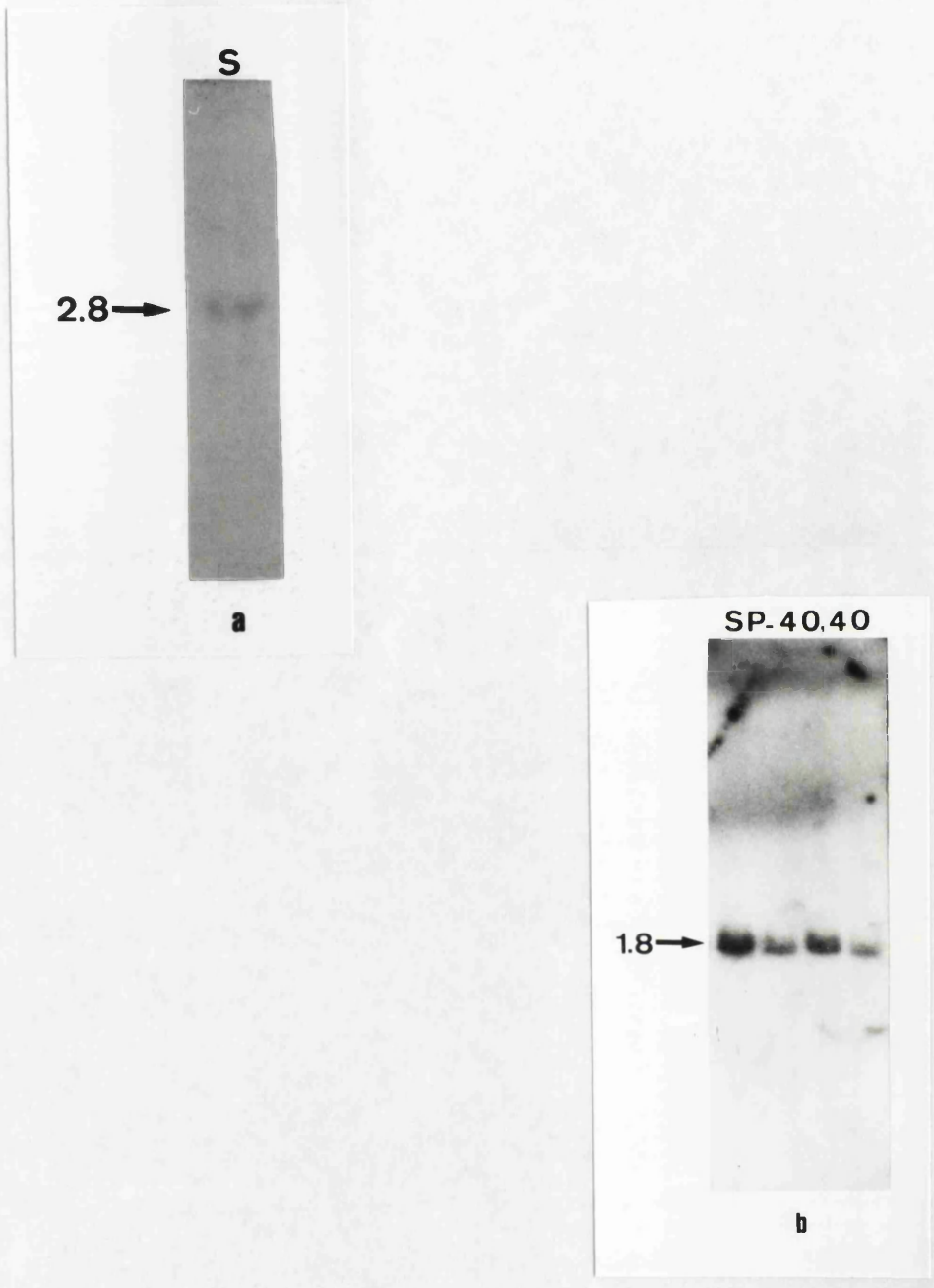


Figure 3.28: Autoradiographs of Northern blot analyses of RNA derived from cultured endothelial cells. The blots were hybridized with probes for **a)** S protein and **b)** SP-40,40. The sizes of the mRNAs were: S protein (2.8 kb), SP-40,40 (1.8 kb).

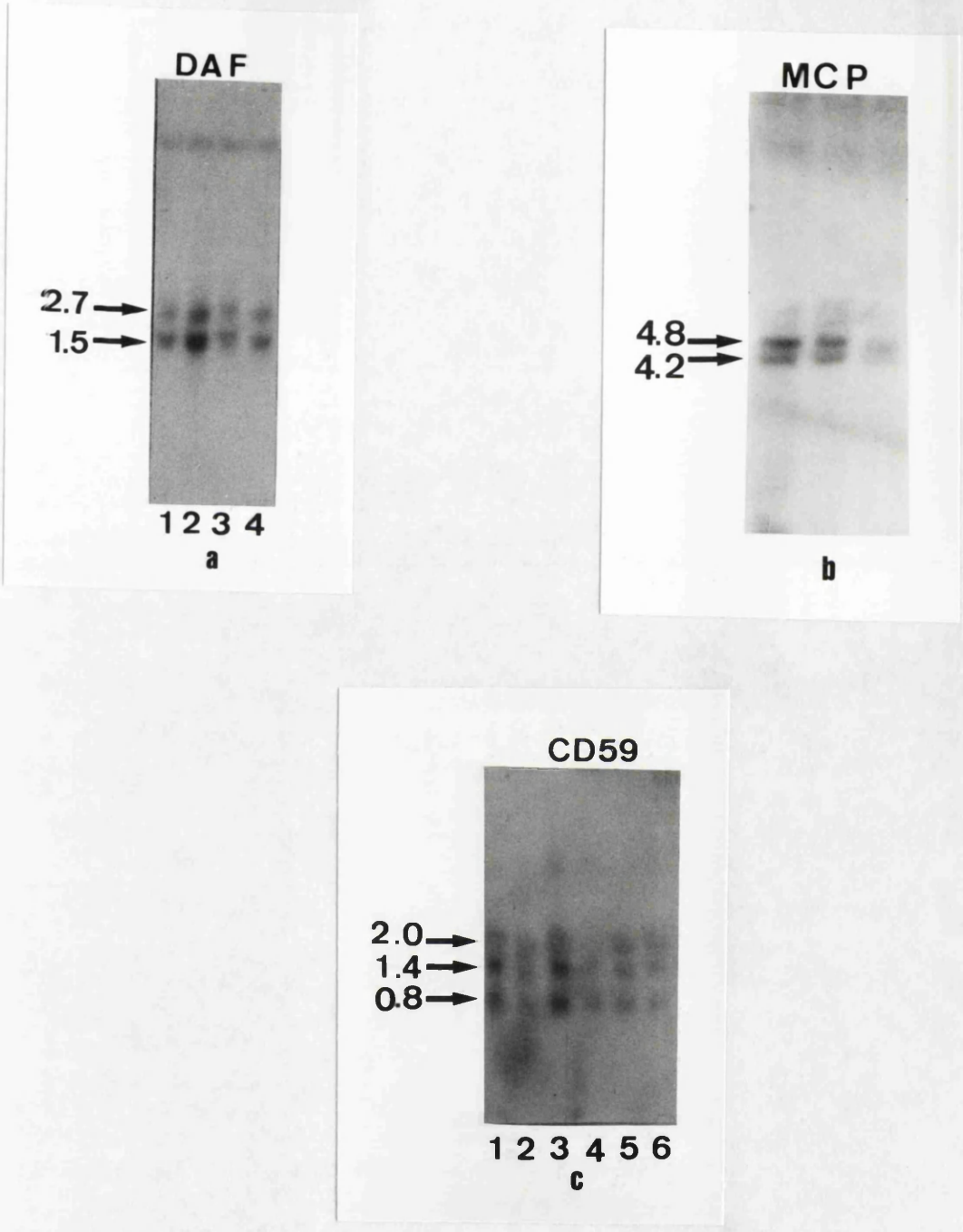


Figure 3.29: Autoradiographs of Northern blot analyses of RNA derived from cultured endothelial cells. The blots were hybridized with probes for **a)** DAF, **b)** MCP and **c)** CD59. The RNAs loaded on Lanes 1 and 3 for DAF and Lanes 3 and 5 for CD59 were isolated after treatment of the cells with cycloheximide (2.5 μg/ml) for 48 hours. The sizes of the mRNAs were: S protein (2.8 kb) and SP-40,40 (1.8 kb).

RATIO	HUVEC	N	OA	RA
1.5kb/2.7kb	2.0 ± 1.0	0.80 ± 0.2	0.73 ± 0.2	0.90 ± 0.6

Table 3.5: The ratio of the abundances of 1.5 kb to 2.7kb species of DAF mRNA in human umblical cord endothelial cells (HUVEC) and normal (N), osteoarthritis (OA) and rheumatoid arthritis (RA) fibroblasts. Each value represents the mean ± SD of three experiments.

summarized in Table 3.6. All the fibroblast RNAs contained a greater preponderance of the highest molecular weight mRNA species, in contrast to endothelial cells, but again there was no difference between the normal, OA and RA fibroblasts. These data suggest that there are cell-specific differences in the processing of the mRNAs for these regulatory proteins. The same appears to be true for MCP mRNA since this occurs as a doublet in RNA from endothelial cells, but not in fibroblast RNA.

The Northern blot data are in agreement with the data obtained from ELISA and flow cytometry measurements. In particular, no trace of C1-INH, C4-bp or CR1 was found at either the protein or the mRNA level.

RATIO	HUVEC	N	OA	RA
1.4kb/2.0kb	1.17 ± 0.3 *	0.65 ± 0.1	0.87 ± 0.5	0.78 ± 0.2
0.8kb/2.0kb	1.35 ± 0.8	0.77 ± 0.3	0.90 ± 0.9	0.60 ± 0.5

Table 3.6: The ratio of the abundances of 1.4 kb to 2.0kb and 0.8 kb to 2.0 kb species of CD59 mRNA in human umblical cord endothelial cells (HUVEC) and normal (N), osteoarthritis (OA) and rheumatoid arthritis (RA) fibroblasts. Each value represents the mean ± SD of three experiments. * Significantly different than normal ($p<0.04$).

3.6. SYNTHESIS OF REGULATORY COMPLEMENT PROTEINS BY MONOCYTES

3.6.1. Analysis of Secreted Regulatory Proteins by ELISA.

Mononuclear phagocytes are the third type of cell in synovium which I have studied by using peripheral blood monocytes prepared from human buffy coat preparations of blood donations. Monocytes were cultured for seven days as described in section 2.3.2. Culture supernatants were collected at the days 1, 3, 5 and 7. The samples were assayed for the complement components C1-INH, C4-bp, factor H and factor I by ELISA. C1-INH, C4-bp and factor H, but not factor I, were secreted by monocytes. The results from three separate experiments are summarized in Figures 3.30 and 3.31 and the secretion rates (calculated as molecule/cell/min) in Table 3.3. These show that monocytes secrete about twice as much C1-INH than factor H and about three times as much factor H than C4-bp (Table 3.3.) and they are the only cell type so far examined which is capable of synthesizing and secreting C4-bp *in vitro*. The presence of cycloheximide (2.5 μg/ml) inhibited approximately 80 % of the synthesis of secretory proteins by monocytes (data not shown). This result indicates that the accumulation of C1-INH, C4-bp and factor H in the culture supernatants was due to *de novo* synthesis and not the secretion of preformed protein.

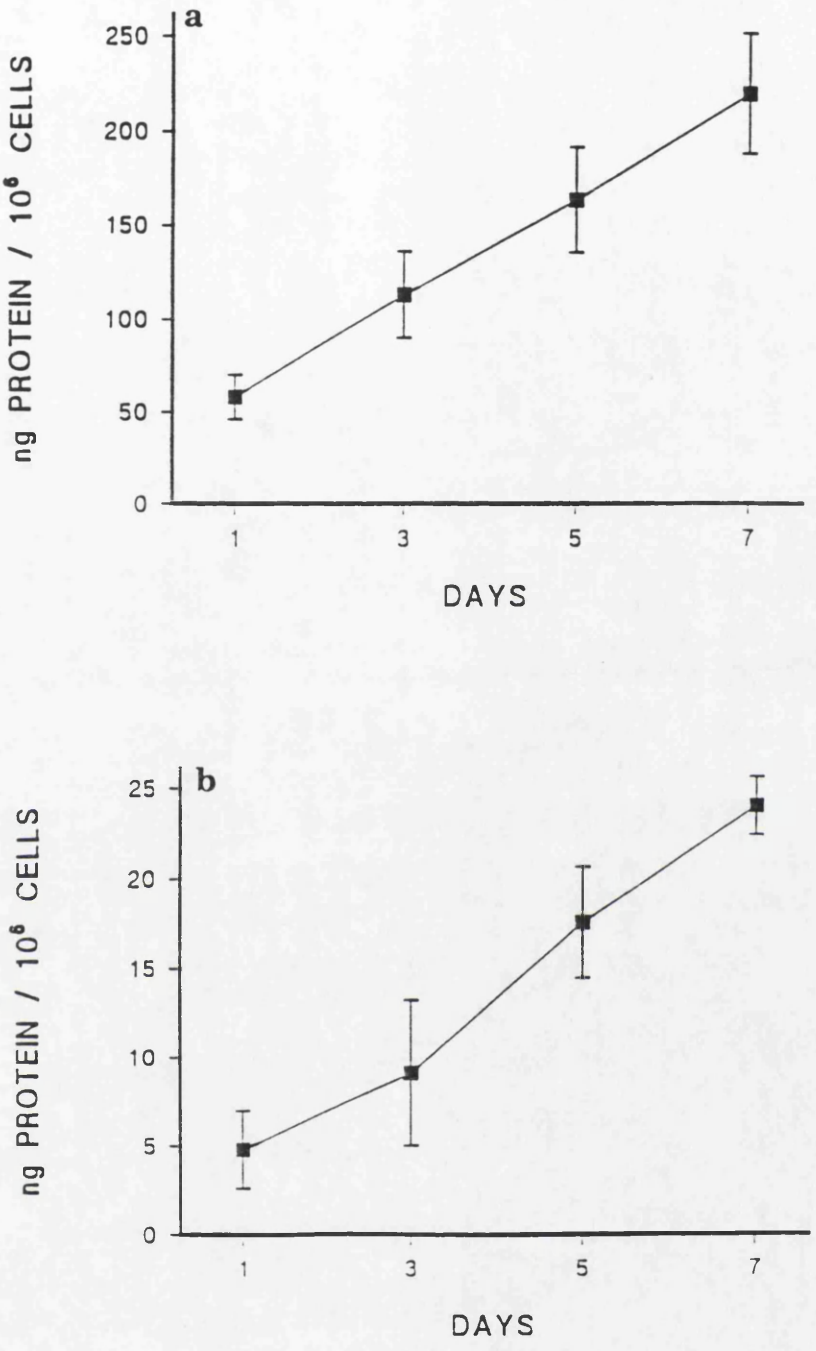


Figure 3.30: ELISA analyses for **a)** C1-INH and **b)** C4-bp secreted by monocytes. The culture medium was removed completely at day 1, 3, 5 and 7. The amount of C1-INH and C4-bp secreted into the culture medium at the indicated days was measured. After seven days in culture, the results were plotted as cumulative synthesis (ng/10^6 cells). Each point represents the mean with the standard deviation of three experiments.

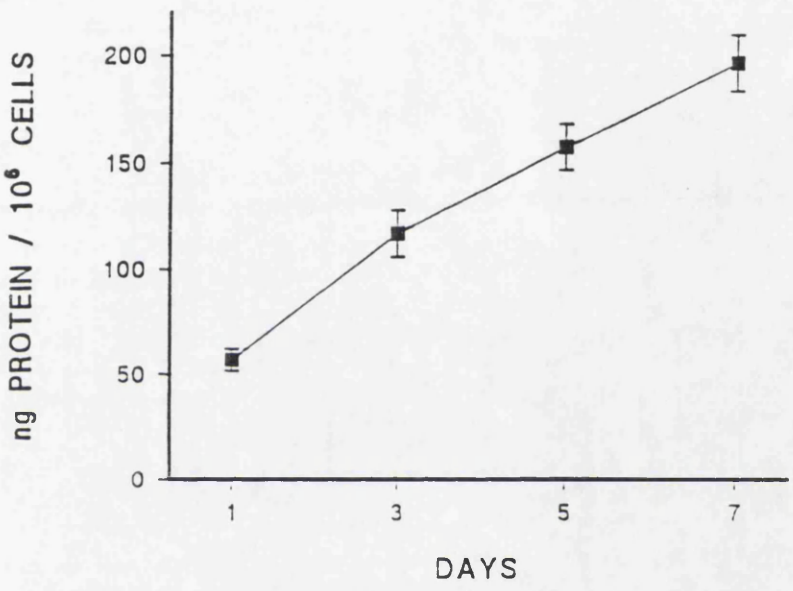


Figure 3.31: ELISA analyses for factor H secreted by monocytes. The culture medium was removed completely at day 1, 3, 5 and 7. The amount of factor H secreted into the culture medium at the indicated days was measured. After seven days in culture, the results were plotted as cumulative synthesis (ng/10^6 cells). Each point represent the mean with the standard deviation of three experiments.

3.6.2. Immunohistochemical Analysis of Membrane Regulatory Proteins.

The presence of regulatory membrane proteins was investigated by immunohistochemical staining. Monocytes were grown on glass slides in a petri dish under standard culture conditions as described in section 2.3.2. Cells were fixed by acetone (section 2.11.1.) and stained with monoclonal antibodies against DAF (IA10H6) (Figure 3.32 b); MCP (E4.3) and CD59 (YTH53.1) (Figure 3.33 a and b); CR1 (E11) and MIP (anti-MIP) (Figure 3.34 a and b) by the alkaline phosphatase method. Intensity of staining for DAF, CD59 and MIP was stronger than for MCP and CR1. Positive staining of monocytes for CR1 is in contrast to fibroblasts and endothelial cells which were negative.

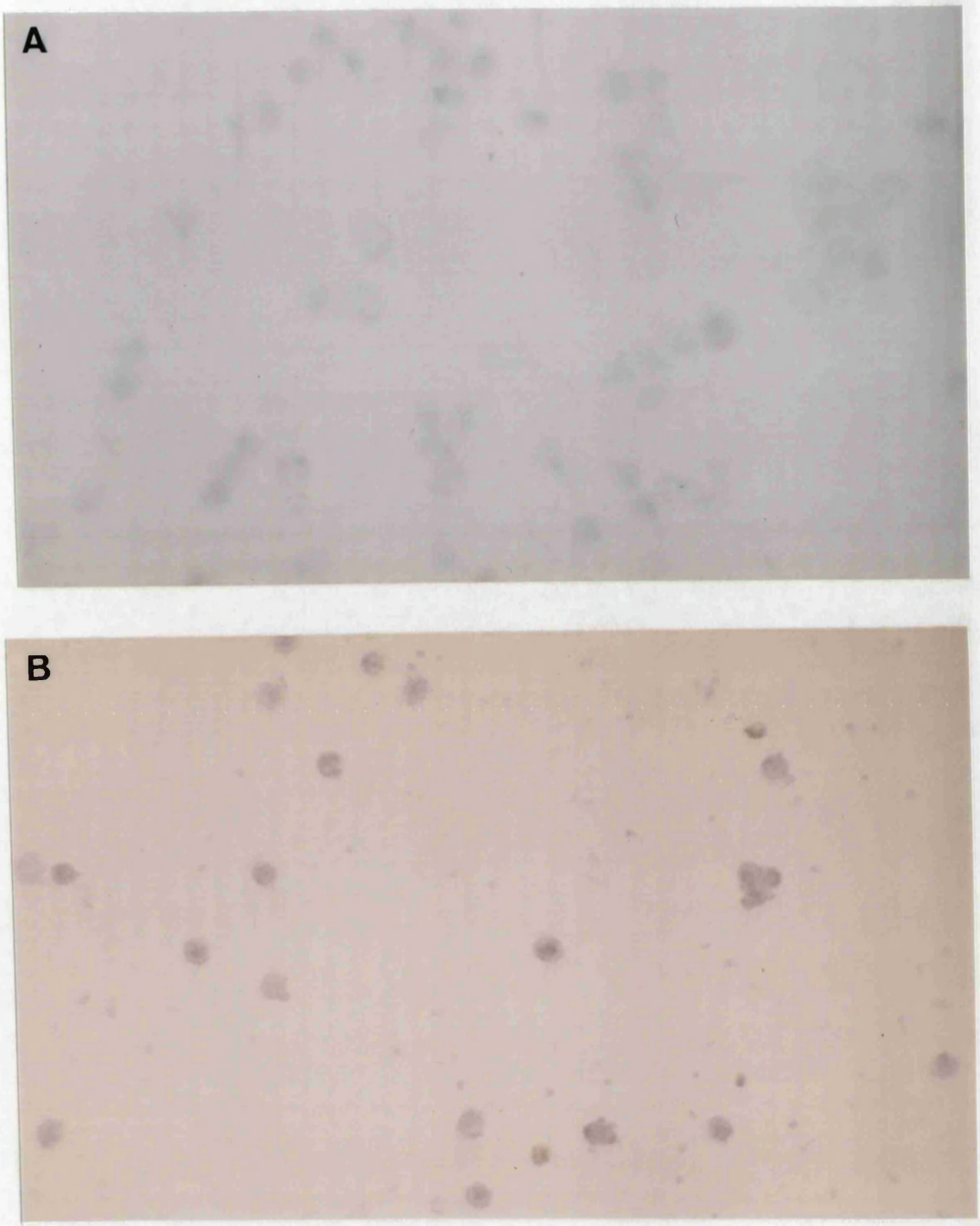


Figure 3.32: Imunohistochemical analyses of monocytes. Cells grown on glass microscope slides were stained by the alkaline phosphatase method. **a)** Mouse IgG was used at 1/100 dilution as control. **b)** Monoclonal antibody to human DAF (IA10H6) was used at 1/100 dilution. DAF localized on monocytes (red colour) (x 250).

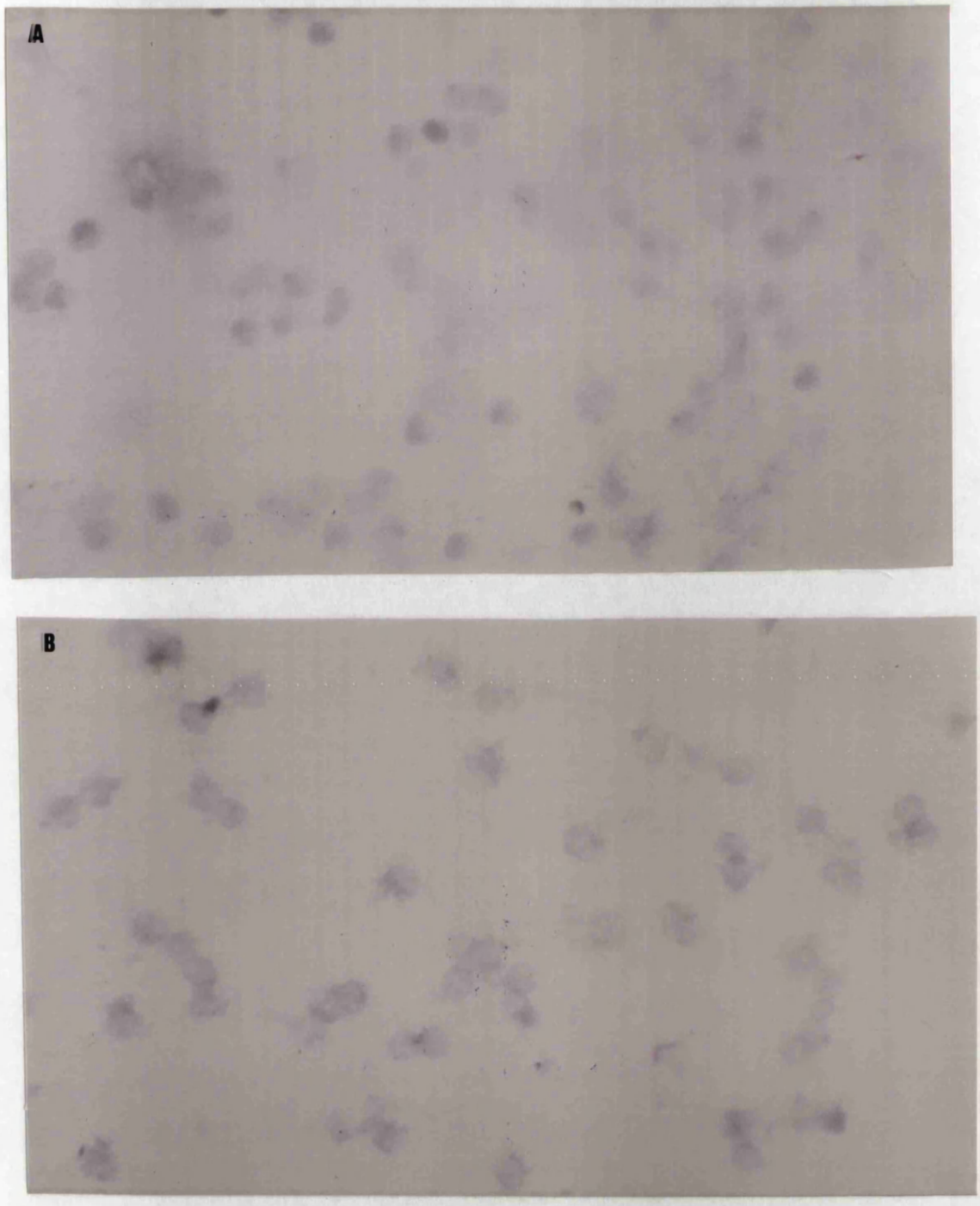


Figure 3.33: Immunohistochemical analyses of monocytes. Cells grown on glass microscope slides were stained by the alkaline phosphatase method. **a)** Monoclonal antibody to human MCP (E4.3) was used at 1/100 dilution. **b)** Monoclonal antibody to human CD59 (YTH53.1) was used at 1/100 dilution. MCP and CD59 localized on monocytes (red colour) (x 250).

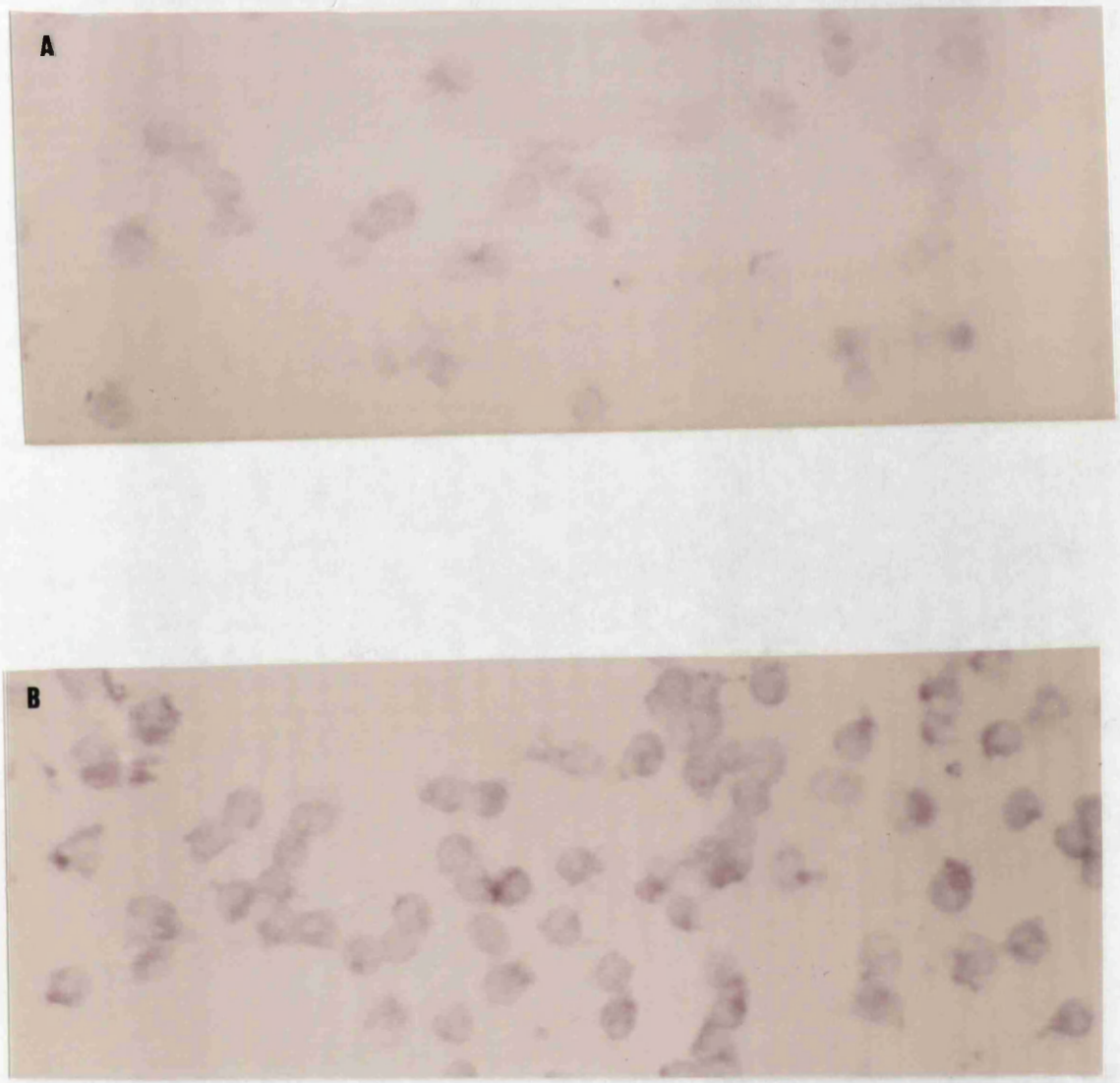


Figure 3.34: Immunohistochemical analyses of monocytes. Cells grown on microscope slides were stained by the alkaline phosphatase method. **a)** Monoclonal antibody to human CR1 (E11) was used at 1/100 dilution. localized on monocytes (pink colour) **b)** Monoclonal antibody to human MIP (anti-MIP) was used at 1/100 dilution. MIP localized on monocytes (red colour) (x 250).

3.6.3. Abundances of mRNAs Encoding the Regulatory Complement Proteins.

To investigate further the synthesis of complement regulatory proteins, monocytes were analyzed for the presence of mRNAs encoding each of these proteins. Total cellular RNA extracted from cultured monocytes was analyzed by Northern blots and dot-blots hybridized with ^{32}P-labelled probes for each of the mRNAs (C1-INH, C4-bp, factor H, factor I, S Protein, SP-40,40, DAF, MCP, CD59 and CR1). The results are summarized in Figures 3.35, 3.36 and 3.37.

A single species of mRNA was observed for C1-INH (2.1 kb), C4-bp (2.5.kb) and SP-40,40 (1.8 kb), whereas two bands were observed for factor H (4.3 kb, 1.8 kb). In monocytes factor H mRNA could not be detected unless cycloheximide (2.5 μg/ml) was present in the cultures. Due to the scarcity of RNA, dot-blots were used for the hybridization with probes for DAF, MCP, CD59 and CR1 mRNAs and positive signals were detected for all of these regulatory membrane proteins. Factor I and S protein mRNAs were not detected on Northern blots of monocyte RNA.

Northern and dot blot data and the data obtained from ELISA and immunohistochemical staining were in agreement in showing that C4-bp and CR1 were expressed in monocytes but not in fibroblasts or endothelial cells.

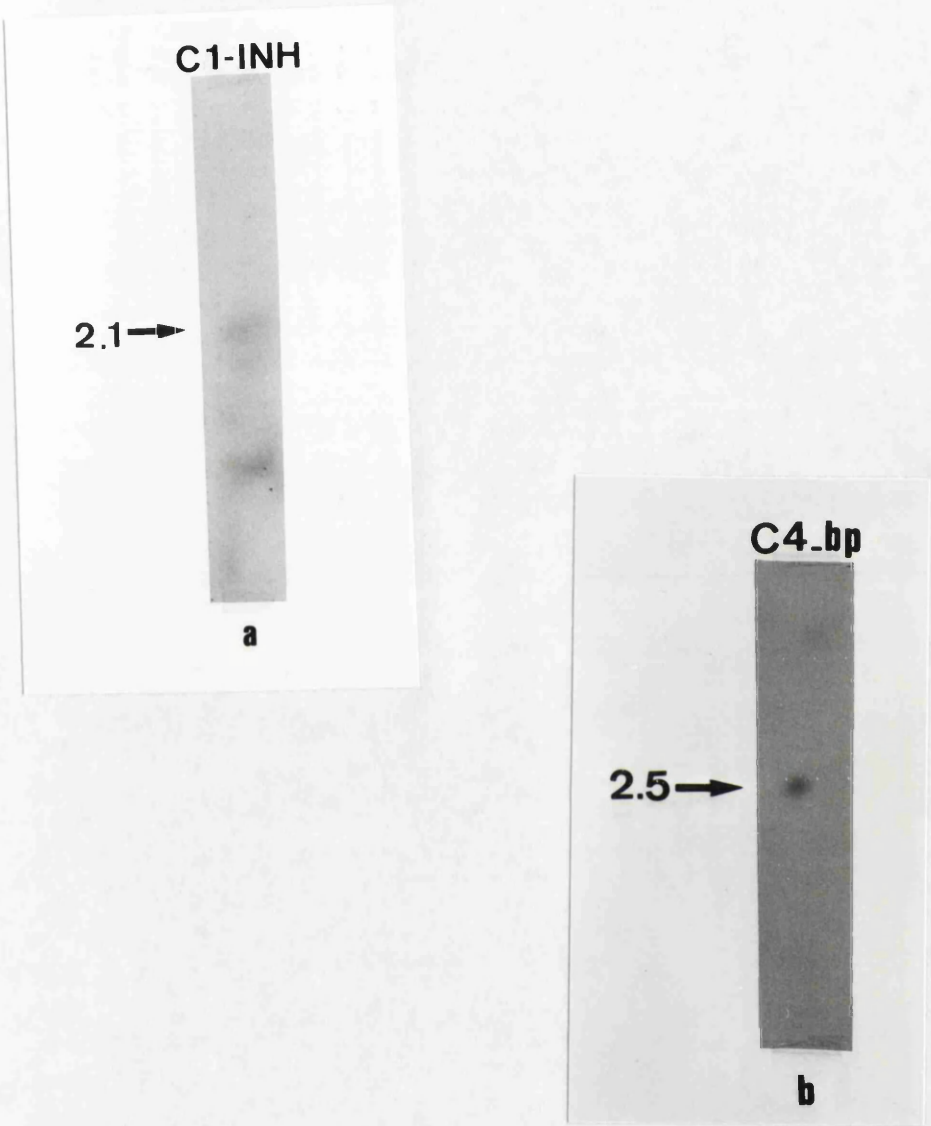
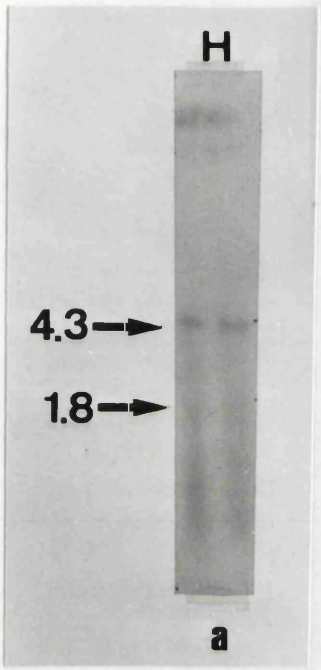


Figure 3.35: Autoradiographs of Northern blot analyses of RNA derived from cultured monocytes. The blots were hybridized with probes for **a)** C1-INH and **b)** C4-bp. The sizes of the mRNA were: C1-INH (2.1 kb) and C4-bp (2.4 kb)



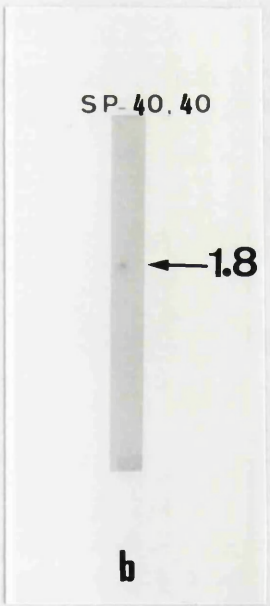


Figure 3.36: Autoradiographs of Northern blot analyses of RNA derived from cultured monocytes. The blots were hybridized with probes for **a)** factor H (total RNA was isolated after treatment of the cells with cycloheximide (2.5 μg/ml) for 48 hours) and **b)** SP-40,40. The sizes of the mRNAs were: factor H (4.3 kb, 1.8 kb) and SP-40,40 (1.8 kb).

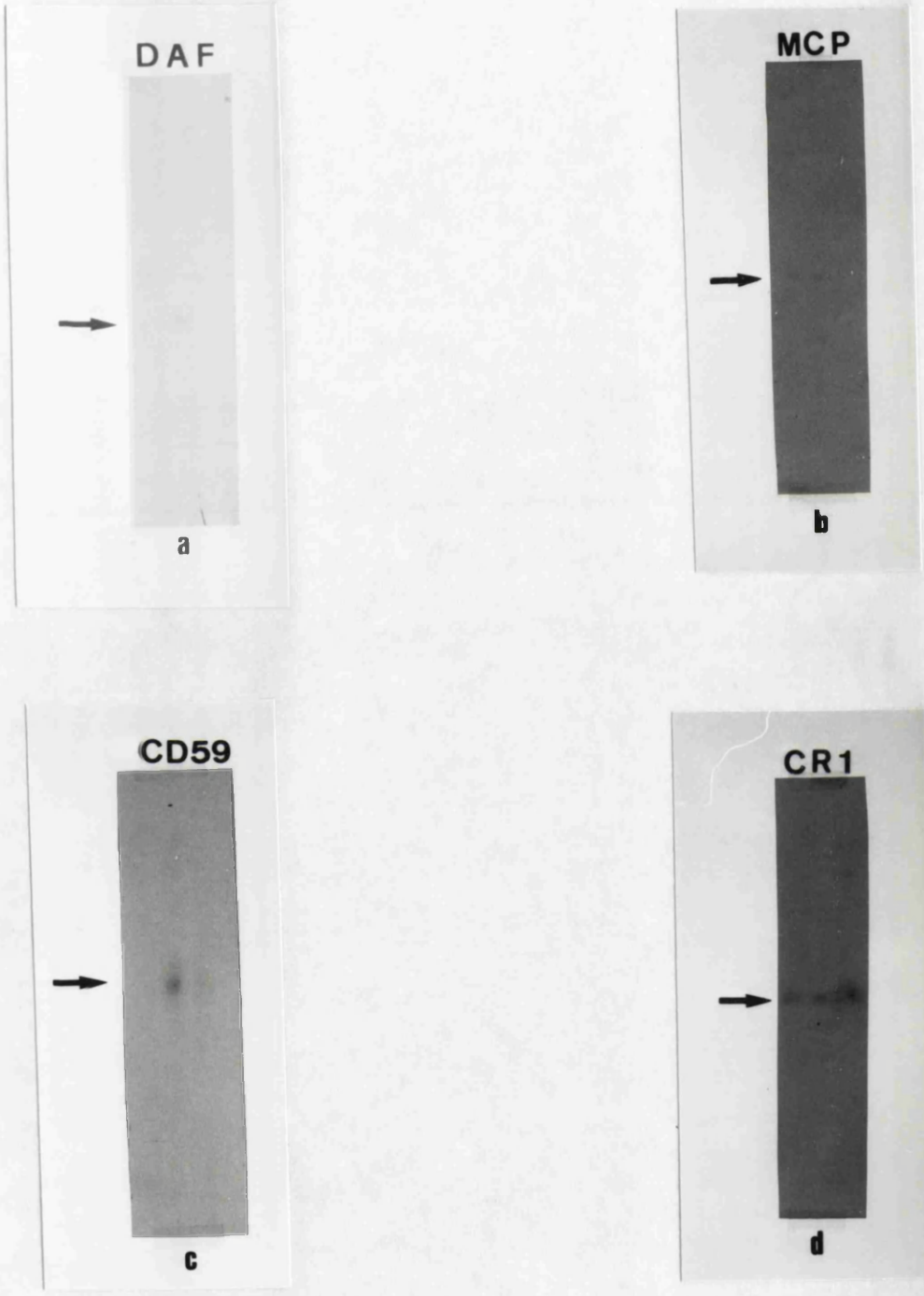


Figure 3.37: Autoradiographs of dot blot analyses of RNA (5 μg) deived from cultured monocytes. The blots were hybridised with probes for **a)** DAF; **b)**MCP; **c)** CD59 and **d)** CR1 mRNAs.

3.7. SYNTHESIS OF REGULATORY COMPLEMENT PROTEINS BY LYMPHOCYTES

3.7.1. Analysis of the Secreted Regulatory Proteins by ELISA.

3.7.1.1. *Peripheral blood lymphocytes cultured under standard culture conditions.*

Lymphocytes are a fourth cell type in synovium and the last one investigated in this thesis in order to understand the regulation of complement activation at sites of inflammation. Lymphocytes were isolated from human buffy coat and were cultured for seven days under standard culture conditions as described in section 2.3.3. Culture supernatants were collected at days 1, 3, 5 and 7. The samples were assayed for the complement components C1-INH, C4-bp, factor H and factor I by ELISA. C1-INH, factor H and factor I were detectable at very low levels after one day. The supernatants which were collected at days 3, 5 and 7 were negative for all components (C1-INH, C4-bp, factor H and factor I). The results from three separate experiments were calculated as molecule/cell/min and summarized in Table 3.7.

It was thought that the proteins detected previously may due the monocyte contamination in the lymphocyte culture or perhaps monocyte-derived cytokines may have stimulated the lymphocytes to secrete these components. So lymphocyte preparations were depleted of monocytes by preincubation in a culture flask at 37°C three to four hours. Then non-adherent cells were transferred to a fresh culture flask and cultured as before. Similar experiments were done with monocyte-depleted lymphocyte cultures. Except for factor H none of the regulatory proteins were detected in the monocyte-depleted lymphocyte culture supernatants

	CONTROL	PHA	PWM	Con A
C1-INH	2.7 ± 1.5	3.3 ± 0.5	1.6 ± 01	2.4 ± 0.9
C4-bp	0	0	0	0.9 ± 0.8
Factor I	1.2 ± 0.4	0	1.2 ± 0.7	31.0 ± 12.0
Factor H	2.5 ± 1.1	1.4 ± 0.6	4.0 ± 0.1	1.4 ± 0.5

Table 3.7. Secretion rates of fluid-phase regulatory complement proteins C1-INH, C4-bp, Factor H and Factor I (molecule/cell/min) in lymphocytes cultured under standard condition (control) and stimulated by Phytohaemagglutinin (PHA), Pokeweed mitogen (PWM) and Concanavalin A (Con A). Each value represents the mean ± SD of three experiments.

under unstimulated conditions (Table 3.8). Factor H was not detectable after one day.

The failure to detect any regulatory complement component in the supernatants after one day of culture led us to stimulate the lymphocytes with mitogens like phytohaemagglutinin (PHA), Pokeweed mitogen (PWM) and Concanavalin A (Con A). Mitogen treatment induces mitosis and proliferation and may stimulate the cells to synthesize complement proteins.

3.7.1.2. *Peripheral blood lymphocytes stimulated with mitogens.*

Lymphocyte cultures were stimulated with phytohaemagglutinin (PHA) (120 µg/ml) for 72 hours, culture supernatants were collected after 1, 3, 5 and 7 days and the samples were assayed for the complement components C1-INH, C4-bp, factor H and factor I by ELISA. The results from three separate experiments were calculated as molecule/cell/min and summarized in Table 3.7. C1-INH and factor H were detected after one day at very low levels. The stimulation with PHA did not increase the amounts significantly. Factor I was not detectable in PHA stimulated lymphocytes.

Similar experiments were done with lymphocytes stimulated with Pokeweed mitogen (PWM) (100 µg/ml) for 72 hours and Concavalin A (Con A) (100 µg/ml) for 72 hours, and the culture supernatants assayed by ELISA. The results, calculated as molecule/cell/min and summarized in Table 3.7. These show that although treatment with PWM did not increase the amounts of proteins significantly, following the stimulation with Con A the synthesis of factor I was increased around 25-fold which suggests that Con A upregulates synthesis and secretion of factor I by peripheral blood lymphocytes.

COMPONENTS	CONTROL	PHA	PWM	Con A
C1-INH	0	0	0	11.0
C4-bp	0	0	0	10.0
Factor I	0	0	0	10.0
Factor H	7.2	15.5	3.9	7.1

Table 3.8. Secretion rates of fluid phase regulatory complement proteins of C1-INH, C4-bp, factor H and factor I (molecule/cell/min) in monocyte depleted lymphocytes cultured under standard condition (control) and stimulated with Phytohaemagglutinin (PHA), Pokeweed mitogen (PWM) and Concanavalin A (Con A). These values are from a single experiment.

Monocyte depleted lymphocyte cultures were stimulated with the same mitogens at the same concentrations. The supernatants were examined for secreted C1-INH, C4-bp, factor H and factor I by ELISA. Only factor H was detected in the monocyte depleted lymphocyte culture supernatants even after stimulation with PWM and PHA (Table 3.8). Interestingly all the secreted regulatory proteins were detected following the stimulation with Con A (100 μg/ml) (Table 3.8.).

These data suggested that although complement proteins disappear after monocyte depletion Con A stimulates the synthesis of secreted proteins (C1-INH, C4-bp, factor H and factor I) by lymphocytes. Perhaps lymphocytes secrete those proteins at very low levels and ELISA is not sensitive enough to detect them or lymphocytes may only begin to synthesize those proteins following the stimulation by Con A or possibly cytokines released at site of inflammation by monocytes and other cell types.

3.7.2. Immunohistochemical Analysis of Membrane Regulatory Proteins.

Lymphocytes were isolated from buffy coats and resuspended in PBS. Cytospin preparations were fixed on glass slides with acetone (see section 2.11.1.) and reacted with monoclonal antibodies against DAF (IA10H6) (Figure 3.38 b), CD59 (YTH53.1) and MIP (anti-MIP) (Figure 3.39 a and b), and against MCP (E4.3) and CR1 (E11) (Figure 3.40 a and b). Although the cells did not react very satisfactorily for DAF, CD59, MCP and CR1, they did give weak positive results. Lymphocytes stained strongly for MIP.

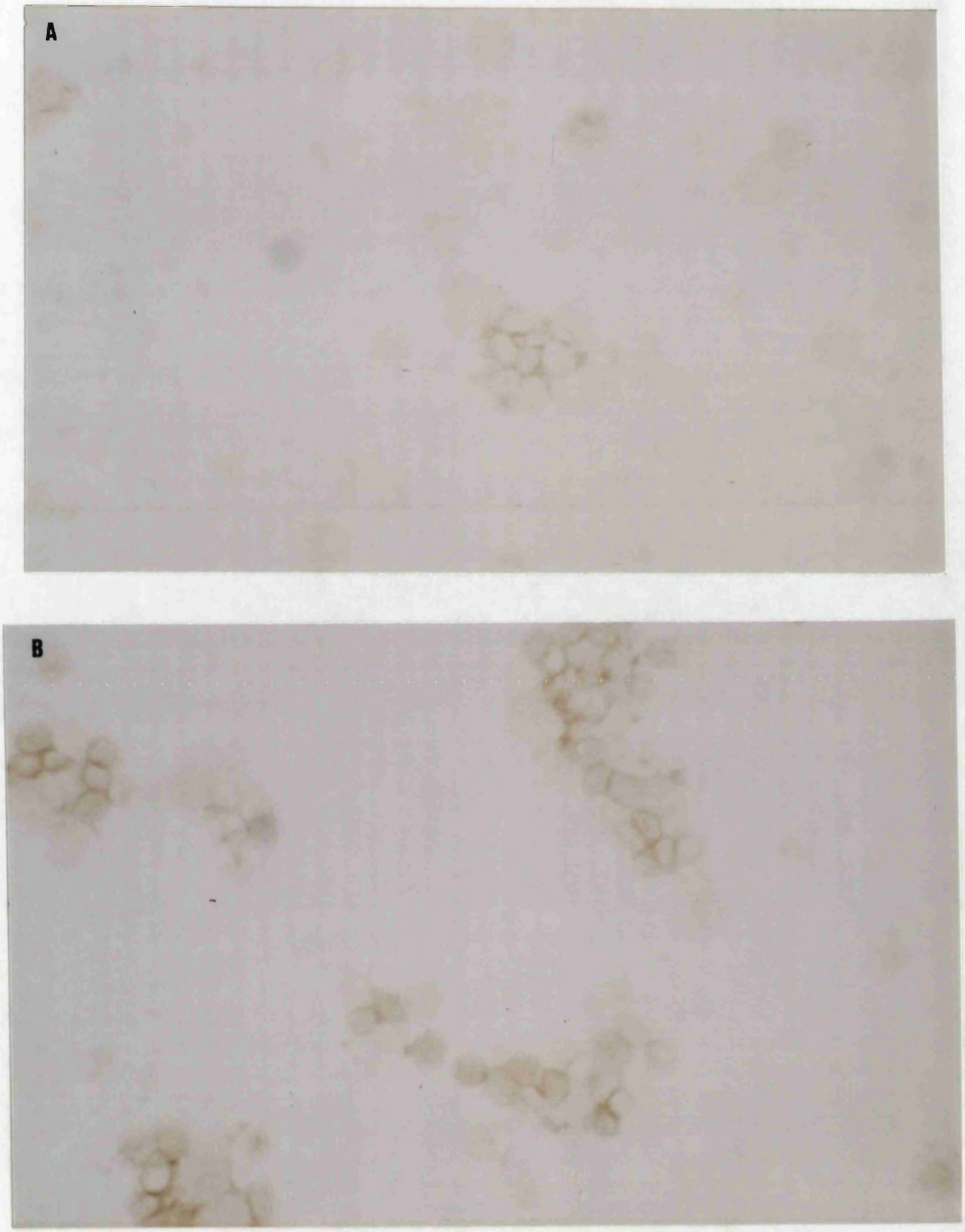


Figure 3.38: Imunohistochemical analyses of lymphocytes. Cytospin preparations of cells were fixed and stained by the immunoperoxidase method. **a)** Mouse IgG was used at 1/100 dilution as control. **b)** Monoclonal antibody to human DAF (IA10H6) was used at 1/100 dilution. DAF localized on lymphocytes (brown colour). x 250

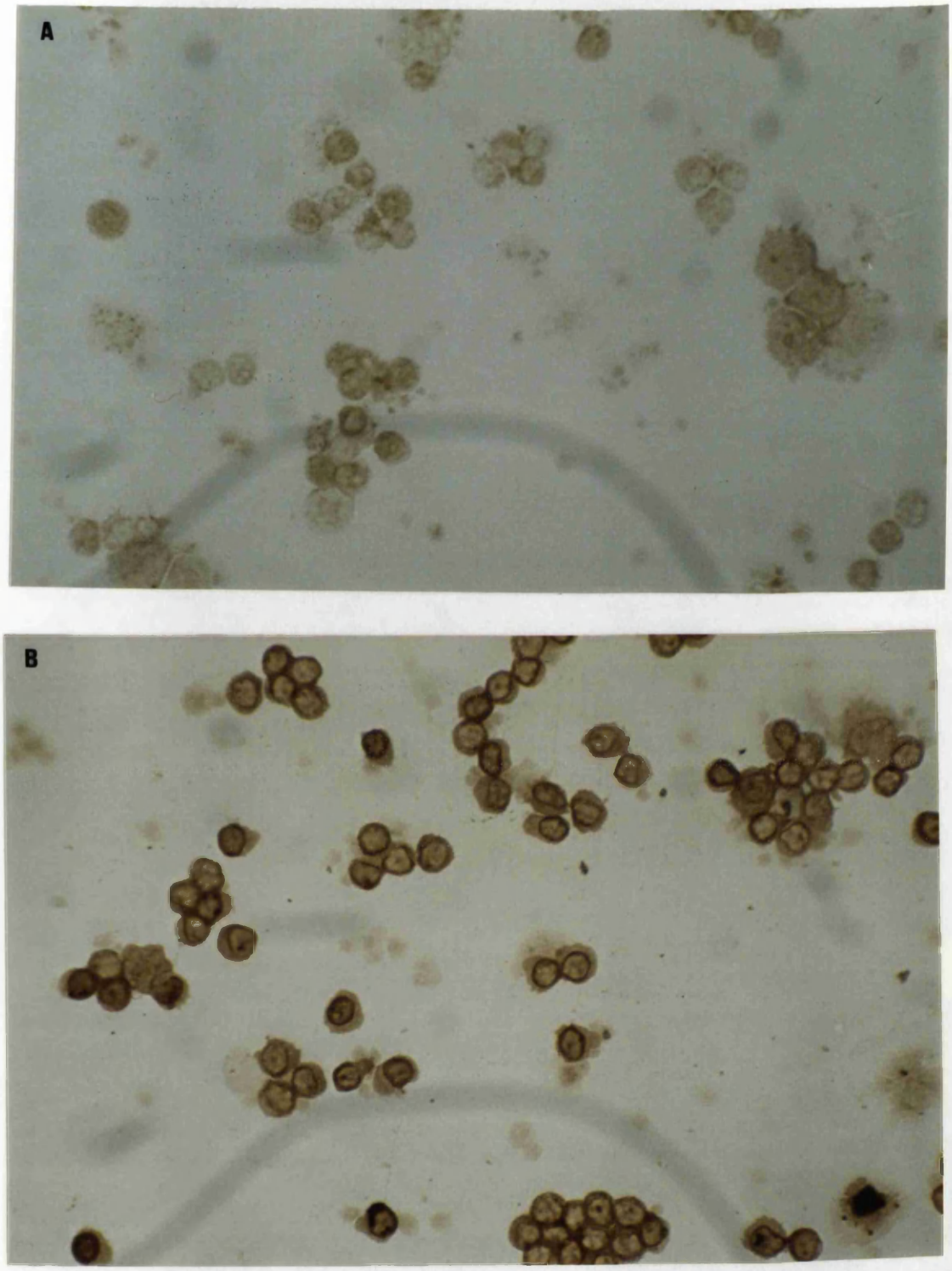


Figure 3.39: Immunohistochemical analyses of lymphocytes. Cytospin preparations of cells were fixed and stained by the immunoperoxidase method. **a)** Monoclonal antibody to human CD59 (YTH53.1) was used at 1/100 dilution. CD59 localized on lymphocytes (brown colour). **b)** Monoclonal antibody to human MIP (anti-MIP) was used at 1/100 dilution. MIP localized on lymphocytes (brown colour) (x 250).

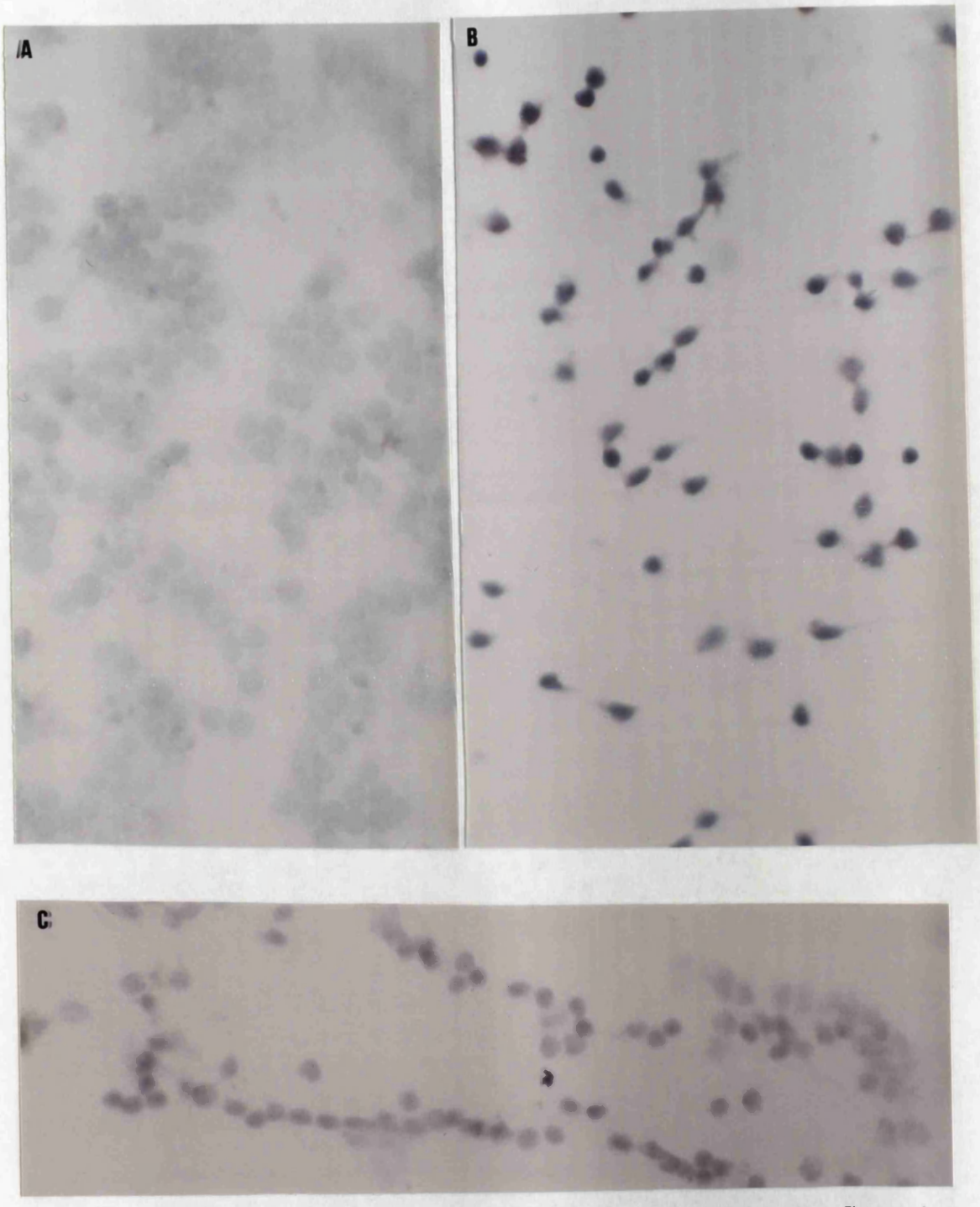


Figure 3.40: Immunohistochemical analyses of lymphocytes. Cytospin preparations of cells were fixed and stained by the alkaline phosphatase method. **a)** Mouse IgG was used at 1/100 dilution as control. **b)** Monoclonal antibody to human MCP (E4.3) was used at 1/100 dilution (pink colour). **c)** Monoclonal antibody to human CR1 (E11) was used at 1/100 dilution (pink colour) (x 250).

3.7.3. Abundance of mRNAs Encoding Regulatory Proteins.

Total cellular RNA extracted from lymphocytes was almost always degraded. Using the RNA immediately after extracting it, and treating the solutions and equipment with diethyl pyrocarbonate (DEPC), did not alter the result. Northern blots were performed as described in section 2.9.1. with the best preparations of RNA. Blots were hybridized with ^{32}P-labelled probes for C1-INH, C4-bp, factor H and factor I mRNAs but none of them gave positive signals. Although smears were detected on the blots hybridized with probes for factor H and C1-INH, it may be deceptive to comment about these data and further investigation is necessary.

3.8. EFFECT OF CYTOKINES ON ENDOTHELIAL CELLS

3.8.1. Analysis of Secreted Regulatory Proteins by ELISA in Endothelial Cells Following Cytokine Stimulation

Cytokines are cell-derived mediators of acute and, particularly, chronic inflammation. Interferon γ (IFNγ) is important in macrophage activation. Interleukin 1 (IL-1) is essential for lymphocyte proliferation in response to antigens; it stimulates fibroblast proliferation and is responsible with interleukin 6 (IL-6) for increased hepatic protein synthesis during the acut phase response. Cells at sites of chronic inflammation are subject to the effects of cytokines.

Endothelial cells were treated with IFNγ, IFNα, IL-1 and IL-6 to better understand the events occurring at sites of inflammation. These cytokines were added to the endothelial cell culture medium at 0.01 ng/ml and 10 ng/ml. The supernatants were collected 24, 48 and 72 hours later. The samples were assayed for the complement components C1-INH, C4-bp, factor H and factor I by ELISA. The results from three separate experiments are summarized in Figures 3.41 and 3.42 and the secretion rates (calculated as molecule/cell/min) are summarized in Table 3.9. The amounts of factor H secreted were increased following the stimulation with IFNγ. Furthermore, although secretion of C1-INH was not detected under standard culture conditions, C1-INH became detectable following stimulation by IFNγ. Addition of IL-1 and IL-6 into the culture medium did not upregulate the secreted complement regulatory proteins; in contrast, a slight suppression was observed. Similarly, following the stimulation of endothelial cells with IFNα, a slight decrease was observed in the amount of secreted factor H. As found with IFNγ, IFNα-treated endothelial cells also secreted C1-INH (35 molecule/cell/minute) at 24, 48

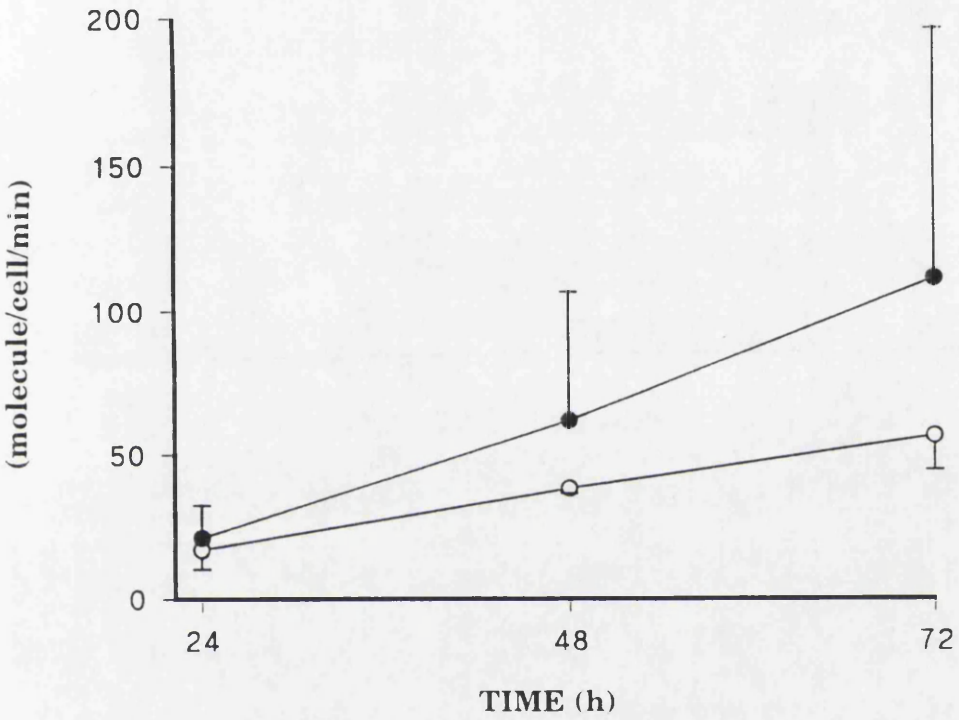


Figure 3.41: ELISA analyses for factor H secreted by endothelial cells. The culture medium was removed completely at 24, 48 and 72 hours. The amount of factor H secreted into the culture medium at the indicated hours was measured. After three days in culture, the results were plotted as (molecule/cell/min). Untreated endothelial cells (open circles), endothelial cells treated with IFNγ (10 ng/ml) (solid circles). Each point represents the mean with the standard deviation of three experiments.

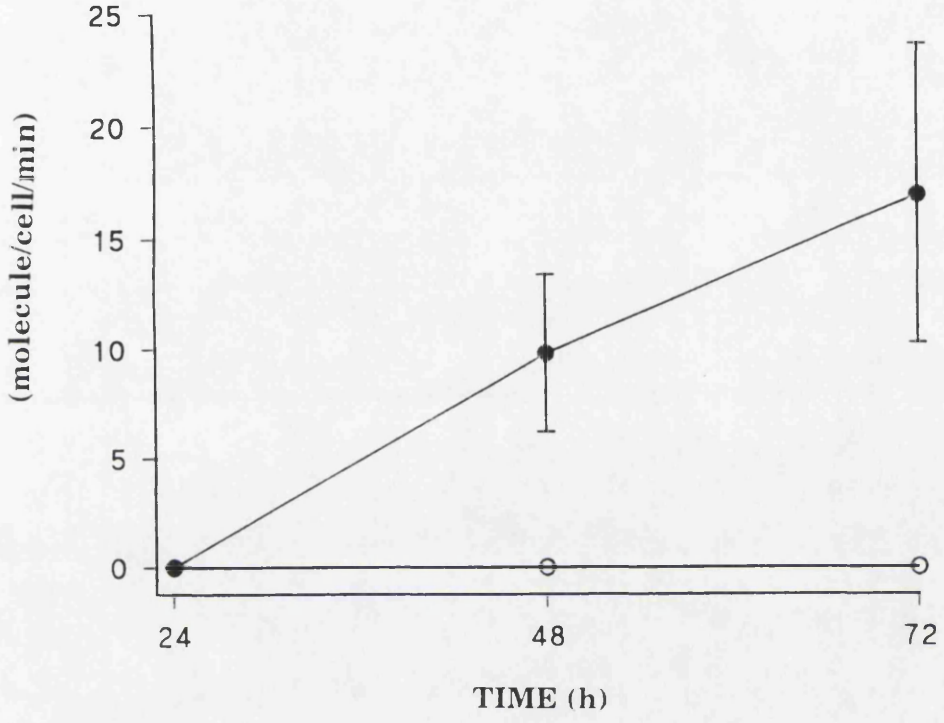


Figure 3.42: ELISA analyses for C1-INH secreted by endothelial cells. The culture medium was removed completely at 24, 48 and 72 hours. The amount of C1-INH secreted into the culture medium at the indicated hours was measured. After three days in culture, the results were plotted as (molecule/cell/min). Untreated endothelial cells (open circles), endothelial cells treated with IFNγ (10 ng/ml) (solid circles). Each point represents the mean with the standard deviation of three experiments.

	CONTROL	0.01 ng/ml	10 ng/ml
IFNγ	53.0 ± 19.0	65.0 ± 25.0	89.0 ± 52.0
IFNα	53.0 ± 19.0	32.0 ± 8.0	39.0 ± 17.0
IL-1β	39.0 ± 22.0	24.0 ± 13.0	30.0 ± 9.4
IL-6	39.0 ± 22.0	17.2 ± 2.8	23.0 ± 19.0

Table 3.9. Secretion rates of factor H expressed as molecule/cell/min in endothelial cells treated with 0.01 ng/ml and 10 ng/ml of IFNγ, IFNα, IL-1β and IL-6 for 24 hours. Each value represents the mean ± SD of three experiments.

and 72 hours. C4-bp not detected in the supernatants of cytokine treated cells.

These data suggested that IFNγ upregulates secreted regulatory proteins in endothelial cells in both the classical (C1-INH) and the alternative (factor H) pathway. Moreover, the secretion of some components like C1-INH becomes detectable only after stimulation with IFNγ. The upregulation observed is dose dependent and the effect develops after 24 hours stimulation by IFNγ. Furthermore, the study made in our laboratory showed that IFNγ also upregulates the transcription rate of the C1-INH and factor H genes in endothelial cells (Lappin et al., 1992). Unlike IFNγ, the other cytokines tested (IL-1 and IL-6) did not upregulate complement regulatory proteins, and although IFNα upregulates CI-INH it did not upregulate factor H. As a result, it was decided to concentrate on a study of the effects of IFNγ on the synthesis of regulatory membrane proteins by endothelial cells.

3.8.2. Analysis of IFNγ-stimulated Membrane Regulatory Proteins by Flow Cytometry.

Endothelial cells were grown until the fourth passage and confluent cells were treated with 10 ng/ml of IFNγ. IFNγ-treated endothelial cells become fusiform in contrast to the characteristic cobblestone appearance of untreated endothelial cells (Figure 3.43). One group of cells was stimulated with IFNγ for 24 hours and the other group used as a control. Cells were prepared for flow cytometry as described in section 2.12. They were treated with monoclonal antibodies against DAF (BRIC 110), MCP (J4-48), CD59 (BRIC 229) and CR1 (E11). The results are summarized in Figure 3.44.

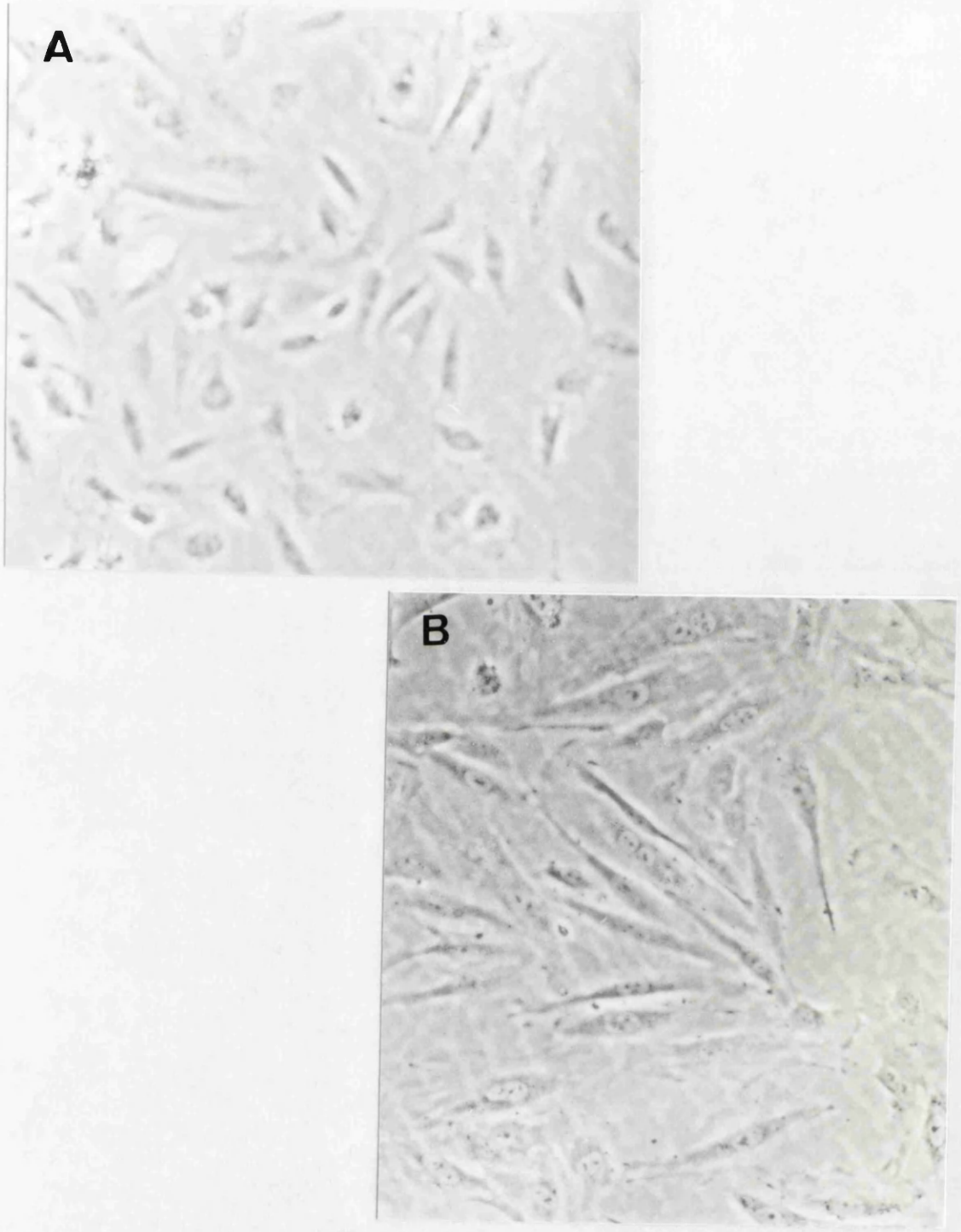


Figure 3.43: a) Characteristic cobblestone appearance of endothelial cells grown under standard culture conditions. b) Following INFγ (10 ng/ml) treatment endothelial cell morphology changed and cells were showed a fusiform appearence. Photographs were taken under light microscopy (x 250).

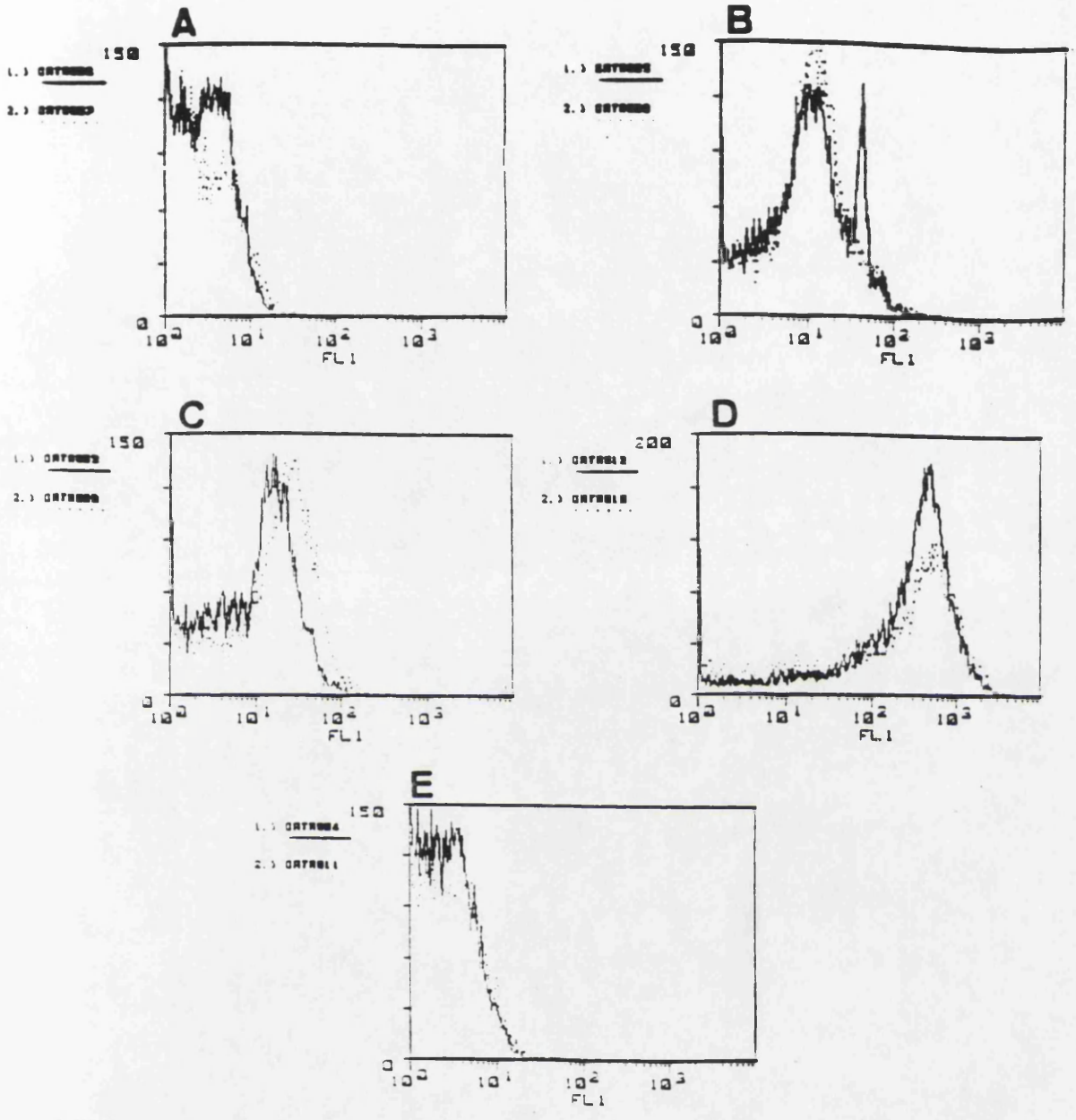


Figure 3.44: Flow-cytometric analyses of endothelial cells during the fourth passage. Dotted lines represent untreated control group, continuous lines represent cells treated with INFγ (10 ng/ml) for 24 hours. Both group of cells were treated with **a)** BSA (0.2%, w/v) in PBS as a background control **b)** anti-DAF (BRIC110, IgG1 mouse), **c)** anti-MCP (J4-48, IgG1 mouse), **d)** anti-CD59 (BRIC229, IgG2b mouse) and **e)** anti-CR1 (E11, IgG1 mouse) and FITC-conjugated anti-mouse Ig. Fluorescence intensity is plotted on a logarithmic scale (x-axis), and cell number on a linear scale (y-axis).

The data suggested that there were no differences in the number of DAF molecules on the majority of the cells after stimulation by IFNγ. However, a small proportion of the cells showed a sharp peak in the fluorescence pattern, which indicates the presence of a sub-set of endothelial cells that upregulate DAF in response to IFNγ. This might be related to the age of the endothelial cells, since this sub-set was absent in higher passages of cells. The fluorescence intensity with anti-MCP was slightly higher in the control group compared to that in IFNγ-stimulated cells. Unlike the secreted regulatory proteins, MCP was downregulated by IFNγ. Nevertheless, although there was no increase in the fluorescence intensity with anti-CD59, the number of cells displaying CD59 on their surfaces was greater in the IFNγ-stimulated cultures. CR1 was not detectable in control or IFNγ-treated cells.

3.8.3. Abundances of mRNAs Encoding Membrane Regulatory Proteins

The effects of IFNγ treatment on protein levels differed between different membrane proteins, as described previously. To investigate if these differences are reflections of the mRNA abundances, total cellular RNA was assayed from control and IFNγ-stimulated endothelial cells.

Endothelial cells in their fourth passage were treated with 0.01, 10 and 100 ng/ml of IFNγ for 24 hours. Total cellular RNA was extracted, and subjected to Northern blot analysis. The blots were hybridized with ^{32}P-labelled probes for the mRNAs encoding DAF, MCP, CD59 and CR1. Multiple bands were observed for DAF (2.7 kb, 1.5 kb), MCP (4.8 kb, 4.2 kb) and CD59 (2.0 kb, 1.4 kb, 0.8 kb). Although similar blots were also probed for CR1 no signals were detected. The Northern blots were stripped and rehybridized with a probe for 28S rRNA to allow corrections

for unequal loading to be made. The results are summarized in Figures 3.45, and 3.46; the abundances of the mRNAs relative to those in unstimulated endothelial cells and corrected for loading errors are summarized in Table 3.10.

These data indicate that the abundance of CD59 mRNA increased after IFNγ stimulation. The increment was not great. The data are in agreement with the flow cytometry results since the number of cells displaying CD59 on their surfaces was more in the IFNγ-stimulated cultures. The abundance of MCP mRNA in IFNγ-treated cultures was slightly decreased compared to untreated cells, also in agreement with the flow cytometry results. A slight increase in mRNA (2.7 kb species) abundances were also observed for DAF in IFNγ-treated cells. The small proportion of responsive cells detected by flow cytometry (see Figure 3.44) seems inadequate to make a difference in mRNA abundances.

3.8.4. The Effect of IFNγ Stimulation on Transcription Rates.

The nuclear extracts were isolated from untreated and IFNγ-treated (10 ng/ml for 24 hours) endothelial cells as described in section 2.7.4. The nascent transcripts were elongated for 30 minutes *in vitro* in the presence of [α-^{32}P]UTP. The labelled RNA was then isolated from the nuclei by RNAzol (see section 2.7.4.). Equal amounts of DAF, MCP and CD59 and vector pUC19 DNAs were immobilized on nylon membranes (see section 2.9.3.). Hybridization with labelled transcripts was carried out as described in section 2.9.4. The results from two experiments are summarized in Figure 4.47.

The data indicate that the transcription rates were increased 60 % for DAF, 90 % for MCP and 3.4 fold for CD59 by IFNγ stimulation. The increase in transcription rates did not correspond with the changes in

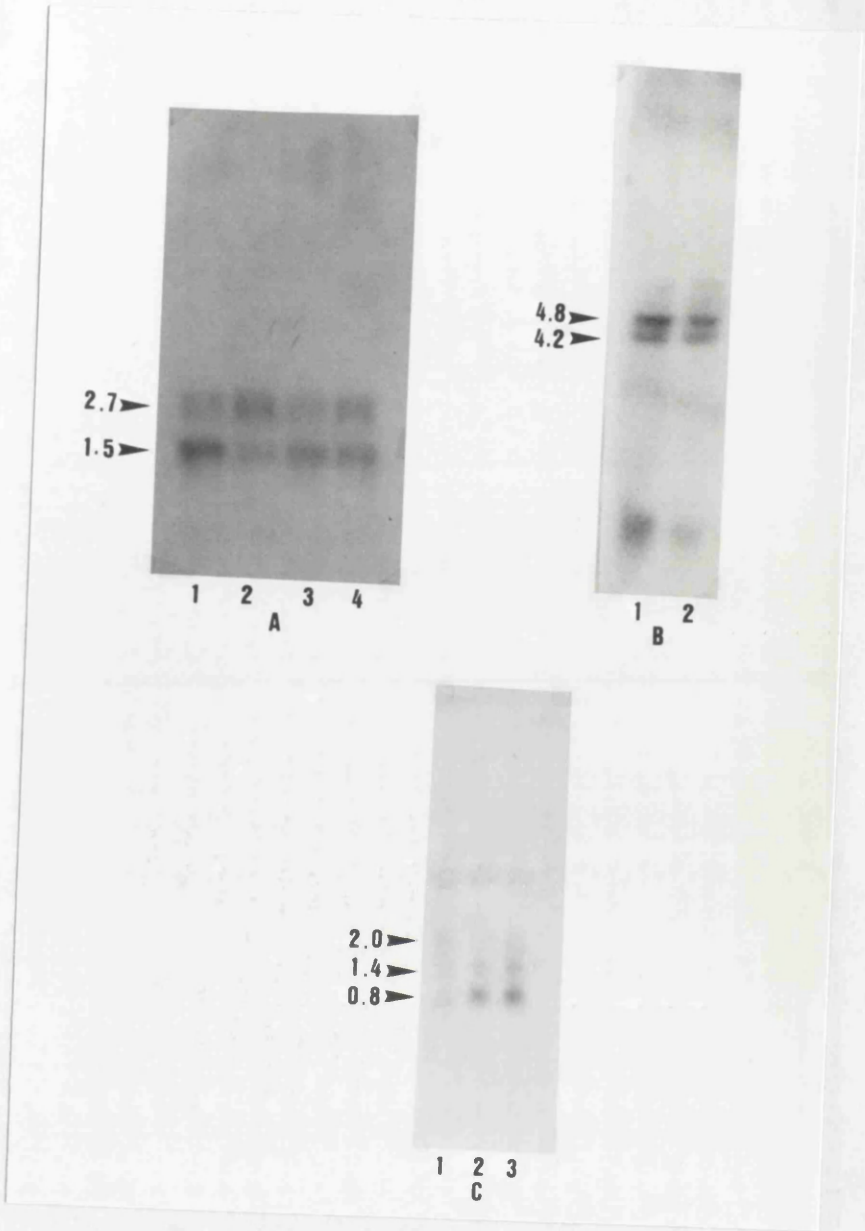


Figure 3.45: Autoradiographs of Northern blot analyses of RNA derived from cultured endothelial cells. The blots were hybridized with ^{32}P-labelled probes for:

a) DAF (lane 1 control; lanes 2, 3 and 4; 0.01, 1 and 10 ng/ml INFγ treated cells RNA respectively).

b) MCP (lane 1 control, lane 2, 10 ng/ml INFγ treated cells RNA).

c) CD59 (lane 1 control, lane 2 and 3; 1 and 100 ng/ml INFγ treated cells RNA). The sizes of mRNA were; DAF (2.7 kb, 1.5 kb), MCP (4.8 kb and 4.2 kb) and CD59 (2.0 kb, 1.4 kb and 0.8 kb).

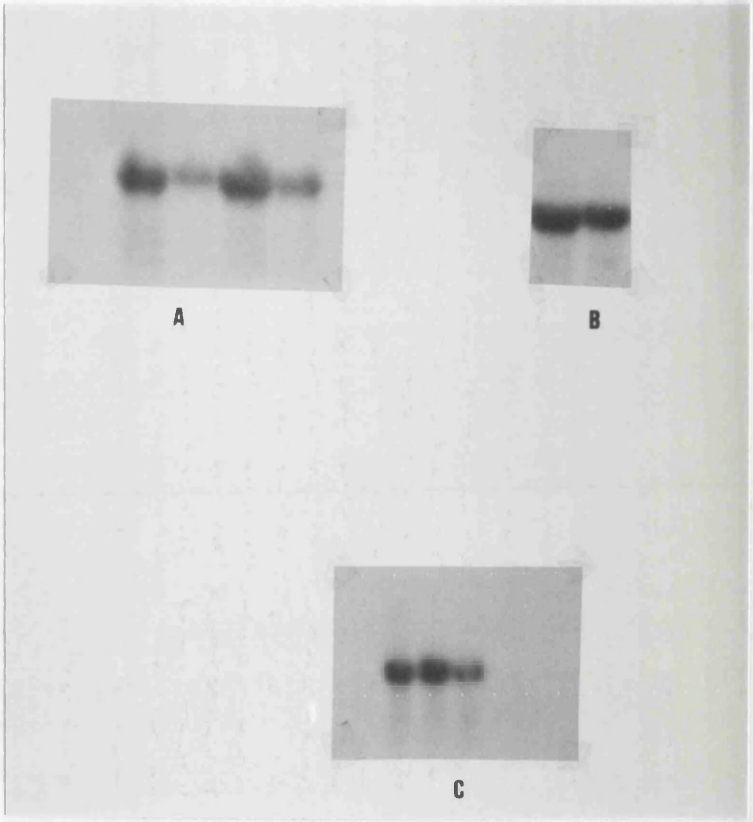


Figure 3.46: Rehybridization of Northern blots shown in Figure 3.45 (in a, b and c) with a probe for 28S rRNA. Northern blots had previously been hybridized with probes for **a)** DAF, **b)** MCP and **c)** CD59.

		CONTROL	10 ng IFNγ
CD59			
	2.0 kb	1.0 ± 0.50	1.48 ± 0.89
	1.4 kb	1.0 ± 0.48	1.36 ± 0.1
	0.8 kb	1.0 ± 0.43	1.67 ± 1.0
DAF			
	2.7 kb	1.0 ± 0.25	1.10 ± 1.0
	1.5 kb	1.0 ± 0.27	0.70 ± 0.36
MCP			
	4.8 kb	1.0 ± 0.35	0.95 ± 0.33
	4.2 kb	1.0 ± 0.40	0.97 ± 0.38

Table 3.10. Relative mRNA abundances for CD59, DAF and MCP in IFNγ-treated (10ng/ml for 24 hours) and untreated endothelial cells. The abundance of each mRNA in untreated cells was given an arbitrary value of 1 unit. Each value represents the mean ± SD of three experiments.

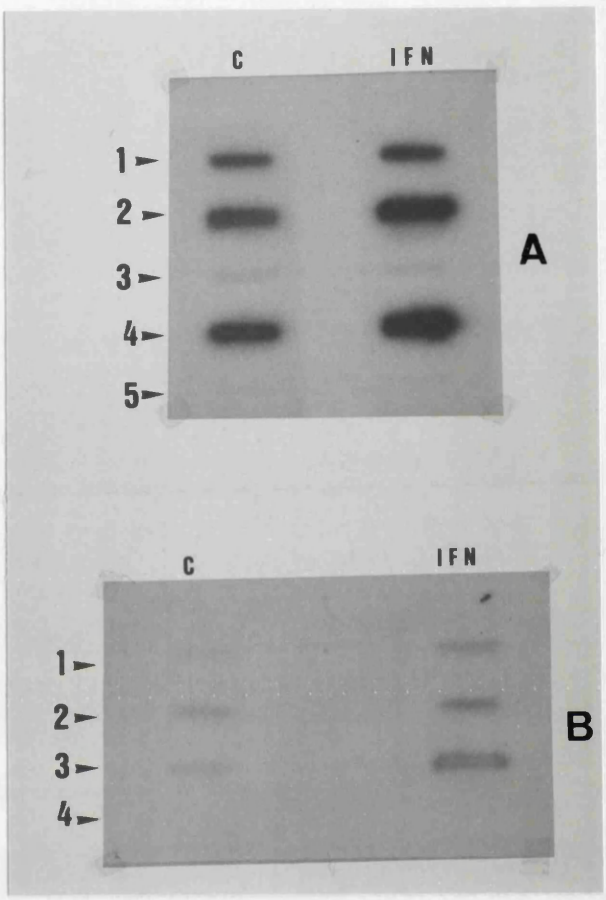


Figure 3.47: Transcriptional activity of regulatory membrane protein genes. Endothelial cells were incubated with INFγ for 24 hours. Subsequently nuclei were isolated for run-on experiments. ^{32}P-labelled transcripts were hybridized to immobilized DNAs, bound to nylon membrane. Plasmids containing the following DNA fragments were used **1)** DAF (in A and B); **2)** MCP (in A and B); **3)** CD59 (in A and B); **4)** SP-40,40 (in A), vector DNA pUC19 (in B); **5)** vector DNA puc19 in A. In the left lane nuclei of untreated endothelial cells, in the right lane nuclei of endothelial cells treated with INFγ (10 ng/ml).

mRNA abundances and protein concentration as shown by the Northern blot and flow cytometry data. It is possible that changes in mRNA stabilities might be an explanation for this discrepancy.

3.8.5. The Effect of IFNγ Stimulation on mRNA Stability.

Endothelial cells were grown until fourth passage. One group of cells were treated with IFNγ for 24 hours and one group used as a control. Both group of cells were treated with Actinomycin D (5 μg/ml), which prevents RNA synthesis, for different times (0, 1, 2, 4, 6, 8, and 24 hours) as described in section 2.3.7.2. Total cellular RNA was extracted and blotted on Hybond N^+ membranes as described in section 2.9.2. Dot blots were hybridized with ^{32}P-labelled probes for the mRNAs of DAF, MCP and CD59, then stripped and rehybridized with a probe for 28S rRNA to allow corrections for unequal loading to be made. The half-lives of DAF, MCP and CD59 mRNAs were calculated to compare their stabilities in untreated and IFNγ-treated cells. The results are summarized in Table 3.11.

These data suggested that CD59 mRNA has longer half-life than MCP and DAF. IFNγ-treatment did not made great differences in the half-lives of membrane regulatory proteins. DAF and CD59 mRNA half-lives showed 25% and 10% decrease respectively wheras MCP mRNA half-life did not change. Although small decreases was observed in mRNA half-lives following IFNγ this is not enough to explaine the contradiction between the increase in transcription rates and the changes in mRNA abundances and protein concentration. A block in the transcription of the membrane regulatory genes may cause the contradiction observed. The increase in transcription rate of DAF, MCP and CD59 following IFNγ-treatment may depend on some transcriptionally active initial exons.

	DAF	MCP	CD59
IFNγ (-)	2.1	1.7	3.9
IFNγ (+)	1.6	1.6	3.5

Table 3.11. The effects of IFNγ (10 ng/ml for 24 hours) on mRNA half-lives (in hours) of DAF, MCP and CD59 in endothelial cells. These values are from a single experiment.

However, in the later part of those genes (encoding DAF, MCP and CD59) transcription rate may reduce, thus run-on transcription assays shows increase transcription but the mRNA abundances remain steady. Similar observation have been reported for c-*myc* (Eick and Bornkamm, 1986) and c-*myb* (Reddy and Reddy, 1989) gene expressions. Further studies on transcription rate of DAF, MCP and CD59 by using a number of probes along these genes needs to be done.

CHAPTER 4. DISCUSSION

4.1. EXTRAHEPATIC COMPLEMENT SYNTHESIS

Activation of the complement system plays a major role in the host defence against infections and in inflammatory responses. Rheumatoid arthritis (RA) is a classical example of chronic inflammation. Local complement activation occurs in RA joints as witnessed by increased levels of activation products including C3dg and C5b-9 membrane attack complex in synovial fluid (Mollnes et al., 1986). These data show that C3 and C5 convertases are formed and that the terminal part of the complement cascade is also involved in activation.

Although the liver is the major site of synthesis for most circulating complement components, it is now well established that other cells including monocytes, macrophages (Littman and Ruddy, 1977, Whaley, 1980), fibroblasts (Katz and Strunk, 1988a, 1988b, 1989a), endothelial cells (Ripoche et al., 1988) and intestinal epithelial cells (Colten, 1976) synthesize complement components. *In vitro* studies of synovial fluid macrophages and adherent synovial membrane cells showed that they synthesize complement components (De Ceulaer et al., 1980). Moreover, metabolic studies with radiolabelled ^{125}I-C3 showed that approximately 50 per cent of the intra-articular C3 in the RA joint was synthesized locally (Ruddy and Colten, 1974). Katz and Strunk (1988a) established that complement protein synthesis is a constitutive function of fibroblasts from normal, osteoarthritis (OA) and RA synovial membranes and thus showed that the capacity to synthesize complement does not require the presence of inflammation. Later studies revealing the presence of mRNAs encoding C4, C2, C3, factor B and C1-INH in

synovial membrane from normal joints as well as OA and RA joints have provided conclusive evidence that synthesis of these complement components occurs locally within normal and inflamed synovium *in vivo* (Moffat et al., 1989). However, the purpose of local synthesis of complement components is unknown. In normal tissues it may play a role in the host defence whereas in inflamed tissues it may contribute to the inflammatory process.

At the time of starting the work described in this thesis, there was abundant evidence that complement activation occurred in RA joints and that some complement components were synthesized locally. In this potentially cytolytic environment, regulation of complement activation is essential for the protection of host cells. The present study is the first thorough investigation of the synthesis of secreted and all the regulatory membrane-bound complement proteins involved in the regulation of complement activation in synovial tissue from normal individuals, and patients with RA and OA. The contributions of some of the different types of cell, which are present in synovium, were also studied using cultures of endothelial cells, fibroblasts, monocytes and lymphocytes, in order to extend the understanding of regulation of complement activation in normal and inflamed synovial membrane.

Complement activation occurs in a cascade manner, and each step is controlled by regulatory proteins in both the classical and the alternative pathways and in the membrane attack sequence. In total there are eleven regulatory proteins either in the fluid-phase or on cell membranes. C1-INH, C4-bp and factor I control the classical pathway in the fluid-phase. Factor H and factor I are also fluid-phase proteins which are responsible for alternative pathway regulation. Membrane attack

sequence regulatory proteins are S protein and SP-40,40 (Choi et al., 1989).

The membrane-bound regulatory proteins DAF, MCP and CR1 affect either the assembly or stability of C3/C5 convertases in both classical and alternative pathways, whereas CD59 and MIP prevent the insertion of membrane attack complexes into cell membranes (Lachmann, 1991).

4.2. COMPLEMENT SYNTHESIS IN SYNOVIAL TISSUE

4.2.1. Dot-blot Analysis of mRNA abundances

In this study the synthesis of regulatory complement proteins was based upon the detection of specific mRNAs by dot-blot analysis. This technique was used as the complement mRNAs were found to be degraded when analyzed by Northern blot analysis. Thus, quantification of the relative abundances of these mRNAs could not be assessed by Northern blotting. Similar technical problems were previously reported by Moffat (1989). However, he concluded that the mRNAs which code for the complement components are unstable in synovial tissue *in vivo* since the probing of the Northern blots with β_2-microglobulin and actin cDNAs showed these mRNAs to be undegraded. Another difficulty of studying the synovial tissue was the synovial membrane tissue samples were limited and the amount of each available tissue sample was small. This limited the number of experiments which could be performed.

Human cDNA probes were used throughout the dot-blot analysis. A determination of the size of the mRNAs was not possible with dot-blots,

but non-specific hybridization was eliminated by washing the filters to high stringency (0.1 x SSC containing 0.1 % SDS at 65°C for 30 minutes), which should only allow the formation of specific DNA-RNA hybrids on the filters.

Densitometric analysis of the dot-blot autoradiographs for DAF, MCP and CR1 did not show significant differences in the abundances of mRNAs from normal synovial membrane compared to OA and RA synovial membrane. This may indicate that these membrane-bound proteins, which are effective on the early steps of complement activation, do not really play a vital role for the inhibition of complement activation in inflamed synovium.

The abundances of the mRNAs encoding the classical and alternative pathway secreted proteins were elevated in RA synovium, as was that of CD59 mRNA. These increases might be related to increased synthesis of these regulatory proteins in an attempt to regulate the activation of complement at the early stages of both the classical and alternative pathways. Thus, cells in synovium protect themselves from the lytic attack of complement by increasing the fluid-phase secreted regulatory proteins rather than the membrane-bound regulatory proteins.

Increased abundance of CD59 mRNA in synovial membrane from RA patients might also show that, if some activated components escape from the inhibition in the fluid-phase, increased synthesis of CD59 on cell membrane might prevent the formation of MAC. This indicates that the cells at sites of inflammation might be well protected against homologous complement attack. However, the data on the secreted regulatory proteins and CD59 must be interpreted cautiously as only one assay for

mRNA abundance could be determined in each type of tissue due to the limited amount of tissue available.

The presence of SC5b-9 complex in the synovial fluid of RA patients and in cultured synovial cells has been reported (Morgan et al., 1988a and 1988b). Although S protein and SP-40,40 act on the soluble C5b-7 complex to prevent its insertion into cell membranes, there was not a significant increase in the abundances of their mRNAs in contrast to that of CD59 mRNA. However, it must be remembered that the rate of translation, and so the amount of protein secreted can be increased even when the abundance of the mRNA is unaltered.

Membrane-bound proteins generally protect only those cells on which they are expressed (Oglesby et al., 1992, Wing et al., 1992). In contrast, all the cells in the inflammatory zone can benefit from the regulatory effects of fluid-phase proteins. Synovial membrane contains different types of cell each of which secretes a different range of the control proteins of the classical and alternative pathways (see sections, 3.4., 3.5, 3.6., 3.7.). Thus complete regulation of activation of the early stage in the fluid-phase depend upon mononuclear phagocytes (C1-INH, C4-bp, factor H), fibroblasts (C1-INH, factor H, factor I), endothelial cells (factor H and factor I). However, regulation of complement at the convertase levels is not completely effective so that regulation of the membrane attack complex on the cell membrane is also required. Thus, elevated levels of secreted classical and alternative pathway proteins and CD59 in RA synovium might provide a broad spectrum of protection for the cells at both the early and at the late stages of complement activation at sites of inflammation.

Although the dot-blot analyses demonstrated that the mRNAs encoding for all the known complement regulatory proteins were present in normal, RA and OA synovial membranes, many mRNAs are subject to translational control and thus the presence of mRNA may not necessarily indicate synthesis of protein. For instance, in unfertilized sea urchin eggs maternal mRNAs are stored as translationally repressed mRNPs (Grainger and Winkler, 1987). Also, as the tissue samples were small, (under agarose) they might only reflect the local changes in mRNA abundances and not the abundances in the whole synovial membrane.

4.2.2. Analysis of Culture Supernatants

Synthesis of C1-INH, C4-bp, factor I and factor H in synovial membranes from normal individuals and patients with RA and OA was also investigated by studying the accumulation of the proteins in tissue culture supernatants. There was great variation between experiments, but all the components measured accumulated rapidly in the culture medium during the first and often the second day of culture. After the second day the rate of accumulation of each components decreased as has been reported for C3 and C4 previously (Moffat, 1989). It is possible that a feed-back inhibition mechanism could be responsible for the decreased synthesis in the later stages of culture as has been suggested for C4 synthesis by guinea pig peritoneal macrophages (Auerbach et al., 1983). Addition of cycloheximide caused variable, but incomplete inhibition of proteins. So, part at least of regulatory complement protein accumulation in medium probably due to release of protein absorbed from

plasma *in vivo*. Washing was not effective to change the accumulation pattern in medium.

The partial inhibition of protein accumulation in culture supernatants observed in some cultures implies that a proportion of the components measured had been synthesized locally by synovial membrane indicating that, in some circumstances at least, the regulatory complement protein mRNAs are translated in cultured fragments of synovial membranes.

4.2.3. Immunohistochemical Analysis

Immunohistochemical staining of frozen sections of synovial membrane from RA patients showed that CD59, MIP and DAF were expressed on most cells, particularly on cells lining the synovium, endothelial cells and lymphocytes. Staining of the synovial lining cells for CD59 and MIP was very strong. The high expression of these membrane-bound proteins may protect these cells from cytolysis and this could help explain their survival despite complement activation in the synovial membrane in the adjacent synovial fluid. The importance of CD59 in the protection of cells against complement attack is demonstrated by the severe complement-mediated haemolysis which occurs in patient with paroxysmal nocturnal haemoglobinuria. These patients have a defect in forming phosphatidyl glycolipid anchors and so lack membrane CD59 (Holguin et al., 1989; Pangburn et al., 1983).

In keeping with the finding of increased abundance of CD59 mRNA on dot-blot analysis of RA synovium, immunohistochemistry results also showed abundant CD59 in synovial lining cells. These observations also

suggest that increased expression of CD59 may be involved in cytolysis in inflamed joints.

It has been reported that DAF is expressed only a small extent in hyperplastic and multilayered synovial lining cells in RA synovitis whereas it is expressed highly in normal synovium (Itoh et al., 1991). In contrast, I have found a wide DAF expression in cells lining the RA synovium. Moreover, in the same study Itoh et al. (1991) HRF20 (CD59) and CR1 were not detected in either the flat or hyperplastic synovial lining cell while I have found a high concentration of CD59 in synovial lining cells. Immunohistochemical studies of cells lining normal and inflamed hyperplastic synovium showed that the degree of hyperplasia varied considerably in different specimens of RA synovium and that the synovial membrane hyperplasia in OA patients was patchy (Athanasou et al., 1988). These observations might explain some of the contradictions between the results of my study and those reported by Itoh et al. (1991). However, I can not explain the deficiency of CD59 reported in normal and inflamed tissue by Itoh et al. (1991). Since cells lining the synovium include two major cell types the macrophage-like (A type) and the fibroblast -like (B type) (Graabek, 1984), and CD59 is widely expressed on peripheral blood monocytes (Philbrick et al., 1990), one would expect this protein to be expressed on A type cells. Furthermore, CD59 mRNA was detected by Northern blotting of fibroblast RNA and membrane expression of CD59 was detected by immunohistochemistry in this study (see sections 3.4.2., 3.4.3.).

Immunohistochemical analysis of normal and OA synovial membrane in addition to RA synovial membrane may clarify any differences in the distribution and the level of expression of membrane-

bound complement regulatory proteins. However, it is clear that cells in synovial membrane are capable of expressing membrane-bound regulatory complement proteins *in vivo* (3.3.2.).

4.3. CELLS RESPONSIBLE FOR COMPLEMENT REGULATORY PROTEIN SYNTHESIS IN SYNOVIAL MEMBRANE

There are various cell types in synovial membrane which could be responsible for extrahepatic synthesis of complement regulatory proteins. These cells are fibroblasts, endothelial cells, mononuclear phagocytes, lymphocytes and macrophage-like (type A) and fibroblast-like (type B) synovial lining cells.

Two approaches could be applied to determine the range of regulatory complement components synthesized by each of these cells. First is isolation of these cells directly from synovial membrane. This is technically possible. Isolation of synovial fibroblasts (Dayer et al., 1976), synovial membrane macrophages (De Ceulaer et al., 1980) and synovial microvascular endothelial cells (Jackson et al., 1989) has been reported. However, as mentioned before, the availability of the synovial membrane tissue samples was very restricted and isolation of all the cell types from synovium was not practical. Instead, fibroblasts were isolated from synovial membrane and model cell cultures chosen for the other cell types.

4.3.1. Complement Regulatory Protein Synthesis by Fibroblasts

Fibroblasts were isolated from synovial membrane from normal individuals and patients with OA and RA and the cells were cultured and passaged until sufficient number was obtained to perform the experiments. The drawback to this multiple passages may select a sub-population of cells that is not representative of the fibroblasts of synovial membrane; again lack of the material dictated this course.

4.3.1.1. mRNA analysis

All regulatory protein mRNAs, except for C4-bp and CR1, were detected in RNA from fibroblasts from all three types of synovial membrane. These findings support the suggestion that complement protein synthesis is a constitutive function of fibroblasts and that the capacity to synthesize these proteins does not require the presence of inflammation (Katz and Strunk, 1988a). However, all that data show is that genes encoding regulatory complement proteins are transcribed and the synovial membrane fibroblasts contain mature mRNAs encoding these proteins *in vitro*.

This is the first report of the detection of factor I mRNA in fibroblasts. The abundance of this mRNA was found to be significantly low in RA fibroblasts compared to normal fibroblasts. It is not known if there is a decrease in the transcription rate of the factor I gene in RA fibroblasts or a decreased stability of its mRNA. As with the factor H mRNA findings, these results imply a persistence of the *in vivo* phenotype in cultured cells. Factor I levels are reduced in RA synovial

fluid (Whaley et al., 1975) whereas levels of factor H are not reduced. These data were originally interpreted in terms of the catabolism of these components, whereas my data suggest that changes in synthesis might play a role in producing these alterations. Reduction of factor I levels in synovial tissue might be expected to result in increased complement activation because of reduced inactivation of C3b and C4b. Failure to inactivate C3b may result in its binding to host cells, with alternative pathway activation occurring unless the membrane regulatory proteins (DAF and MCP) can cope with this increased demand (reviewed in Parker, 1992). If not chronic tissue damage will result.

Factor H mRNA abundance was significantly higher in RA fibroblasts than in normal or OA fibroblasts. However, caution must be excersed in the interpretation of these data as they imply that some of the phenotypic characteristics that fibroblasts express in inflamed synovial membrane persist in culture *in vitro*. Furthermore, the secretion of factor H was not significantly increased in RA synovial fibroblasts (see 4.3.1.2.). There was no difference in C1-INH mRNA abundance between normal, RA and OA synovial fibroblasts; C4-bp does not appear to be synthesized by these cells.

Previously biosynthetic labelling experiments showed that synovial fibroblasts synthesize seven complement components including C1-INH (Katz and Strunk, 1988a). Later, ELISA analysis of synovial membrane fibroblast culture supernatants confirmed the synthesis of C1-INH in these cells (Moffat, 1989). Recently, the occurrence of C1-INH and factor H mRNAs in, and secretion of the proteins from skin fibroblasts have been reported (Lappin et al., 1992).

A strong association between the presence of SP-40,40 and membrane attack complex (MAC) occurs in glomeruli in which immunoglobulin deposits are present, and investigators suggested that MAC found in the absence of SP-40,40 is cytolytically active (French et al., 1992). In RA fibroblasts the abundance of SP-40,40 mRNA is increased (section 3.4.3.). The presence of the TCC (the soluble form of the C5b-9 MAC which contains S protein and/or SP-40,40) in RA joints has also been reported (Mollnes et al., 1986; Morgan et al., 1988a). Thus, up-regulation of SP-40,40 synthesis may prevent the formation of the lytic MAC and so protect the host cells against homologous complement attack.

Apart from its cytolytic activity, non-lethal amounts of MAC stimulate the production of oxygen metabolites from cultured human synoviocytes (Morgan et al., 1988b). Thus, sub-lethal amounts of MAC stimulate the formation of these toxic metabolites which could contribute to tissue damage. The abundance of the mRNA for the other MAC regulatory protein (S protein) was reduced in RA synovial fibroblasts. Assuming that the relative abundances of S protein and SP-40,40 mRNAs reflect the synthetic rates of these two proteins, it is possible that increased synthesis of SP-40,40 could compensate for decreased S protein synthesis.

The relative abundances of the mRNAs for membrane regulatory proteins DAF, MCP and CD59 in OA, RA and normal synovial membrane fibroblasts were not different. However, there were differences in the distribution of the different species of CD59 mRNA in the three types of fibroblasts. The relative abundance of the 2.0 kb species of CD59 mRNA in OA was significantly lower than in RA or normal fibroblasts. However,

the functional significance of this is unclear as it has been suggested that the higher molecular-weight bands simply represent relatively stable, albeit nonfunctional, processing intermediates (Philbrick et al., 1990). If this conclusion is correct, the decreased abundance of this mRNA species may not necessarily cause a deficiency in CD59 expression on the cell surface and thus may not cause a functional defect. Cell-specific expression of different mRNA species of CD59 were also detected in fibroblasts and endothelial cells and will be discused in more detail in section 4.5. Recently, tissue-specific expression of CD59 mRNA species was also reported in liver, brain, lung tissues and placenta (Kumar et al., 1993). Whether these differences are related to differences in the abundance of protein is unknown. Although CD59 was isolated as a complement regulatory protein, there are reports which suggest that CD59 participates in a transmembrane signalling pathway which results in cell activation (reviewed in Lublin, 1992). Moreover, CD59 with MIP have been reported to be stress proteins (Schiren et al., 1991). Further investigations are required on the level of expression of the membrane regulatory proteins and their functional activity in the three types of fibroblasts and the significance, if any, of relative abundances of the different mRNA species of CD59.

4.3.1.2. Analysis of culture supernatants

Analysis of culture supernatants from synovial fibroblasts derived from three normal, three RA and three OA patients indicated that these fibroblasts synthesize C1-INH, factor H and factor I. The secretion of proteins increased linearly throughout the seven-day culture period.

Addition of cycloheximide to culture media inhibited the synthesis of these proteins. This indicates *de novo* synthesis of the secreted regulatory proteins in cell culture.

There was no difference in the amounts of C1-INH secreted by normal and RA synovial membrane fibroblasts. This is in agreement with the finding of similar abundances of C1-INH mRNA in normal and RA fibroblasts. However, C1-INH secretion was significantly lower from OA fibroblasts compared to normal fibroblasts. Although C1-INH mRNA abundance was also lower in OA than normal fibroblasts, the difference was not statistically significantly.

Despite the increased abundance of factor H mRNA in RA fibroblasts, there were no significant differences in the amounts of factor H secreted from normal, RA and OA fibroblasts. It is possible that in RA fibroblasts the mRNA encoding factor H is masked by proteins in stable ribonuclear particles which protect them against degradation, but which also prevent translation. If this is the correct explanation then the *in vitro* culture conditions must not allow the unmasking of mRNAs, possibly because of the absence of a factor which stimulates translation and which is only present *in vivo*. The occurrence of such masked mRNAs has previously been shown to explain events during, for the example, the fertilization of sea urchin eggs (Grainger and Winkler, 1987).

There was a good agreement between the abundance of factor I mRNA and the excretion of this protein. As mentioned above, the abundance of factor I mRNA was lower in RA fibroblasts than in normal synovial fibroblasts. In fact, although the secretion of factor I was detected in all normal synovial fibroblast cell culture supernatants, I detected factor I in only one of the three RA fibroblast culture

supernatants. Statistical analysis of the data showed significant differences between normal and RA fibroblasts in the amounts of factor I secreted. Furthermore, the secretion rate of factor I in fibroblasts was very low compared with those of C1-INH and factor H.

The double-antibody ELISA assays performed with anti-S protein antibody did not give satisfactory results as considerable amounts of S protein bound non-specifically to the plastic surface despite the use of blocking agents and detergents. Similar problems have been reported by other investigators (Hogasen et al., 1991). This problem occurred even when different types of ELISA plates were used and suggest that the adhesion properties of S protein (vitronectin) may be involved. SP-40,40 antibody was not available. Thus the secretion rates of these two proteins could not be determined.

4.3.1.3. Immunohistochemical analysis

Normal synovial fibroblasts stained positively for DAF, MCP, CD59 and MIP. CD59 and MIP showed the strongest staining while MCP was weakest. It is unknown what is the relative contributions of CD59 and MIP for protection against complement-mediated damage as compared with DAF and MCP. Functional comparison studies need to be performed. If the expression pattern observed is correct it would appear that protection against insertion of the MAC is more important than protection at the level of the C3 and C5 convertases. Expression of MCP, CD59 and MIP has been reported previously in skin and synovial fibroblasts (McNearney et al., 1989, Schieren et al., 1991). These data are in agreement with those in section 3.4.2. of this thesis.

Because of lack of time staining was not performed on RA and OA fibroblasts. Also it is known that the presence of inflammatory mediators modulate the expression of membrane-bound proteins, *e.g.* IFNγ, upregulates MIP and CD59 in fibroblast (Schieren et al., 1991). So, additional tissue sections from intact synovial membrane, which include the connective tissue, rather than isolated fibroblast, may give a better idea for the expression pattern of membrane-bound proteins in normal and inflamed tissue synovial fibroblasts.

4.3.2. Complement Regulatory Protein Synthesis by Monocytes

Monocytes/macrophages are a major source of extrahepatic complement protein as they synthesize more complement components than any cells apart from hepatocytes. They are the only cell type examined in this study that synthesize C4-bp. Although they do not synthesize factor I, they do synthesize C1-INH, so they secrete all of the regulatory components of the classical pathway, which may be important in the regulation of the complement activation in the normal synovial joint, and in the synovial joint affected by OA and RA.

Although factor H secretion was detected in monocyte culture supernatants, factor H mRNA was not detectable by Northern blot analysis of RNA from monocytes. This failure to detect factor H mRNA was probably due to its instability in monocytes. Factor H mRNA was detected when the cells had been cultured in the presence of cycloheximide, which selectively stabilizes mRNAs by arresting their translation on ribosomes (Alberts et al., 1989). The half-life of factor H mRNA in monocytes was found to be very short (12 minutes) compared

with over 3 hours in fibroblasts and endothelial cells (Lappin et al., 1992). These cell-specific differences in mRNA stability could be due to a labile destabilizing factor, such as that described for *c-myc* mRNA (Brewer and Ross, 1989). It is probable that the instability of factor H mRNA in monocytes contributes to the low secretion rate of this protein from these cells.

It has also been reported that IFNγ or synthetic glucocorticoids (dexamethasone) increase factor H mRNA abundance in monocytes (Lappin et al., 1992) and endothelial cells (Dauchel et al., 1990) probably by stabilizing factor H mRNA. It is possible that the therapeutic effects of both glucocorticoids and IFNγ in rheumatoid arthritis patients (Sanz and Alboukerk, 1991; Machold et al., 1992) may partially due to the increased synthesis of factor H by the cells in synovial membrane which would regulate complement activation at the level of the alternative pathway C3 convertase and/or at the level of the C5 convertase of the classical and alternative pathways.

Neither factor I protein nor factor I mRNA were detected in monocytes. Previously, the ability of monocyte-conditioned media to inactivate C3b was taken as evidence of the synthesis and secretion of factor I by monocytes (Whaley, 1980). However, my data suggest that such C3b inactivation is due to a protease(s) other than factor I.

Using Western blotting and ELISA, S protein has been demonstrated in the supernatants of monocytes and alveolar and peritoneal macrophages cultured in serum-free medium (Hetland et al., 1989). However, I was unable to detect S protein mRNA in Northern blots. A low transcription rate or short half-life or both may be the reason for my failure to detect S protein mRNA by Northern blots. Measures to

increase S protein mRNA abundance could have been taken. These include the addition of cytokines to increase transcription of the S protein gene and/or cycloheximide to stabilize S protein mRNA. These measures have been used successfully to detect factor H mRNA in monocytes (section 3.6) and C1-INH secretion in endothelial cells (section 3.8).

As well as showing for the first time that monocytes expressed SP-40,40, my data confirm previous observations on the ability of monocytes to synthesize C1-INH (Yeung-Laiwah et al., 1985), C4-bp (Lappin and Whaley, 1990), factor H (Whaley, 1980), DAF (Kinoshita et al., 1985), MCP (Seya et al., 1988), CD59 (Hideshima et al., 1990) and CR1 (Fearon, 1980).

4.3.3. Complement Regulatory Protein Synthesis by Lymphocytes.

In this study human peripheral blood lymphocytes were used as a model cell. Although C1-INH, factor H and factor I secretion was detected in culture supernatants from peripheral blood lymphocytes by ELISA, following monocyte depletion only small amount of factor H was present. Thus it is probable that most of the complement components detected were synthesized by contaminating monocytes. It is known that addition of antigen-stimulated lymphocyte culture supernatants to monocyte cultures increases C2 synthesis (Littman and Rudy, 1977). Similar factors present in the culture supernatant may stimulate lymphocytes to secrete complement regulatory proteins in the presence of monocytes. Although phytohaemagglutinin and concanavalinA stimulate human T cells, and pokeweed mitogen stimulates both human T and B cells (Lydyard and Grossi, 1990), only concanavalinA treatment significantly

stimulated factor I synthesis in lymphocyte cultures. Factor I synthesis by lymphocytes has been reported previously (Lambris et al., 1982). Several attempts to detect complement mRNAs in lymphocyte RNA were unsuccessful, which could have been due to the degradation of the mRNAs during extraction or their low abundance as a result of their instability *in vivo*. Further studies are required to resolve this issue.

Although complement regulatory proteins might not be secreted by lymphocytes, these cells could contribute to the regulation of complement in inflamed sites by secreting cytokines (*e.g.* IFNγ) which would act on other cell types to up-regulate the synthesis of regulatory components. Therefore, lymphocytes might play an indirect regulatory role in complement synthesis at sites of inflammation.

4.3.4. Complement Regulatory Protein Synthesis by Endothelial Cells.

Angiogenesis, the process of generation of new blood vessels, is an important feature, not only of normal phenomena such as embryonic development and wound healing, but also pathological states such as tumour growth and chronic inflammation (Folkman, 1985). In immunogenically mediated chronic inflammation such as RA synovitis, a significant increase in the number of capillaries and postcapillary venules has been observed (Folkman et al., 1985). The vessel wall is frequently exposed to proteolytic enzymes that are generated in plasma and within vascular tissue at sites of inflammation. Protection of endothelial cells depends upon the production and/or expression of inhibitors of proteolytic enzymes. Despite complement activation at sites of inflammation

endothelial cells are well protected against homologous attack by complement.

In this study, human umbilical vein endothelial cells were characterized and used as a model to investigate the regulation of complement activation.

4.3.4.1. mRNA analysis

Analysis of Northern blots indicated that unstimulated endothelial cells do not express any classical pathway regulators. However, IFNγ stimulates C1-INH synthesis in endothelial cells (see section 3.8.1.). This suggests the occurrence of a fine regulation of complement regulatory protein synthesis at sites of inflammation. Previously, Schimaier et al. (1989) reported the expression of C1-INH in endothelial cells. However, although they detected the mRNA by slot blotting, the message was not apparent on their Northern blots.

Messenger RNAs for all the membrane regulatory proteins except CR1 were detected by Northern blotting of endothelial cell RNA. Interestingly, cell-specific differences in the abundance of different mRNA species were observed, and they are discussed in section 4.5.

To my knowledge, this (section 3.5.3) is the first report of the detection of the mRNAs for S protein and SP-40,40 in endothelial cells. The synthesis of S protein from endothelial cells has been detected previously by radioimmunoassay using a combination of monoclonal and polyclonal S-protein antibodies (Berge et al., 1992).

4.3.4.2. Analysis of culture supernatants

Synthesis of factor H and factor I by endothelial cells was confirmed by the detection of these proteins in culture supernatants (section 3.5.1). The secretion rate of factor H was twice than of factor I. As endothelial cells are in contact with the blood their ability to synthesize complement components could be important *in vivo* as such synthesis could contribute to the circulating plasma levels. However, it is more probable that the purpose of the release of factor H and factor I by endothelial cells *in vivo* is to produce a local increase in their concentrations close to the endothelial cell surface, so that these proteins, together with the membrane-bound molecules DAF, MCP and CD59 protect endothelium from complement-mediated damage.

Although I did not detect C1-INH in culture supernatants from unstimulated endothelial cells, the secretion of C1-INH by endothelial cells has been reported by Schmaier et al. (1989). This could be explained by a possible difference in the sensitivity of ELISA assays and by the use of conditioned media in that study which could lead to the accumulation of synthesized protein into the culture supernatant. However, treatment of endothelial cells with IFNγ stimulated C1-INH synthesis and revealed the protein in culture supernatants (see section 3.8.1.). This suggests that in inflammation endothelial cells contribute to the early stages of classical pathway activation (C1 activation) as well as to the later stages of the alternative and classical pathways (C3 and C5 convertases).

4.3.4.3. Flow cytometric analysis

DAF, MCP and CD59 were detected on the surfaces of endothelial cells. The intensity of the signal from the CD59 antibody was higher than from the DAF and MCP. Although a sub-population of cells responsive to IFNγ for DAF expression was detected by flow cytometry at the fourth-passage of endothelial cells, unstimulated cells did not show such sub-population for any membrane-bound regulatory complement protein. It is not known if this sub-population also express CD59 and MCP. Also it is not known if they possess any functional significance against complement attack. Further studies need to be performed to bring answers to these questions. IFNγ-responsive sub-population of endothelial cells are discussed more in section 4.4.1.

These data suggest that, in addition to S protein and SP-40,40, CD59 also plays a role in the control of the membrane attack sequence. Tight control of this lytic sequence is required to protect endothelial cells from the lytic attacks of complement activation.

In the early studies of endothelial cells, expression of MCP (McNearney et al., 1989), DAF (Asch et al., 1986) and CD59 (Hamilton et al., 1990; Brooimans et al., 1992) was shown. Biosynthetic studies of endothelial cells have shown that CR1 is not present on endothelial cells (Julen et al., 1992). These data are in agreement with those in section 3.5.

4.4. THE EFFECTS OF CYTOKINES ON ENDOTHELIAL CELLS

The expression of secreted complement regulatory proteins in synovial membrane was demonstrated at the mRNA level (dot blots) and some of the cells which could be responsible for this synthesis were identified by studying different cell types in culture. Cytokines are likely to play a role in the pathogenesis of RA (Arend and Dayer, 1990). Synovial tissue and synovial fluid are rich sources of these mediators and ample data have accrued supporting the notion that cytokines can account for some of the functional properties of cells in synovium (Firestein et al., 1990). One of these is the modulation of the synthesis of complement components.

It was feasible to study only one cell type in detail so I concentrated on endothelial cells for the following reasons:

1) They form the interface between blood and tissues and are therefore key cells in the inflammatory response.

2) Control of complement in the immediate vicinity of the endothelial cells is essential to protect the endothelial cells from the powerful cytolytic effects of complement.

3) There is little information available on complement synthesis in these cells.

I selected to study the cytokines IFNγ, IFNα, IL-1 and IL-6 as they are known to be present at sites of inflammation and they regulate the synthesis of complement components in some cells.

Preliminary studies showed that IFNγ and IFNα increased the synthesis of the secreted regulatory complement proteins in endothelial cells. It has been reported previously that treatment with IFNγ increases

the secretion and the mRNA abundances of factor H and factor I (Ripoche et al., 1988) and C1-INH (Lappin et al., 1992). This report show that the increased synthesis of C1-INH was due to increase in transcriptional rate. I detected an inhibition in factor H protein levels in the presence of IL-1β or IL-6 (section 3.8.1). The inhibitory effect of IL-1 on factor H has been reported previously by Broomimans et al. (1990). The opposing effects of IFNγ and IL-1/IL-6 on the secretion of fluid-phase regulatory proteins indicates that the relative concentrations of these cytokines in the immediate vicinity of endothelial cells affect the level of synthesis of the complement proteins and might either amplify or down-regulate the inflammatory response.

4.4.1. The Effect of IFNγ on Membrane Protein synthesis

The production of mRNA in eukaryotic cells is a complex process involving both transcriptional and post-transcriptional mechanisms. Modulation of the abundances of mRNAs can occur as a result of regulation at a number of stages, though changes in rates of gene transcription and mRNA turnover and translation appear to have the greater effects (Darnell, 1982).

This part of the study was therefore undertaken to determine the effects of IFNγ on the transcription of the genes encoding DAF, MCP and CD59 in endothelial cells, to investigate the effects of IFNγ on the stability of the mRNA encoding DAF, MCP and CD59, and to determined the effects of IFNγ on the expression of these proteins on the cell surface.

IFNγ increased the rate of transcription of genes encoding DAF, MCP and CD59 (section 3.8.4). Many studies have shown that IFNγ

stimulated the transcription of a large number of genes (Larner et al., 1986), including complement regulatory genes C1-INH, factor H and C4-bp (Lappin et al., 1992). The DNA sequences involved in the regulation of transcription by IFNγ are thought to be principally located in the regions upstream from the coding sequence of IFN-responsive genes. A computer search for conserved sequences in the upstream region of human interferon-sensitive genes has identified interferon-stimulated response elements (ISRE) which are located in the flanking regions of these genes (Levy et al., 1988, Shirayashi et al., 1988) including the factor B gene (Wu et al., 1987). The role of such consensus sequences in the action of interferons on the transcription of DAF, MCP and CD59 genes is unclear. However, there are no published data regarding the presence of IFNγ response elements in DAF, MCP and CD59 genes. There are controversial reports about the inducibility of CD59 expression with IFNγ. Philbrick et al. (1990) found no effect of IFNγ on CD59 message levels in transformed cell lines of lymphoid origin and T cells. In contrast, Tandon et al. (1992) showed that the expression of CD59 in human thyroid cells is enhanced by IFNγ.

Although IFNγ induced increases in the rates of transcription of the genes encoding DAF and MCP, it had no effect on the abundances of these mRNAs. Moreover, a 3-fold increase in the rate at which the CD59 gene was transcribed effected no more than a 50% increase in mRNA abundance. However, increased transcription in response to IFNγ was associated with a reduction in the half-lives of DAF and CD59 mRNAs. Though, the reduction was insufficient to explain the discrepancy between transcription rate and mRNA abundance. An arrest in transcription may explain this discrepancy. If this speculation is

correct, hybridization of the labelled RNA to dot blots containing cloned fragments of different regions of the genes would indicate the site of the arrest. A block to transcription elongation has previously been reported for the *c-myc* (Eick and Bronkamm, 1986) and for *c-myb* genes (Redy and Redy, 1989).

A sub-population of cells responsive to IFNγ for DAF expression was detected by flow cytometry of fourth-passage endothelial cells. It is unlikely that these cells arose solely as an artefact. Such an artefact would have affected the expression of the other regulatory components in IFNγ-treated cells, since cells harvested from the same culture flask and then subjected to antibodies against membrane-bound regulatory proteins and this sub-population was only present for DAF. Moreover, there was not such sub-population in control cells. This sub-population might arise from expansion of inherently IFNγ-sensitive cells that normally are present in low numbers. However, my data showed that some endothelial cells are responsive to IFNγ-stimulation for DAF expression. Interestingly, this sub-set disappeared after several more passages of the cells. It is not known if the proportion of cells that is sensitive to IFNγ is greater during the first passage. It has been reported that prolonged cell culture causes a significant reduction in immunogenicity in endothelial cells (Dhesi et al., 1992), and the secretion of factor H by endothelial cells was reduced when endothelial cells were passaged repeatedly (Brooimans et al., 1989). This could be due either to a change in phenotype as cells proliferate *in vitro*, or to the IFNγ-responsive cells being at a proliferative disadvantage. These observations indicate one of the problems of using *in vitro* culture to draw conclusions regarding events *in vivo*.

After treatment of endothelial cells with IFNγ there was an increase in the proportion of cells expressing CD59, but again this was only seen at low passage numbers.

These results suggest that IFNγ upregulates secretion of fluid-phase regulatory complement proteins and, possibly, the expression of the membrane-bound regulatory proteins. As IFNγ induces remission in some RA patients (Machold et al., 1992), there is the possibility that increased synthesis of fluid-phase complement proteins could contribute to this by down-regulating complement activation. (This point has been discussed previously). It is interesting that, although cells responsible for the production of IFNγ are present in RA synovial joint, previous studies have shown that, cultured synovial tissue fragments produce very little IFNγ (Firestein and Zvaifler, 1987). Furthermore, the quantitative analysis of cytokine gene expression in RA by *in situ* hybridization showed that, in contrast to high levels of IL-1β, IL-6 and TNFα mRNs in synovial tissue, there was little or no IFNγ mRNA detected (Firestein et al 1990). Since IFNγ favours the synthesis of the regulatory proteins of complement (section 3.8.1), and suppresses the synthesis of C3 (Lappin et al., 1990), the key activation component in both classical and alternative pathways, the absence of IFNγ synthesis in RA synovium might favour activation of complement, therefore leading to chronicity of the inflammation.

Further studies are required to investigate the effect of IFNγ on the expression of the membrane-bound regulatory proteins and the ability of IFNγ-treated cells to resist complement attack. Also, the role of interferon-responsive elements in the regulation of transcription of these interferon-sensitive genes, and the mechanisms by which IFNγ modulates

the stabilities of the mRNAs encoded by C1-INH, factor H, DAF, MCP and CD59 genes, should be investigated.

4.5. CELL-SPECIFIC EXPRESSION OF THE REGULATORY COMPLEMENT PROTEINS

Several of the complement regulatory proteins, DAF, MCP, CR1, factor H and C4-bp constitute the regulators of the complement activation (RCA) gene family and are located at a single chromosomal site (reviewed in Hourcade et al., 1989). Although no detailed analysis is yet available of the nucleotide structure of the promoter and enhancer elements of the cluster of RCA genes, immunohistochemical means of detection of the gene products suggest that there is cell- and tissue-specific regulation of their expression. However, the ubiquitous tissue expression of DAF and MCP are also shown (McNearney et al., 1989; Kinoshita et al., 1985; Asch et al., 1986). It is clear that gene duplication has played an important part in the evolutionary development of the RCA cluster (Hourcade et al., 1989), and homologies in the transcriptional regulatory regions might also be found among the RCA family members.

In my study, cell-specific differences were detected in a number of the complement regulatory proteins secreted. Cell-specific differences in the secretion rates of some proteins were also observed. C1-INH secretion was much higher from fibroblasts than from monocytes, and the secretion of C1-INH was only detectable in the presence of IFNγ in endothelial cells. Furthermore, factor H was very low in monocytes compared with fibroblasts. However, factor H was the only regulatory complement protein to be secreted by all cells under standard culture conditions.

Despite these differences, DAF, MCP, CD59 and MIP were always present on the membranes of the fibroblasts, endothelial cells, monocytes and lymphocytes.

Cell-specific differences in the stabilities of the mRNAs for C2, factor B, C3, C4-bp, C1-INH and factor H in Hep G2 cells, monocytes, skin fibroblasts and endothelial cells have been reported by Lappin et al. (1992). It was thought that these differences might derive from cell-specific expression of a labile destabilizing factor, such as that described for *c-myc* mRNA (Brewer and Ross, 1989).

In addition to these, cell-specific differences in the relative abundances of the various species of mRNAs for DAF, MCP and CD59 mRNAs were seen in endothelial cells and fibroblasts. Fibroblasts from normal and inflamed synovium expressed a greater preponderance of the highest molecular size CD59 mRNA species than endothelial cells. In contrast, endothelial cells expressed a greater relative abundance of the lower molecular size species of DAF mRNA than fibroblasts. Furthermore, MCP occurs as a doublet in RNA from endothelial cells but not in fibroblast RNA. These data suggest that there are cell-specific differences in the processing of the pre-mRNAs encoding these regulatory proteins. Tissue-specific polymorphism of MCP has been demonstrated and shown to be due to differential splicing (Russel et al., 1992). It is not known whether the larger mRNA species have to be processed further before being translated, or whether they are translated both give rise to the same protein, or to give different proteins. Therefore, it has yet to be determined whether the tissue-specific differences in the abundances of the various mRNA species for DAF, MCP and CD59 have an effect on the expression or the activity of the protein products. It will

be important to sequence the different mRNA species for each of these proteins in order to gain some insight into the nature of each of them.

Recently, investigators have shown that the abundances of the various species of CD59 mRNA differ between brain, liver, lung and placenta (Kumar et al., 1993). In the same study they also suggested an exclusive relationship between the expression of SP-40,40, CD59 and DAF in normal tissues: tissues showing abundant SP-40,40 mRNA had low abundances of CD59 and DAF mRNAs, and vice versa. There may be a positive or negative feed-back mechanism between cells controlling the expression of different regulatory complement proteins. Cells may interact with each other to obtain the levels of regulatory proteins sufficient to protect themselves and surrounding cells in the tissue from the lytic activation of complement system. Further studies need to be performed to determine interactions among cells for the expression of the complement regulatory proteins.

4.6. GENERAL CONCLUSIONS

The results obtained in this study indicated that tissues from synovial membrane from normal individuals, osteoarthritis and rheumatoid arthritis patients synthesize all regulatory complement proteins: C1-INH, C4-bp, factor H, factor I, S protein, SP-40,40, DAF, MCP, CD59 and MIP. This shows:

1) Locally synthesized complement regulatory components could regulate complement activation in normal and inflamed synovial joints.

2) Complement regulatory protein synthesis is a constitutive function of cells and such synthesis does not require the presence of inflammation.

Membrane-bound proteins DAF, MCP, CD59 and MIP were expressed in all cells. Thus, even when activation may not be inhibited in the fluid-phase, the ubiquitous presence of these membrane regulatory proteins should provide a tight control of complement activation.

IFNγ regulates the control of complement activation by increasing secretion of the fluid-phase proteins although further work must be undertaken to confirm this observation.

The extrahepatic synthesis of complement regulatory proteins at sites of inflammation may play a major role in the protection of host cells from the lytic attack of complement. Furthermore, manipulation of the expression of the genes encoding the regulatory proteins by IFNγ may be helpful in down-regulating inflammation and reducing tissue damage.

However, the results obtained in my experiments were determined under *in vitro* conditions and may not necessarily reflect the *in vivo* situation.

The work described in this thesis contributes to the understanding of the regulation of the complement system in normal and inflamed tissues. The following studies should be undertaken as a continuation of this work.

1. *In situ* hybridization and immunohistochemistry of normal and rheumatoid synovial tissues to show which cells synthesize the complement regulatory proteins *in vivo*.

2. Studies of the cell-specific differences in the expression of the different mRNA species for DAF, MCP and CD59 should be performed in order to understand the processing of each of these mRNAs and in order to determine whether differences in the structure, level of expression and function of the protein are dependent on the cell-specific differences in the relative abundances of different mRNA species

3. In terms of understanding the regulation of the expression of the complement genes the transcriptional and post-transcriptional effects of cytokines on each gene in different cell types must be evaluated.

CHAPTER 5. REFERENCES

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