

FcγRIIIa: Tissue Distribution and Regulation of Expression

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Dedication

To mum and dad

Acknowledgements

First of all I am indebted to Professor Edwards whose specialist knowledge guided me throughout the duration of this research. I am also grateful to Jo Cambridge, Selina Blades, Vikki Abrahams and Geoffrey Laurent for their technical assistance and support in helping me acquire the necessary laboratory skills to perform the experimental procedures required for this research.

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Abstract

Fc γ RIIIa is an IgG Fc receptor described on large granular lymphocytes, natural killer cells, approximately 5 percent of peripheral blood monocytes (Ravetch J V, Kinet J P *et al*, 1991), macrophages in the synovial intima of adult synovium, macrophages in dermis exposed to mechanical stretch (Edwards J C W, Blades S *et al*, 1997) and on Kupffer cells within normal liver tissue (Tuijnman W B, Van Wichen D F *et al*, 1993). The expression of Fc γ RIIIa by macrophages in other normal human tissues has been poorly documented.

Small immune complexes are present in the sera of patients with rheumatoid arthritis (Mannik M, Nardella F A, 1985). These complexes fix complement poorly (Brown P B, Nardella F A *et al*, 1982) and are small enough to be expected to pass through vessel walls and access tissue macrophages. The prominent expression of Fc γ RIIIa on synovial intimal macrophages first led to the hypothesis that inflammation in rheumatoid arthritis might be due to an interaction between small complexes and Fc γ RIIIa.

The cross-linking of 2 or 3 Fc γ RIIIa but not Fc γ RI or Fc γ RIIa receptors with respective anti-Fc γ R monoclonal antibodies results in the release of TNF- α , IL1- α and reactive oxygen species from mature macrophages *in vitro* (Abrahams V M, Cambridge G *et al*, 2000). Binding of small immune complexes to Fc γ RIIIa on macrophages with release of proinflammatory cytokines would explain

inflammation in rheumatoid disease, but only if the tissue distribution of FcγRIIIa proved to be consistent with the clinical picture.

The first aim of this study was to assess the distribution of FcγRIIIa in a range of normal human tissues using immunohistochemical techniques. FcγRIIIa expression in normal human tissues was assessed semiquantitatively using microdensitometry.

Results from these experiments revealed high levels of FcγRIIIa expression in tissues targeted by rheumatoid arthritis. Factors responsible for the induction of FcγRIIIa on tissue macrophages have not been clearly defined.

The second aim of this study was to investigate the factors responsible for the induction of FcγRIIIa on tissue monocyte-macrophages. Rheumatoid arthritis has a predilection for sites where mechanical stretch occurs. These are also sites where FcγRIIIa expression is present. Some cell types release TGFβ in response to mechanical stretch (Riser B L, Cortecs P *et al*, 1996). TGFβ₁ has been reported to selectively induce FcγRIIIa on monocytes *in vitro* (Welch G R, Wong H L *et al*, 1990). Therefore TGFβ produced as a result of mechanical stretch or mechanical stretch itself might directly induce FcγRIIIa expression on macrophages. Other cytokines, neuropeptides and/or the interaction of monocytes with extracellular matrix proteins might also be responsible for FcγRIIIa induction.

To investigate the factors responsible for the induction of FcγRIIIa on tissue monocyte-macrophages, peripheral blood monocytes were isolated from normal

donors and cultured on different substrates. The cytokines TGF β and IL-10 were added to cultured monocytes at a concentration of 10 ng/ml. Stretching experiments were undertaken using the flex I, McKeesport System. Synovial fibroblasts were also subjected to stretch and the resulting conditioned medium was added to cultured monocytes.

The induction of Fc γ RIIIa was assessed by using murine monoclonal anti- Fc γ RIII antibody 3G8 followed by FACS analysis.

The effect of cytokines, matrix proteins, neuropeptides, conditioned medium from stretched and non-stretched synovial fibroblasts and other factors on the induction of Fc γ RIIIa on cultured monocytes is summarized in the table below.

Table A: Effect of Different Factors on the Induction of Fc γ RIIIa Expression on Cultured Monocytes

	Effect on the Induction of Fc γ RIIIa Expression on Cultured Monocytes
TGF β ₁	↓ (substrate/matrix independent)
IL-10	↑ (substrate/matrix independent)
Matrix Proteins: collagen I, collagen IV, fibronectin and laminin	No effect
Substance P	↑ (on adherent monocytes only)
M-CSF	↓
GM-CSF/Mechanical Stretch/Hyaluronan	No effect
Conditioned medium from non-stretched synovial fibroblasts	↑↑
Sodium Aurothiomalate	↓
Methotrexate	↑
Hydrocortisone	Cytotoxic at high concentrations
Osteoarthritic Synovial Fluid	Slight ↓

The soluble factor in conditioned fibroblast medium responsible for the induction of FcγRIIIa was found to be a heat stable, acid labile protein with a molecular weight of more than 100 kDa.

This fibroblast factor may therefore be responsible for the selective induction of FcγRIIIa on monocyte-macrophages *in vivo* in tissues targeted by rheumatoid arthritis.

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INTRODUCTION

RHEUMATOID ARTHRITIS

Classification

Rheumatoid arthritis is classified according to the criteria of the American Rheumatism Association (revised criteria Arnett F C, Edworthy S M *et al*, 1987). The American Rheumatism Association's criteria are helpful for both epidemiological work and for classification of patients for clinical trials. To be defined as having rheumatoid arthritis according to the American Rheumatism Association's criteria, patients must have 4 or more of the criteria listed below. Criteria 1 through to 4 must also have been present for at least 6 weeks.

- 1.) Morning stiffness in and around joints lasting at least 1 hour before maximal improvement.
- 2.) Soft tissue swelling (arthritis) of 3 or more joint areas observed by a physician.
- 3.) Swelling (arthritis) of the proximal interphalangeal, metacarpophalangeal, or wrist joints.
- 4.) Symmetric swelling (arthritis)
- 5.) Rheumatoid nodules
- 6.) The presence of rheumatoid factor
- 7.) Radiographic erosions and/or periarticular osteopenia in hand and/or wrist joints

Epidemiology

Using the criteria above in a prospective population of 450000 people in Norfolk (United Kingdom), the incidence of rheumatoid arthritis was found to be 3.4 in woman and 1.4/10000 in men per annum (Symmons D P, Barrett E M *et al*, 1994). The incidence increased sharply with age in men from age 45. It increased in women until age 45, then plateaued and fell after age 75. The estimated prevalence of rheumatoid arthritis in the United States of America (USA) is 0.9%. 6.5 to 12% of patients with rheumatoid arthritis are severely disabled and between one and two-thirds of previously employed patients have a reduced work capacity. In 1991 the estimated lifetime cost of medical care charges for a single patient with rheumatoid arthritis in the USA was predicted to be approximately \$US 12,578 (Allaire S H, Prashker M J *et al*, 1994).

Articular Features of Rheumatoid Arthritis

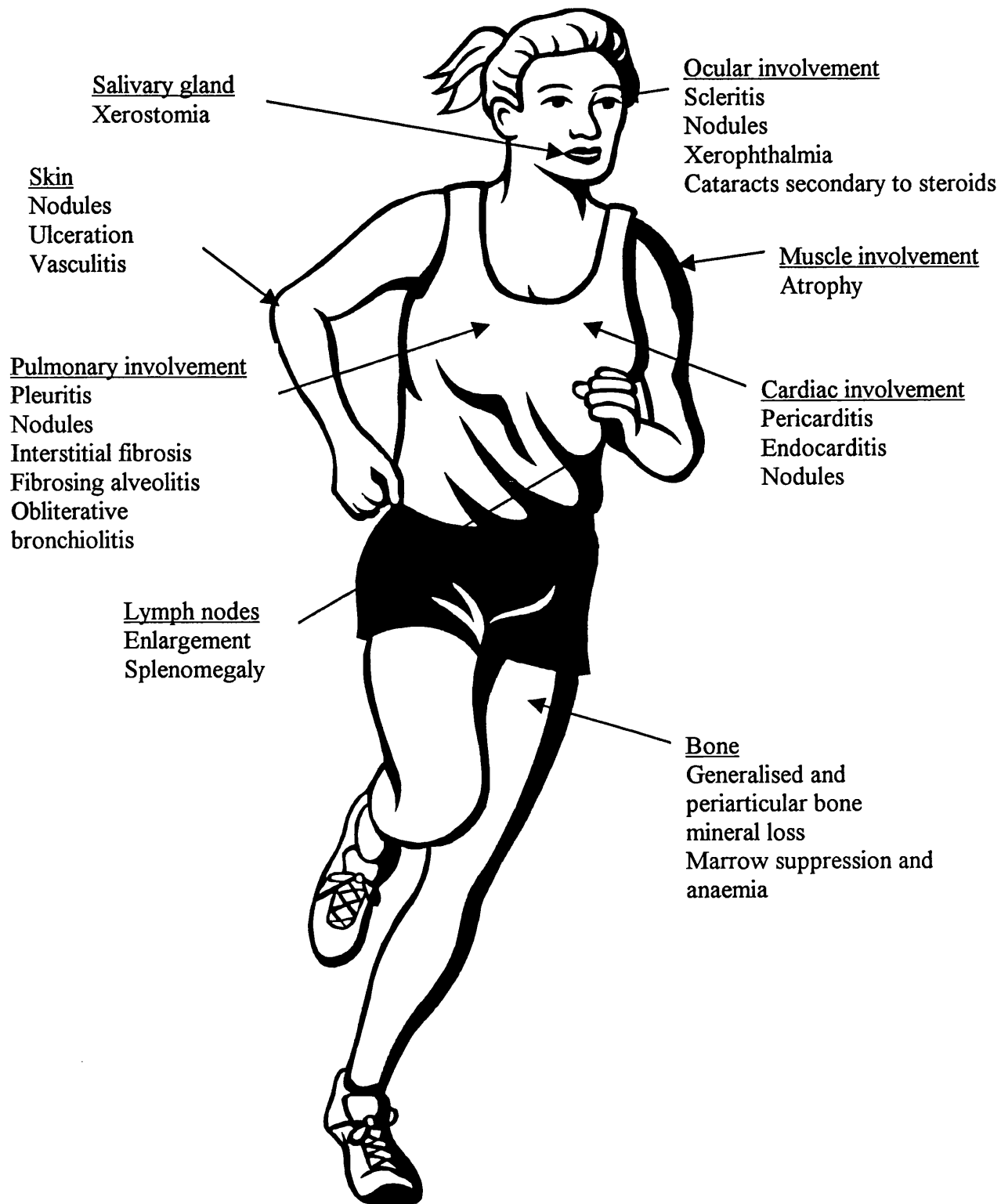
Synovitis causes pain, stiffness and swelling of the affected joint. Any diarthrodial joint can be affected in rheumatoid arthritis but usually the wrists, the metacarpophalangeal joints, the proximal interphalangeal joints, the ankles, the metatarsophalangeal joints, the knees, the shoulders, the hips and the elbows are involved. The joints most commonly affected are the metatarsophalangeal joints and those least often affected are the hip joints (Maddison P J, Isenberg D A *et al*, 1998). Rheumatoid arthritis characteristically involves joints symmetrically, although the dominant side is often more severely affected (Owsianik W D J, Kundi A *et al*, 1980). However, it is well recognized that

synovitis is reduced in the joints of paralyzed extremities, as, for example, following a cerebro-vascular event (Smith R D, 1979; Bland J H, Eddy W M, 1968). Immobilization of joints by casting or splinting also results in a reduction in synovitis (Harris R, Copp E P, 1962; Partridge R E H, Duthie J J R, 1963; Gault S J, Spyker J M, 1969). These clinical observations suggest that a neurogenic or mechanical mechanism may modulate the clinical features of rheumatoid arthritis. Rheumatoid arthritis also affects non-articular sites (extra-articular manifestations).

Extra-articular Features of Rheumatoid Arthritis

The occurrence of extra-articular manifestations varies but they tend to be more common in those patients that are rheumatoid factor positive. The true incidence of extra-articular disease is difficult to determine, as it can remain sub-clinical. For example, at post-mortem up to a third of patients with rheumatoid arthritis have evidence of pericarditis that was not clinically apparent (Yamakido M, Ishioka S *et al*, 1992). Figure 1.1 overleaf shows the extra-articular sites affected by rheumatoid disease.

Figure 1.1: Extra-articular Features of Rheumatoid Arthritis



Rheumatoid Nodules

Rheumatoid nodules are a common extra-articular feature of rheumatoid arthritis occurring in 20% of patients with rheumatoid arthritis. Patients who are positive for rheumatoid factor have a higher incidence of rheumatoid nodules (Schumacher H R, Gall E P, 1988). Nodules are most commonly found on extensor surfaces. However, they may more rarely be found in internal organs such as the lung, heart and gallbladder. A temporal relationship exists between mild repetitive trauma and the appearance of rheumatoid nodules (Ben-Chetir E, Enk C *et al*, 1992). This supports the role of local pressure or mechanical stretch in the pathogenesis of rheumatoid nodules.

Patients commenced on methotrexate sometimes develop new nodules. Other extra-articular manifestations of rheumatoid arthritis in the form of nailfold lesions, cutaneous ulcers, pleuritis and pericarditis have also been reported with the onset of methotrexate therapy (Abu-Shakra, Nicol P *et al*, 1993). Nodules regress when methotrexate is stopped and recur after rechallenge with methotrexate (Kerstens P J S M, Boerbooms A M T *et al*, 1991; Segal R, Caspi D *et al*, 1988).

Laboratory Investigations

Laboratory investigations do not in isolation confirm a diagnosis of rheumatoid arthritis, but can be useful in the overall assessment of a patient in terms of their prognosis and

response to therapeutic interventions. Below is a list of investigations commonly performed.

Rheumatoid factors

Rheumatoid factors are antibodies with their specificity directed to antigenic determinants on the Fc regions of immunoglobulin G. These antibodies may exist in all classes of immunoglobulins and are therefore called IgG, IgA and IgM rheumatoid factors. Waaler discovered the occurrence of rheumatoid factors in patients with rheumatoid arthritis in 1937. He observed that serum from a patient with rheumatoid arthritis caused agglutination of sheep erythrocytes that had been sensitised with rabbit antibodies to sheep red blood cells. These “agglutination factors” were termed rheumatoid factors by Pike *et al* in 1949.

In 1961 Kunkel *et al* demonstrated the presence of IgG rheumatoid factors and Schrohenloher in 1966 confirmed this. In 1974 Pope *et al* discovered the presence of rheumatoid factor dimers in the serum of a patient with rheumatoid arthritis. He proposed that two IgG rheumatoid factors form a dimer, whereby one antibody-combining site of each molecule reacts with the Fc region of the other molecule. In this model the other antibody combining site and antigenic determinant on the Fc region of each IgG rheumatoid factor molecule remain free. Approximately 80% of patients with rheumatoid arthritis have circulating IgG rheumatoid factors (Carson D A, 1993). Those patients without circulating rheumatoid factors often have rheumatoid factors in the

joints. In rheumatoid synovium rheumatoid factors are produced by B-lymphocytes and plasma cells (Kobayashi I, Ziff M, 1973; Harris E D Jr., 1990; Natvig J B, Randen I *et al*, 1989; Smiley J D, Sachs C *et al*, 1968; Bankhurst A D, Husby G *et al*, 1976).

Rheumatoid factors are found in the circulation of normal individuals, in people immunized with foreign antigen and in those with a chronic infection (Welch M J, Fong S *et al*, 1983). Assuming rheumatoid factors in rheumatoid arthritis are pathogenic, this suggests that a subset of rheumatoid factors are involved in the pathogenesis of rheumatoid disease.

The reaction of IgM or IgG with an antigen causes a conformational change in the Fc region of the antibody to reveal a binding site for the first component of complement, C1q. C1q is composed of six subunits, resembling a 'bunch of tulips'. C1q reacts with Fc via its globular heads but attachment by two critically spaced binding sites is required for activation (Chapel H, Haeney M 1988). The Fc regions of pentameric IgM are so spaced that one IgM molecule can activate C1q. In contrast IgG is relatively inefficient because the chance of two randomly sited IgG molecules being the critical distance apart to activate C1q is relatively low. Complement activation by self-associating IgG rheumatoid factors has been demonstrated (Mannik M, Nardella F A, 1985). Thus large immune complexes may be able to bind complement and contribute to the inflammatory response of rheumatoid arthritis. However, the complement receptor 1 (CR1) on red blood cells should clear large immune complexes.

Complement activation by IgG rheumatoid factor dimers or oligomers is ineffective (Brown P B, Nardella F A *et al*, 1982). These rheumatoid factor dimers tend not to be cleared and are small enough to be expected to cross over to the extravascular space where they may interact with tissue macrophages.

The interaction of dimeric IgG complexes with Fc γ RIIIa on natural killer cells induces cell signalling with the production of TNF- α (Hendrich C, Kuipers J G *et al*, 1991). Cross-linking of Fc γ RIIIa on cultured macrophages using an anti- Fc γ RIII antibody results in the release of TNF- α , IL-1 and reactive oxygen species (Abrahams V M, Cambridge G *et al*, 2000; Trezzini C, Jungi T W *et al*, 1990). Intimal synovial macrophages selectively express Fc γ RIIIa (Edwards J C W, Blades S *et al*, 1997). Synovial inflammation in rheumatoid arthritis may therefore be initiated by small IgG rheumatoid factor complexes binding to Fc γ RIIIa on intimal macrophages causing local release of inflammatory mediators that result in tissue inflammation.

Small rheumatoid factor complexes of the IgG class may therefore be pathogenic in rheumatoid arthritis.

Erythrocyte Sedimentation Rate

The erythrocyte sedimentation rate is a measurement of the rate at which red blood cells settle. It is usually elevated in patients with rheumatoid arthritis and in conjunction with patients symptoms be used to follow a patient's response to therapeutic intervention.

C-reactive protein

The C-reactive protein is an acute-phase protein that is synthesized in the liver and increased inflammatory conditions in response to increased levels of TNF- α and IL-6. Its serum concentration can be used in conjunction with a patient's symptoms to follow their disease activity and response to treatment.

Anaemia

Anaemia of chronic disease is the commonest extra-articular manifestation of rheumatoid arthritis. The severity of the anaemia correlates with the activity of rheumatoid disease. The cytokines TNF- α and IL-1 cause suppression of the bone marrow (Johnson R A, Waddelow T A *et al*, 1989b). This contributes to the anaemia of chronic disease in rheumatoid arthritis. It also explains the reversal of anaemia that occurs in patients receiving monoclonal antibodies against TNF- α (Elliot M J *et al*, 1994). Patients with rheumatoid arthritis may be anaemic for other reasons and these include:

- Iron deficiency anaemia
- Bone marrow suppression secondary to disease modifying anti-rheumatic drugs
- Haemolysis
- Hypersplenism

- Chronic renal failure
- Vitamin B12 or folate deficiency

SYNOVIUM

Normal Synovium

The synovium is a specialized tissue that encloses diarthrodial joints and is absent from the contact surfaces of articular cartilage (Ghadially F N, 1978; Henderson B, Edwards J C W, 1987). In rheumatoid arthritis an inflammatory response is directed at the synovium.

The innermost part of normal synovium that faces the joint space consists of a thin layer of cells approximately 3 cells deep and about 50 µm thick. This is referred to as the synovial intima or lining layer (Stevens C R, Blake D R *et al*, 1991a). Beneath this is a larger region called the subintima.

The cellular component of the intima consists of synovial monocyte-macrophages and synovial fibroblasts (Henderson B, Edwards J C W, 1987; Revell P A, 1989; Athanasou N A, Quinn J, 1991; Wilkinson L S, Pitsillides A A *et al*, 1992). These cells have been termed type A and B cells respectively and are collectively referred to as synoviocytes. Synovial macrophages reach the synovium via the circulatory system from the bone marrow during embryogenesis and in inflammation (Edwards J C W, Willoughby D A, 1982). They express the macrophage lysosome-associated marker CD68 and the IgG Fc receptor FcγRIIIa (Edwards J C W, Blades S *et al*, 1997).

Synovial fibroblasts are different from fibroblasts found in most other tissues. Synovial fibroblasts express a number of enzymes, such as hyaluronan synthase and uridine diphosphoglucuronate dehydrogenase (Edwards J C W, 1994). Normal synovial intimal fibroblasts have also been shown to express molecules associated with B-lymphocyte survival and differentiation such as vascular cell adhesion molecule-1 (VCAM-1), and decay accelerating factor (DAF) (Edwards J C W, Leigh R D *et al*, 1997). Cultured synovial fibroblasts in the presence of TNF- α show a rapid up regulation of mRNA encoding VCAM-1 (Marlor C W, Webb D L *et al*, 1992). These experimental results suggest that TNF- α *in vivo* will induce VCAM-1 on synovial fibroblasts. VCAM-1 supports the survival and differentiation of B-lymphocytes in rheumatoid synovium and their production of rheumatoid factor (Youinou P Y, Morrow J W *et al*, 1984; Dechanet J, Merville P *et al*, 1995).

The synovial lining cells are separated by spaces 1-2 μm wide (Levick J R, McDonald J N, 1989b). Extracellular matrix proteins secreted by synovial fibroblasts occupy these spaces. In rheumatoid arthritis synovial fibroblasts secrete matrix-degrading enzymes such as collagenase (Lohmander S, 1994). There is no basement membrane separating the intima and subintima. No morphological differences exist in the synovial intima within a joint or between different joints.

The subintimal tissue contains scattered fibroblasts, macrophages, mast cells and adipocytes. It is richly supplied with blood vessels and lymphatics (Davies D V, 1946).

The degree of vascularization and composition of the extracellular matrix of the subintima varies between joints.

Capillaries are also situated in the intima and at the junction of the intima with the subintima. Endothelial cells have fenestrae in their plasma membrane. (Knight A D, Levick J R, 1984). These windows allow the rapid transfer of water, lipid-insoluble solutes like electrolytes, glucose, amino acids and non-protein bound drugs to the synovium and synovial fluid. The windows resist the passage of plasma proteins (Knight A D, Levick J R *et al*, 1988).

Synovial lymphatics remove plasma proteins, bound drugs, immune complexes and degraded cartilage macromolecules from the joint. Smaller molecules such as cytokines and carbon dioxide are cleared by diffusion into capillaries (Bauer W, Short C L *et al*, 1933; Simkin P A, Benedict R S *et al*, 1990; Brown T J, Laurent U B G *et al*, 1991).

The synovium contains a good supply of myelinated (1-5 μm diameter) and unmyelinated nerve fibres (<1 μm diameter) (Mapp P I, 1995). The myelinated nerve fibres are postganglionic adrenergic sympathetic fibres that are located around blood vessels and thus control articular blood flow. Unmyelinated C fibres extend into the intima of the synovium (Barry W, 1981). The cell bodies of these unmyelinated nerve fibres synthesize neuropeptides principally substance P and calcitonin gene-related peptide (CRGP) (Mapp P I, Kidd B L *et al*, 1990).

Rheumatoid Synovium

In rheumatoid arthritis the synovium is the target of an inflammatory response. The synovial intima becomes thicker owing to an influx of monocytes from the peripheral circulation and to an increase in the size of synoviocytes (Henderson B, Edwards J C W, 1987; Revell P A, 1989). Both intimal and subintimal macrophages increase in number. Lymphocytes accumulate in perivascular areas and develop into lymphoid follicles with germinal centres containing B-lymphocytes. These B-lymphocytes together with plasma cells are found in the stroma where they produce rheumatoid factors (Munthe E, Natvig J B, 1972; Youinou P Y, Morrow J W *et al*, 1984). T-lymphocytes are found in clusters and as isolated cells deep in the synovium. The majority of these T cells are CD4 positive, in comparison to synovial fluid where CD8 positive T cells predominate (Lydyard P M, Hanglow A *et al*, 1982).

As a consequence of cellular infiltration and necrosis, the vascularity of the superficial region of rheumatoid synovium is reduced (Stevens C R, Blake D R *et al*, 1991b). This results in functional hypoperfusion of rheumatoid synovium.

The innervation of the synovium undergoes changes in rheumatoid arthritis. The number of nerve fibres is greatly reduced. Both postganglionic adrenergic sympathetic and unmyelinated C fibres are absent from the superficial synovium and areas of inflammation. Nerve fibres in deeper tissues remain but show a reduction in specific

neuropeptides (Gronbald M, Konttinen Y T *et al*, 1988; Mapp P I, Kidd B L *et al*, 1990; Pereria da Silva A, Carmo-Fonseca M, 1990).

SYNOVIAL FLUID

Synovial fluid is found within synovial joints (diarthroses). The amount of synovial fluid in normal joints is difficult to measure because the volume of fluid is very small in relation to the internal surface area of the joint. The normal knee joint contains about 0.5ml of aspirable fluid. The fluid is spread over the intra-articular space covering the synovium and articular cartilage to form a thin film approximately 24 μ m thick (Levick R, Price F M *et al*, 1996).

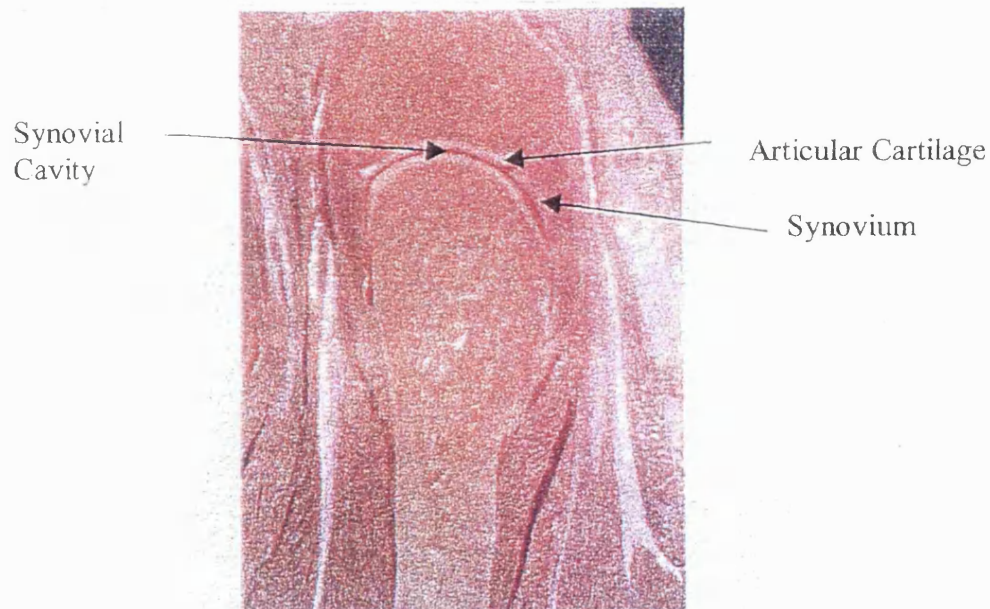


Figure 1.2: Coronal Section Through a Metacarpophalangeal Joint. Reproduced From Human Anatomy, Gosling J A *et al*. Gower Medical Publishing, London, 1985.

Synovial fluid is in close contact with intimal cells of the synovium. Normal intimal macrophages express Fc γ RIIIa on their cell surface (Edwards J C W, Blades S, 1997). It is possible that factors in synovial fluid may be responsible for the induction of Fc γ RIIIa on intimal macrophages. The composition of normal synovial fluid together with its function is discussed below.

Synovial fluid is a passive ultrafiltrate of plasma across the walls of synovial lining capillaries (Levick J R, 1983). Therefore, the composition of synovial fluid is partly dependant on the molecular weight of solute molecules. Small molecular weight solutes (<10kDa) in plasma equilibrate with those in synovial fluid by the process of passive diffusion.

The concentration of electrolytes in synovial fluid is similar to that of plasma (Bauer W, Ropes M W *et al*, 1940). The concentration of glucose in synovial fluid is only two thirds that of plasma owing to intra-articular consumption (Ropes M W, Muller A F *et al*, 1960). The protein concentration in synovial fluid is 13mg/L, which is only 20 percent of that, found in plasma (Weinberger A, Simkin P A *et al*, 1989). Small molecular weight proteins are present in higher concentrations than large molecular weight proteins. Plasma proteins enter synovial fluid by passive diffusion. The predominant protein present in synovial fluid is albumin. Other proteins are synthesized locally and released into synovial fluid from synovial fibroblasts and macrophages. These proteins are diverse and include cytokines and glycoproteins. Proteins are cleared from synovial fluid by crossing the synovial intima after which they may reach lymph channels or undergo local degradation (Bauer W, Short C L *et al*, 1933 and Simkin P A, 1985).

The low friction characteristics of the joint are secondary to a combination of three molecules; lubricin (lubricating glycoprotein-1), water and hyaluronan.

Lubricin is a 166 kDa glycoprotein found in human synovial fluid at a concentration of 50 µg/ml (Swann D A, Silver F H *et al*, 1985). It has the shape of a flexible rod 173-197 nm long and 1-2 nm wide with numerous oligosaccharide side chains. *In vitro* friction experiments have demonstrated the lubricating ability of lubricin at concentrations of 30-50 µg/ml. The lubricating ability of lubricin has been shown in part to be dependant on its ability to bind articular cartilage (Swann D A, Hendren R B *et al*, 1981). It has also been suggested that lipids in synovial fluid may contribute to the lubrication of joints. The total lipid concentration of synovial fluid is 0.24 g/dl, approximately 45% of this is made up of a lipid called dipalmitoyl phosphatidylcholine. This lipid is adsorbed onto articular cartilage and also acts as a lubricant in synovial joints (Williams P F, Powell G L *et al*, 1993).

Hyaluronan is a glycoaminoglycan synthesized by normal intimal synovial fibroblasts (Yielding K L, Tomkins G M *et al*, 1957; Pitsillides A A, Wilkinson L S *et al*, 1991b). It has a concentration in normal synovial fluid of approximately 3mg/ml. Hyaluronan is a linear glycoaminoglycan composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine with a molecular weight of approximately 10×10^6 daltons. The glycoaminoglycan chains form tangled coils in solution and this contributes to the viscous properties of synovial fluid (Bothner H, Wik O, 1987). The viscous properties of hyaluronan are believed to be dependant on the molecular weight of hyaluronan and the phospholipid concentration of synovial fluid. High molecular weight preparations of hyaluronan exhibit increased stiffness. It is proposed that phospholipid binding to

hydrophobic pockets of hyaluronan cause a change in the tertiary structure of hyaluronan allowing closed loops to open, resulting in a more flexible form of hyaluronan and as a consequence of this, less viscous synovial fluid (Scott J E, Cummings C *et al*, 1991; Ghosh P, Hutadilok, 1994).

Hyaluronan maintains the film of synovial fluid within the joint. Water entering the synovial cavity combines with hyaluronan. If water leaves synovial fluid it has to take hyaluronan with it but the synovial intima restricts the passage of hyaluronan out of the joint. This process is known as solute polarisation.

In addition to the role of hyaluronan in maintaining a film of synovial fluid, it also modulates the function of leukocytes and fibroblasts through a specific cell surface receptor called CD44.

CD44 is a 90kDa transmembrane glycoprotein widely distributed on T lymphocytes, granulocytes, monocytes, fibroblasts, keratinocytes and epithelial cells (Culty M, Miyaka K *et al*, 1990; Aruffo A, Stamenkovic M *et al*, 1990). The distribution of CD44 in normal synovium is largely restricted to the intimal layer. CD44 has many isoforms that differ both in amino acid sequence and carbohydrate composition (Borland G, Ross J A *et al*, 1998). The precise cellular functions and role of CD44 and its isoforms remain uncertain, but ligation of CD44 on monocytes *in vitro* by specific monoclonal antibodies induces the production of IL-1 and TNF α (Webb D S, Shimizu Y *et al*, 1990). A hyaluronan-CD44 interaction is responsible for fibroblast production of IL-

1 β , TNF α and IL-8. Thus the CD44 receptor-ligand interaction can transmit the necessary signals for the release of cytokines. The CD44-hyaluronan interaction may therefore be a mechanism of regulating the production of cytokines *in vivo*. This regulation of cytokine production is dependant on the molecular weight of hyaluronan. The molecular weight of hyaluronan can vary from 10*10⁶ Daltons to as little as 2*10⁵ Daltons (Balazs E A, Watson D *et al*, 1967). High molecular preparations act in an anti-inflammatory manner and are more effective at reducing the release of cytokines from monocytes and fibroblasts. High molecular weight preparations also mediate anti-inflammatory effects by being more effective at inhibiting leukocyte migration compared to low molecular weight preparations (Forrester J V, Wilkinson P C, 1981).

The cytokines synthesized and released by synovial monocytes, fibroblasts, lymphocytes and endothelial cells are present in synovial fluid. Literature on the cytokine profile of normal synovial fluid is scant as normal synovial fluid is rarely aspirated. However fluid aspirated from patients with osteoarthritis or rheumatoid arthritis contains a wide spectrum of different cytokines. Inflammatory synovial fluid has the greatest concentration of cytokines, TGF β , IL-1 α , IL-1 β , IL-6, IL-8, IL-10, IL-12, TNF α , IL-4, IFN- γ and growth factors are all raised. The above cytokines are found in osteoarthritic fluid but at a much lower concentration compared to inflammatory fluid (Schlaak J F, Pfers I *et al*, 1996).

Proteoglycans are present in normal synovial fluid and arise mainly from articular cartilage. In inflammation the concentration of proteoglycans is elevated reflecting increased mobilization of glycoaminoglycans (Heimer R, Sporer R *et al*, 1992).

Additional proteins may be synthesized by the synovium: for example, IgG rheumatoid factor synthesized and released by B lymphocytes and plasma cells in rheumatoid synovium can be found in inflammatory synovial fluid. Other proteins are depleted by local consumption for example the components of complement.

Soluble fibronectin is found in normal synovial fluid at a concentration of 172 ± 69 $\mu\text{g/ml}$. Data shows that fibronectin in synovial fluid differs from that found in plasma suggesting that synovial fluid fibronectin is locally synthesized by synovial fibroblasts (Carnemolla B, Cutolo M *et al*, 1984).

Human IgG Fc Receptors

Fc γ receptors are transmembrane proteins that bind the Fc region of IgG. Fc receptors are involved in phagocytosis, the generation of oxygen free radicals, antibody dependent cytotoxicity to tumour cells, antigen presentation and in triggering the release of lysosomal enzymes, TNF- α , IL-1 and IL-6 (Van de Winkel J G J, Capel P J A, 1993). Specific functions can be attributed to distinct Fc γ receptors. Fc receptors are composed of two or three extracellular domains that contain immunoglobulin like regions and a single membrane-spanning segment (Mellman I, Koch T *et al*, 1988). Fc receptors capable of triggering cellular activation have intracellular activation motifs that resemble those of the B-cell receptor and T-cell receptor signal transduction subunits (Reth M G, 1989). The signal transduction units contain a motif called the immunoreceptor tyrosine-based activation motif (ITAM). This interacts with tyrosine kinases to mediate signal transduction (Lin C T, Shen Z *et al*, 1994).

Three classes of Fc γ receptors have been identified Fc γ RI (CD64), Fc γ RII (CD32) and Fc γ RIII (CD16). Two or three genes encode each Fc γ receptor and therefore different isoforms of each class exist (de Haas M, Vossebeld P J M *et al*, 1995).

Fc γ RI

Fc γ RI is a 72 kDa glycoprotein that is constitutively expressed on monocytes and macrophages and can be induced on neutrophils and eosinophils by IFN- γ or G-CSF (Fanger M W, Shen L *et al*, 1989; Ravetch J V, Kinet J P, 1991; Van de Winkel J G

J, Anderson C L, 1991; Hartnell A, Kay A B *et al*, 1992). IFN- γ also up regulates Fc γ RI expression on monocytes and macrophages (Arend W P, Ammons J T *et al*, 1987; Rossman M D, Chien P *et al*, 1986; Girad M T, Hjaltadottir S *et al*, 1987, Girad M T *et al*, 1987; Guyre P M, Morganelli P M *et al*, 1983). There is weak expression of Fc γ RI on synovial intimal cells (Bröker B M, Edwards J C W *et al*, 1990). Fc γ RI binds monomeric IgG with high affinity. The affinity of Fc γ RI for the different IgG subclasses varies as follows: IgG₃ > IgG₁ > IgG₄ > IgG₂ (Huber H, Douglas S D *et al*, 1971). Under most conditions Fc γ RI may be saturated with monomeric IgG.

Fc γ RII

Fc γ RII is a 40-kDa glycoprotein that is present on monocytes, platelets, neutrophils and B cells (Tettero P A T, van der Schoot F J *et al*, 1987). There is weak expression of Fc γ RII on intimal synovial cells and in the subintima (Bröker B M, Edwards J C W *et al*, 1990). Fc γ RII has low affinity for monomeric IgG and therefore appears to be specific for immune complexes. The order of binding activity to Fc γ RII by different IgG subclasses is as follows: IgG₁ > IgG₂ = IgG₄ > IgG₃ (Stengelin S, Stamenkovic I *et al*, 1988). Fc γ RII interacts with Fc γ RIIIb on neutrophils in phagocytosis and immune-complex induced actin assembly and calcium flux (Naziruddin B, Duffy B F *et al*, 1992). Therefore there is evidence to suggest that on neutrophils Fc γ RII needs to interact with Fc γ RIIIb to produce a biological response to immune complexes.

FcγRIII

FcγRIII is a 50-70 kDa glycoprotein and occurs as two isoforms FcγRIIIa and FcγRIIIb (figure 1.3) each the product of a different gene (Fleit H B, Wright S D *et al*, 1982, Ravetch J V, Perussia B, 1989; Scanlon B J, Scigliano E *et al*, 1989).

FcγRIIIb is present on neutrophils and eosinophils. Neutrophils contain a large intracellular pool of FcγRIIIb, which is localised in easily mobilizable secretory vesicles (Tosi M F, Zakem H *et al*, 1992 and de Haas M, Kerst J M *et al*, 1994). The extracellular domains of FcγRIIIa and FcγRIIIb are similar (Moxley G, Ruddy S, 1993). FcγRIIIb has a glycosphosphoinositol linkage (Ravetch J V, Kinet J P, 1991). A role for FcγRIIIa and CR3 in FcγRIIIb signalling has been proposed (Brunkhorst B A, Strohmeier G *et al*, 1992; Naziruddin B, Duffy B F *et al*, 1992; Petty H R, Todd R F, 1993). Exposure of neutrophils to TNF-α results in a reduction of FcγRIIIb. FcγRIIIb binds only complexed IgG₁ and IgG₃ with no binding to IgG₂ or IgG₄ (Huizinga T W J, van Kemenade F *et al*, 1989). Acting in synergy with FcγRIIIa, FcγRIIIb is involved in IgG-dependant or complement dependant phagocytosis and in the production of reactive oxygen species (Vossebeld P J M, Kessler J *et al*, 1995; Edberg J C, Kimberly R P *et al*, 1994)

FcγRIIIa is expressed on natural killer cells, a small proportion of peripheral blood monocytes (≈5%), a subset of T-lymphocytes. Cultured peripheral blood monocytes adherent to polystyrene show an increase in the expression of FcγRIIIa. This increased expression reaches a plateau after three days of culture (Klaassen R J L,

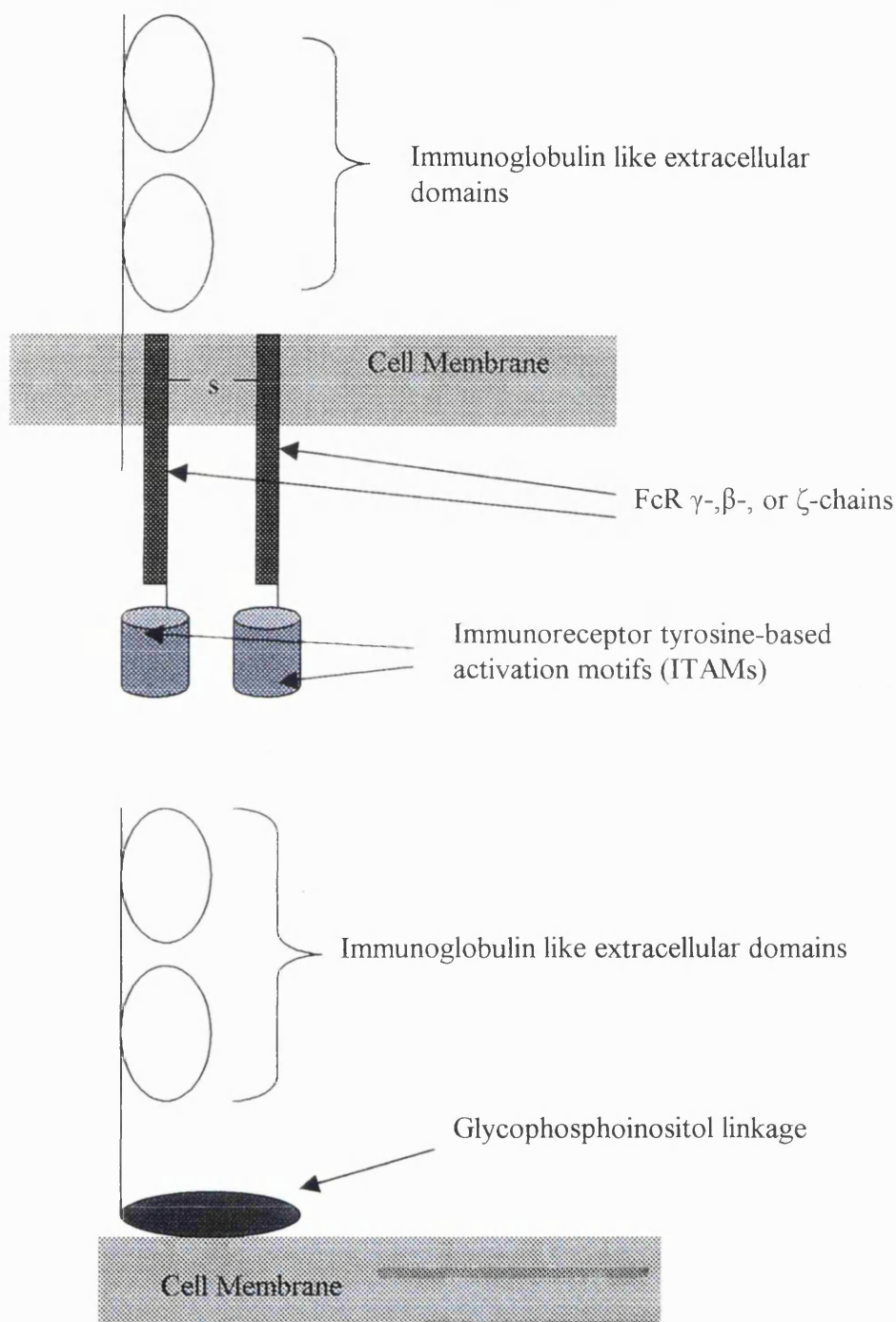
Ouwehand W H *et al*, 1990). Monocytes cultured on Teflon plates also show an increase in the expression of FcγRIIIa. However in contrast to monocytes cultured on polystyrene the increased expression of FcγRIIIa reaches a plateau after fourteen days of culture (Clarkson S B, Ory P A, 1988). Monocytes adhere more strongly to polystyrene (Andreessen R, Picht J *et al*, 1983). This probably accounts for the shorter duration of time observed for FcγRIIIa expression to reach a plateau when monocytes are cultured on polystyrene.

In vitro experiments have shown that FcγRIIIa can be induced by the cytokines TGFβ₁ and IL-10 (Welch G R, Wong H L *et al*, 1990 and Calzada-Wack J C, Frankenberger M *et al*, 1996) and down regulated by IL-4 (Wong H L, Welch G R *et al*, 1991; Wahl S M, Allen J B *et al*, 1992).

Within tissues FcγRIIIa has been found on macrophages in normal adult and fetal synovium in the intimal region (Edwards J C W, Blades S *et al*, 1997). FcγRIIIa is also present on dermal macrophages that have been exposed to mechanical stretch (Edwards J C W, Blades S *et al*, 1997), Kupffer cells and alveolar macrophages (Bordessoule D, Jones M *et al*, 1993; Tuijnman W B, Van-Wichen D F *et al*, 1993). It is likely that the immediate microenvironment at these sites is responsible for the induction of FcγRIIIa. FcγRIIIa is of moderate affinity and its IgG subclass specificity is IgG₁ = IgG₃ > IgG₂ = IgG₄ (Vance B A, Huizinga T W J *et al*, 1993). Polymorphisms in the extracellular domain of FcγRIIIa have been identified. These polymorphisms affect the affinity of FcγRIIIa for particular subclasses of IgG. Upon engagement of certain FcγRIIIa polymorphisms there is a greater rise of intracellular

calcium and thus a greater degree of cell activation (Wu J, Edberg J C *et al*, 1997). A low binding phenotype of Fc γ RIIIa (substitution of valine in place of phenylalanine at amino acid position 176 in the extracellular domain 2 of Fc γ RIIIa) is strongly associated with patients who have systemic lupus erythematosus and in particular those with nephritis (Wu J, Edberg J C *et al*, 1997). The addition of anti-Fc γ RIIIa monoclonal antibodies to cultured monocytes expressing Fc γ RIIIa results in the release of TNF- α , IL-1 and reactive oxygen species. Monoclonal antibodies to Fc γ RI and Fc γ RII do not induce TNF- α release (Abraham's V M, Cambridge G *et al*, 2000; Trezzini C, Jungi T W *et al*, 1991). TNF- α release has only been achieved via Fc γ RI and Fc γ RII by secondary cross-linkage following ligation of the receptors by IgG Fc (Debets J M H, van de Winkel J G J *et al*, 1990). Fc γ RIIIa is able to induce signalling in response to a ligand binding two or at most three receptors, whereas Fc γ RI and Fc γ RII require multiple cross-linking in order to induce signalling.

Figure 1.3: Schematic Structure of Fc γ RIIIa (top) and Fc γ RIIIb (bottom)



Both Fc γ RIIIa and Fc γ RIIIb have two extracellular regions comprised of immunoglobulin like domains. Fc γ RIIIa exists as an oligomer associated with FcR γ -, β - or ζ -chains, which contain immunoreceptor tyrosine-based activation motifs. These interact with tyrosine kinases to mediate signal transduction. Fc γ RIIIb is expressed as a phosphatidylinositol-glycan linked glycoprotein. Fc γ RIIIb has no intracellular signalling mechanism. A role for Fc γ RIIIa and CR3 in Fc γ RIIIb signalling has been proposed (Brunkhorst B A, Strohmeier G *et al*, 1992).

THE EXTRACELLULAR MATRIX OF SYNOVIUM

Synovial cells are surrounded by a complex organization of extracellular matrix proteins. Distinct domains on matrix proteins interact with specific cellular receptors to affect cellular activities including cell migration, proliferation, differentiation and tissue stromal organization (Lévesque J P, Hatzfeld A *et al*, 1991; De Sousa M, Tilney N L *et al*, 1991; Savino W, Villa-Verde D M S, 1993). The cell surface receptors that bind matrix proteins are called cell-adhesion molecules. In addition to the role of cell-adhesion, these molecules can also affect the function of cells. Cell-adhesion molecules belong to four families of proteins, the selectins, the mucins, the integrins and the immunoglobulin superfamily.

The extracellular matrix proteins in the synovial intima may be directly responsible for inducing FcγRIIIa on synovial monocyte-macrophages. Alternatively FcγRIIIa induction may be dependant on immunoregulatory molecules such as cytokines or other macromolecules bound to extracellular matrix proteins. The extracellular matrix proteins found in the synovial intima where FcγRIIIa is preferentially expressed (Edwards J C W, Blades S *et al*, 1997) are different from those of the subintima where FcγRIIIa expression is sparse. López-Moratello *et al* demonstrated that an increased percentage of human peripheral blood monocytes cultured in the presence of fibronectin or a peptide of fifteen amino acids (found in the extracellular matrix proteins fibronectin, laminin, tenascin, undulin, type IV collagen and type VIII collagen) expressed FcγRIIIa compared to control monocyte cultures. This data supports the

hypothesis that extracellular matrix proteins in the synovial intima may induce FcγRIIIa on intimal monocyte-macrophages *in vivo*.

Quantitative data on the precise composition of matrix components within synovial tissue is scanty because of the difficulties in quantifying immunohistochemical data, the difficulty of performing accurate microdissections of the synovial intima without contamination with underlying tissue and the difficulty of obtaining large amounts of tissue required for the sensitive assay methods required to quantify matrix proteins. The matrix proteins found in synovium are discussed in more detail below.

Collagens

Type I, III, IV, V, and VI collagens are found in synovium. Results from immunohistochemical studies of normal synovium demonstrate the expression of collagen VI in the synovial intima and around blood vessel walls. In addition to these sites type VI collagen is found in the subintima of rheumatoid synovium. (Wolf J, Carsons S E, 1991; Okada Y, Naka K *et al*, 1990). In rheumatoid synovium fibronectin codistributes with type VI collagen (Wolf J, Carsons S E, 1991). Type VI collagen occurs as microfibrils that are formed from triple helical monomers of type VI collagen. The term microfibril specifies a particular subgroup of fibrils with a diameter less than 20 nm, without the 67 nm banding pattern of collagen fibres (Maddox B K, Sakai L Y *et al*, 1989). The triple helical monomers of type VI collagen assemble laterally to form dimers and then tetramers. These associate in an end to end fashion to form double

beaded microfibrils with a periodicity of 100 nm (Bruns R R, Press W *et al*, 1986) and a diameter of 3-5 nm (Engvall E, Hesse H *et al*, 1986).

Beneath the synovial lining types I, III and V collagen form 67 nm striated fibrils. These fibrils are grouped into large bundles that are orientated parallel to the synovial surface (Eyre D R, Muir H, 1975; Rittig M, Tittor F *et al*, 1992). Type III collagen has been demonstrated in the intima and subintimal regions of synovium associated with thin cross-striated collagen fibrils described above. Type I collagen is found only in the subintima (Okada Y, Naka K *et al*, 1990). Type II collagen is absent in synovium.

Type IV collagen is found around synovial intimal cells in a pericellular distribution and at the basement membrane of blood vessels. No type IV collagen is present around deeper cells (Pollock L E, Lalor P *et al*, 1990). Type IV collagen has a molecular weight of 550-600 kDa. It consists of two different peptide chains and is non-fibril forming. It is believed that type IV collagen aggregates into a rhomboid form which builds up into a network resembling “chicken wire”. This is thought to confer the property of mechanical stability to synovium (Timpl R, Wiedemann H *et al*, 1981).

Fibrillin

Histochemical studies reveal a second type of microfibril in the synovial lining. The principal structural component of these microfibrils is the glycoprotein fibrillin. Fibrillin has been characterized as a single chained protein with a molecular weight of

350 kDa (Sakai L Y, Keene D R *et al*, 1986). These fibrillin containing microfibrils have a diameter of 10-13 nm and an average periodicity of 50 nm (Keene D R, Maddox B K *et al*, 1991). Fibrillin based microfibrils bind a wide range of immunologically active ligands, including latent TGF- β binding protein (Bashir M M, Han M D *et al*, 1996; Reinhardt D P, Chalberg S C *et al*, 1995; Gibson M A, Hatzinikolas G *et al*, 1995; Pfaff M, Reinhardt D P *et al*, 1996; Sakamoto H, Broekelmann T *et al*, 1996). Fibrillin is present in the extracellular matrix of the skin, aorta, lung, muscle, cornea, placenta and ciliary zonule (Sakai L Y, Keene D R *et al*, 1986).

Laminin

Laminin (M.W. 800 kDa) consists of three distinct polypeptide chains folded into a cruciform structure. The polypeptide chains are designated A, B1 and B2 and are stabilized by interchain disulfide bonds. The A and B1 chains of laminin can in certain tissues be replaced by distinct but homologous polypeptides. Laminin may therefore differ in its biological activity depending on its location (Paulsson M, 1992). Laminin is found at the basement membrane of tissues. Within synovium it is found around intimal cells and at the basement membrane of blood vessels in a similar distribution to collagen IV (Pollock L E, Lalor *et al*, 1990). The integrin family of cell adhesion molecules bind to laminin.

The integrin family of cell adhesion molecules are heterodimeric transmembrane proteins with an α and β chain. Several different α and β chains may combine that give

rise to integrins with different ligand properties. Cells expressing integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_4$ and $\alpha_v\beta_3$ have been found to bind laminin (Mercurio A M and Shaw L M, 1991). Some of these integrins are promiscuous and bind to ligands other than laminin. The role of laminin on cellular differentiation has been studied on neuronal cells where laminin has been shown to promote neurite outgrowth and survival (Edgar D, Timpl R *et al*, 1984; Engvall E, Davis G E *et al*, 1986; Alder R, Jerdan J *et al*, 1985). The effects of laminin in neuronal systems have been shown to be synergistic with growth factors such as nerve growth factor and fibroblast growth factor (Edgar D, Timpl R *et al*, 1984; Drago J, Nurcombe V *et al*, 1991). Given these findings it has been suggested that matrix proteins and cytokines may act in concert in regulating growth and differentiation of cells *in vivo* (Nathan C, Sporn M, 1991).

Fibronectin

Fibronectin is synthesized by synovial fibroblasts. It is released as a soluble disulfide-bonded glycoprotein dimer with a molecular weight of 440 kDa (Matsubara T, Spycher M A *et al*, 1983; Mapp P I, Stevens C *et al*, 1993; Revell P A, 1989). Oxidisation of cysteine residues leads to the formation of more disulfide bonds converting fibronectin into a multimer. The formation of multimers contributes to the insolubility of fibronectin. Fibronectin exists in a soluble form as dimers and monomers in body fluids such as synovial fluid, cerebrospinal fluid, plasma and amniotic fluid. It occurs as an insoluble form within tissues.

Fibronectin has been demonstrated in the intimal cell layer of the synovium and at the basement membrane of blood vessels in normal human synovium (Pollock L E, Lalor P *et al*, 1990; Clemmensen I, Hølund B, 1983). In rheumatoid synovium fibronectin codistributes with type VI collagen at the synovial intima, around blood vessels and in the subintima (Wolf J, Carsons S E, 1991). Cell surface fibronectin has various organizational forms ranging from thick fibrils on lung fibroblasts to thinner fibrils in skin and a punctate appearance on kidney cells (Vaheri A, Ruoslahti E *et al*, 1976; Mosher D F, Saksela O *et al*, 1977).

Fibronectin is also distributed on the basement membranes in the lung, (Bray B A, 1978), exocrine glands, mucous membranes, sweat, sebaceous, mammary and parotid glands (Matsuda M, Yoshida N *et al*, 1978), spleen, intestines (Crouch E, Balien G *et al*, 1978; Linder E, Stenman S *et al*, 1978), liver sinusoids and the sarcolemma of striated muscle (Stenman S, Vaheri A *et al*, 1978).

In vivo experiments have demonstrated that fibronectin co-distributes with type III collagen in fetal dermis (Linder E, Stenman S *et al*, 1978) and the collagen component of connective tissue in basement membranes (Linder E, Stenman S *et al*, 1978; Stenman S, Vaheri A, 1978; Wartiovaara J, Linder E *et al*, 1974). Treatment of alveolar basement membrane with collagenase results in the release of fibronectin (Bray B A, 1978). *In vitro* experiments have demonstrated fibronectin binding to collagen I, II, and III (Pearlstein E, Gold L I *et al*, 1980). The function of fibronectin *in vivo* may therefore be dependant on its association with collagen. It has been postulated that the binding of

fibronectin to collagen induces conformational changes in fibronectin that results in the exposure of active domains on fibronectin (Pearlstein E, 1978). Fibronectin fibrils run parallel to the long axis of cells in a pattern similar to intracellular microfilament bundles (Wartiovaara J, Linder E *et al*, 1974; Hynes R O, Destree A T, 1978). The arrangement of intracellular microfilaments is related with the distribution of fibronectin on the cell surface. Antibodies to fibronectin result in a disruption of intracellular microfilaments. Treatment of cultured cells with cytochalasin B disrupts microfilaments and causes release of surface associated fibronectin and prevents fibril reformation (Hynes R O, Ali I U *et al*, 1978; Ali I U, Hynes R O, 1977; Kurkinen M, Wartiovaara J *et al*, 1978). These observations suggest a role for microfilaments in maintaining fibronectin organization on the cell surface. Colchicine and vinblastine sulfate antagonists of microtubules have no effect on fibronectin release (Ali I U, Hynes R O, 1977; Kurkinen M, Wartiovaara J *et al*, 1978). Several workers have demonstrated that fibronectin preferentially accumulates in areas of cell-cell contact (Chen L B, Maitland N *et al*, 1977; Chen L B, Burridge K *et al*, 1978; Chen L B, Gallimore P H *et al*, 1976; Mautner V, Hynes R O *et al*, 1977). *In vitro* experiments have demonstrated that attachment of cells to collagen is dependent on the presence of fibronectin (Klebe R J, 1974). Fibronectin mediated cell adhesion involves three steps (Klebe R J, 1975; Pearlstein E, 1976), as outlined below:

- Fibronectin complexing to collagen
- Cell attachment to the complex
- Cell spreading on the complex

In common with other extracellular matrix proteins fibronectin is composed of structural domains that contain binding sites for other macromolecules and cells. In addition to binding to collagen fibronectin binds to fibrin, DNA, integrins and heparin. Fibronectin has three heparin binding sites Hep I, Hep II and Hep III (Ingham K C, Brew S A *et al*, 1990; Moyano J V, Camemolla B *et al*, 1999). The integrin $\alpha_4\beta_1$ (VLA-4) is a receptor that is expressed on monocytes, eosinophils and lymphocytes, it binds the Hep I, Hep II, connecting segment 1(CS-1) and RGD sequence on fibronectin (Sharma A, Askari J A *et al*, 1999). The RGD sequence and Hep III domain on fibronectin bind only activated $\alpha_4\beta_1$ (Sánchez-Aparicio P, Domínguez-Jiménez C *et al*, 1994; Moyano J V, Camemolla B *et al*, 1999).

The Hep I domain is mainly involved in the formation of fibronectin matrices (Hynes R O, 1990; Zang Q, Mosher D F *et al*, 1996). The Hep II domain has the highest avidity for heparin and contains specific sequences that bind proteoglycans and/or the $\alpha_4\beta_1$ integrin at the cell surface and induce cell adhesion (Iida J, Skubitz A *et al*, 1992; Mooradian D L, McCarthy J B *et al*, 1993; Woods A, McCarthy J B *et al*, 1993). Within the Hep II domain is a site called H1 that is a ligand for the integrin $\alpha_4\beta_1$ on lymphoid cells.

The Hep III domain binds heparin only at low salt concentrations, mediates adhesion of T and B-lymphocytes and contains a sequence that binds activated $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins (Moyano J V, Camemolla B *et al*, 1999).

The CS-1 sequence consists of 25 amino acids encoded by an alternatively spliced fibronectin transcript (Humphries M J, Akiyama S K *et al*, 1986). CS-1 is found only on a few scattered synoviocytes in the intima and subintima of osteoarthritic synovium (Müller-Lander U, Elices M J *et al*, 1997). In rheumatoid synovium CS-1 fibronectin mRNA is detected in synovial fibroblasts (Elices M J, Tsai V *et al*, 1994). CS-1 fibronectin is also found decorating the lumen of endothelial cells in rheumatoid synovium. CS-1 facilitates $\alpha_4\beta_1$ dependent migration of cells (Elices M J, Tsai V *et al*, 1994; Van Dinther-Janssen A C, Pals S T *et al*, 1998).

It has been demonstrated that the $\alpha_4\beta_1$ ligands H1 and RGD induce different intracellular responses than CS-1 (Moyano J V, Carnemolla B *et al*, 1999; Domínguez-Jiménez C, Sánchez-Aparicio P *et al*, 1996). The physiological response of the fibronectin- $\alpha_4\beta_1$ integrin interaction will therefore be dependant on which fibronectin ligand binds the $\alpha_4\beta_1$ integrin.

Several biological functions of fibronectin have been shown to be present in proteolytic fragments but not in intact fibronectin (Clark R A F, Wikner N E *et al*, 1988; Hocking D C, Sottile J *et al*, 1994). It is believed that certain fragments or cryptic sites that become exposed upon fibronectin degradation or conformational change may serve to recruit activated cells to sites and provide a substrate for their attachment (Moyano J V, Carnemolla B *et al*, 1999).

The data from López-Moratello *et al* would support the hypothesis for a role of extracellular matrix proteins in inducing FcγRIIIa on intimal synovial monocyte-macrophages. Distinct extracellular matrix proteins are found around cells in the synovial intima. These matrix proteins may be either directly or indirectly responsible for the induction of FcγRIIIa on synovial monocyte-macrophages.

CYTOKINES AND OTHER INFLAMMATORY MEDIATORS

The term cytokine encompasses a wide variety of mediators that are individually called interferons, interleukins and growth factors. Cytokines are low molecular weight proteins that are secreted by a variety of different cell types (J Kuby, 1997).

Cytokines generally exert their effects locally where they are released, by binding to specific receptors. When a cytokine binds to a receptor on a target cell, intracellular signals within the cell lead to changes in gene expression. In this way cytokines can influence the function and action of immune cells.

Cytokines can affect the same cells from which they are released. In this case they are said to exert an autocrine action. Alternatively a cytokine may be released from one cell and exert an effect on another cell close by. In this case the cytokine is said to have a paracrine action. Less commonly cytokines can be released into the circulation where they can exert an effect on cells far away from where they are released. In this case they exert an endocrine action.

The actions of cytokines are widespread. They are involved in regulating both the cellular and humoral immune response. Cytokines can also stimulate or inhibit the activation, proliferation and differentiation of cells and are therefore involved in the induction of an inflammatory response, the induction of wound healing and the regulation of haematopoiesis. Some cytokines are produced constitutively by cells and

modulate the local environment of a tissue (Seitz M, Loetscher P *et al*, 1994). This modulation may contribute to the efficient functioning of the tissue. This may, however, be at the cost of producing a microenvironment within a tissue that makes it susceptible to a disease.

In the context of rheumatoid arthritis the local cytokine milieu may explain the presence of FcγRIIIa that is found on intimal synovial macrophages (Edwards J C W, Blades S *et al*, 1997). The cytokines that may be responsible for inducing FcγRIIIa expression on macrophages are discussed in more detail below.

Transforming Growth factor beta (TGFβ)

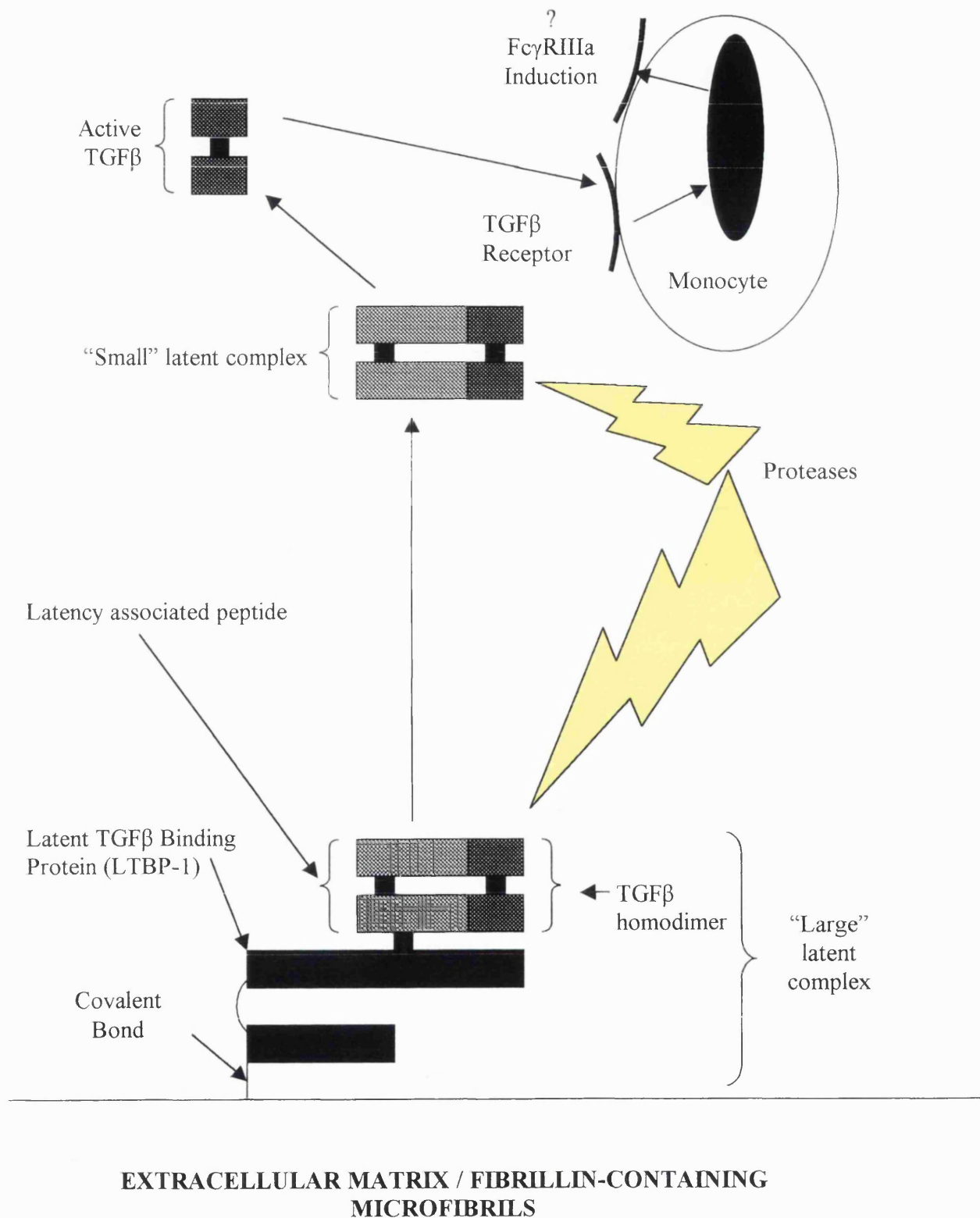
TGFβ₁ has been shown to selectively induce FcγRIIIa on monocytes *in vitro* (Welch G R, Wong H L *et al*, 1990). Mechanical stretch may serve as a stimulus for TGFβ synthesis and release (Riser B L, Cortecs P *et al*, 1996). Normal macrophages in the synovial intima and mechanically stretched dermis express FcγRIIIa (Edwards J C W, Blades S, 1997). These tissues are both subject to mechanical stretch in normal physiological conditions. Thus TGFβ may be responsible for providing a microenvironment in tissues that induces FcγRIIIa on monocytes-macrophages and the stimulus for TGFβ synthesis and release may be mechanical stretch.

TGF β is a cytokine that is released from most cell types. There are three isoforms in human tissue TGF β_1 , TGF β_2 and TGF β_3 (Wataya-Kaeda M, Hashimoto K *et al*, 1994). The isoforms are differentially expressed in human tissues. The predominant isoform in bone and synovium is TGF β_1 , TGF β_2 and TGF β_3 are not found in normal or rheumatoid synovial tissue (Taketazu F, Kato M *et al*, 1994). Fifty percent of TGF β in the kidney is TGF β_2 (Marra F, Bonewald L F *et al*, 1996). Virtually all cells express receptors for TGF β (Burmester J K, Qian S W *et al*, 1998).

TGF β is found in the highest concentration in bone, platelets and serum (Wakefield L M, Smith D M *et al*, 1988), but the TGF β at these sites is mostly in a latent inactive form. Active TGF β is a disulfide-bonded homodimer with a molecular weight of 12.5 kDa. It exists in two latent forms. The first is TGF β noncovalently associated with latency-associated peptide. This is called a “small latent complex” with a molecular weight of 100kDa. The second latent form of TGF β is called the “large latent complex” and consists of the small latent complex bound to protein known as latent TGF β binding protein (LTBP) (Dallas S L, Park-Snyder S *et al*, 1994).

The large latent complex is covalently associated with fibrillin containing microfibrils. Proteases can cleave LTBP-1 from the matrix releasing the latent complex. Further proteolysis of the small latent complex then results in the release of active TGF β (Dallas S L, Saharianen J *et al*, 1997).

Figure 1.4: Transforming Growth Factor Beta and the Putative Induction of FcγRIIIa on Tissue Monocyte-macrophages



There are three receptors that bind TGF β (Derynck R, 1994) . TGF β initially binds the type II receptor. The type I receptor is then recruited to form a complex with the ligated type II receptor. The activated type II receptor then phosphorylates the type I receptor. This results in the phosphorylation of transcription factors called Smads 2,3 and 4. The Smad family of transcription factors regulates the transcription of target genes within the nuclei of cells (Massague J, Hata A *et al*, 1997). It is believed that the type III TGF β receptor does not signal and serves as a reservoir for TGF β , presenting TGF β to the type II receptor (Lopez-Casillas F, Wrana J L *et al*, 1993). The type II TGF β receptor has a 100-fold higher affinity for TGF β ₁ and TGF β ₃ compared with TGF β ₂ (Lin H Y, Wang X-F *et al*, 1992) , the type III receptor has equal affinity for all three isoforms (Lopez-Casillas F, Wrana J L *et al*, 1993). A transmembrane glycoprotein called endoglin has a higher affinity for TGF β ₂ than for TGF β ₁ or TGF β ₃. Endoglin is the most abundant TGF β binding protein on endothelial cells (McAllister K A, Johnson D W *et al*, 1994). The type II TGF β receptor is present on normal synovial intimal cells and its expression on these cells is up regulated in rheumatoid arthritis (Taketazu F, Kato M *et al*, 1994).

The role of TGF β is diverse. Mice that have been genetically modified not to express TGF β ₁ (knockout mice), die of a massive inflammatory response early in life (Kulkarni A B, Huh C G *et al*, 1993). These mice also have mineralization defects in their bones. A variant of the TGF β ₁ gene has been shown to be associated with low bone mass in osteoporotic women (Langdahl B L, Knudsen J Y *et al*, 1997). Mice that lack TGF β ₂ or

TGF β ₃ have a wide range of developmental defects (Sanford L P, Ormbsby I *et al*, 1997; Proetzel G, Pawlowski S A *et al*, 1995).

The major biological functions of TGF β are inhibition of cell proliferation, regulation of cell differentiation, cell adhesion and extracellular matrix deposition. Clinical interest in TGF β , therefore, focuses on wound healing, fibrosis, inflammation, autoimmunity and neoplasia.

TGF β is released from cells in response to a variety of stimuli and below is a table that summarizes the cells from which TGF β is released, the stimuli that results in TGF β release and the action of TGF β .

Table 1.1: Summary of TGF β

CELL	STIMULI FOR TGF β RELEASE	LOCAL EFFECT OF TGF β
Osteoblasts (Klein-Nulend J <i>et al</i> , 1995)	Mechanical stimulation	Regulation of bone metabolism:
Endothelial cells (Phan S H <i>et al</i> , 1992)	Tissue injury, IL-1 β , TNF α	Regulation of inflammatory process and wound repair: - Chemotactic for monocytes - Decreased synthesis of IL-1, IL-6 and TNF (Kitamura M <i>et al</i> 1996)
Cardiac fibroblasts (Lee A A <i>et al</i> , 1995)	Angiotensin II	Myocardial fibrosis
Monocyte-macrophages (Toosi Z <i>et al</i> , 1995)	Infection e.g. Mycobacterium tuberculosis	Resolution of infection and regulation of the inflammatory process
Epithelial cells (Danielpour D <i>et al</i> , 1996)	All-trans-retinoic acid and 1 alpha, 25 dihydroxycholecalciferol (vitamin D3)	Inhibition of cellular proliferation i.e. antitumour action
Mesangial cells (Riser B L <i>et al</i> , 1996)	Mechanical stretch (increased glomerular capillary pressure)	Extracellular matrix deposition, fibrosis and scarring
Keratinocytes (Piazza G A <i>et al</i> , 1995)	Lysophosphatidic acid	Increased extracellular matrix deposition

TGF β may be responsible for the *in vivo* induction of Fc γ RIIIa on synovial macrophages. Synovial fibroblasts may release TGF β . In this context TGF β will be acting in a paracrine fashion. Alternatively TGF β may be released from synovial macrophages, and thus act in an autocrine fashion. The production of TGF β may be a constitutive property of synovial cells. However, mechanical stretch may serve as a stimulus for TGF β production in the same way as it serves as stimulus for TGF β production in mesangial cells (Riser B L *et al*, 1996). It is therefore important to confirm the data by Welch *et al* that demonstrates Fc γ RIIIa induction on cultured monocytes. The effect of TGF β may ultimately be dependent on the interaction of extracellular proteins, other cells and additional cytokines in the local milieu.

Interleukin 10

Interleukin 10 (IL-10) is a homodimer with a molecular weight of 39 kDa (Windsor W T, Syto R *et al*, 1993), it can be produced by CD4⁺ T lymphocytes, some CD8⁺ T lymphocytes, B lymphocytes, monocytes, dendritic cells, keratinocytes, mesangial cells, hepatocytes and bronchial epithelial cells (Vieira P, De Waal Malefyt *et al*, 1991). The functions of IL-10 are summarized in the tables below.

Immunostimulatory Effects:

- Enhanced proliferation and cytolytic activity of CD8⁺ T lymphocytes (Chen W F, Zlotnik A *et al*, 1991)
- Stimulation of natural killer cell activity and augmentation of IL-2 induced natural killer cell proliferation, cytotoxicity and cytokine production (Kuby J, 1997)
- Enhanced B lymphocyte proliferation and synthesis of IgM, IgG and IgA (Rousset F, Garcia E *et al*, 1992)
- Upregulation of FcγRIIIa receptor expression on monocytes (Calzada-Wack J C, Frankenberger M *et al*, 1996)

Anti-inflammatory Actions:

- Inhibition of the synthesis of TNF, IL-1, IL-6, IL-8 by monocytes/macrophages, neutrophils, eosinophils and mast cells (De Waal Malefyt R, Abrams J *et al*, 1991; Cassatella M A, Meda L *et al*, 1993; Arock M, Zuanzyamorium C *et al*, 1996)
- Suppression of free oxygen radical release, nitric oxide production and prostagalandin production by macrophages (Bogdan C, Vodovotz Y *et al*, 1991)

Immunosuppressive Properties:

- Downregulation of major histocompatibility complex (MHC) class II, intracellular adhesion molecule 1 and B7 on monocytes and macrophages (de Waal Malefyt R, Haanen J *et al*, 1991; Ding L, Linsey P S *et al*, 1993; Willems F, Marchant A *et al*, 1994)
- Downregulation of MHC II on dendritic cells (Macontonia S E, Doherty T M *et al*, 1993)
- Inhibition of CD4⁺ lymphocytes with reduced secretion of IL-2 and IL-5 (Malefyt R D, Yssel H *et al*, 1993; Schandene L, Alonsovega C *et al*, 1994)

The ability of IL-10 to induce FcγRIIIa on monocytes *in vitro* suggests that IL-10 may be responsible for the expression of FcγRIIIa on normal intimal synovial macrophages. However there is currently no data to suggest that IL-10 is present around tissue macrophages expressing FcγRIIIa casting doubt on IL-10 as a factor responsible for FcγRIIIa expression on tissue macrophages *in vivo*.

A phase I dose escalating double blind placebo controlled trial with IL-10 given to patients with rheumatoid arthritis has been performed. The small sample size of this study precluded any conclusions about clinical efficacy (St Clair E W, 1999). Further clinical trials are being conducted at present to evaluate the role of IL-10 in improving patients symptoms in rheumatoid arthritis, but at present no data is available from these trials.

Haemopoietic Growth Factors

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) are growth factors involved in progenitor cell proliferation and differentiation in the bone marrow. Growth factors are also present outside the bone marrow microenvironment where they have roles other than the regulation of hematopoiesis.

GM-CSF has a molecular weight of 14 to 35 kDa (Gasson J, 1991) and is released from activated T lymphocytes (Thompson C B, Lindsten T *et al*, 1989), activated fibroblasts (Lipsky P E, Davis L S *et al*, 1989), endothelial cells (Broudy V C, Kaushansky *et al*, 1987), macrophages (Munker R, Gasson J *et al*, 1986) and mast cells (Wodnar-Filipowicz, Heusser C H *et al*, 1989). GM-CSF induces the proliferation and differentiation of myeloid precursors (Gasson J, 1991). In addition to this GM-CSF influences the function of mature cells of the granulocytic and monocytic lineages (Metcalf D, 1985). M-CSF augments the functional activities and survival of monocytes and macrophages (Clark S C, Kamen R *et al*, 1987; Das S K, Stanley E R *et al*, 1982).

M-CSF has a molecular weight of between 45 to 60 kDa (Stanley E R, Guilbert L J, 1981). As apposed to GM-CSF, M-CSF is produced constitutively from synovial fibroblasts (Seitz M, Loetscher P *et al*, 1994). It is also released from activated macrophages, secretory epithelial cells of the endometrium, bone marrow stromal cells and cytokine activated endothelial cells. Normal unstimulated human mesothelial cells

in culture also express biologically active M-CSF (Demetri G D, Zenzie B W *et al*, 1989). Normal human monocytes cultured with M-CSF for 10 days have been reported to yield more FcγRIIIa positive cells than monocytes cultured with IL-3 or GM-CSF (Young D A, Lowe *et al*, 1990). However, in this report no comparison was made with untreated cultured monocytes, so it is difficult to draw a conclusion as to the ability of M-CSF to induce FcγRIIIa on monocytes. GM-CSF has an inhibitory effect on FcγRIIIa expression on normal human monocytes *in vitro* (Kruger M, Coorevits T P M *et al*, 1996). M-CSF may be responsible for the long-term influx of monocytes to the synovial intima and its constitutive expression by synovial fibroblasts may be responsible for inducing FcγRIIIa in a paracrine fashion on monocytes within tissues.

Interferons

Interferons (IFN) are classified as type I or type II. Type II interferon is now called IFN- γ .

Type I interferons are characterised by their lack of inactivation after exposure to an acidic environment (pH 2), as compared to IFN- γ which is acid labile. Type I interferons are further subdivided to IFN- α , IFN- β , IFN- ω and IFN- τ . 13 genes code for structurally different forms of IFN- α . IFN- β and IFN- ω are encoded by separate single genes. IFN- τ is found in sheep and cattle in the cytotrophoblast, where it creates the conditioned necessary for the implantation of the ovum. In 1994 Whaley *et al* reported the presence of DNA sequences in human cytotrophoblastic cells that encoded for an IFN with close similarity to bovine IFN- τ , but later work to isolate the IFN- τ gene in humans has failed, casting doubt on the presence of IFN- τ in humans.

The production of type I IFNs is not a specialised function and all cells can produce them. Viruses or double stranded RNA stimulate IFN synthesis. Certain cytokines can induce IFN- α or IFN- β synthesis. IL-1 and TNF induce the synthesis of human IFN- β from fibroblasts (Reis L F, Lee T H *et al*, 1989). Fibroblasts cultured *in vitro* in the absence of infection produce IFN- β (Pilling D, Akbar A *et al*, 1999) thus IFN- β production by fibroblasts may occur *in vivo* in the absence of viral infection. To support this Pilling *et al* have found that fibroblasts in osteoarthritic synovium express IFN- β .

Type I interferons share a common receptor that recruits cytoplasmic tyrosine kinases as it does not contain its own functional enzyme (Uzé G, Lutfalla G *et al*, 1995). Type I interferons induce a number of different proteins, the function of type I IFNs is summarised below:

- Inhibition of viral activity by inhibition of viral penetration, transcription, transduction, maturation and budding from cell surface.
- Inhibition of replication of normal and tumour cells.
- Antitumour activity secondary to the stimulation of macrophages, T cells and NK cells.
- Regulation of immune function by increased MHC I expression thereby improving the presentation of viral antigens to CD8 positive cytotoxic T cells and consequently increasing the lytic activity of CD8 positive T cells.
- Increased NK cell activity.
- Rescue of activated T cells from apoptosis by IFN- β .

Interferons are used clinically in the treatment of patients with different forms of carcinoma, metastatic melanoma, myeloma, ovarian cancers, chronic myelogenous leukaemia, haemangioma and kaposi sarcoma. Other examples of the applications of interferons is in the treatment of viral infections such as hepatitis B, C and D and in the relapsing remitting form of multiple sclerosis.

Fibroblasts are a common source of type-1 interferons especially IFN- β especially after stimulation by viruses. Immunohistochemistry on sections of osteoarthritic synovium reveals the presence of IFN- β producing fibroblasts (Pilling D, Akbar A N *et al*, 1999). These IFN- β producing synovial fibroblasts may in an autocrine fashion induce Fc γ RIIIa on synovial monocytes-macrophages.

Tumour Necrosis Factor Alpha

Tumour necrosis factor-alpha (TNF- α) derives its name from its ability to induce necrosis of tumours when it is injected into tumour-bearing animals (Coley \approx 1890s).

At inflammatory sites TNF- α is produced mainly by monocyte/macrophages. TNF- α is increased in synovial fluid and synovial tissue of patients with rheumatoid arthritis (Saxne T, Palladino M *et al*, 1988). Other cells produce TNF- α but to a lesser extent and these include B and T lymphocytes, natural killer cells, fibroblasts, epithelial cells and neurons.

TNF- α is a homotrimer that occurs as a membrane bound form. Cleavage of this by a specific metalloproteinase called TNF- α converting enzyme results in the release of the soluble TNF- α homotrimer with a molecular weight of 51 kDa (Van Ostade, Tavernier J *et al*, 1994; Tracey K J, Vlassara H *et al*, 1989).

TNF- α production by macrophages occurs after macrophages have been stimulated with products of bacteria, such as gram-negative lipopolysaccharide (Barrios-Rodiles M, Tiraloché G *et al*, 1999). Ligation of Fc γ RIIIa on natural killer cells results in the release of TNF- α (Anegón I, Cuturi G *et al*, 1988). In the context of rheumatoid arthritis, small immune complex (IgG rheumatoid factor dimers) ligation of Fc γ RIIIa on macrophages at sites where rheumatoid disease occurs may therefore result in TNF- α release by macrophages.

TNF- α contains three TNF receptor-binding sites. When a molecule of TNF binds two cell-surface receptors signal transduction occurs. Two types of TNF receptors occur, type I and type II, also called p55 or p60 and p80 or p75 respectively (Bazzoni F, Beutler B, 1996; Banner D W, D'Arcy A *et al*, 1993). These receptors are found on polymorphonuclear leucocytes, vascular endothelial cells and fibroblasts. Soluble forms of these receptors also occur and are elevated in the sera and synovial fluid of patients with rheumatoid arthritis (Cope A P, Aderka D *et al*, 1992). The soluble forms of these receptors are thought to serve an inhibitory function by competing with cell surface receptors for TNF- α . TNF causes inflammation in the tissues where it is expressed through a number of mechanisms outlined below:

- Induction of cyclooxygenase 2, thereby increasing the local synthesis of prostaglandins, leukotrienes, platelet activating factor, nitric oxide and reactive oxygen species (Cao C, Matsumura K *et al*, 1998).
- Enhanced expression of adhesion molecules ICAM-1, ELAM-1 and VCAM-1 on endothelial cells that increases neutrophil and leucocyte binding to vascular endothelium (Pober J S, Gimbrone N A *et al*, 1986; Bevilacqua M P, Pober J S *et al*, 1987; Osborn L, Hession C *et al*, 1989).
- Induction of IL-1, GM-CSF, IL-8 and IL-6; the former three are chemotactic for neutrophils and monocytes, IL-6 causes increased synthesis of an acute-phase protein in the liver called C-reactive protein.

- Induction of metalloproteinase enzymes from neutrophils, fibroblasts and chondrocytes that result in resorption of cartilage and bone (Bertolini D R, Nedwin G E *et al*, 1986).
- Induction of VCAM-1, decay accelerating factor and complement receptor 2 on synovial fibroblasts. These are involved in sustaining B lymphocyte survival and therefore rheumatoid factor production within the synovium (Edwards J C W, Leigh R D *et al*, 1997).

Further evidence for TNF contributing to the signs and symptoms of rheumatoid arthritis comes from data of anti-TNF therapy in patients with rheumatoid arthritis. There are two anti-TNF agents at present that have been evaluated in clinical trials in patients with rheumatoid arthritis. The first is a chimeric monoclonal anti-TNF- α antibody called infliximab (Remicaide[®]) and the second a recombinant p75-TNF-receptor fusion protein called etanercept (Enbrel[®]) that acts as a form of soluble receptor that binds TNF and blocks its interaction with receptors on cells thereby interfering with the biological activity of TNF.

A two year phase III, double blind, randomised, placebo-controlled trial assessing infliximab (in 4 different dosing regimes, all with methotrexate) in patients with active rheumatoid arthritis showed that at 30 weeks approximately 60% of patients receiving infliximab showed a significant clinical response (ACR20) (Maini R N, St Clair *et al*, 1999).

A phase III placebo-controlled study over 6 months with entanercept alone showed a similar significant clinical response (ACR20) at 6 months, when entanercept was combined with methotrexate, a slightly higher proportion of patients, approximately 70% achieved a 20% ACR response (Moreland L W, Schiff M H *et al*, 1999; Weinblatt M E, Kremer J M *et al*, 1999).

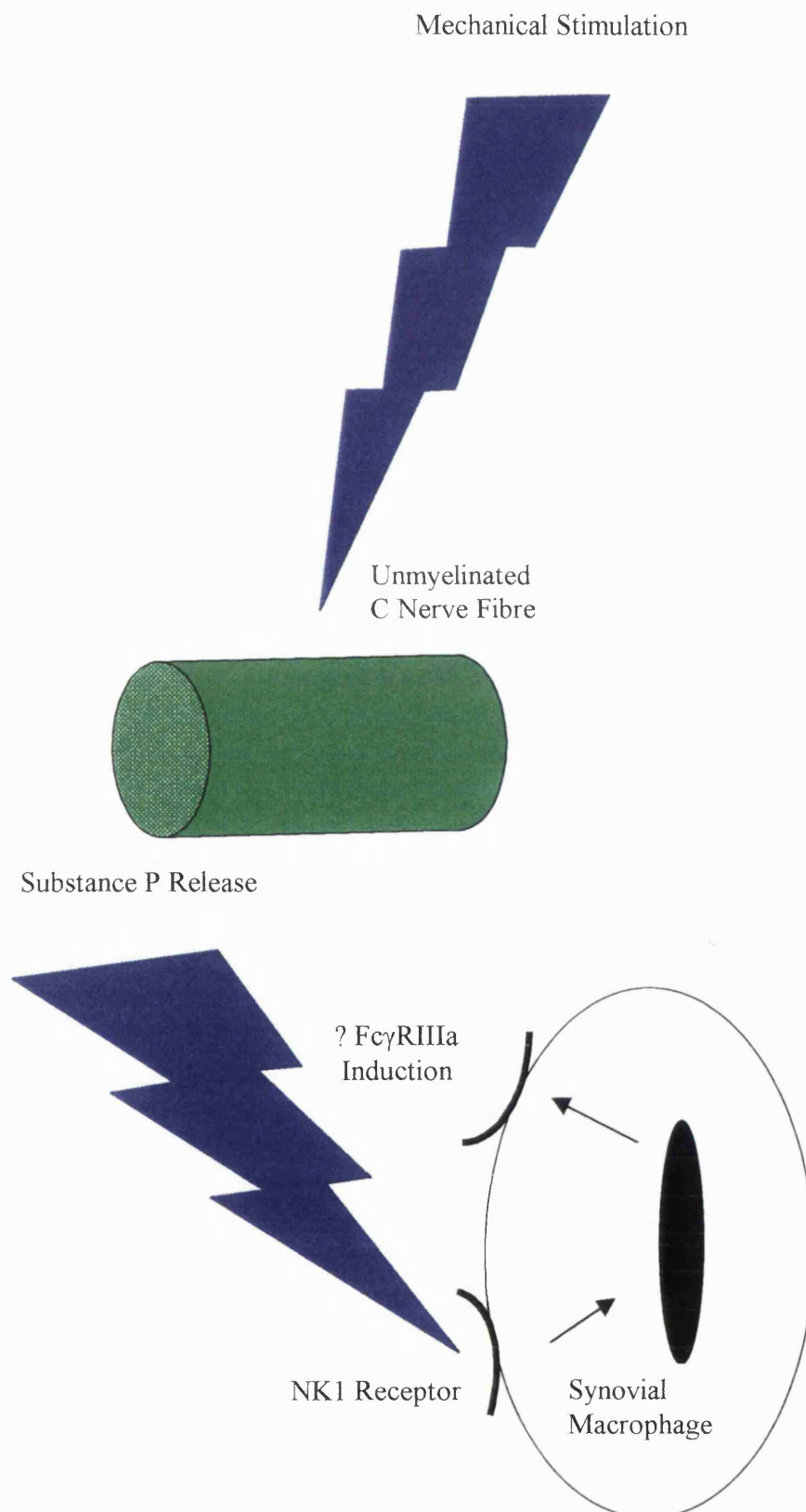
Efficacy of these anti-TNF therapies however appears to be transient with disease activity rising if treatment is discontinued.

Substance P

Substance P is a polypeptide of 11 amino acids and belongs to a family of peptides called the tachykinins (Chang M M, Leeman S E *et al*, 1971; Tregar G W, Niall H D *et al*, 1971). The cell bodies of unmyelinated C fibres that extend into the synovial intima synthesize substance P (Barry W, 1981; Mapp P I, Kidd B L *et al*, 1990). Mechanical stimulation causes substance P release (Kuraishi Y, Hirota N *et al*, 1989). Substance P binds to three receptors called neurokinin 1 (NK1), neurokinin 2 (NK2) and neurokinin 3 (NK3). Substance P has greatest affinity for NK1 (Regoli D, Nantel F *et al*, 1991) but at high concentrations may bind NK2 and NK3. Following peripheral stimulation of primary afferent neurons, substance P is released into the dorsal horn of the spinal cord. Substance P is considered to be instrumental in increased pain sensitivity (hyperalgesia and allodynia). Stimulation of afferent neurons also causes the peripheral release of neuropeptides. Intimal synoviocytes and endothelial cells in osteoarthritic synovium constitutively express the NK1 receptor (Sakai K, Matsuno H *et al*, 1998).

In vitro experiments reveal that Substance P stimulates macrophages to release prostaglandins and thromboxane. The dissociation constant of substance P with its receptor indicates that the physiological concentration of substance P is in the nanomolar range.

Figure 1.5: Substance P and the Putative Induction of FcγRIIIa on Tissue Monocyte-macrophages



DISEASE MODIFYING ANTI-RHEUMATIC DRUGS

Gold Salts

Gold salts have been used as an effective treatment in patients with rheumatoid arthritis since the 1930s. Relatively little is known about how gold salts alleviate rheumatoid disease.

During active chrysotherapy the presence of gold can be demonstrated within synovial intimal macrophages and in the subsynovial connective tissues. In addition to the synovium gold deposition occurs within the macrophages of many organs (liver, spleen, marrow, lymph nodes, adrenal glands, dermis, testes, ovaries, skeletal and cardiac muscle, the gastrointestinal tract, salivary glands, and lungs), renal tubular epithelium, and after recent chrysotherapy, in seminiferous tubules, hepatocytes and adrenal cortical cells (Vernon-Roberts B, Doré J L *et al*, 1976). Gold persists in the synovium for up to 23 years after the cessation of therapy.

In vitro gold salts impair monocyte-macrophage function. Littman B *et al*, (1984) have demonstrated the effect of gold sodium thiomalate on three functional correlates of human monocyte maturation outlined below.

- i. Human monocytes contain myeloperoxidase and this peroxidase activity is usually lost as monocytes mature. The loss of monocyte peroxidase activity was inhibited by gold salts.

- ii. Human monocytes do not spontaneously synthesize or secrete the second component of complement (C2), but as monocytes mature they synthesize C2. The addition of gold sodium thiomalate to monocyte cultures inhibited C2 synthesis.
- iii. Human monocytes acquire the ability to lyse chicken red blood cells after 4-5 days of incubation. This activity reaches a peak after 7 days of incubation. Gold sodium thiomalate inhibits spontaneous monocyte-mediated cytotoxicity for chicken erythrocytes.

The beneficial effect of gold salts in rheumatoid disease may result from its ability to impair the expression of FcγRIIIa on tissue monocyte-macrophages.

Gold salts have also been shown to reduce the circulating levels of IL-6 and monocyte derived cytokines such as IL-1, TNF and IL-6 in rheumatoid synovium (Barrera P, Boerbooms A M *et al*, 1996).

In addition to the above mechanism gold salts reduce immunoglobulin production from cultured lymphocytes. Gold salts may therefore reduce the amount of pathogenic rheumatoid factor thereby alleviating the symptoms of rheumatoid disease. (Coughlan R J, Richter M B *et al*, 1984).

Methotrexate

In controlled clinical trials methotrexate has been shown to be efficacious in the treatment of polyarthritis associated with rheumatoid disease (Weinblatt M E, Colblyn J S *et al*, 1985; Williams H J, Wilkens R F *et al*, 1985; Weinblatt M E, Weissman B N *et al*, 1992). The influence of methotrexate on the extra-articular manifestations of rheumatoid disease remains a matter of debate. Accelerated nodulosis occurs in approximately 8 percent of patients commenced on methotrexate for rheumatoid disease (Kerstens P J S M, Boerbooms A M T *et al*, 1992). The mode of action of methotrexate is poorly understood. *In vitro* experiments have proposed several possible mechanisms by which methotrexate may suppress rheumatoid arthritis. These are listed below:

- Methotrexate enhances the expression of IL-1 receptor antagonist and the soluble tumour necrosis factor receptor p75 from the monocytic U937 cell line (Seitz M, Zwicker M *et al*, 1998).
- Increased expression of IL-1 receptor antagonist and inhibition of IL-1 β synthesis from peripheral blood monocytes (Seitz M, Zwicker M *et al*, 1998).
- Inhibition of neutrophil chemotaxis (Cream J J, Pole D S, 1980).

The effects of methotrexate have been shown to be mediated by an increase in intra and extracellular adenosine (Seitz M, 1999). In stimulated neutrophils occupancy of adenosine receptors inhibits, the oxidative burst (Cronstein B N, Kramer S B *et al*,

1983; Cronstein B N, Levin R E *et al*, 1992), adhesion to endothelial cells (Cronstein B N, Levin R E *et al*, 1992), and production of leukotriene B₄ and TNF- α (Krump E, Lemay G *et al*, 1996; Thei M, Chouker A *et al*, 1995). In monocyte-macrophages, adenosine inhibits the release of TNF- α , IL-6 and IL-8 (Sajjadi F G, Takabayashi K *et al*, 1996; Bouma M G, Stad R K *et al*, 1994) and stimulates the transcription of IL-1 receptor antagonist and IL-10 (Cronstein B N, 1997; Le Moine O, Stordeur P *et al*, 1996).

A possible explanation for the exacerbation of rheumatoid nodules in some patients taking methotrexate might be explained by an up-regulation of Fc γ RIIIa on tissue macrophages. *In vitro* experiments demonstrate that methotrexate increases the expression of Fc γ RIIIa on monocytic U937 cells (Seitz M, Zwicker M *et al*, 1998). Methotrexate also stimulates the transcription of IL-10 (Le Moine O, Stordeur P *et al*, 1996). IL-10 selectively induces Fc γ RIIIa on cultured monocyte/macrophages (Calzada-Wack J C, Frankenberger M *et al*, 1996). Thus methotrexate may induce Fc γ RIIIa on monocytes/macrophages by stimulating IL-10 synthesis and release.

Although methotrexate effectively suppresses synovial inflammation, its ability to induce the extra-articular features of rheumatoid arthritis may be explained by the induction of Fc γ RIIIa on tissue monocytes/macrophages *in vivo*.

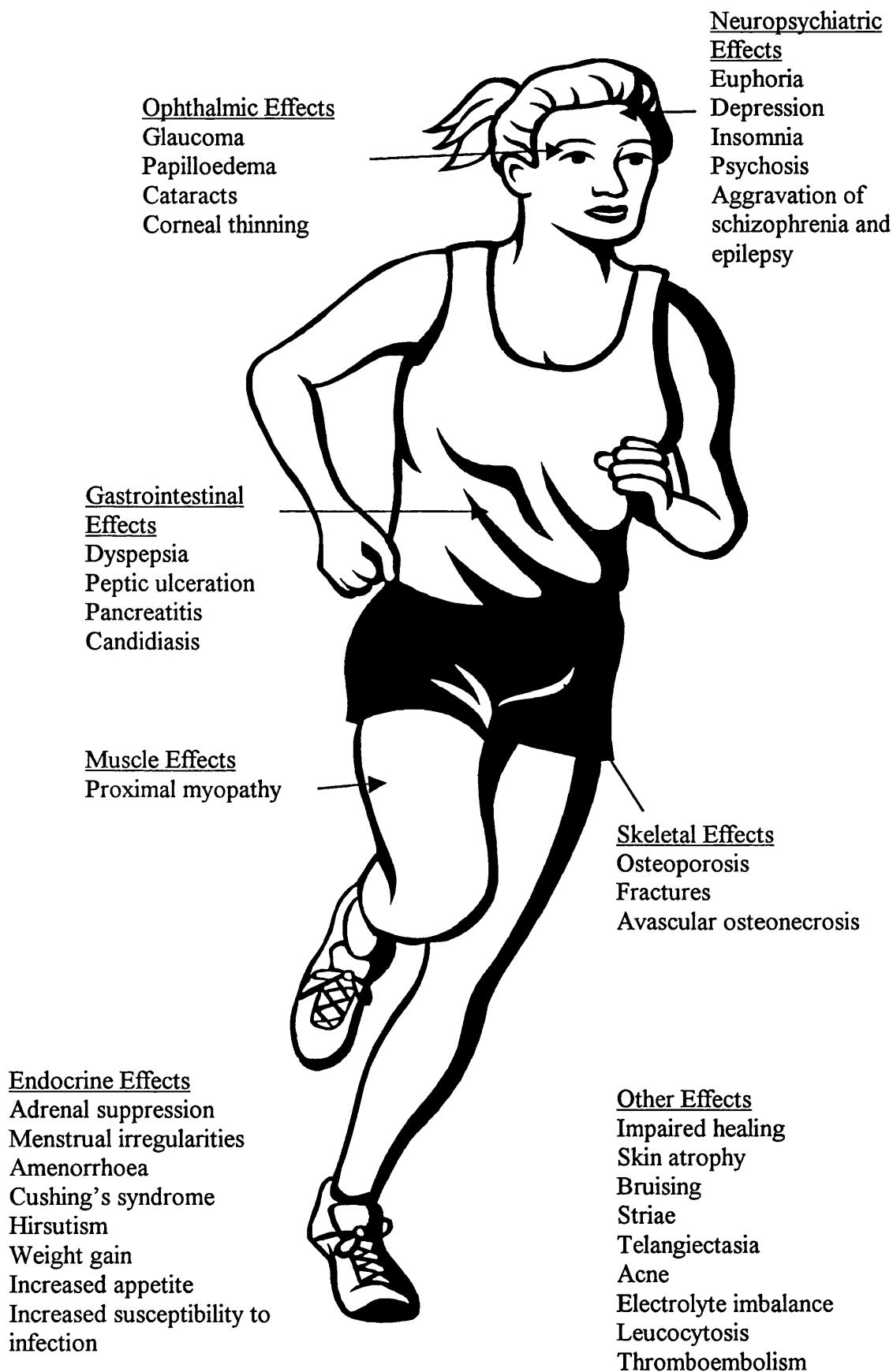
Anti-TNF Agents

See page 72

Glucocorticoids

A glucocorticoid in the form of cortisone was first used to treat a patient with rheumatoid arthritis in 1948 by Philip S Hench. Glucocorticoids have become a widely accepted form of treatment for patients with rheumatoid arthritis. Glucocorticoids have been shown to be beneficial both at low doses and at high intravenous doses (Kirwan J R, 1995; Youssef P P, Cormack J *et al*, 1996; Youssef P P, Haynes D R *et al*, 1997). Glucocorticoids however tend not to be used in individual patients for a long period of time because of the side effects associated with chronic steroid intake. These side effects are summarised in Figure 1.6.

Figure 1.6: Side Effects of Glucocorticoid Therapy §



§ (British national formulary 1999)

Glucocorticoids pass through cell membranes and bind glucocorticoid receptors within the cytoplasm of cells (Barnes P J, Adcock I, 1993). This steroid receptor complex then enters into the cell nucleus where it binds to specific DNA sites called glucocorticoid-responsive elements (Truss M, Beato M, 1993; Tsai M J, O'Malley B W 1994; Bamberger C M, Schulte H M *et al*, 1996; Onate S A, Tsai S Y *et al*, 1995). Transcription and translation of certain genes leads to increased production of target proteins. Transcription of other genes is inhibited by glucocorticoids.

Glucocorticoids increase the production of lipocortin 1 (annexin 1), which inhibits phospholipase A₂. Inhibition of phospholipase A₂ prevents the conversion of arachidonic acid to inflammatory mediators (Goulding N J, Guyre P M, 1993). Glucocorticoids inhibit the synthesis of IL-2, IL-6 and TNF- α (Buttgereit F, Brink I *et al*, 1995). In addition to this, the induction of inducible cyclooxygenase 2 (COX-2) is inhibited and the stability of COX-2 mRNA is reduced by glucocorticoids (Boumpas D T, 1996; Ristimäki A, Narko K *et al*, 1996). These effects further reduce the production of inflammatory mediators from arachidonic acid contributing to the anti-inflammatory effect of glucocorticoids.

The *in vitro* addition of dexamethasone to human osteoblast cells results in a dose-dependent activation of up to ninety percent of latent TGF β . Dexamethasone has no effect on TGF β mRNA or total protein production (Oursler M J, Riggs B L *et al*, 1993; Bodine P V, Riggs B L *et al*, 1995). The above mechanisms of glucocorticoids are referred to as the “genomic actions”.

Glucocorticoids can cause rapid effects that are insensitive to inhibitors of transcription and protein synthesis these are termed “nongenomic actions”. These actions are thought to be mediated via novel steroid-selective cell surface receptors. The relevance of these actions is unclear as the concentration of steroid required for these effects is not physiological or pharmacological (Moore F L, Orchinik M *et al*, 1994; Orchinik M, Murray T F *et al*, 1991; Moore F L, Orchinik M *et al*, 1995).

In addition to the genomic and nongenomic mechanisms of action, glucocorticoids also have nongenomic physiochemical actions. Clinically relevant concentrations of methylprednisolone inhibit calcium and sodium cycling across plasma membranes and decrease the intracellular free calcium concentration (Buttgereit F, Brand M D *et al*, 1993). Through this mechanism it is thought that high doses of glucocorticoids may interfere with processes that are essential for the activation and maintenance of lymphocytes (Buttgereit F, Wehling M *et al*, 1998).

The effect of glucocorticoids on FcγRIIIa expression on monocyte/macrophages is not known. TGFβ selectively induces FcγRIIIa on monocytes in vitro (Welch G R, Wong H L *et al*, 1990). Glucocorticoids may therefore be expected to induce FcγRIIIa as a consequence of their ability to activate latent TGFβ. The genomic effects of glucocorticoids will account for their anti-inflammatory properties.

THE SOURCE OF TNF- α IN RHEUMATOID JOINTS

TNF- α is increased in synovial fluid and synovial tissue of patients with rheumatoid arthritis (Saxne T, Palladino M *et al*, 1988). TNF- α is therefore very likely to be the key cytokine responsible for causing inflammation of the synovium in rheumatoid arthritis. Further evidence for TNF- α contributing to the signs and symptoms of rheumatoid arthritis come from data of anti-TNF therapy in patients with rheumatoid arthritis. A significant clinical response to anti-TNF therapy can be demonstrated in patients with rheumatoid arthritis (pages 74-75).

In the early 1980's it had been shown that immune complexes, including those derived from rheumatoid factor, could stimulate macrophages to produce cytokines capable of inducing extracellular matrix degradation (Nardella F A, Dayer J M *et al*, 1983). The mechanism by which immune complexes stimulated macrophages at the time was not clear although it was postulated that immune complexes may cause inflammation through their ability to activate the classic pathway of the complement system.

The reaction of IgM or IgG with an antigen causes a conformational change in the Fc region of the antibody to reveal a binding site for the first component of complement, C1q. C1q is composed of six subunits, resembling a 'bunch of tulips'. C1q reacts with Fc via its globular heads but attachment by two critically spaced binding sites is required for activation (Chapel H, Haeney M 1988). The Fc regions of pentameric IgM are so spaced that one IgM molecule can activate C1q. In

contrast IgG is relatively inefficient because the chance of two randomly sited IgG molecules being the critical distance apart to activate C1q is relatively low. Complement activation by self-associating IgG rheumatoid factors has been demonstrated (Mannik M, Nardella F A, 1985). Thus large immune complexes may be able to bind complement and contribute to the inflammatory response of rheumatoid arthritis. However, the complement receptor 1 (CR1) on red blood cells should clear large immune complexes.

Complement activation by IgG rheumatoid factor dimers or oligomers is ineffective (Brown P B, Nardella F A *et al*, 1982). These rheumatoid factor dimers tend not to be cleared and are small enough to be expected to cross over to the extravascular space where they may interact with tissue macrophages.

Approximately 80% of patients with rheumatoid arthritis have circulating IgG rheumatoid factors (Carson D A, 1993). Those patients without circulating rheumatoid factors often have rheumatoid factors in the joints. In 1974 Pope *et al* discovered the presence of rheumatoid factor dimers in the serum of a patient with rheumatoid arthritis. He proposed that two IgG rheumatoid factors form a dimer, whereby one antibody-combining site of each molecule reacts with the Fc region of the other molecule. In this model the other antibody combining site and antigenic determinant on the Fc region of each IgG rheumatoid factor molecule remain free.

In rheumatoid synovium rheumatoid factors are produced by B-lymphocytes and plasma cells (Kobayashi I, Ziff M, 1973; Harris E D Jr., 1990; Natvig J B, Randen I *et al*, 1989; Smiley J D, Sachs C *et al*, 1968; Bankhurst A D, Husby G *et al*, 1976).

Work by Fanger, Lydyard, Huizinga, van de Winkel and others led to a clear understanding of the structure and function of IgG Fc receptors. An interaction between IgG rheumatoid factor and Fc receptors being responsible for the source of TNF- α in joints seemed plausible. Synovial intimal, serosal, alveolar, salivary gland and placental macrophages, Kupffer cells, and macrophages in mechanically stressed dermis express high levels of the receptor Fc γ RIIIa (see appendix 4).

The ability of small IgG rheumatoid factor complexes to interact with Fc γ RIIIa and induce cell signalling with the production of TNF- α was first suggested by studies on natural killer cells which only express this IgG Fc receptor (Hendrich C, Kuipers J G *et al*, 1991). Moreover recently it has been shown that the cross-linking of two or three Fc γ RIIIa receptors with an anti-Fc γ RIIIa monoclonal antibody is sufficient to trigger macrophages to release TNF- α , IL-1 α and reactive oxygen species. (Abrahams V M, Cambridge G *et al*, 2000; Trezzini C, Jungi T W *et al*, 1990). Synovial inflammation in rheumatoid arthritis may therefore be initiated by small IgG rheumatoid factor complexes binding to Fc γ RIIIa on intimal macrophages causing local release of inflammatory mediators that result in tissue inflammation.

In summary self-associating dimeric complexes of IgG rheumatoid factor are small enough to evade clearance by complement receptors and to pass through vessel

walls to the extravascular space. These complexes may then activate macrophages bearing the receptor Fc γ RIIIa. As a consequence of this TNF- α may be released which will result in inflammation. In the synovium, TNF- α also induces changes in fibroblasts which facilitate the local accumulation of ectopic lymphoid tissue and plasma cells, with the generation of high local levels of rheumatoid factor and the formation of larger immune complex aggregates which induce further inflammatory events, including complement activation.

The involvement of T lymphocytes in rheumatoid arthritis has been suggested by the presence of T cell infiltrates in the synovium and by the genetic association with HLA-DR1 and DR4 haplotypes (Panayi G S, Lanchbury J S *et al*, 1992; Alcaro G S, 1995). In spite of intensive efforts, T cells responsive to a single antigen, or restricted populations of T cells, have not been identified in rheumatoid synovium. T cells in rheumatoid synovium are hyporesponsive (Zvaifler N J, Firestein G S *et al*, 1998; Breedveld F C, Verweij C L *et al*, 1997). Clinical trials with various anti-T cell monoclonal antibodies have failed to lead to a sustained suppression of disease activity.

The mechanisms governing the survival of rheumatoid factor committed B lymphocytes may differ from those for other autoreactive B lymphocytes (Kyburz D, Corr M *et al*, 1999). B lymphocytes producing rheumatoid factor may obtain 'bypass' help from T lymphocytes recognizing foreign antigens (Roosnek E, Lanzavecchia A, 1991). T lymphocytes in synovium may therefore be responding to

foreign antigen and provide 'bypass' help to B lymphocytes. This 'bypass' T cell help may perpetuate rheumatoid factor producing B lymphocytes.

HYPOTHESIS

Hypothesis

Binding of small immune complexes to Fc γ RIIIa on macrophages with release of proinflammatory cytokines would explain inflammation in rheumatoid disease, but only if the distribution of Fc γ RIIIa proved to be consistent with the clinical picture. Therefore, the first hypothesis was that Fc γ RIIIa would solely be expressed in tissues targeted by rheumatoid arthritis.

The second hypothesis was that the immunological microenvironment of normal synovium may be responsible for inducing Fc γ RIIIa on synovial monocyte-macrophages. The factor or factors responsible for inducing Fc γ RIIIa *in vivo* are uncertain. With a detailed knowledge of the local tissue environment surrounding intimal monocyte-macrophages, clinical observations of rheumatoid disease, and published work on the induction of Fc γ RIIIa, it is possible to contemplate what factors may be responsible for the induction of Fc γ RIIIa *in vivo*. Based on current information it is possible to list factors that may be responsible for the induction of Fc γ RIIIa on intimal monocyte-macrophages *in vivo*. These factors are listed below with references to pages where they are explained in more detail.

- The cytokine TGF β ₁ (page 57)
- Mechanical stretch either directly or indirectly as a consequence of TGF β ₁ synthesis or release. (page 57)
- The cytokine interleukin-10 (page 64)
- Growth factors such as GM-CSF or M-CSF (page 67)

- Neuropeptides such as Substance P (page 76)
- Extracellular matrix proteins-: (page 46)
 - Fibrillin-1 microfibrils
 - Collagen IV
 - Laminin
 - Fibronectin

Synovial fluid is in close contact with synovial intimal macrophages. It is therefore possible that a substance found in normal synovial fluid is responsible for inducing Fc γ RIIIa on synovial macrophages. Below is a list of possible factors in synovial fluid that may be responsible for the induction of Fc γ RIIIa.

- Cytokines in particular IL-10 and TGF β (see page 57 and 64 for more information).
- Extracellular matrix proteins synthesized and released into the synovial fluid by intimal fibroblasts (see page 46 for more information).
- Hyaluronan

Experiments on cultured blood monocytes with the above factors were undertaken to investigate which might be responsible for the induction of Fc γ RIIIa *in vivo*.

AIMS

Aims

- To compare the expression of FcγRIIIa in a wide range of normal tissues in a semiquantitative fashion and to analyze the relationship between FcγRIIIa, DAF and specific matrix elements.
- To investigate the factors responsible for the induction of FcγRIIIa in tissue monocyte-macrophages.

The role of cytokines (TGFβ₁, IL-10, M-CSF and GM-CSF), substance P, mechanical stretch, synovial fluid, hyaluronan, matrix proteins (laminin, collagen IV and fibronectin) and conditioned medium from synovial fibroblasts were studied. The effect of hydrocortisone, sodium aurothiomalate and methotrexate on FcγRIIIa expression was also examined.

METHODS

TISSUES

Normal human tissues were obtained from surgical procedures performed at University College London Hospitals, not relating to pathology of the tissue obtained, and post-mortem (Tissue Bank, Addenbrookes Hospital, Cambridge). Normality was confirmed histologically. Tissue samples were embedded in OCT compound, snap frozen in *n*-hexane and stored at -80° C. Two specimens were examined of each of the following tissues except where numbers are indicated in brackets: synovium (5), lung, intestine, spleen, breast, myocardium, pericardium, brain, prostate, kidney, uterus, thyroid, liver, spinal disc and entheses, placenta, umbilical cord, flexor forearm skin, skin exposed to mechanical stress; at the elbow (1) and over a toe interphalangeal joint (1), fingernail (1), salivary gland (1), gall bladder (1), tonsil (4), bone marrow aspirate smear (1) and skeletal muscle (5).

IMMUNOHISTOCHEMISTRY

Tissues were sectioned to a thickness of 5 μm at -25°C , taken up onto slides, air dried for 1 hour and fixed with cold acetone for 10 minutes. Between all further steps tissues were washed in two changes of phosphate-buffered saline (PBS) for 5 minutes each.

Serial sections of each tissue were incubated with either a mouse monoclonal IgG1 anti-human pan-Fc γ RIII at 25 $\mu\text{g/ml}$ (in duplicate), a mouse monoclonal IgM anti-human DAF at 14 $\mu\text{g/ml}$ or a mouse monoclonal IgG1 anti-human CD68 at 10 $\mu\text{g/ml}$. Synovial sections were also incubated with a purified mouse monoclonal IgG1 anti-human Fc γ RI at 15 $\mu\text{g/ml}$ and a purified mouse monoclonal IgG2b anti-human Fc γ RII at 12 $\mu\text{g/ml}$. All antibodies were diluted in PBS and incubated for 60 minutes at room temperature. Controls were incubated in PBS. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide in methanol. Sections were then incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins at 40 $\mu\text{g/ml}$ for 30 min followed by diaminobenzidine hydrochloride for 10 minutes, washed in tap water, counterstained in Harris's haematoxylin (except for one of each pair stained for Fc γ RIIIa) dehydrated in alcohol, cleared in Histoclear and mounted in DepeX.

Uncounterstained sections stained for Fc γ RIIIa were analysed by microdensitometry.

MICRODENSITOMETRY

Semiquantitative analysis of Fc γ RIIIa expression was performed on sections stained with the immunoperoxidase technique. As far as possible two specimens for each tissue were analysed microdensitometrically. Sections were stained using supraoptimal concentrations of primary antibody and rigidly standardized incubation times. Analysis was performed using an M85 Vickers scanning microdensitometer. Absorption was measured at 550 nm with a mask diameter corresponding to a 5 μ m section. Absorption was measured as a relative extinction, in arbitrary units, using unstained areas of the section as reference. Measurements were taken over the cytoplasm of macrophages showing maximum staining in each of 10 randomly selected high-power fields. Mean readings and standard errors were obtained for each sample. Measurements from each staining batch were normalized by defining 1 unit as the value obtained for macrophages in a normal synovial intima included in each batch.

MONOCYTE PREPARATION

To isolate peripheral white blood cells, human venous blood was obtained from normal donors. Blood was collected in Fenwal[®] single blood pack units containing a citrate anticoagulant solution.

Buffy coat was obtained by placing whole blood onto a solution of 5% Dextran and 0.9% sodium chloride in a ratio of blood to dextran of 10:1. Fifteen millilitres of buffy coat was then carefully layered onto eight mls of Ficoll-Hypaque[®] (density 1.077) in 20 millilitre polystyrene universals. Fourteen universals prepared in this way were then centrifuged at 400g at 21⁰C for thirty minutes. After centrifugation the band containing monocytes and lymphocytes was aspirated with a Pasteur pipette and suspended in RPMI to a total volume of 20 millilitres. This suspension was then centrifuged at 400g at 21⁰C for 10 minutes. The resulting supernatant was aspirated and discarded. The pellet of white blood cells was then resuspended in RPMI. Three further washes as above were performed at 175g and 21⁰C for 10 minutes. The final white blood cell pellets were resuspended in 20-30 millilitres of RPMI supplemented with 10% of fetal calf serum, 1% glutamine, 1% Penicillin and Streptomycin, 0.2% Gentamicin, 2% non-essential amino acids and 1% pyruvate and placed in polystyrene six well plates and incubated at 37⁰C for two hours. The plates were washed six times with RPMI to remove non-adherent cells.

Following this fresh growth media was added to the adherent monocytes. The monocytes were then removed using a rubber policeman. The concentration of monocytes was adjusted to 1 million cells/ml. These monocytes were then cultured under different conditions to investigate the factors responsible for the induction of FcγRIIIa.

MECHANICAL STRETCHING OF MONOCYTES

Human peripheral blood monocytes in RPMI supplemented with 10% fetal calf serum, 1% glutamine, 1% penicillin, 1% streptomycin, 0.2% gentamicin, 2% non-essential amino acids and 1% pyruvate were seeded onto flexible bottomed Flex I[®] six well culture plates at a concentration of 1×10^6 cells per millilitre. The monocytes were then incubated at 37°C overnight in a humidified incubator with 5% carbon dioxide.

Non-adherent cells were then removed by washing with RPMI and fresh growth medium was placed into each well. Cells adhered to the Flex I[®] plates are difficult to see unless the refractive index of the air/silastic interface is modified. Therefore monocytes were viewed by placing the Flex I[®] six well plate in the lid of a standard 35 mm 6 well culture plate filled with water, the water comes into contact with the bottom of the silastic membrane. This procedure of viewing cells corrects the refractive index changes in the silastic membrane of the Flex I[®] culture plates and permits clear viewing of cells with a standard inverted cell culture light microscope (x10 magnification). Flex I[®] plates treated with type I collagen and fibronectin were used.

The Flex I[®] flexible bottomed culture plates were cyclically stretched by applying a negative pressure to the substratum of the plates. This causes a downward deformation of the silastic membranes of the plate. Cells adherent to the silastic membrane are therefore stretched. The amount of elongation an adherent cell

experiences depends on its location on the substrate and can be calculated mathematically. Stretch is greatest at the periphery of each well in the plate and is least at the centre of each well.

Using a custom made electrical unit designed and made by Dr. Warren Williams (1997) a negative pressure at the substratum of the Flex I[®] plate was generated. Withdrawing the plunger of a 10-millilitre syringe generated this negative pressure. The negative pressure was then transduced to the substratum of the Flex I[®] plate via a 3 mm polypropylene tube connected to a 45 mm luer-lock cannula. The tip of the cannula was placed at the substratum of the Flex I[®] plate. In order to create an airtight seal at the substratum, the plate was seated on a foam bed that was sealed at the edge with petroleum jelly. The six well plate was secured on the foam bed by elasticated bands. Stretching of cells took place in a humidified incubator at 37⁰C with 5% carbon dioxide.

One cycle of stretch and return of the silastic membrane to an unstretched form took 3 minutes. Controlling the level of negative pressure at the substratum regulated the degree of cell stretch. Setting of a time switch enabled the duration to which cells were exposed to stretch to be controlled. Thus both the degree of stretch and the duration of stretch were regulated. Cells placed in Flex I[®] plates that were not stretched served as controls.

When cells had been stretched for the required length of time the stretching machine was switched off. EDTA at a concentration of 3 mM was used to remove

monocytes from the Flex I[®] plates. The monocytes were then washed and their concentration adjusted to 1×10^6 / ml. These cells were stained for Fc γ RIIIa expression and subjected to FACS analysis.

ASSESSMENT OF FcγRIIIa EXPRESSION ON CULTURED MONOCYTE-MACROPHAGES

Cultured adherent monocytes were removed using a rubber policeman. Using a haematocytometer the concentration of monocytes was adjusted to 1 million cells per millilitre. 100 µl of monocytes at 1×10^6 cells/ml were placed in a single well of a 96 round bottom well plate at 4°C. The monocytes were washed with a 150 µl of 1% bovine serum albumin (BSA) and 0.1% sodium azide in PBS. The 96 well plate was centrifuged at 300 g for 5 minutes at 4°C. The resultant supernatant was aspirated using a glass Pasteur pipette. The monocytes were incubated in duplicate with a mouse IgG₁ monoclonal anti-FcγRIII antibody (3G8 clone), at a concentration of 2 µg/ml for 45 minutes and a non reactive mouse IgG₁ antibody at a concentration of 2 µg/ml that served as an isotype control. At the end of the incubation the monocytes were washed twice with 150 µl of 1% BSA and 0.1% sodium azide in PBS as above.

The monocytes were then incubated for a further 45 minutes at 4°C with a FITC-conjugated F (ab')₂ fragment of goat anti-mouse immunoglobulins at a concentration of 0.05 g/l. All antibodies were diluted to the required concentration in 1% BSA and 0.1% sodium azide. Duplicate wells of monocytes were also incubated with a FITC-conjugated monoclonal IgG2a mouse anti-human CD14 (clone TÜK4) at a concentration of 100 µg/ml and a non reactive FITC-conjugated mouse IgG2a at a concentration of 100 µg/ml for 45 minutes at 4°C, these served as a positive and isotype negative control respectively. All incubations with FITC-conjugated

reagents were performed in the dark. At the end of the incubation the monocytes were washed twice with 1% BSA and 0.1% sodium azide.

The monocytes were then fixed in 250 μ l of 2% paraformaldehyde for 20 minutes before being analysed on the flow cytometer.

Data is presented as the mean fluorescence intensity of Fc γ RIIIa or the percentage change of the mean fluorescence intensity of Fc γ RIIIa. Where possible data is analysed using the Student's *t* test (Swinscow T D, Statistics, BMJ publishing 1983, 36-38) to give p values.

FLOW CYTOMETRIC ANALYSIS

Analysis was performed on a Becton-Dickinson FACScan equipped with an argon ion laser emitting 15 mV at 488 nm and capable of detection at 530 nm (FITC). The FACScan is supported by a Mint computer (model Pentium 200 MMX) for data acquisition and analysis with a commercial computer software package WinFCM (Applied Cytometer Systems UK). Monocytes were selected on the basis of forward angle (FSC) and side angle (SSC). Data was collected for 10000-20000 events of interest.

Using WinMDI (version 2.7 1998 J. Trotter) the monocytes were analyzed. Data was expressed as the mean fluorescence intensity of 10000 to 200000 cells.

FIBROBLAST ISOLATION AND CELL CULTURE

Synovial tissue was taken from patients with osteoarthritis undergoing hip replacement surgery. The synovial tissue was dissected from the joint and washed in 0.1% bovine serum albumin in Dulbecco's modified Eagles medium (DMEM). The tissue was chopped into small pieces (1mm³) and washed again. The tissue was then digested in serum free DMEM with 2mg/ml collagenase and 0.2mg/ml deoxyribonuclease I in a shaking waterbath at 37°C for 1 hour. The resulting suspension was then passed through a fine nylon sieve and resuspended in DMEM containing 10% fetal calf serum and centrifuged at 80g for 5 minutes. The resulting cell pellet was resuspended in DMEM with 10% fetal calf serum and seeded out in 75 cm² Falcon flasks. The isolated fibroblasts were cultured in a humidified 5% Carbon Dioxide atmosphere at 37°C overnight. To remove non-adherent cells the flasks were washed 3 times with DMEM supplemented with 10% fetal calf serum. Fresh DMEM supplemented with 10% fetal calf serum, 1% glutamine, 1% Penicillin and Streptomycin, 0.2% Gentamicin, 2% non-essential amino acids and 1% pyruvate was added. Media was changed twice a week. When the fibroblast cultures were confluent and between passages 3-6 the supernatant or conditioned fibroblast media was removed and stored at -80°C. At confluence, the cells were trypsinized, split in a 1:3 ratio and recultured.

CONCENTRATION OF CONDITIONED FIBROBLAST MEDIUM BY MOECULAR WEIGHT

Concentration of conditioned fibroblast medium according to molecular weight was achieved by using Centricon® centrifugal filter devices. Two millilitres of fibroblast-conditioned medium was inserted into the sample reservoir of the centrifugal filter device. The device was then covered with the retentate vial and placed into a counterbalanced centrifuge. Centrifugal filter devices with the ability to separate medium into fractions more than and less than 100 kDa, 3 kDa, and 30 kDa were used. These devices were spun at 1000 g for 30 minutes, 7500 g for 2 hours and 5000 g for 1 hour respectively.

After centrifugation the device was removed. The filtrate contained fractions of a lower molecular weight than the retentate. The filtrate was stored and placing the retentate vial over the sample reservoir, inverting the unit, and centrifuging the device for 2 minutes at 300 g achieved recovery of the retentate. The recovered retentate was made up to the volume of the original sample.

The retentate and filtrate were then used as culture medium for freshly isolated adherent monocytes in six well polystyrene plates. The monocytes were cultured for 36 to 46 hours before being removed and analysed for FcγRIIIa expression. Unfractionated conditioned fibroblast medium, unfractionated normal growth medium and fractionated normal growth medium were also used for monocytes cultures in these experiments to serve as controls and help in the interpretation of the results.

TREATMENT OF CONDITIONED FIBROBLAST MEDIUM WITH PROTEASE

Two hundred micrograms of protease was added to one millilitre of conditioned fibroblast medium and left for 8 hours at 37°C, a further 100 µg of protease was added to the conditioned fibroblast medium and left at 37°C overnight.

This protease treated fibroblast-conditioned medium was used as a culture medium for freshly isolated adherent monocytes. Fibroblast conditioned medium and normal growth medium not treated with protease and normal growth medium treated with protease in the same method as outlined above was also used as culture medium for adherent monocytes and served as controls. Monocytes were cultured for 36 to 46 hours in a humidified incubator at 37°C with 5% carbon dioxide. Monocytes were then removed and analysed for FcγRIIIa expression.

SUMMARY OF RESULTS

Fc γ RIIIa Expression on Monocytes-Macrophages

- Fc γ RIIIa is present on macrophages only in normal human tissues targeted by rheumatoid arthritis (pages 115-130).
- Approximately 5 percent of peripheral blood monocytes express Fc γ RIIIa (pages 131-139).
- Fc γ RIIIa expression increases on cultured adherent monocytes over time and plateaus after 3 days. Non-adherent cultured monocytes express less Fc γ RIIIa over the same duration of time (pages 131-139).

The Effect of Cytokines, Matrix Proteins and Substance P on Fc γ RIIIa Expression

- TGF β ₁ suppresses the expression of Fc γ RIIIa on cultured adherent and non-adherent monocytes (pages 139-148)
- IL-10 induces the expression of Fc γ RIIIa on cultured adherent monocytes (pages 148-152)
- Fc γ RIIIa expression is similar on monocytes cultured on laminin, polystyrene, collagen I, collagen IV and fibronectin (pages 167-173)

- The effect of TGF β_1 and IL-10 on Fc γ RIIIa expression is independent of the substrate to which monocytes are adhered (pages 167-173).
- Substance P induces the expression of Fc γ RIIIa on cultured adherent monocytes but not on non-adherent monocytes (pages 152-158).
- Neither GM-CSF nor M-CSF induced Fc γ RIIIa expression on cultured monocytes (pages 158-167)

Synovial Fluid and Fc γ RIIIa Expression

- Synovial fluid monocytes express similar levels of Fc γ RIIIa compared to monocytes in peripheral blood (pages 192-200).
- The addition of osteoarthritic synovial fluid to cultured adherent monocytes did not increase Fc γ RIIIa expression compared to monocytes cultured in the absence of synovial fluid (pages 192-200).
- The addition of hyaluronan to monocytes *in vitro* did not result in a greater elevation of Fc γ RIIIa compared to monocyte cultures without hyaluronan. (pages 192-200).

Mechanical Stretch and FcγRIIIa Expression

- Cultured monocytes subjected to mechanical stretch did not express more FcγRIIIa than non-stretched monocytes (pages 173-1180).
- Conditioned medium from non-stretched fibroblasts increased the level of FcγRIIIa expression on cultured monocytes (pages 200-217).
- The increased expression of FcγRIIIa on monocytes cultured with conditioned fibroblast medium is partially aborogated by neutralising antibodies to IL-10 (pages201-217).
- Conditioned medium from non-stretched fibroblasts contains a soluble acid labile, heat stable protein with a molecular weight of more than 100 kD that induces the expression of FcγRIIIa on monocytes *in vitro* (pages 200-217).

The Effect ofDisease Modifying Anti-Rheumatic Drugs on FcγRIIIa Expression

- Methotrexate increased the expression of FcγRIIIa on cultured monocytes (pages 180-192).
- The expression of FcγRIIIa on cultured monocytes is reduced by sodium aurothiomalate (pages 180-192).
- Hydrocortisone [10^{-6} M] appears to be cytotoxic to monocytes (pages 180-192)

RESULTS

EXPERIMENT 1: THE DIFFERENTIAL DISTRIBUTION OF FcγRIIIa IN NORMAL HUMAN TISSUES AND CO-LOCALISATION WITH DAF AND FIBRILLIN-1

Introduction

FcγRIIIa is an IgG Fc receptor found on large granular lymphocytes, natural killer cells and macrophages. The addition of anti-FcγRIII monoclonal antibodies to cultured monocytes expressing FcγRIIIa results in the release of TNF-α, IL-1 and reactive oxygen species (Abraham's V M, Cambrigde G *et al*, 2000; Trezzini C, Jungi T W *et al*, 1991). Small immune complexes (IgG rheumatoid factor dimers) are present in sera and synovial fluid of patients with rheumatoid arthritis. These complexes can be predicted to evade complement-mediated clearance and pass through vessel walls. Small immune complex binding to FcγRIIIa on tissue macrophages could result in the release of proinflammatory cytokines that may contribute to inflammation in rheumatoid disease.

FcγRIIIa has been found on macrophages in normal adult and fetal synovium in the intimal region (Edwards J C W, Blades S *et al*, 1997). FcγRIIIa is also present on dermal macrophages that have been exposed to mechanical stretch (Edwards J C W, Blades S *et al*, 1997), Kupffer cells and alveolar macrophages (Bordessole D, Jones M *et al*, 1993; Tuijnam W B, Van-Wichen D F *et al*, 1993). Data on the distribution of FcγRIIIa on macrophages in other normal tissues is not available.

The aim of this experiment was to investigate FcγRIIIa expression on macrophages in a complete range of normal human tissues.

Methods

Normal human tissues were obtained as described on page 97. FcγRIIIa expression was identified using immunochemical techniques (page 98). A scanning and integrating microdensitometer was used to make a semiquantitative analysis of FcγRIIIa expression (page 99).

Cellular Staining of FcγRIIIa

Measurable levels of staining for FcγRIIIa were present on synovial intimal, and to a lesser degree subintimal, macrophages, alveolar macrophages, pericardial macrophages, Kupffer cells, macrophages in mechanically stressed dermis, salivary gland macrophages, placental Hofbauer cells (macrophages), and monocytes in umbilical cord vessels (Table 3.1).

Table 3.1, shows the semiquantitative assessment of macrophage FcγRIIIa expression in normal tissues.

Table 3.1: Semiquantitative Assessment of Macrophage FcγRIII Expression in Normal Tissues

Tissues	Macrophage Population	FcγRIIIa Index*
Synovium	Intimal macrophages	1
		1.28 ± 0.09*
		0.72 ± 0.04*
	Subintimal macrophages	0.38 ± 0.02
		0.35 ± 0.04
Lung	Alveolar macrophages	0.99 ± 0.10
		0.99 ± 0.08
Pericardium	Macrophages	1.03 ± 0.07
		0.92 ± 0.08
Liver	Kupffer cells	1.50 ± 0.09
		0.42 ± 0.04
Stressed dermis (elbow)	Macrophages	0.68 ± 0.07
Stressed dermis (toe)		0.27 ± 0.07
Salivary gland	Macrophages	0.43 ± 0.05
Placenta	Hofbauer cells	0.39 ± 0.04
		0.39 ± 0.03
Umbilical cord	Intravascular monocytes	0.86 ± 0.04
Bone marrow	Macrophages/precursors	Not measured†
Skeletal muscle	Macrophages	Not measured
Spleen/tonsil	Macrophage subsets	Not measured
Breast	Macrophages	Undetectable
		Undetectable
Brain	Microglia	Undetectable
		Undetectable
Thyroid	Macrophages	Undetectable
		Undetectable
Spinal disc/enthesis	Macrophages	Undetectable
		Undetectable
Intestine	Macrophages	Undetectable
		Undetectable
Myocardium	Macrophages	Undetectable
		Undetectable
Prostate	Macrophages	Undetectable
		Undetectable
Flexor forearm dermis	Macrophages	Undetectable
		Undetectable
Uterus	Macrophages	Undetectable
		Undetectable
Kidney	Macrophages/ Mesangial cells	Undetectable
		Undetectable
Gallbladder	Macrophages	Undetectable
Nailbed	Macrophages	Undetectable
Unstressed dermis	Macrophages	Undetectable
		Undetectable

* Mean relative extinction for immunoperoxidase staining for FcγRIIIa normalized to synovial intima = 1, ± standard error. Comparative values for two synovial samples given in parentheses.

† See text, index not derived for technical reasons.

Macrophage FcγRIIIa staining was observed in muscle, bone marrow and lymphoid tissue, but was not measured for technical reasons. In other tissues, FcγRIIIa staining was not seen on macrophages despite their presence, as indicated by CD68 staining.

In muscle very few FcγRIIIa⁺ cells were found in comparisons to the number of CD68⁺ cells present. The level of FcγRIIIa staining on this minority of macrophages was significant, but the etiolated profile of the cells precluded reliable measurement using the standard mask. Large FcγRIIIa⁺ mononuclear cells consistent with macrophages were seen in bone marrow and some samples of tonsil and spleen white pulp. However, it was not possible to be certain of the identity and state of maturation of these cells. In other samples of tonsil virtually no staining was seen than on scattered granulocytes.

Extracellular staining of FcγRIII

Extracellular staining for FcγRIII was restricted to synovial intima, mesothelia, skeletal and cardiac perimysium, uterine arterial internal elastic lamina and dermis at sites of mechanical stress. In synovial intima FcγRIII staining was chiefly cellular and pericellular, and less often on isolated fibrillar structures. Pericellular FcγRIII and DAF frequently co-localized around the same cells in a pattern which matched closely to that of fibrillin-1. Extracellular DAF staining was not confined to sites of fibrillin-1 but showed some apparent overlap with pericellular staining for laminin.

Niether DAF nor Fc γ RIII staining co-localized with collagen VI-containing fibrillar structures or with collagen IV, which did not show a pericellular or fibrillar pattern.

In pericardium, and on the peritoneal surface of spleen, linear staining for both Fc γ RIII and DAF was observed. For pericardium, Fc γ RIII and DAF staining was discontinuous with both brightly stained and unstained areas. Linear staining co-localized with fibrillin-1 and not collagen VI. A similar pattern of staining was seen on scattered fibrous septae between cardiac myocytes.

Linear staining for both Fc γ RIII and DAF was observed on skeletal muscle epimysium. Staining patterns for the two molecules demonstrated major overlap and followed the pattern of fibrillin-1 based microfibrils, which formed a discontinuous perimyseal network. Collagen-VI-based microfibrils formed a continuous perimyseal envelope, which, although closely apposed to fibrillin-1-based microfibrils, did not match Fc γ RIII and DAF staining. CD68 staining indicated that only a fraction of epimysial staining for Fc γ RIII and DAF could be accounted for by macrophage cytoplasm/membranes.

A proportion of small to medium-sized uterine arteries showed Fc γ RIII staining of the internal elastic lamina, but vessels in other tissues did not stain.

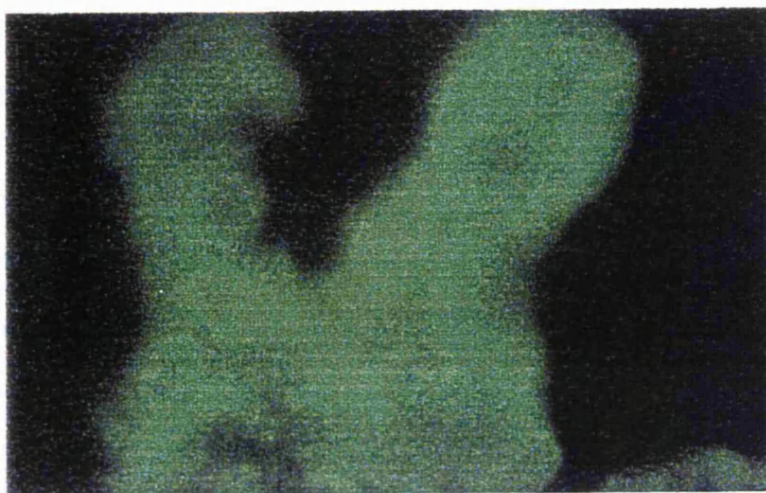
Extracellular matrix-associated Fc γ RIII was assumed to represent Fc γ RIIIa, being associated with Fc γ RIII⁺ macrophages and not granulocytes. In muscle, although Fc γ RIII⁺ macrophages were scarce, CD68 staining occasionally revealed

macrophages with cytoplasm extension of similar extent to a stretch of microfibril-associated Fc γ RIII staining suggesting that Fc γ RIIIa may be deposited by transient macrophage filopodia. In one sample of dermis exposed to mechanical stress granulocytes were present, raising the possibility that fibrillar staining was due to Fc γ RIIIb in this case.

In keeping with previous reports (Medof M E, Walter E I *et al*, 1987), extracellular DAF staining was observed in many tissues, but with major variation in intensity. A proportion of DAF staining precisely co-localized with a subset of fibrillin-1-containing fibrillar elements, including large elastic fibres in tissue stroma but not vascular internal elastic lamina. DAF was also present in epithelial basal laminae. DAF did not co-localize with collagen VI.

Figure 3.1a & b: Synovial intimal villus stained for FcγRIII and DAF showing co-localization of pericellular staining; final magnification x400

a.



b.

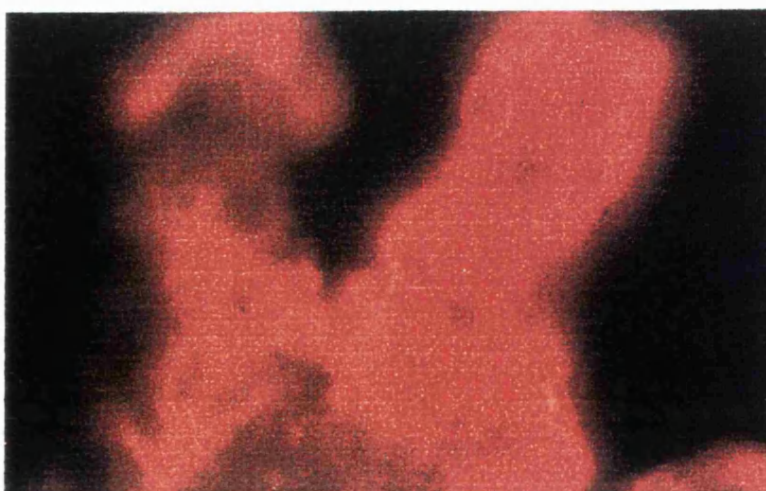
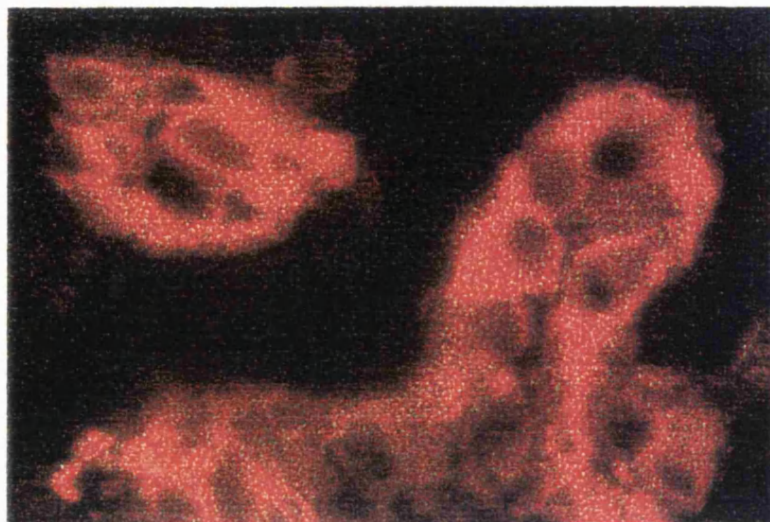


Figure 3.1c & d: Synovial intimal villus stained for DAF and fibrillin-1, both pericellular (middle left) and fibrillar (top right) staining patterns co-localize; final magnification x400

c.



d.

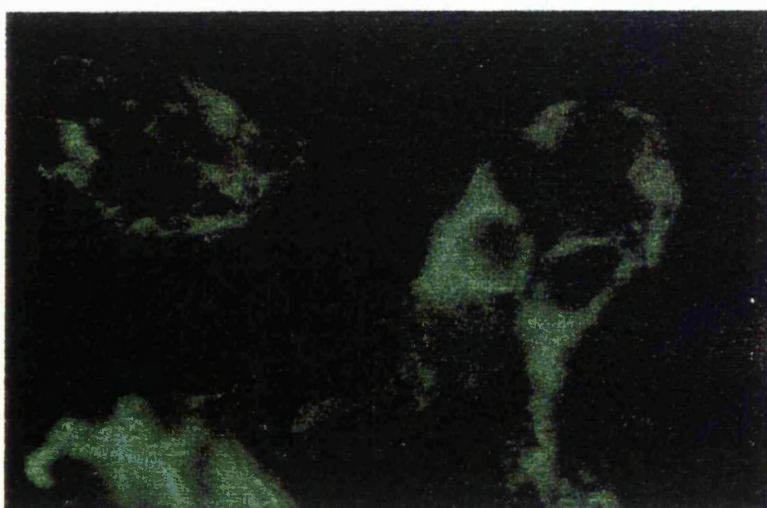
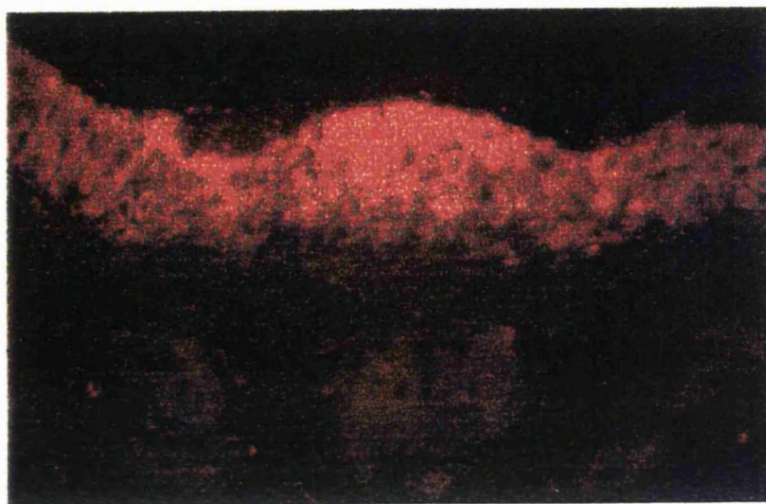


Figure 3.1e and f: Synovium stained for DAF and laminin (intima at top, subintimal vessels below). Co-localization of pericellular staining is less consistent than for DAF and fibrillin-1. Even where staining for DAF and laminin outlines the same cell, the pattern appears concentric rather than identical; final magnification x200.

e.

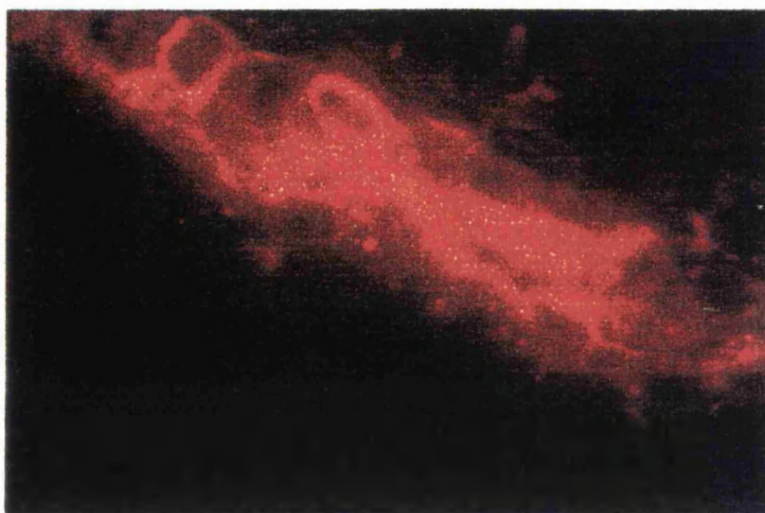


f.



Figure 3.1g and h: Synovial intima stained for DAF and collagen. The images are totally discordant, with the exception of one elliptical cell outline (top centre). On precise registration the outlines of this cell are partially concentric, and not coincident; final magnification x400

g.



h.

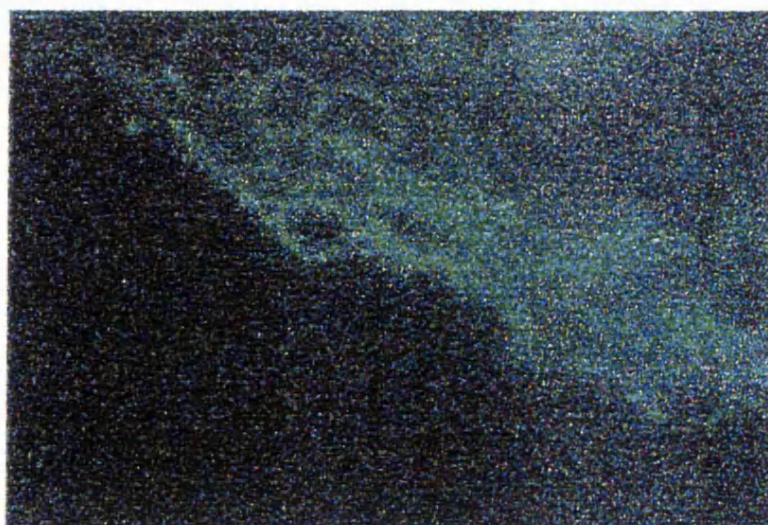
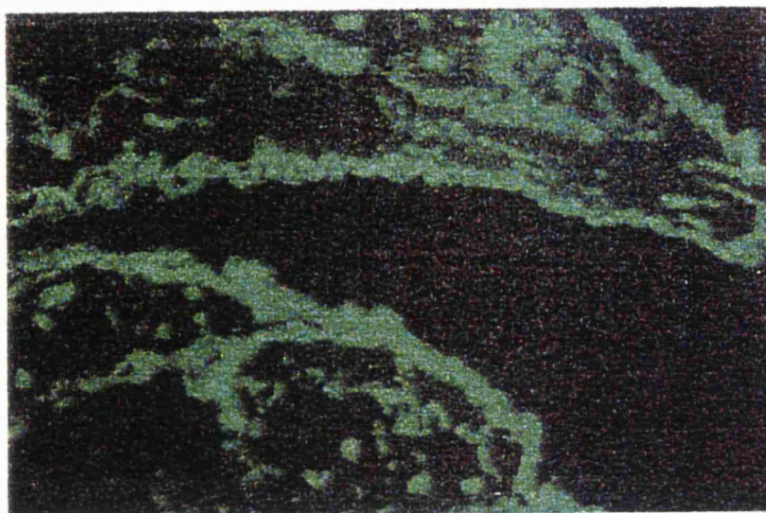


Figure 3.1i and j: Pericardium stained for FcγRIII and DAF showing an area of bright continuous curvilinear staining of the mesothelium and some associated fibrous septae. Macrophages are sparse at this site and account for only a small fraction of FcγRIII staining. Autofluorescent glycogen granules are also present in the underlying tissue, seen more brightly on the rhodamine channel (j); final magnification x200.

i.



j.

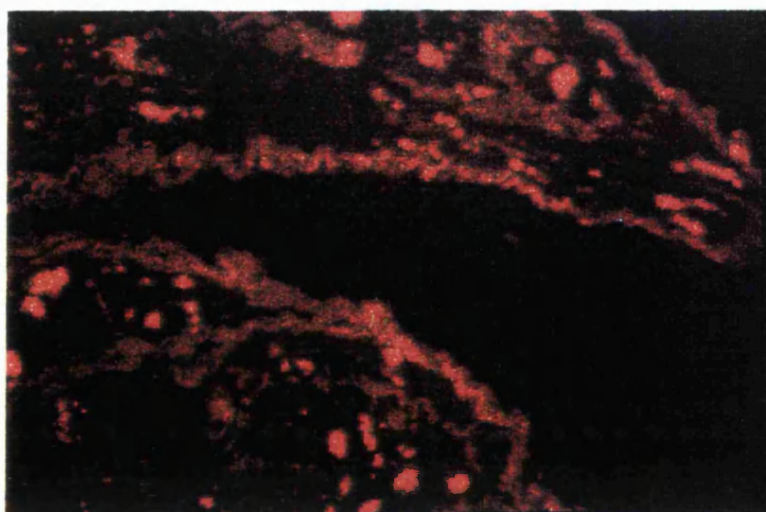
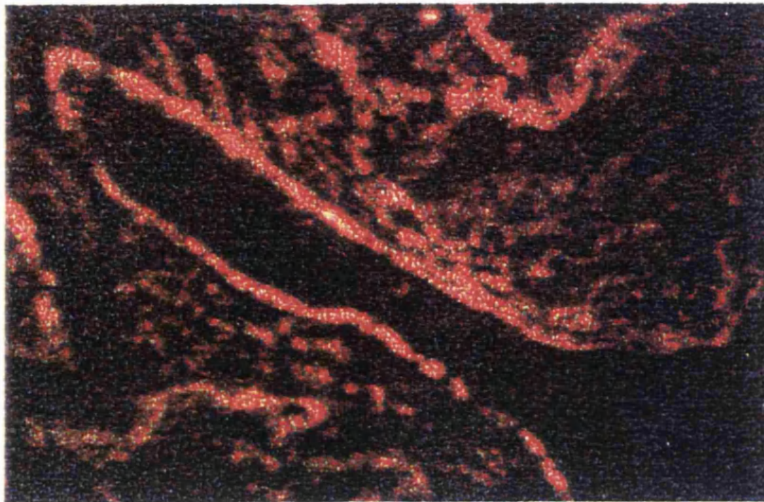


Figure 3.1k and l: Pericardium stained for DAF and fibrillin-1. Pericellular and extracellular linear staining for DAF co-localizes almost entirely with fibrillin-1; final magnification x200

k.



l.

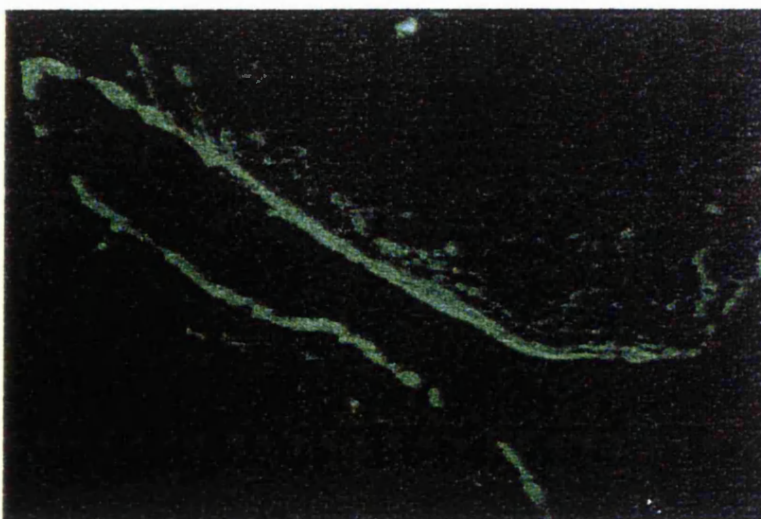
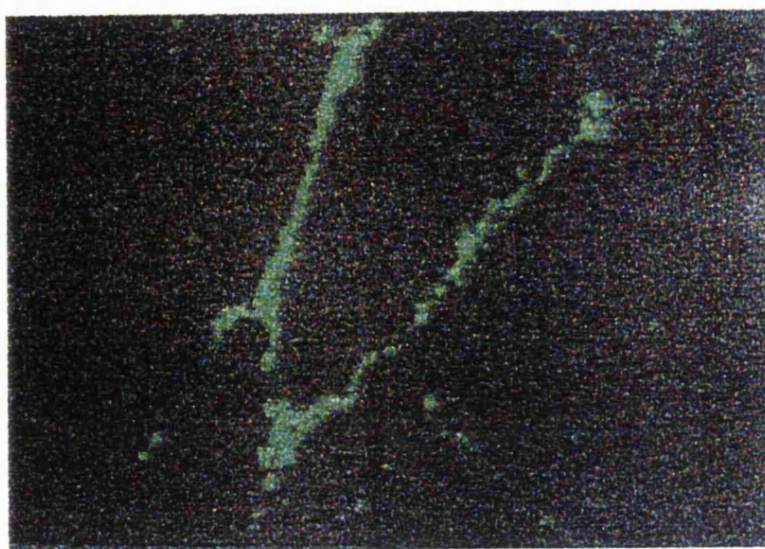


Figure 3.1m and n: Muscle stained for FcγRIII and DAF. The images share many common elements but are not identical; final magnification x200.

m.



n.

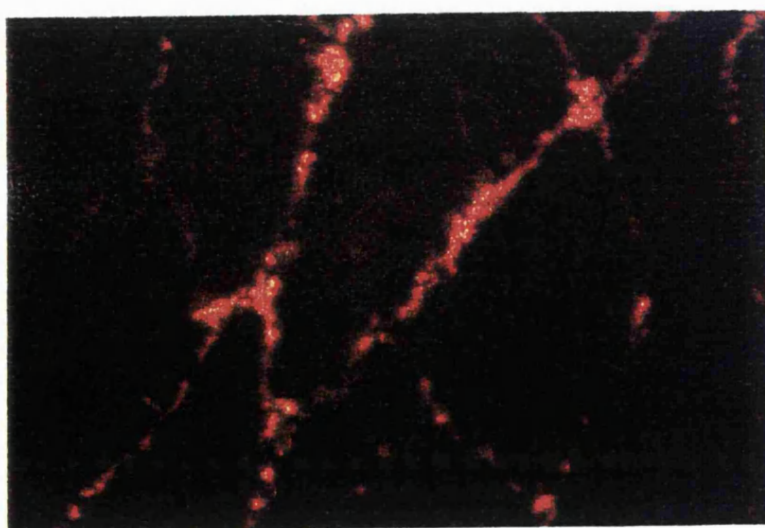
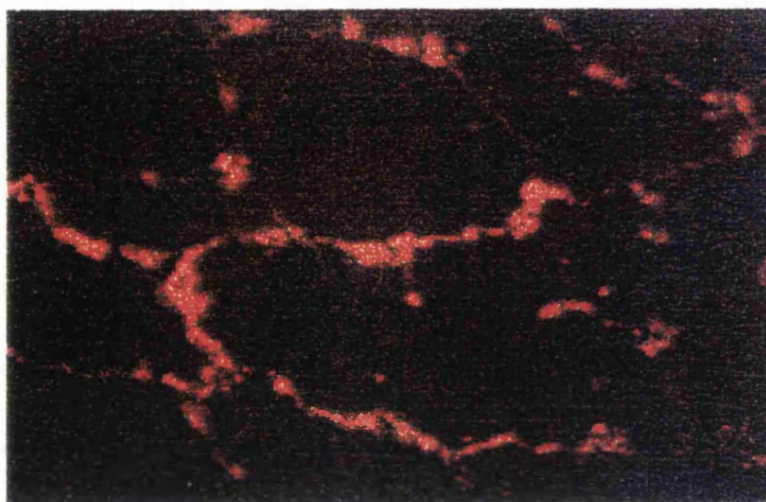


Figure 3.1o and p: Muscle stained for DAF and fibrillin-1. The images show close correspondence, on fibrillar structures outlining individual muscle cells; final magnification x200.

o.



p.

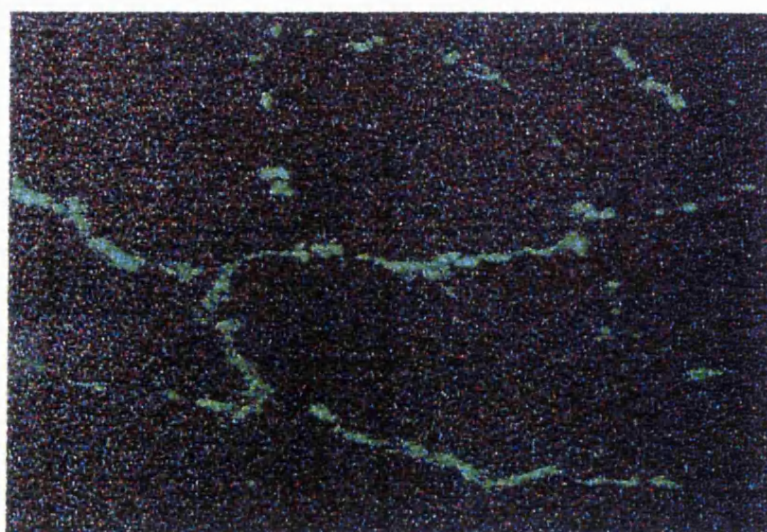


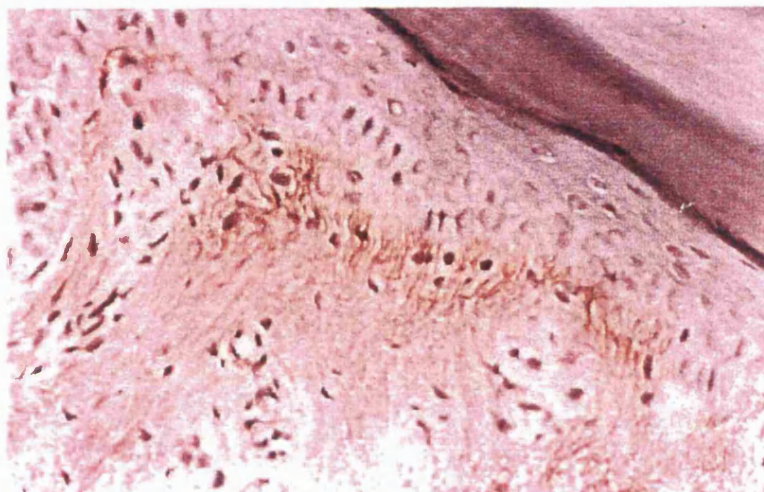
Figure 3.1q: Muscle stained for CD68. This field is of a serial section of the field shown in (o) and (p), demonstrating that the FcγRIII staining in (o) is not accounted for by cellular staining of macrophages; final magnification x200.

q.

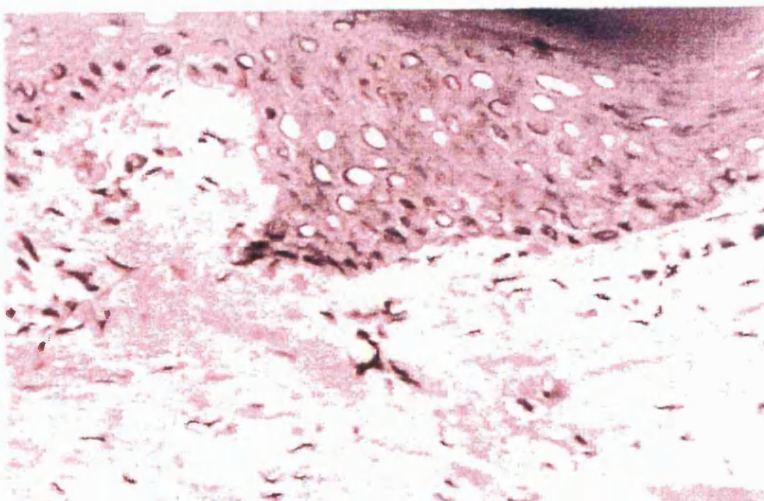


Figure 3.1r and s: Dermis from a bunion and a site 2 inches away stained for FcγRIIIa. Dermal macrophages from bunion site show staining for FcγRIII. Two inches away from the bunion there is no staining for FcγRIIIa; final magnification x400.

r.



s.



EXPERIMENTS 2a-c: FcγRIIIa EXPRESSION ON MONOCYTES *IN VITRO*

Introduction

Approximately five percent of circulating peripheral blood monocytes express FcγRIIIa. Peripheral blood monocytes cultured on a polystyrene substrate show an increase in the expression of FcγRIIIa over time. This increased expression reaches a plateau after three days of culture (Klaassen R J L, Ouwehand W H *et al*, 1990). Monocytes cultured on a Teflon substrate take 14 days for FcγRIIIa expression to plateau (Clarkson S B and Ory P A, 1988). Monocytes adhere more strongly to polystyrene (Andreessen R, Picht J *et al*, 1983). This probably accounts for the shorter duration of time observed for FcγRIIIa expression to reach a plateau when monocytes are cultured on polystyrene.

The aim of experiments 2a-c was to investigate the expression of FcγRIIIa on monocyte-macrophages over time on an adherent (polystyrene) and non-adherent (polypropylene) substrates.

Methods

Peripheral blood monocytes were obtained as described on page 100. They were cultured on polystyrene (adherent) and polypropylene (non-adherent) substrates. Monocytes cultured on a polypropylene substrate were gently agitated to further

prevent monocyte adherence. At different time points monocytes were taken and the level of FcγRIIIa was assessed as described on pages 105-107.

Results

Results from experiment 2a investigating the expression of FcγRIIIa on cultured monocytes on a polystyrene (adherent) substrate over time are shown in table and figure 3.2a.

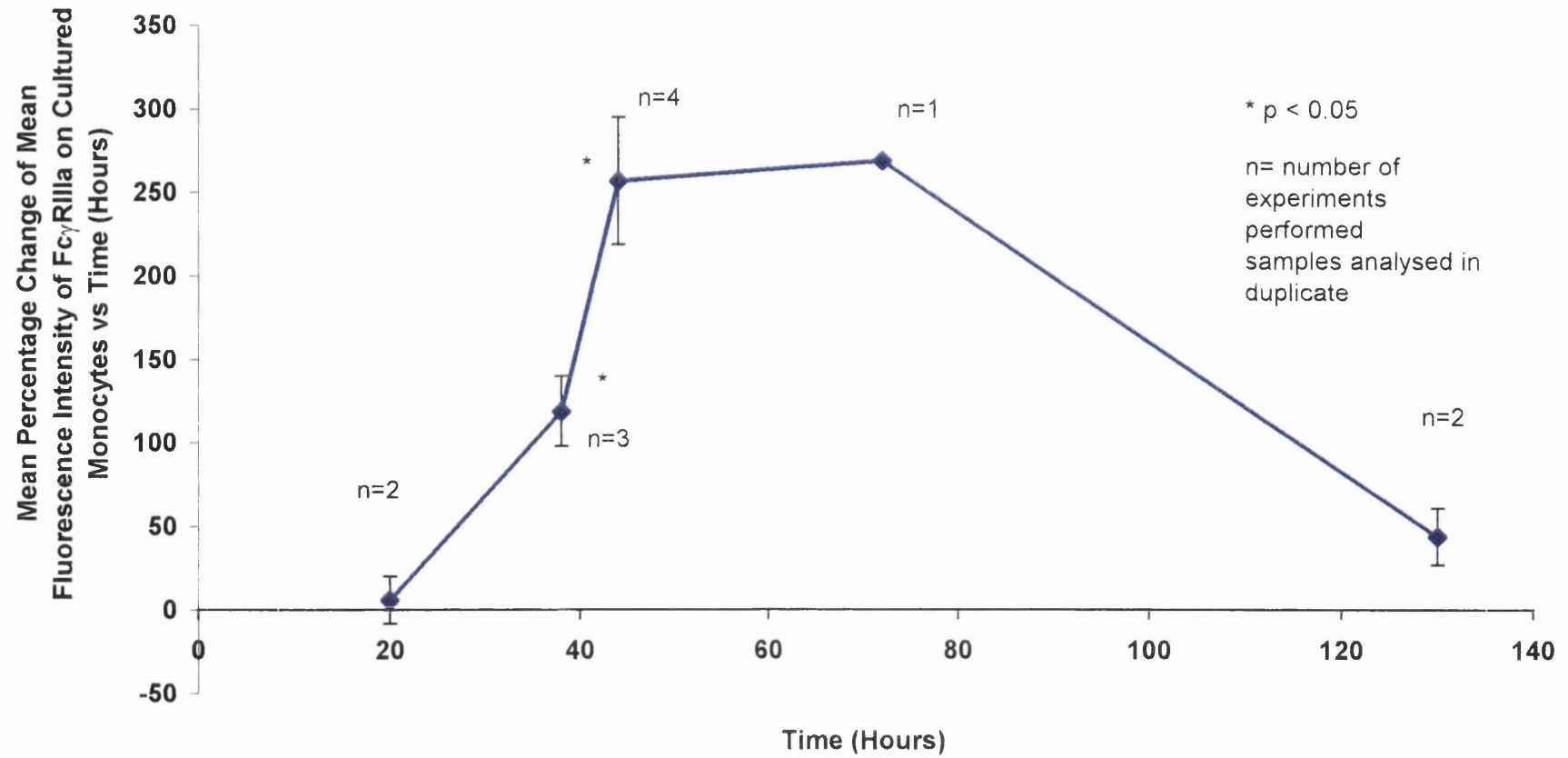
Table 3.2a: Mean percentage change of mean fluorescence intensity of FcγRIIIa on cultured adherent monocytes with time (hours).

Time (Hours)	Mean Percentage Change of Mean Fluorescence Intensity of FcγRIIIa Cultured Adherent Monocytes +/- SEM	
20	6 +/- 14	n=2 (T ₀ 12.6, 39.4; T ₂₀ 11.5, 47.3)
38	119 +/- 21*	n=3 (T ₀ 49.4, 89, 17; T ₃₈ 104, 166, 44)
44	257 +/- 38*	n=4 (T ₀ 12.6, 6.7, 37.4, 61; T ₄₄ 56, 48.5, 127.3, 181.6)
72	269	n=1 (T ₀ 12.6; T ₇₂ 48)
130	44 +/- 17	n=2 (T ₀ 49.4, 28.3; T ₁₃₀ 62, 45)

Samples analysed in duplicate, n=number of experiments performed *p < 0.05
 Baseline MFI values given in brackets respectively
 T=Time with subscript denoting number of hours

Peripheral blood monocytes cultured on a polystyrene substrate show an increase in the expression of FcγRIIIa after twenty hours of incubation. The increase of FcγRIIIa expression continues to rise with time and peaks between forty four to seventy two hours of culture. This is consistent with the data of Klaassen R J L *et al*.

Figure 3.2a: Mean Percentage Change of Mean Fluorescence Intensity of FcγRIIIa on Cultured Adherent Monocytes (+/- SEM) versus Time (Hours)



After an incubation of one hundred and thirty hours the expression of FcγRIIIa falls compared to that at 72 hours. This fall in FcγRIIIa expression may be as a result of the monocytes becoming senescent.

In experiment 2b monocytes were cultured on polystyrene (adherent) and polypropylene (non-adherent) substrate for sixty-five hours. FcγRIIIa expression was then assessed as described on pages 105-107. The data from experiment 2b is shown in table and figure 3.2b.

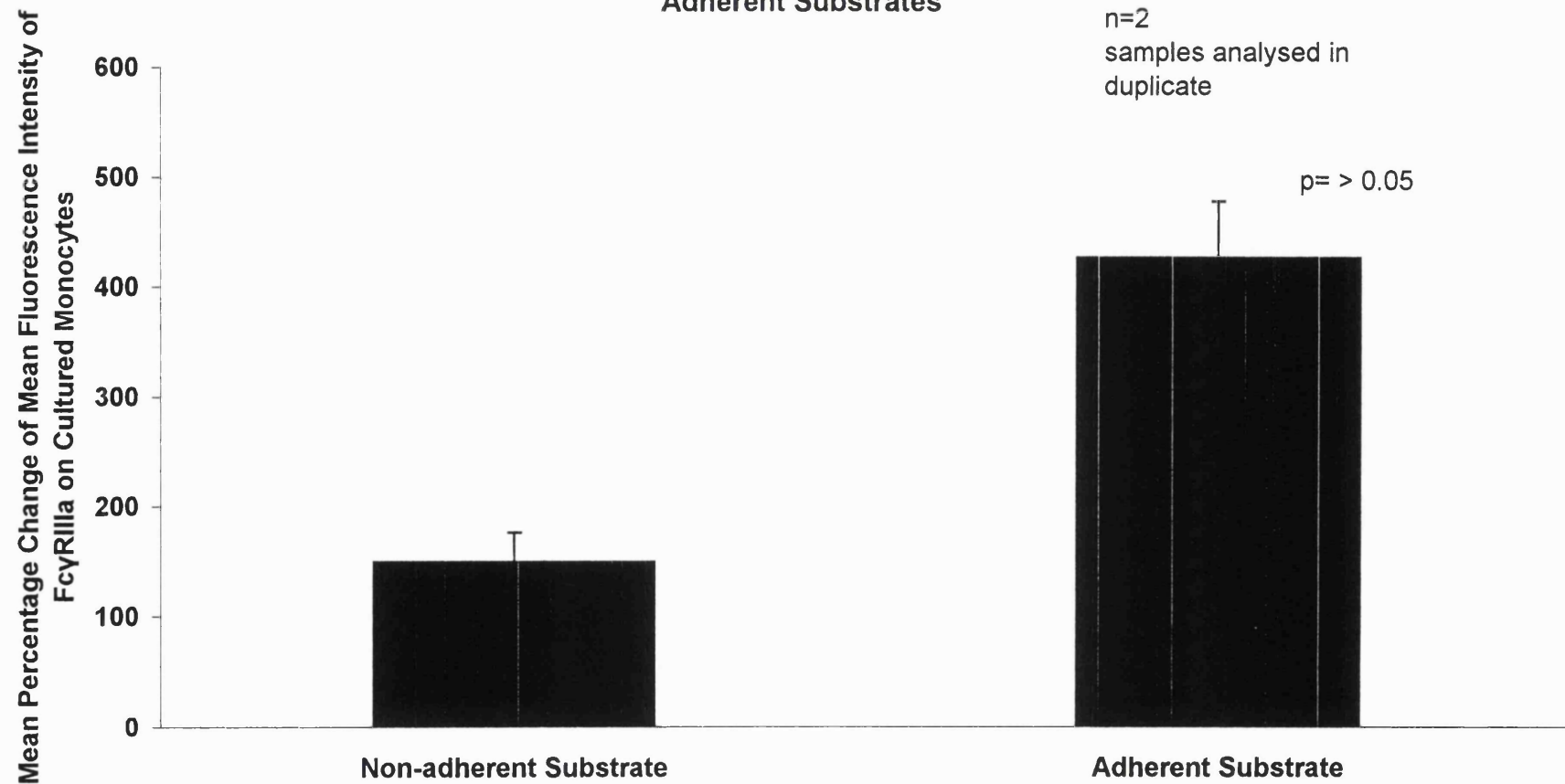
Table 3.2b: Mean percentage change of mean fluorescence Intensity of FcγRIIIa on cultured monocytes after a sixty-five hour incubation on a non-adherent and adherent substrate.

Substrate	Mean Percentage Change of Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM
Non-adherent	149 +/- 27 n=2 (T ₀ 32.3, 35.5; T ₆₅ 88, 80)
Adherent	427 +/- 51* n=2 (T ₀ 32.3, 35.5; T ₆₅ 185,171)

Samples analysed in duplicate, n=number of experiments performed *p > 0.05
Baseline MFI values given in brackets respectively
T=Time with subscript denoting number of hours

Monocytes cultured on a non-adherent substrate show less expression of FcγRIIIa than monocytes concurrently cultured for the same duration of time on an adherent substrate (table and figure 3.2b).

Figure 3.2b: Mean Percentage Change of Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes After a Sixty-five Hour Incubation +/- SEM versus Non-adherent and Adherent Substrates



In experiment 2c peripheral blood monocytes were cultured on both polystyrene (adherent) and polypropylene (non-adherent) substrates. Monocytes were taken at different time points and FcγRIIIa expression was determined as described on pages 105-107. The data from experiment 2c is shown in table and figure 3.2c.

Table 3.2c: Mean percentage change of mean fluorescence intensity of FcγRIIIa on cultured adherent and non-adherent monocytes over time (hours).

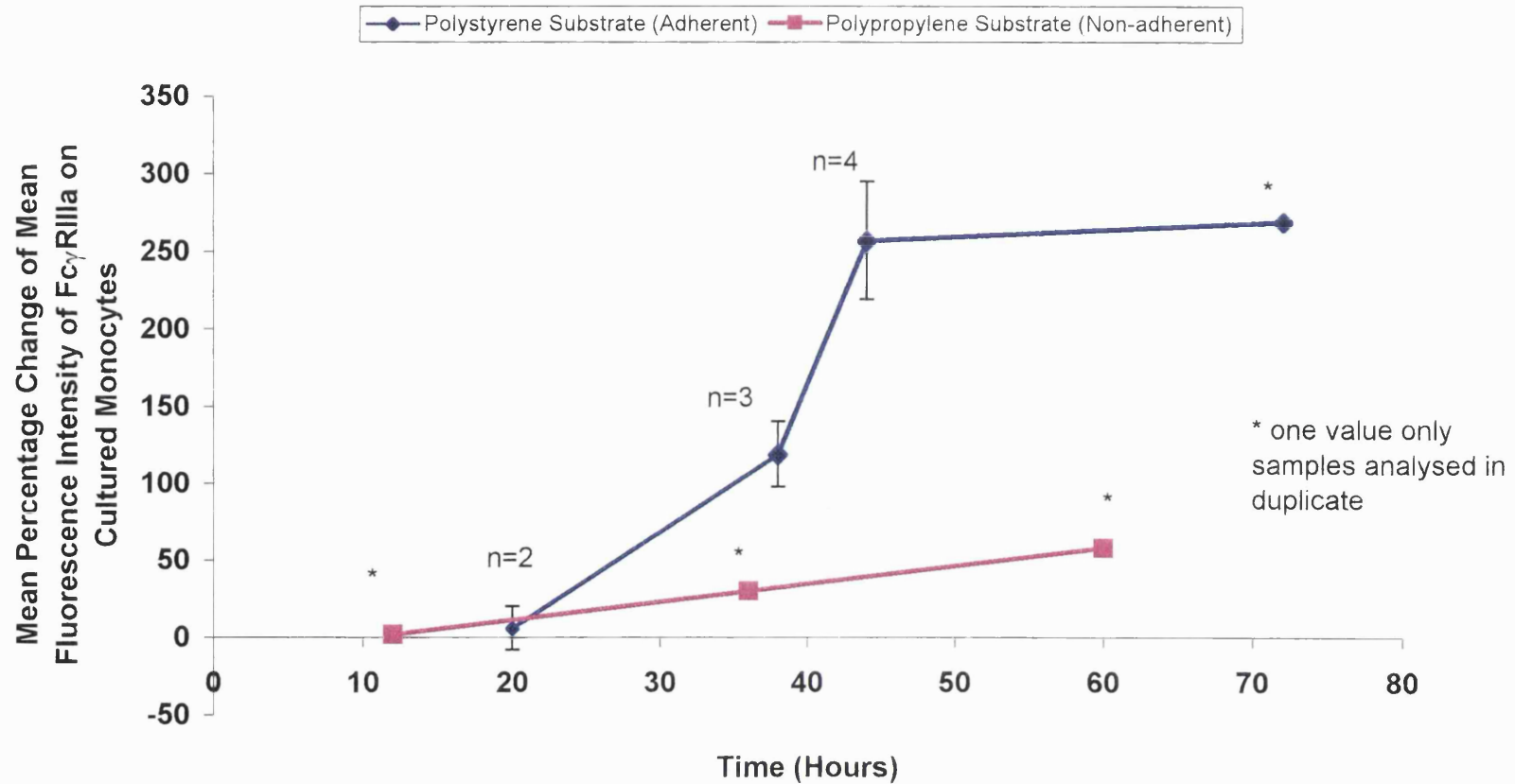
Time (Hours)	Mean Percentage Change of Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM	
	Adherent Substrate	Non-adherent Substrate
0		
12		2* (T ₀ 63.6; T ₁₂ 65)
20	6 +/- 14 n=2 (T ₀ 12.6, 39.4; T ₂₀ 11.5, 47.3)	
36		30* (T ₀ 63.6; T ₃₆ 83)
38	119 +/- 21 n=3 (T ₀ 49.4, 89, 17; T ₃₈ 104, 166, 44)	
44	257 +/- 38 n=4 (T ₀ 12.6, 6.7, 37.4, 61; T ₄₄ 56, 48.5, 127.3, 181.6)	
60		58* (T ₀ 63.6; T ₆₀ 101)
72	269*	

* one value only, n=number of experiments performed
Samples analysed in duplicate, baseline MFI values given in brackets respectively
T=Time with subscript denoting number of hours

Because only one experiment was performed at each time course for non-adherent monocytes it is not possible to perform statistical analysis on this data. However, adherence of peripheral blood monocytes *in vitro* increases the expression of FcγRIIIa over time as shown in figure 3.2a. Monocytes cultured on a non-adherent

substrate show a smaller increase in FcγRIIIa expression over time compared to adherent monocytes as shown in figure 3.2c. A larger number of experiments would need to be performed in order for statistically significant data to be achieved. There is nevertheless a trend revealing that adherence to a polystyrene substrate seems to be a factor that results in the induction of FcγRIIIa on cultured monocytes.

Figure 3.2c: Mean Percentage Change of Mean Fluorescence Intensity of Fc γ R1IIa on Cultured Adherent and Non-adherent Monocytes +/- SEM versus Time (Hours)



EXPERIMENTS 3a-d: TRANSFORMING GROWTH FACTOR BETA AND FcγRIIIa EXPRESSION ON CULTURED MONOCYTES

Introduction

TGFβ₁ has been shown to selectively induce FcγRIIIa on monocytes *in vitro* (Welch G R, Wong *et al*, 1990). Mechanical stretch may serve as a stimulus for TGFβ synthesis and release (Riser B L, Cortecs P *et al*, 1996). Normal macrophages in the synovial intima and mechanically stretched dermis express FcγRIIIa (Edwards J C W, Blades S, 1997). Synovium is exposed to mechanical stretch in physiological conditions. Thus TGFβ may be responsible for providing a microenvironment in tissues that induces FcγRIIIa on monocyte-macrophages and the stimulus for TGFβ synthesis and release may be mechanical stretch.

The aim of experiments 3a-d were to investigate the effect of TGFβ₁ on FcγRIIIa expression on cultured monocytes. Experiments have been performed to investigate whether TGFβ₁ induces FcγRIIIa on cultured monocytes as shown by Welch G R *et al*.

Methods

In experiment 3a and b peripheral blood monocytes were obtained as described on page 100. These monocytes were cultured on a polystyrene substrate with different concentrations of TGFβ for thirty-eight hours. The monocytes were then removed

and FcγRIIIa expression was assessed as described in pages 105-107. The results from experiments 3a and b are shown in table and figures 3.3a and b.

Results

Table 3.3a and b: Mean fluorescence intensity of FcγRIIIa on cultured adherent monocytes versus different concentrations of TGFβ₁ (ng/ml) (n=1)

[TGFβ ₁] ng/ml	Mean Fluorescence Intensity of FcγRIIIa on Cultured Adherent Monocytes	[TGFβ ₁] ng/ml	Mean Fluorescence Intensity of FcγRIIIa on Cultured Adherent Monocytes
0	305	0	38
5	128	2	16
10	130	4	13
20	122	6	12
40	111	8	11
		10	12

n= number of experiments performed
Samples analysed in duplicate

TGFβ₁ at a concentration of 2 ng/ml suppressed the induction of FcγRIIIa on adherent cultured monocytes. This suppression was maintained at higher concentrations of TGFβ₁ up to a concentration of 40 ng/ml.

Experiment 3c investigated the effect of TGFβ₁ at a concentration of 10 ng/ml on FcγRIIIa expression on cultured monocytes over time compared to monocytes concurrently cultured in the absence of TGFβ₁. Monocytes were obtained as described on page 100 and cultured on a polystyrene substrate. At different time

Figure 3.3a: Mean Fluorescence Intensity of Fc γ R11a on Adherent Cultured Monocytes versus [TGF β] ng/ml after a 38 Hour Incubation (n=1)

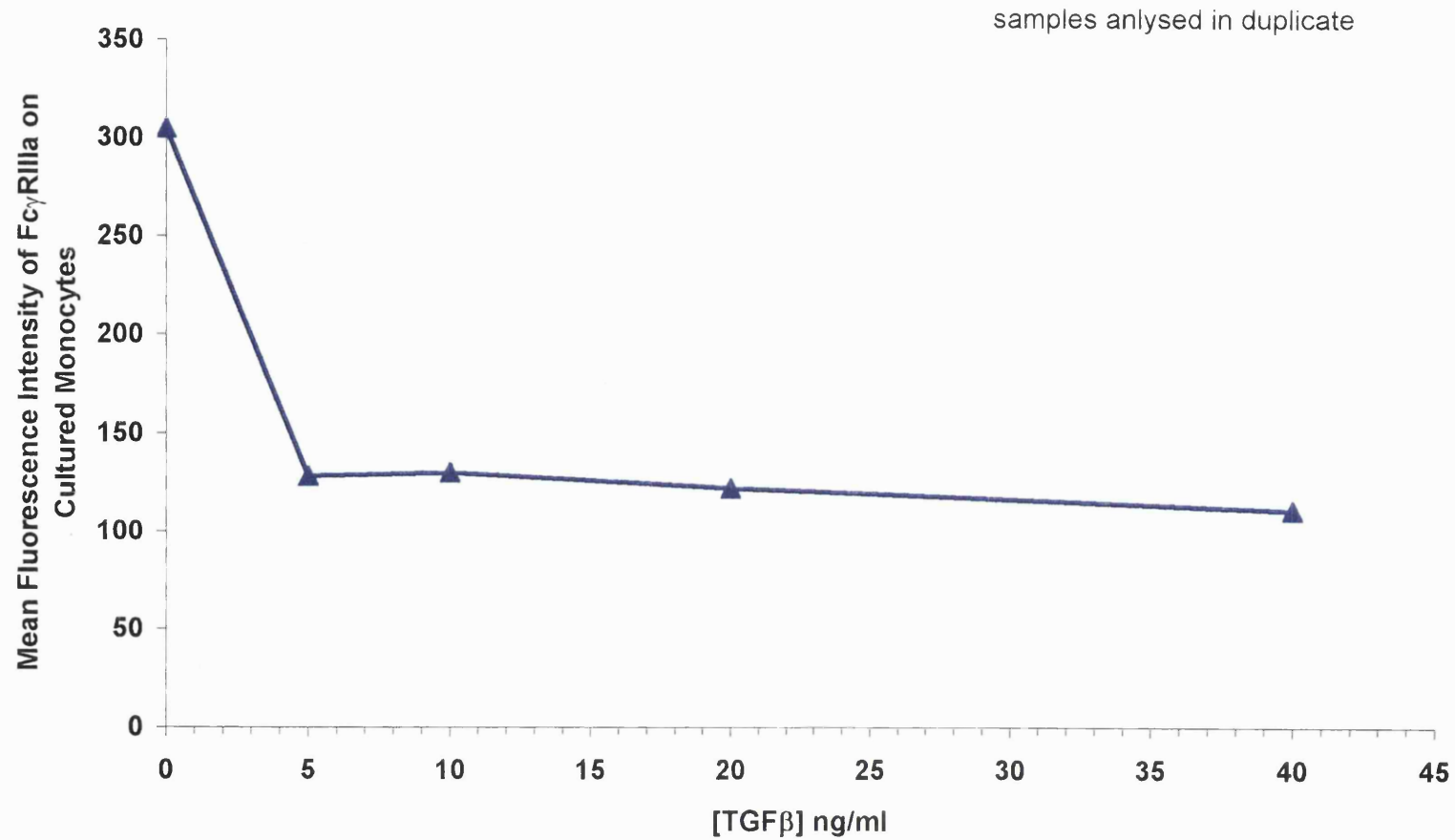
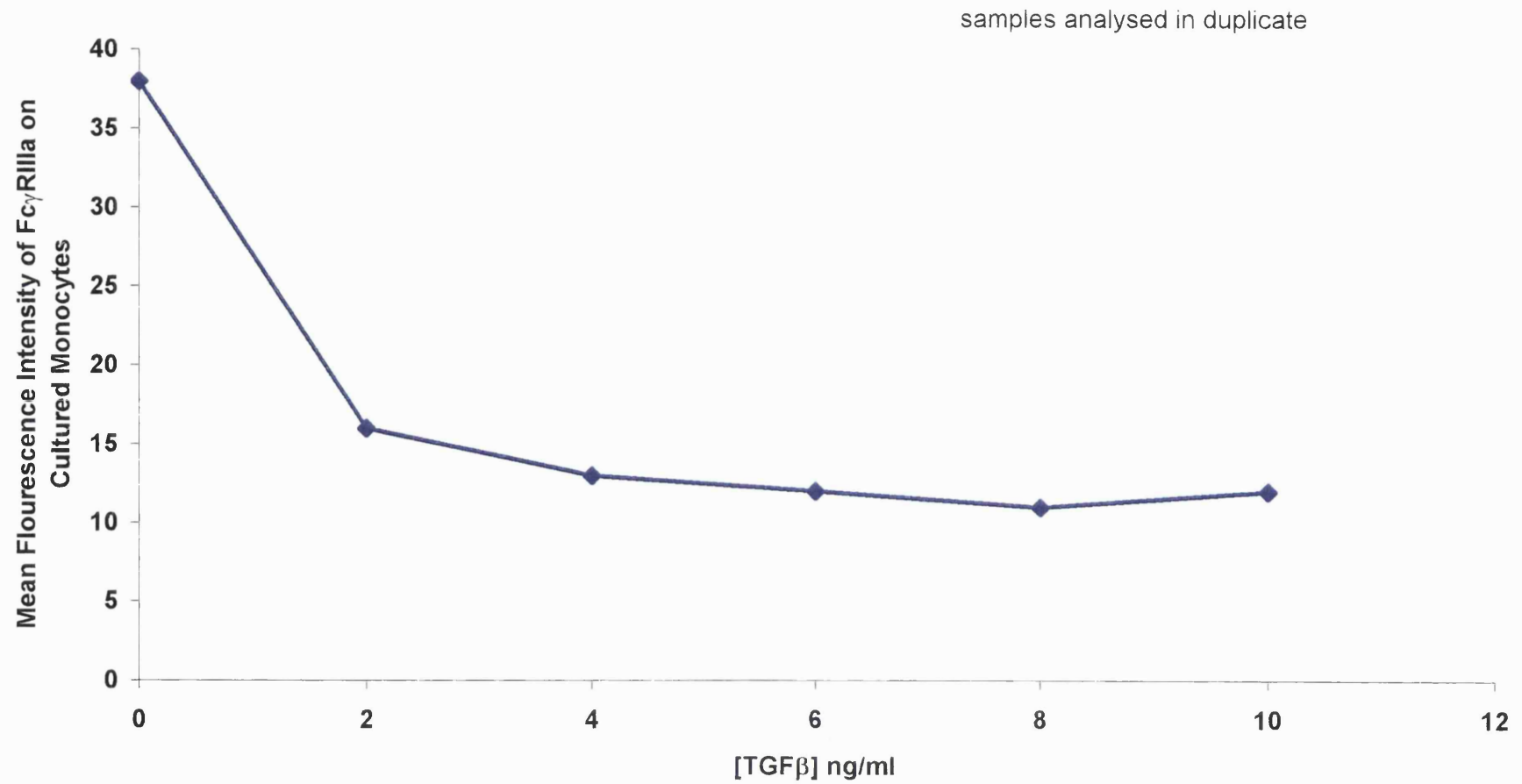


Figure 3.3b: Mean Fluorescence Intensity of Fc γ R1IIa on Adherent Cultured Monocytes versus [TGF β] (ng/ml) after a 38 Hour Incubation (n=1)



points monocytes were removed and FcγRIIIa expression was assessed as described in pages 105-107. The results from experiment 3c are shown in table and figure 3.3c.

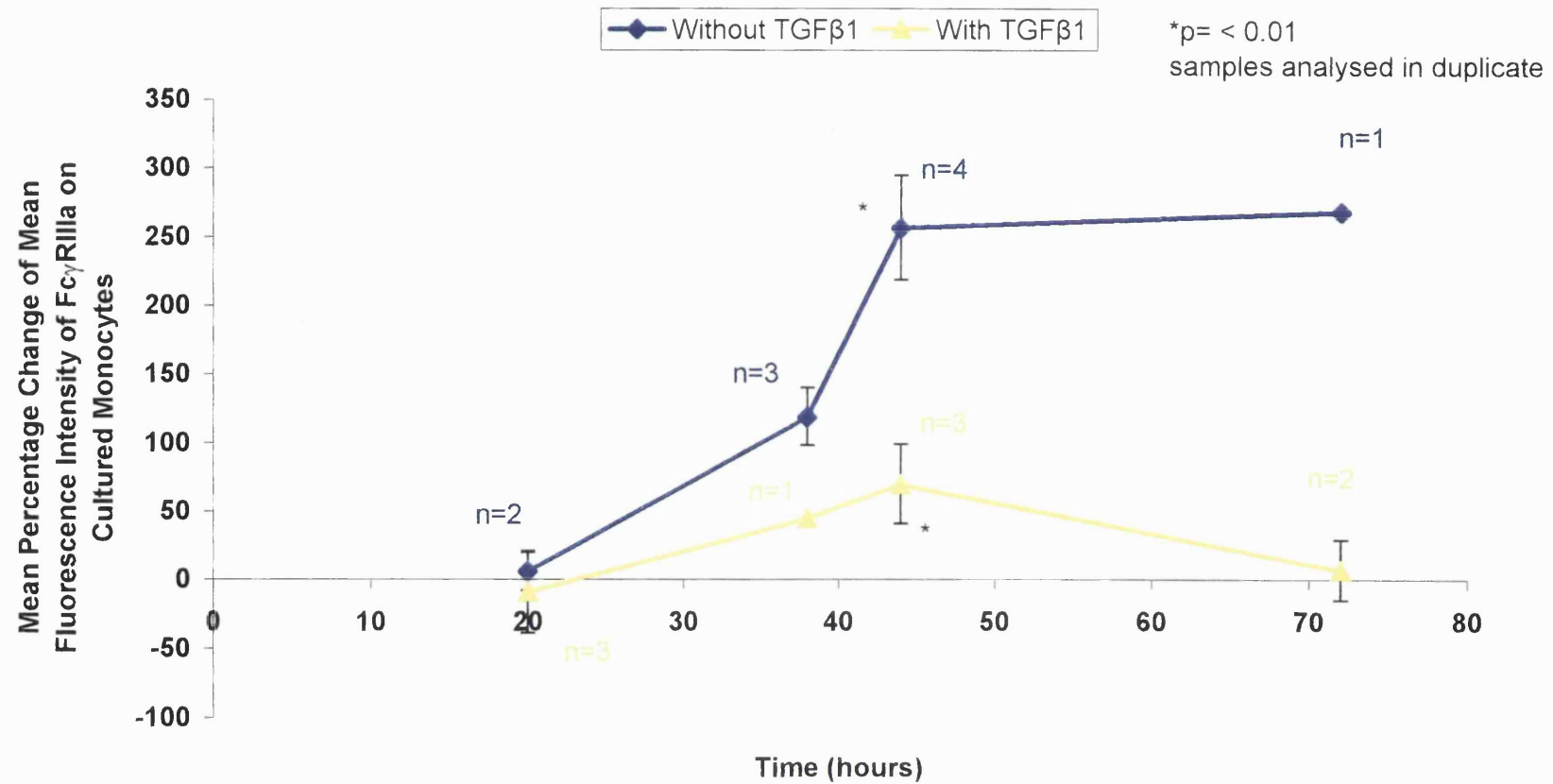
Table 3.3c: Mean percentage change in mean fluorescence intensity of FcγRIIIa on adherent cultured monocytes over time (hours) with and without the addition of TGFβ₁ (10 ng/ml).

Time (Hours)	Mean Percentage Change in Mean Fluorescence Intensity of FcγRIIIa on Adherent Cultured Monocytes +/- SEM	
	Without TGFβ ₁	With TGFβ ₁ (10 ng/ml)
0		
20	6 +/- 14 n=2 (T ₀ 12.6, 39.4; T ₂₀ 11.5, 47.3)	- 9 +/- 30 n=3 (T ₀ 12.6, 7, 61; T ₂₀ 12, 10, 24)
38	119 +/- 21 n=3 (T ₀ 49.4, 89, 17; T ₃₈ 104, 166, 44)	45 n=1 (T ₀ 49; T ₃₈ 71)
44	257 +/- 38 n=4 (T ₀ 12.6, 6.7, 37.4, 61; T ₄₄ 56, 48.5, 127.3, 181.6)	70 +/- 29* n=3 (T ₀ 12.6, 7, 61; T ₄₄ 15, 15, 110.2)
72	269 n=1	7 +/- 22 n=2 (T ₀ 12.6, 7; T ₇₂ 11, 9)

n=number of experiments performed, samples analysed in duplicate *p= < 0.01
Baseline MFI values given in brackets respectively
T=Time with subscript denoting number of hours

TGFβ₁ suppressed the increased expression of FcγRIIIa on cultured monocytes that was seen on monocytes cultured in the absence of TGFβ₁ over time, p= <0.01 after 44 hours of culture.

Figure 3.3c: Mean Percentage Change of Mean Fluorescence Intensity of Fc γ R11a on Adherent Cultured Monocytes +/- SEM Versus Time (hours) With and Without the Addition of TGF β 1 (10 ng/ml).



Experiment 3d investigated whether this suppression also occurred when non-adherent monocytes were cultured with TGF β ₁. In experiment 3d peripheral blood monocytes obtained as described on page 100 were cultured on a polypropylene substrate (non-adherent) with and without TGF β ₁ (10 ng/ml). Monocytes were gently agitated, to further prevent adherence during culture. At different time points monocytes were removed and Fc γ RIIIa expression was assessed as described on pages 105-107. The results from experiment 3d are shown in table and figure 3.3d.

Table 3.3d: Mean fluorescence intensity of Fc γ RIIIa on cultured non-adherent monocytes over time with and without the addition of TGF β ₁ (10 ng/ml). (n=1)

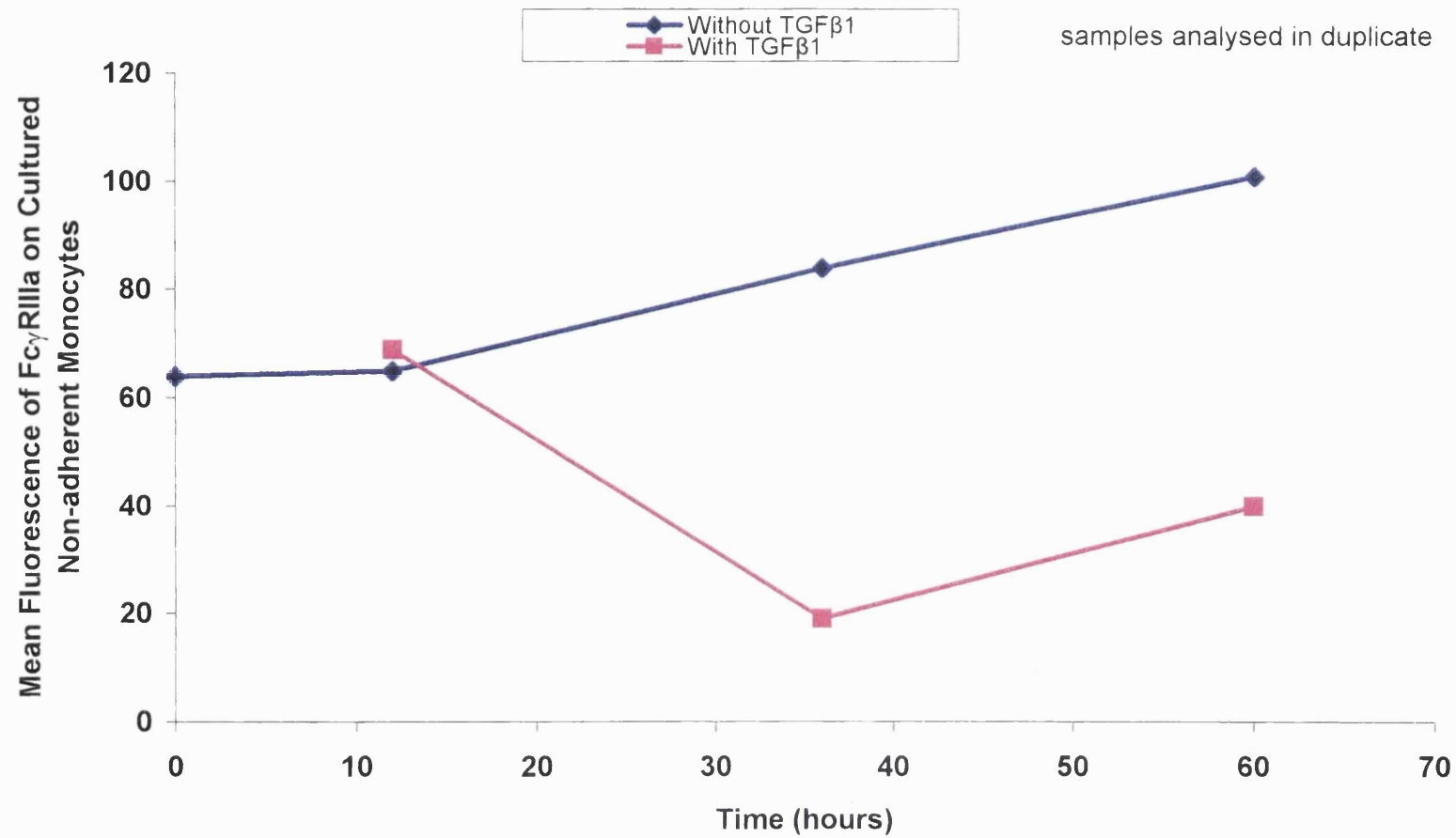
Time (Hours)	Mean Fluorescence Intensity of Fc γ RIIIa on Cultured Non-adherent Monocytes	
	Without TGF β ₁	With TGF β ₁
0	64	
12	65	69
36	84	19
60	101	40

Samples analysed in duplicate

TGF β ₁ suppresses the expression of Fc γ RIIIa on cultured non-adherent and adherent monocytes. This data contrasts that of Welch G R *et al.* Despite following Welch's materials and methods it was not possible to replicate his findings that showed TGF β ₁ selectively induced the expression of Fc γ RIIIa on cultured peripheral blood monocytes.

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Figure 3.3d: Mean Fluorescence Intensity of FcγRIIIa on Cultured Non-adherent Monocytes versus Time (hours) With and Without the Addition of TGFβ1 (10 ng/ml) (n=1)



EXPERIMENT 4a: INTERLEUKIN 10 AND FcγRIIIa EXPRESSION ON CULTURED MONOCYTES

Introduction

IL-10 upregulates FcγRIIIa expression on cultured monocytes (Calzada-Wack J C, Frankenberger M *et al*, 1996). The ability of IL-10 to induce FcγRIIIa on monocytes *in vitro* suggests that IL-10 may be responsible for the expression of FcγRIIIa on normal intimal synovial macrophages.

The aim of experiment 4a was to investigate whether IL-10 induces FcγRIIIa on cultured monocytes as shown by Calzada-Wack J C *et al*.

Methods

Peripheral blood monocytes were obtained as described on page 100. Monocytes were cultured on a polystyrene substrate for forty hours with different concentrations of IL-10. After forty hours the monocytes were removed and FcγRIIIa expression was assessed as described on pages 105-107. The results from experiment 4a are shown in table and figure 3.4a.

Results

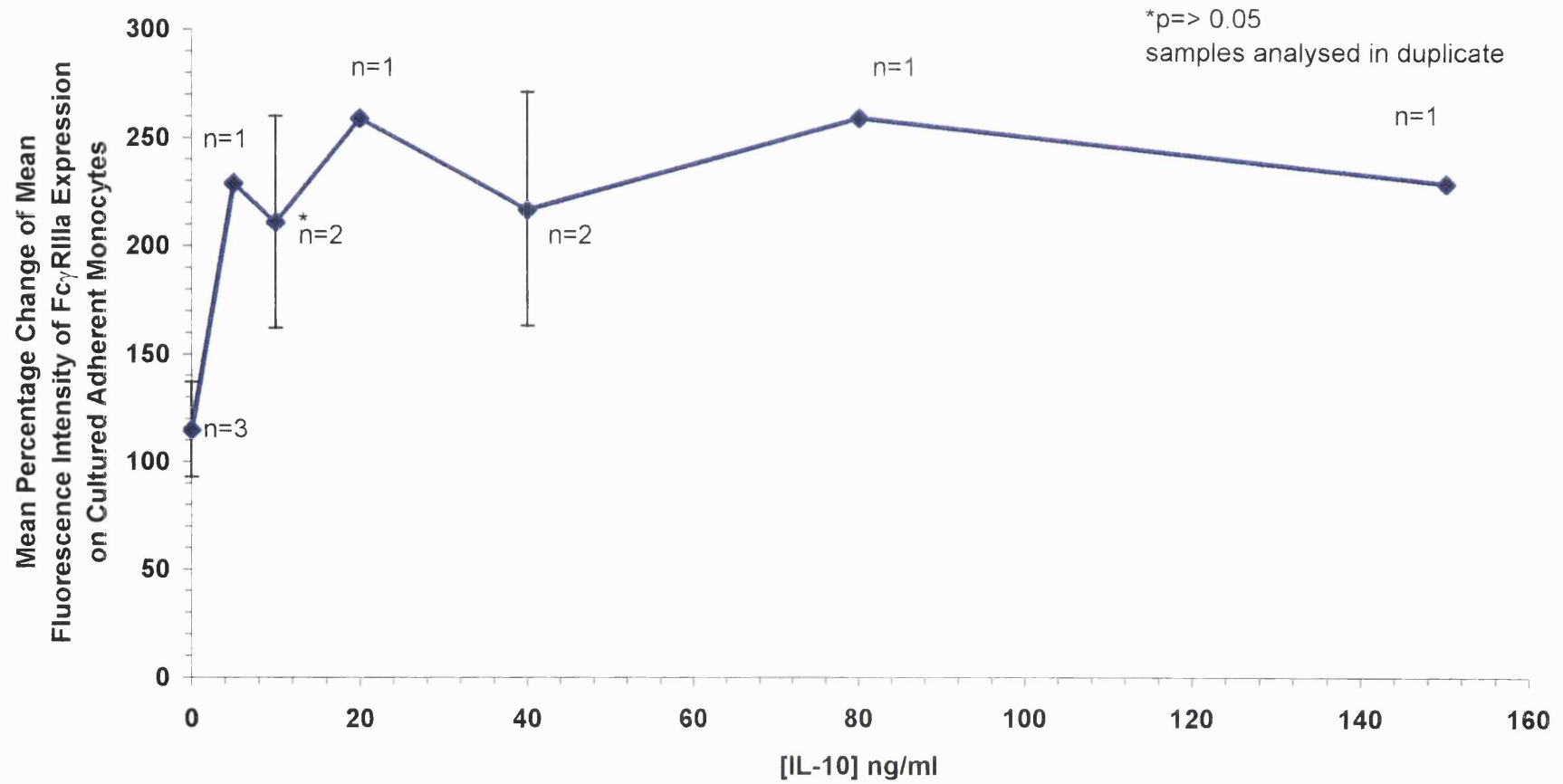
Table 3.4a: Mean Percentage Change of Mean Fluorescence Intensity of FcγRIIIa on Cultured Adherent Monocytes versus [IL-10] ng/ml

[IL-10] ng/ml	Mean Percentage Change of Mean Fluorescence Intensity of FcγRIIIa Expression on Cultured Monocytes +/- SEM
0	115 +/- 22 n=3 (T ₀ 17,89,13; T ₄₀ 44,166,26)
5	229 n=1 (T ₀ 17; T ₄₀ 61)
10	211 +/- 49* n=2 (T ₀ 17,13; T ₄₀ 61, 34)
20	259 n=1 (T ₀ 17; T ₄₀ 61)
40	217 +/- 54 n=2 (T ₀ 17,89; T ₄₀ 63, 234)
80	259 n=1 (T ₀ 17; T ₄₀ 61)
150	229 n=1 (T ₀ 17; T ₄₀ 56)

n=number of experiments performed, samples analysed in duplicate
 Baseline MFI values given in brackets respectively *p= >0.05
 T=Time with subscript denoting number of hours

IL-10 at a concentration of 5 ng/ml induces more expression of FcγRIIIa on adherent cultured monocytes than monocytes cultured without IL-10 after a forty hour incubation. The level of FcγRIIIa expression plateaued on increasing the concentration of IL-10 above 5 ng/ml. Induction of FcγRIIIa on cultured monocytes by IL-10 was evident after approximately forty hours of incubation. Although the p value above is >0.05 the trend demonstrates that IL-10 increases the expression of FcγRIIIa on cultured monocytes. This is consistent with the findings of Calzada-Wack J C *et al* that IL-10 induces the expression of FcγRIIIa on cultured monocytes. The number of experiments performed above may be too small to demonstrate statistical significance for IL-10 increasing the expression of FcγRIIIa on cultured monocytes.

Figure 3.4a: Mean Percentage Change of Mean Fluorescence Intensity of FcγRIIIa Expression on Cultured Adherent Monocytes +/- SEM versus [IL-10] ng/ml



There is currently no data to suggest that IL-10 is present in normal tissues where macrophages express FcγRIIIa. This casts doubt on IL-10 as a factor responsible for FcγRIIIa expression on tissue macrophages *in vivo*.

The administration of IL-10 to patients with rheumatoid arthritis has failed to demonstrate any clinical efficacy (St Clair E W, 1999). This in part may be explained by the possibility of IL-10 up regulating FcγRIIIa expression on synovial macrophages *in vivo*.

EXPERIMENTS 5a AND b: SUBSTANCE P AND FcγRIIIa EXPRESSION ON CULTURED MONOCYTES

Introduction

Clinical observations suggest that a neurogenic or mechanical mechanism may modulate the clinical features of rheumatoid arthritis (pages 20-24 and 76-77).

Mechanical stimulation may serve as a stimulus for substance P release (Kuraishi Y, Hirota N *et al*, 1989). The synovium contains unmyelinated C nerve fibres, whose cell bodies synthesize substance P (Mapp P I, Kidd B L *et al*, 1990). Intimal synoviocytes constitutively express the substance P receptor NK1 (Sakai K, Matsuno H *et al*, 1998).

Substance P may be released in normal synovial intimal tissue as a consequence of mechanical stimulation. It may then act via the NK1 receptor to up regulate FcγRIIIa expression on synovial intimal macrophages (Figure 1.5). The same process may also explain the presence of FcγRIIIa on dermal macrophages that have been subject to mechanical stretch.

The aim of experiments 5a and b were to investigate whether substance P induces FcγRIIIa on cultured monocytes.

Methods

Peripheral blood monocytes were obtained as described on page 100. They were cultured for forty hours with different concentrations of substance P. Experiments were performed on both adherent (polystyrene) and non-adherent (polypropylene) substrates, experiments 5a and 5b respectively. FcγRIIIa expression was assessed as described on pages 105-107. The results from experiment 5a and 5b are shown in table and figure 3.5a and b.

Results

Table 3.5a: The Mean Percentage Change in The Mean Fluorescence Intensity of FcγRIIIa +/- SEM With Different Concentrations of Substance P.

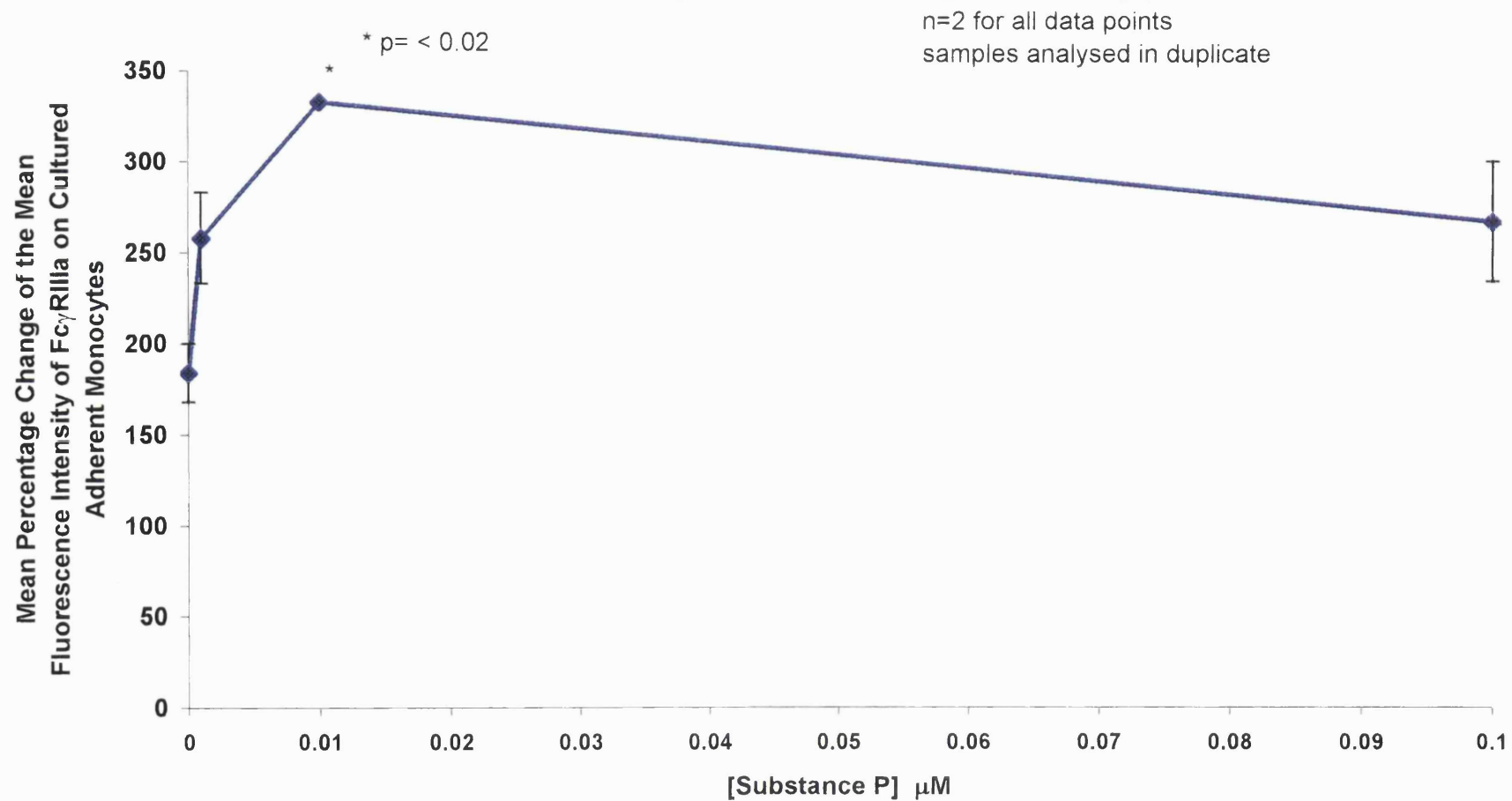
[Substance P] μM	Mean Percentage Change in The Mean Fluorescence Intensity of FcγRIIIa +/- SEM With Different Concentrations of Substance P
0	184 +/- 16 n=2 (T ₀ 6.01, 6.03; T ₄₀ 16.16, 18.11)
0.001	258 +/- 25 n=2 (T ₀ 6.01, 6.03; T ₄₀ 20.24, 23.16)
0.01	333 +/- 0 * n=2 (T ₀ 6.01, 6.03; T ₄₀ 25.63, 25.79)
0.1	267 +/- 33 n=2 (T ₀ 6.01, 6.03; T ₄₀ 23.79, 19.89)
1	225 +/- 8 n=2 (T ₀ 6.01, 6.03; T ₄₀ 20.15, 18.38)
10	250 +/- 17 n=2 (T ₀ 6.01, 6.03; T ₄₀ 19.82, 18.11)

n=number of experiments performed, samples analysed in duplicate *p= < 0.02

Baseline MFI values given in brackets respectively

T=Time with subscript denoting number of hours

Figure 3.5a: Mean Percentage Change of the Mean Fluorescence Intensity of Fc γ R1IIa on Cultured Adherent Monocytes +/- SEM versus [Substance P] μ M



At a physiological concentration (0.01 μM), substance P induces more Fc γ RIIIa on cultured adherent monocytes than monocytes cultured in the absence of substance P ($p = < 0.02$). At supraphysiological concentrations substance P up regulates Fc γ RIIIa expression on cultured monocytes. This elevation of Fc γ RIIIa expression is less than that seen with physiological concentrations of substance P but still more than that of monocytes cultured in the absence of substance P.

Monocytes cultured on a polypropylene substrate (non-adherent) with substance P showed no increase in Fc γ RIIIa expression. This data is shown in table and figure 3.5b.

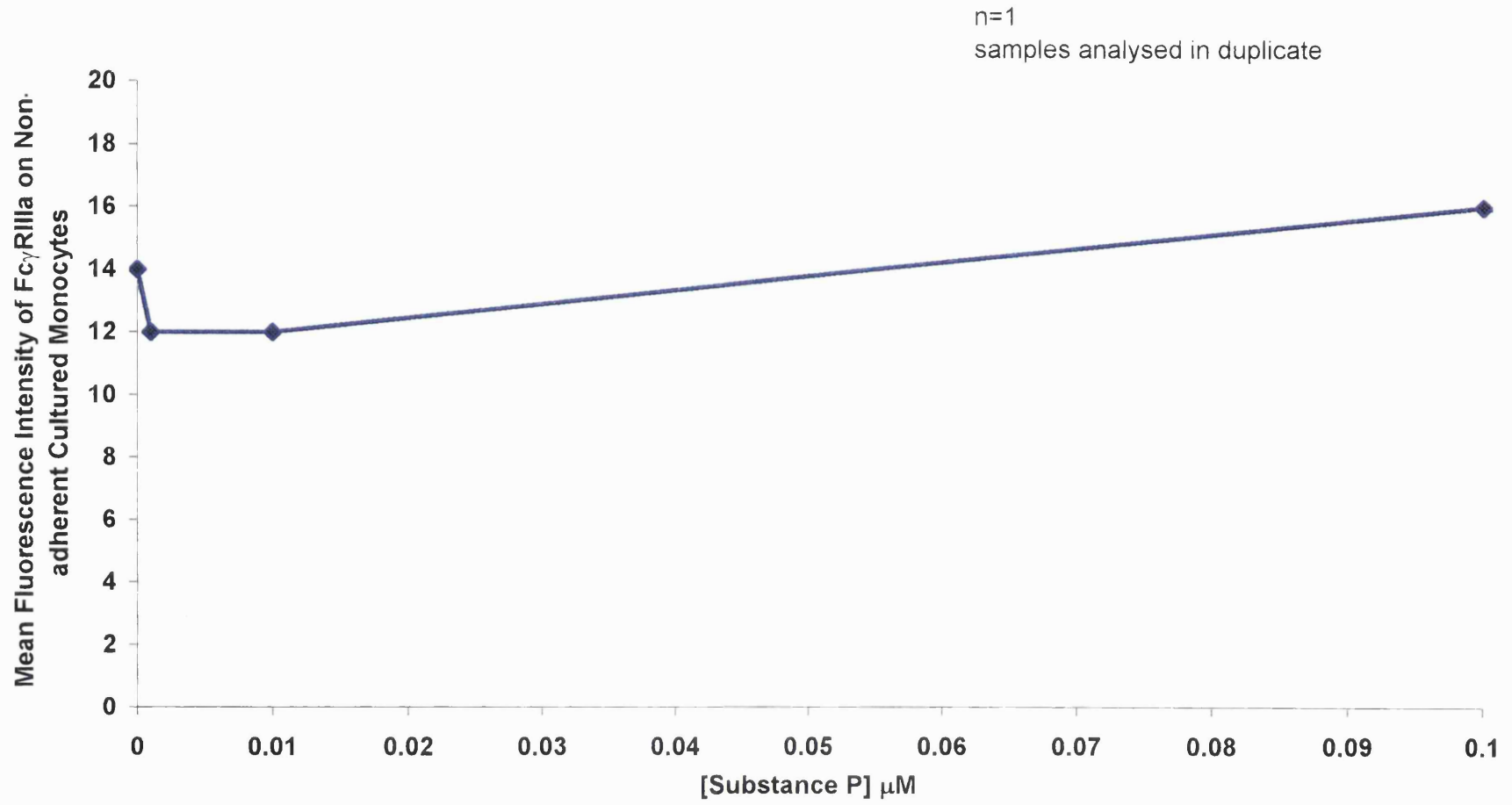
Table 3.5b: The Mean Fluorescence Intensity of Fc γ RIIIa on Cultured Non-adherent Monocytes with Different Concentrations of Substance P.

[Substance P] μM	Mean Fluorescence Intensity of Fc γ RIIIa on Cultured Non-adherent Monocytes
0	14
0.001	12
0.01	12
0.1	16
1	13
10	18

n=1

Samples analysed in duplicate

Figure 3.5b: The Mean Fluorescence Intensity of Fc γ R11a on Cultured Non-adherent Monocytes versus [Substance P] μ M



Substance P may increase FcγRIIIa expression on adherent monocytes by binding and signalling through the NK1 receptor. To investigate this as a putative mechanism in the up regulation of FcγRIIIa by substance P it would be necessary to conduct experiments in the presence of a NK1 receptor antagonist.

Monocytes that are cultured on a non-adherent substrate may not express the NK1 receptor. This would explain why an increase in FcγRIIIa expression on monocytes is not seen when monocytes are cultured with substance P on a non-adherent substrate. However, the experiments with non-adherent monocytes were only performed once. They would need to be repeated before definite conclusions could be drawn from the data.

To investigate this further it would be necessary to conduct experiments examining the expression of the NK1 receptor on freshly isolated monocytes and monocytes cultured on an adherent and non-adherent substrate.

EXPERMENTS 6a-d: HAEMOPOIETIC GROWTH FACTORS AND Fc γ RIIIa EXPRESSION ON CULTURED MONOCYTES

Introduction

M-CSF is produced constitutively from synovial fibroblasts (Seitz M, Loetscher P *et al*, 1994). GM-CSF is produced from activated fibroblasts (Lipsky P E, Davis L S *et al*, 1989) and macrophages (Munker R, Gasson J *et al*, 1986). Normal human monocytes cultured with M-CSF for 10 days have been reported to yield more Fc γ RIIIa positive cells than monocytes cultured with IL-3 or GM-CSF. This report made no comparison with untreated cultured monocytes. It is therefore difficult to draw a conclusion as to the ability of M-CSF to induce Fc γ RIIIa on monocytes. GM-CSF has an inhibitory effect on Fc γ RIIIa expression on normal human monocytes *in vitro* (Kruger M, Coorevitis T P M *et al*, 1996).

The aim of experiments 6a-d was to investigate the effect of haemopoietic growth factors M-CSF and GM-CSF on the expression of Fc γ RIIIa on cultured monocytes.

Methods

Peripheral blood monocytes were obtained as described on page 100. Monocytes were cultured on polystyrene (adherent) and polypropylene (non-adherent) substrates with different concentrations of M-CSF and GM-CSF. Fc γ RIIIa expression was then assessed using the techniques described on pages 105-107.

Experiment 6a was performed to investigate the effect of different concentrations of M-CSF on FcγRIIIa expression on monocytes cultured for 40 hours on an adherent substrate. Results from this experiment are shown in table and figure 3.6a.

Results

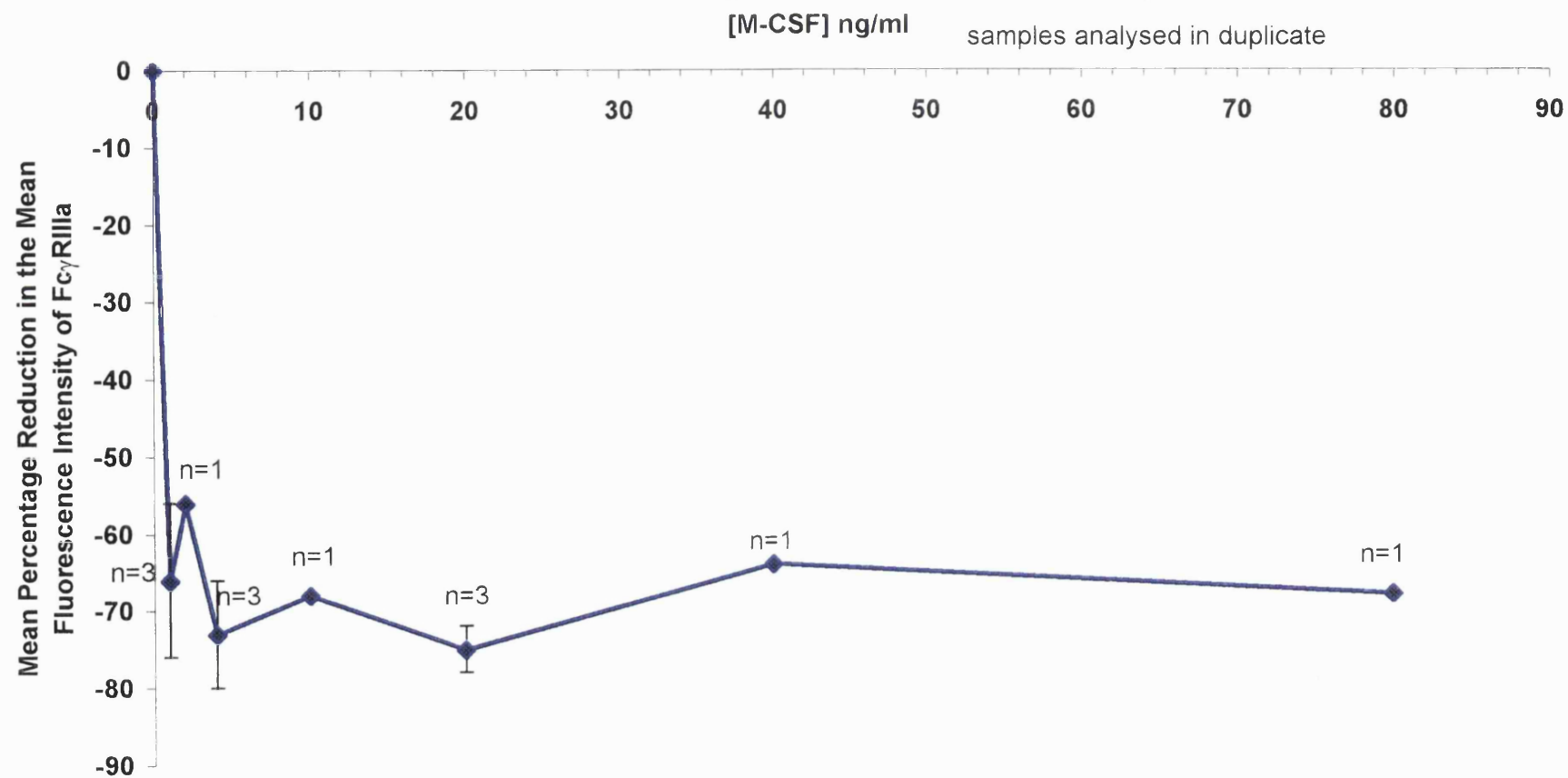
Table 3.6a: Mean Percentage Reduction in the Mean Fluorescence Intensity of FcγRIIIa on Cultured Adherent Monocytes With Different Concentrations of M-CSF.

[M-CSF] ng/ml	Mean Percentage Reduction in the Mean Fluorescence Intensity of FcγRIIIa +/- SEM on Cultured Adherent Monocytes
1	-66 +/- 10 n=3 (T ₀ 25, 43, 32; T ₄₀ 13, 8, 10)
2	-56 n=1 (T ₀ 25; T ₄₀ 11)
4	-73 +/- 7 n=3 (T ₀ 25, 43, 32; T ₄₀ 10, 8, 7)
10	-68 n=1 (T ₀ 25; T ₄₀ 8)
20	-75 +/- 3 n=3 (T ₀ 25, 43, 32; T ₄₀ 8, 7, 7)
40	-64 n=1 (T ₀ 25; T ₄₀ 9)
80	-68 n=1 (T ₀ 25; T ₄₀ 8)

n=number of experiments performed, samples analysed in duplicate
Baseline MFI values given in brackets respectively
T=Time with subscript denoting number of hours

M-CSF at a concentration of 1 ng/ml reduced the expression of FcγRIIIa on cultured adherent monocytes. This reduction plateaued on increasing the concentration of M-CSF further.

Figure 3.6a: Mean Percentage Reduction in the Mean Fluorescence Intensity of Fc γ R1IIa on Cultured Adherent Monocytes +/- SEM versus [M-CSF] ng/ml



Experiment 6b was performed to investigate the effect of different concentrations of M-CSF on FcγRIIIa expression on monocytes cultured on a non-adherent (polypropylene) substrate. Results from experiment 6b are shown in table and figure 3.6b.

Table 3.6b: Mean Reduction in the Mean Fluorescence Intensity of FcγRIIIa on Non-adherent Monocytes +/- SEM with Different Concentrations of M-CSF.

[M-CSF] ng/ml	Mean Reduction in the Mean Fluorescence Intensity of FcγRIIIa on Non-adherent Monocytes with Different Concentrations of M-CSF
1	-56 +/- 4 n=2 (T ₀ 29, 27; T ₄₀ 12, 13)
4	-58 +/- 15 n=2 (T ₀ 29, 27; T ₄₀ 12, 12)

n=number of experiments performed, samples analysed in duplicate
Baseline MFI values given in brackets respectively
T=Time with subscript denoting number of hours

M-CSF also reduced the expression of FcγRIIIa on monocytes cultured on a non-adherent substrate.

Experiment 6c was performed to investigate the *in vitro* effect of GM-CSF on FcγRIIIa expression on cultured adherent monocytes after an incubation period of 40 hours. The results from experiment 6c are shown in table and figure 3.6c.

Figure 3.6b: Mean Reduction in Mean Fluorescence Intensity of Fc γ R1IIa on Non-adherent Cultured Monocytes +/- SEM versus [M-CSF] ng/ml

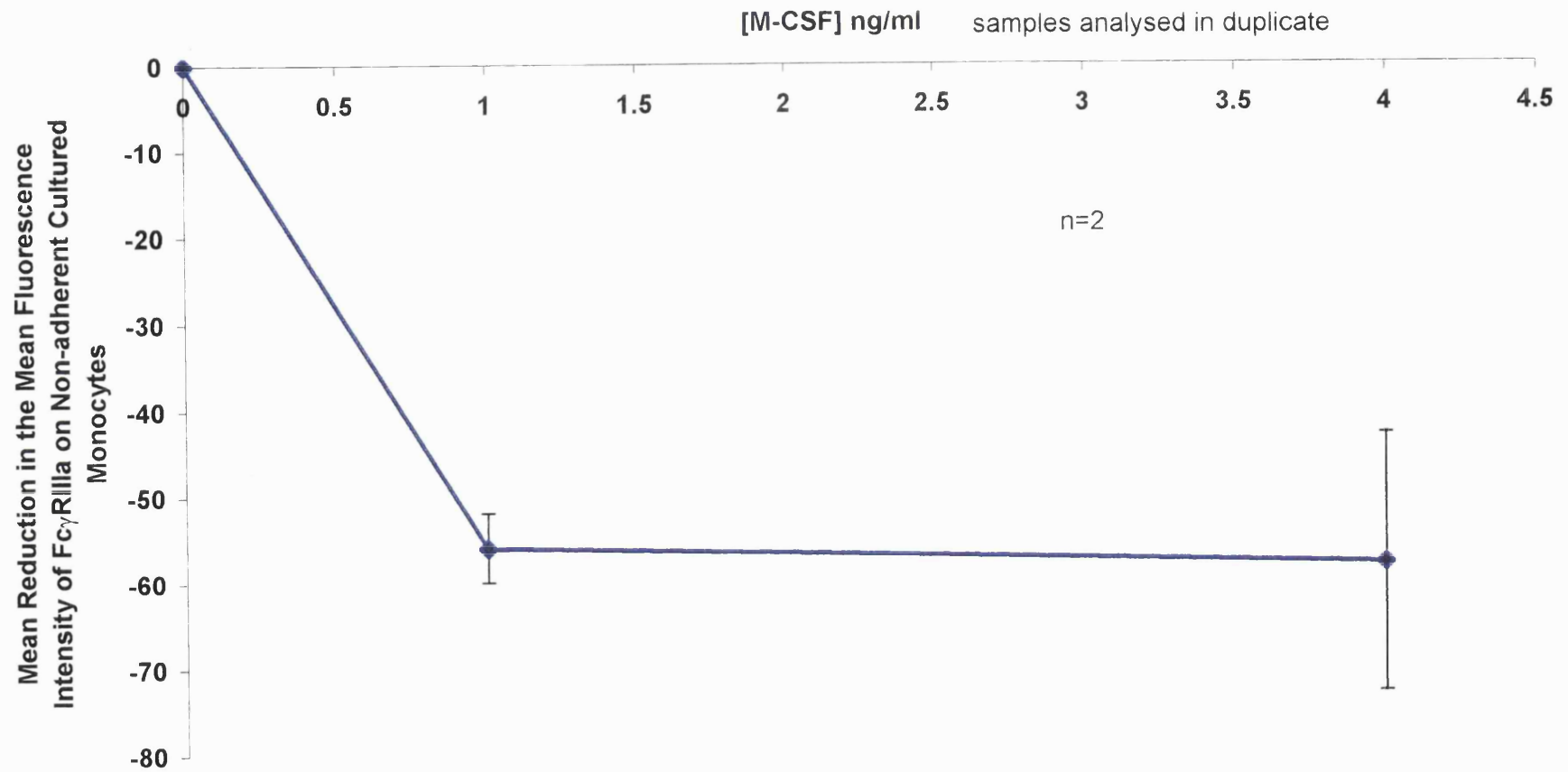


Table 3.6c: Mean Fluorescence Intensity of FcγRIIIa on Cultured Adherent Monocytes With Different Concentrations of GM-CSF.

[GM-CSF] ng/ml	Mean Fluorescence Intensity of FcγRIIIa on Cultured Adherent Monocytes
0	20
3	20
6	22
12	18
24	18
48	19

n=1, samples analysed in duplicate

Experiment 6d was performed to investigate the effect of GM-CSF on FcγRIIIa expression on monocytes cultured on a non-adherent substrate. Results from experiment 6d are shown in table and figure 3.6d.

Table 3.6d: Mean Fluorescence Intensity of FcγRIIIa on Cultured Non-adherent Monocytes With Different Concentrations of GM-CSF.

[GM-CSF] ng/ml	Mean Fluorescence Intensity of FcγRIIIa on Cultured Non-Adherent Monocytes
0	11
3	14
6	14
12	12
24	8
48	12

n=1, samples analysed in duplicate

Figure 3.6c: Mean Fluorescence Intensity of Fc γ R11a versus [GM-CSF] ng/ml

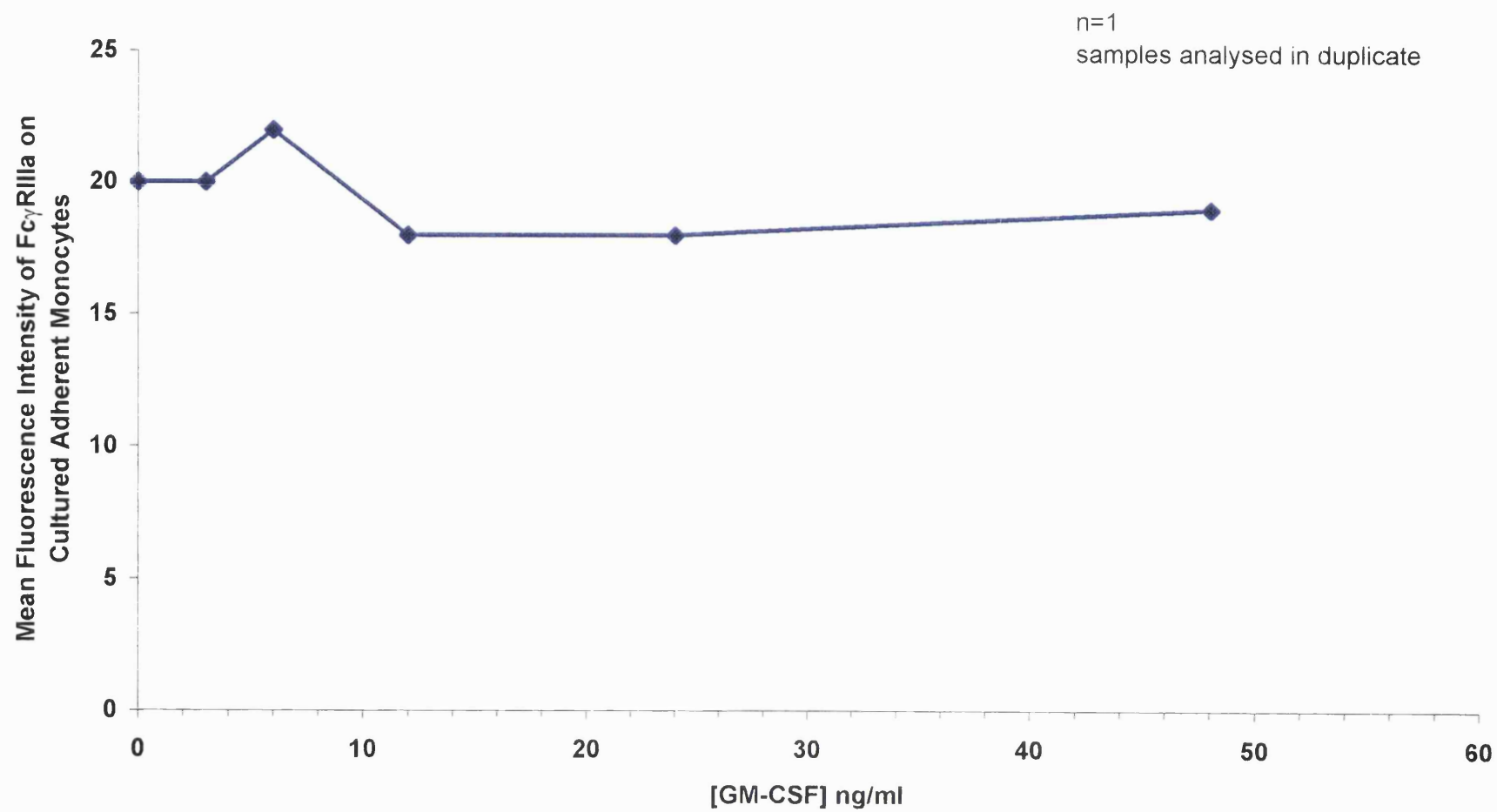
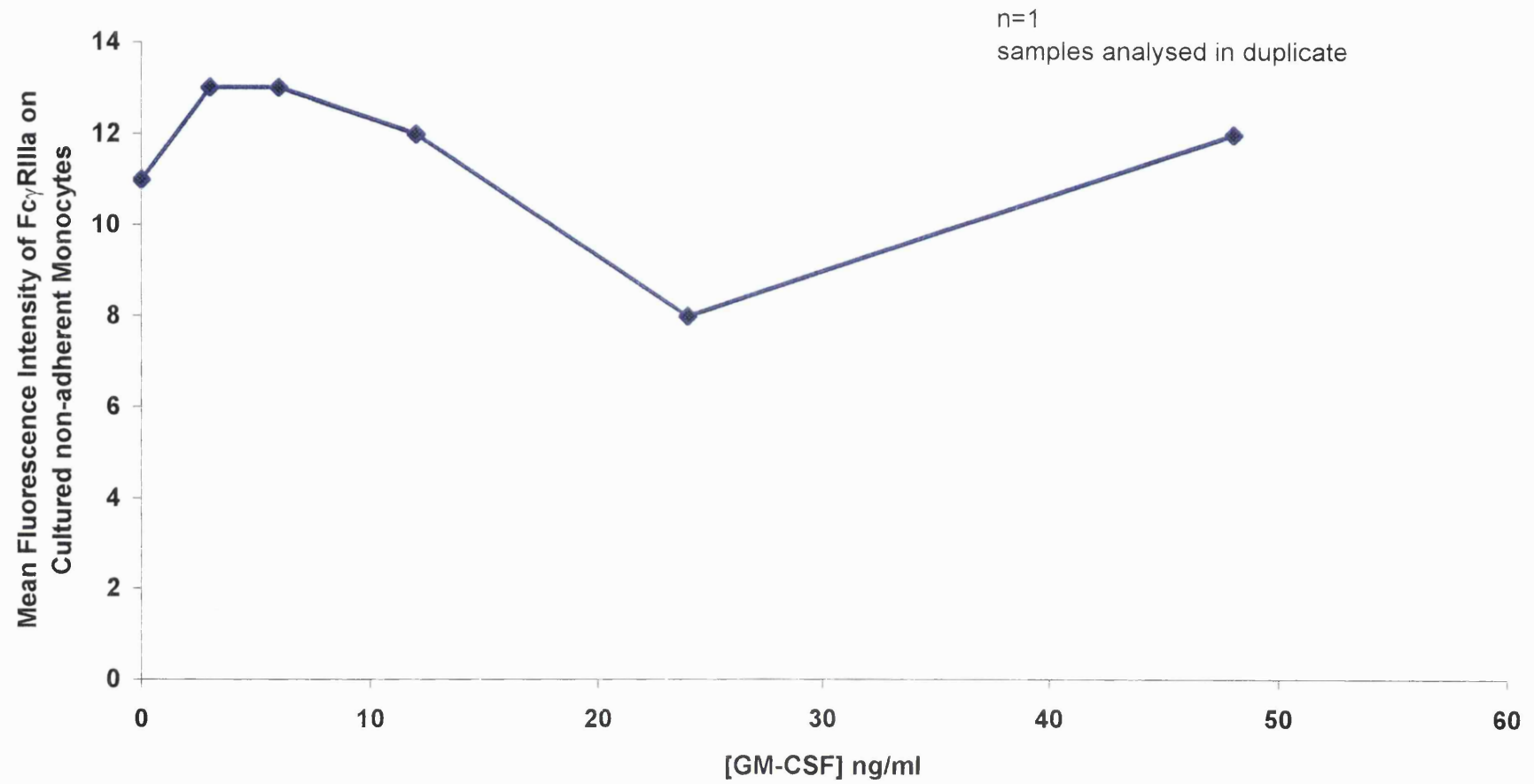


Figure 3.6d: Mean Fluorescence Intensity of Fc γ R11a on Cultured Non-adherent Monocytes versus [GM-CSF] ng/ml



GM-CSF does not upregulate FcγRIIIa expression on cultured monocytes both on an adherent and non-adherent substrate.

Neither M-CSF nor GM-CSF up regulate FcγRIIIa expression on cultured monocytes. It is therefore unlikely that they are responsible for the induction of FcγRIIIa on synovial macrophages *in vivo*.

EXPERIMENTS 7a AND b: THE ROLE OF EXTRACELLULAR MATRIX IN FcγRIIIa EXPRESSION ON CULTURED MONOCYTES

Introduction

Synovial macrophages are surrounded by a complex organization of extracellular matrix proteins. The extracellular matrix proteins found in the synovial intima where FcγRIIIa is preferentially expressed (Edwards J C W, Blades S *et al*, 1997) are different from those of the subintima.

Evidence supporting the role of extracellular matrix proteins in the induction of FcγRIIIa comes from López-Moratello *et al*, 1995. He demonstrated that an increased percentage of human peripheral blood monocytes cultured in the presence of fibronectin or a peptide of fifteen amino acids (found in the extracellular matrix proteins fibronectin, laminin and type IV collagen) expressed FcγRIIIa compared to control monocyte cultures.

The extracellular matrix proteins that are found in synovium are discussed in more detail in pages 46-55.

The aim of experiments 7a and b are to investigate the effect of different matrix proteins on the expression of FcγRIIIa on cultured monocytes.

Methods

Peripheral blood monocytes were obtained as described on page 100. Six-well plates coated in fibronectin, laminin, collagen I and collagen IV were used for monocyte cultures to investigate the effect of the relevant synovial extracellular matrix proteins on the expression of Fc γ RIIIa. Monocytes were cultured for forty-five hours. IL-10, TGF β ₁ separately and in combination with each other were added to monocyte cultures on different extracellular matrix proteins. These experiment were performed to investigate the effect of IL-10 and TGF β ₁ on Fc γ RIIIa expression on monocytes adherent to matrix proteins that are present in the synovium where Fc γ RIIIa is selectively expressed. Fc γ RIIIa expression was assessed as described on pages 105-107.

Results

Experiment 7a was performed to investigate Fc γ RIIIa expression on cultured monocytes adherent to a polystyrene substrate, a fibronectin substrate, a laminin substrate, a collagen IV substrate and a collagen I substrate with and without the addition of TGF β ₁ after a 45 hour incubation. Results from experiment 7a are shown in table and figure 3.7a.

Table 3.7a: The Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes on a Polystyrene, Collagen I, Collagen IV, Laminin and Fibronectin Substrates With and Without the Addition of TGFβ₁ (10 ng/ml).

Substrate	Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes	
	Without TGFβ ₁	With TGFβ ₁ (10 ng/ml)
Polystyrene	127	51
Fibronectin	122	47
Laminin	147	49
Collagen I	143	46
Collagen IV	145	47

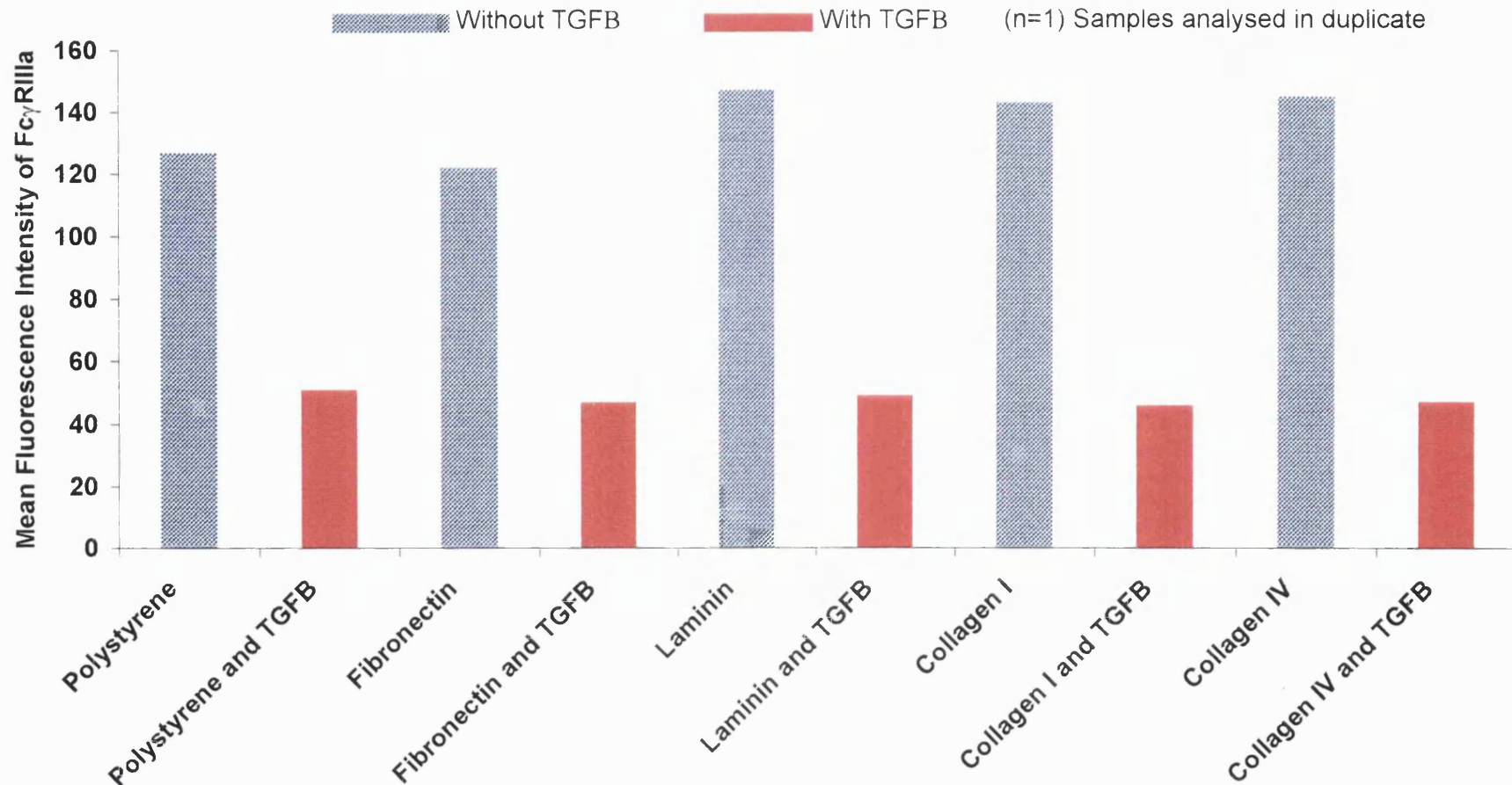
n=1, samples analysed in duplicate

The level of FcγRIIIa expression on cultured monocytes on different matrix proteins is similar. Therefore it does not appear that FcγRIIIa is selectively up regulated by matrix proteins that are found in synovial tissue.

TGFβ₁ uniformly suppressed the level of FcγRIIIa expression on cultured monocytes regardless of the substrate. This shows that the ability of TGFβ₁ to suppress FcγRIIIa is independent of the substrate that monocytes are adherent to.

Experiment 7b was performed to investigate FcγRIIIa expression on cultured monocytes adherent to laminin, fibronectin, polystyrene, collagen I and collagen IV with and without the addition of IL-10 (10 ng/ml) and also with the addition of both

Figure 3.7a: Mean Fluorescence of Fc γ R1IIa on Cultured Monocytes versus Different Substrates With and Without the Addition of TGF β (10 ng/ml)



IL-10 and TGF β ₁ (10 ng/ml) after an incubation period of fifty six hours. Results from experiment 7b are shown in table and figure 3.7b.

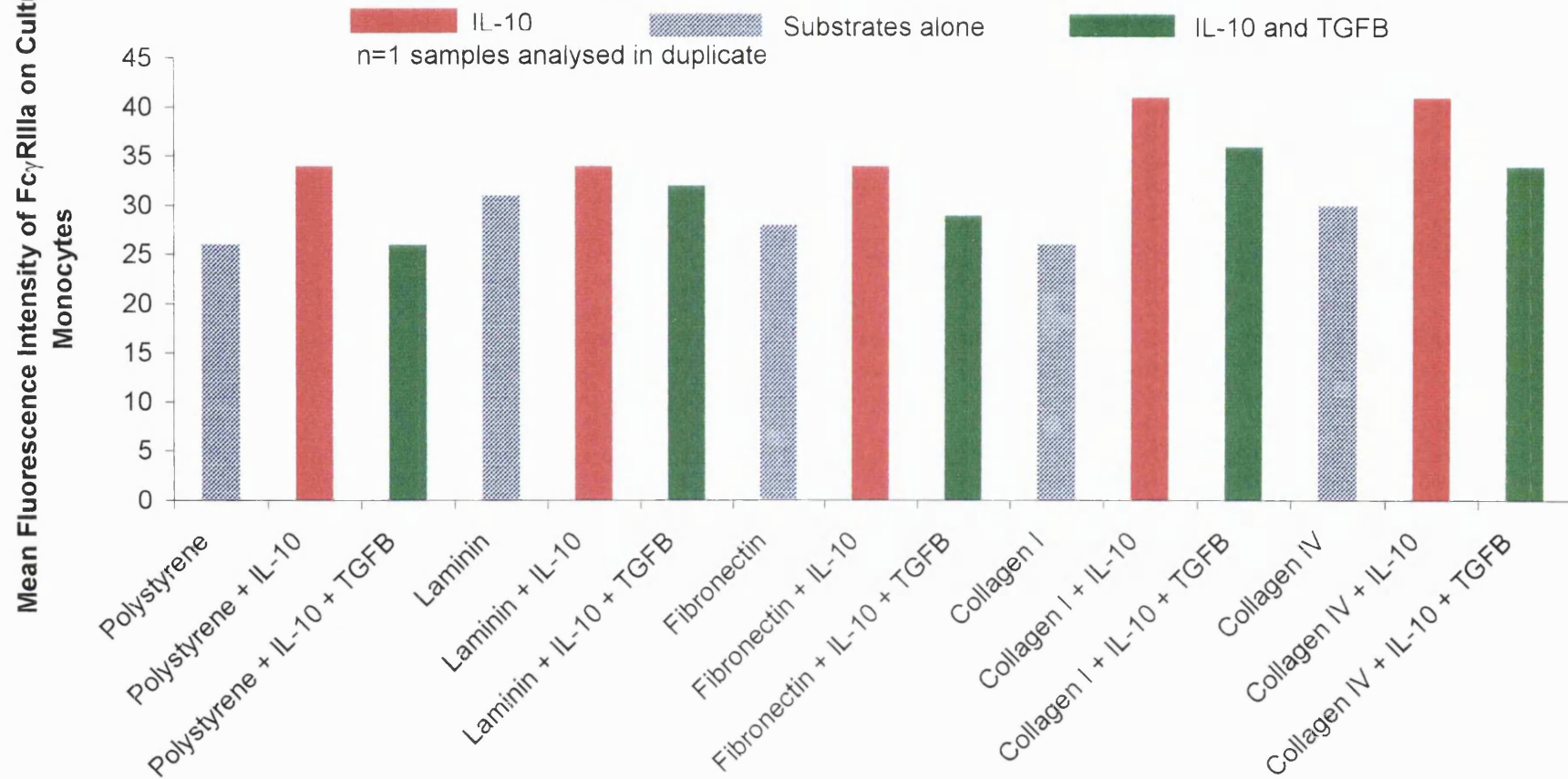
Table 3.7b: The Mean Fluorescence Intensity of Fc γ RIIIa on Cultured Monocytes Adherent to Laminin, Fibronectin, Polystyrene, Collagen I and Collagen IV With and Without the Addition of IL-10 and both IL-10 and TGF β ₁

Substrate	Mean Fluorescence Intensity of Fc γ RIIIa on Cultured Monocytes		
	No Cytokines	IL-10	IL-10 and TGF β ₁
Polystyrene	26	34	26
Laminin	31	34	32
Fibronectin	28	34	29
Collagen I	26	41	36
Collagen IV	30	41	34

n=1, samples analysed in duplicate

IL-10 induces the expression of Fc γ RIIIa on cultured monocytes. The degree of induction of Fc γ RIIIa on cultured monocytes is more or less the same with different substrates. This demonstrates that the induction of Fc γ RIIIa on cultured monocytes by IL-10 is independent of the substrate. The addition of TGF β ₁ either completely or partially abrogates the induction of Fc γ RIIIa on cultured monocytes that is seen with IL-10 alone.

Figure 3.7b: The Mean Fluorescence Intensity of Fc γ R11a on Cultured Monocytes versus Polystyrene, Collagen I, Collagen IV, Laminin and Fibronectin Substrates With and Without the Addition of IL-10 (10 ng/ml) and both IL-10 and TGFB



EXPERIMENT 8a AND b: MECHANICAL STRETCH AND FcγRIIIa EXPRESSION ON CULTURED MONOCYTES

Introduction

In rheumatoid arthritis synovitis is reduced in the joints of paralysed extremities as for example following a cerebro-vascular event (Smith R D, 1979; Bland J H, Eddy W M, 1968). Immobilization of joints by casting or splinting also results in a reduction in synovitis (Harris R, Copp E P, 1962; Partridge R E H, Duthie J J R, 1963; Gault S J, Spyker J M, 1969). These clinical observations suggest that a mechanical mechanism may modulate the clinical features of rheumatoid arthritis.

FcγRIIIa is expressed on normal intimal synovial macrophages and on macrophages in the dermis that have been exposed to mechanical stretch (Edwards J C W, Blades S *et al*, 1997). The sites in the body that are affected by rheumatoid arthritis including the extra-articular sites are all places that are subject to mechanical stretch or fluid flow. This supports the clinical observations that mechanical stretch or shear forces may have a role in the pathogenesis of rheumatoid disease.

The stimulus for FcγRIIIa expression on tissue macrophages in the synovium and mechanically stretched dermis may be mechanical stretch. The exact nature and degree of mechanical stretch that synovium is exposed to has not been identified.

The aim of experiments 8a and b are to investigate the effect of mechanical stretch on FcγRIIIa expression on cultured monocytes.

Methods

Peripheral blood monocytes were obtained as described on page 100. In all the following experiments monocytes were stretched approximately 10 percent of their length. This was the maximum stretch that cultured monocytes tolerated. The frequency of stretch in all the experiments was 0.004 Hz (stretch 2 minutes and relaxation 2 minutes). The method used to stretch cultured monocytes is described in more detail on pages 102-104. FcγRIIIa expression was assessed as described on pages 105-107.

Results

Experiment 8a was performed to investigate the effect of mechanical stretch on FcγRIIIa expression on monocytes cultured on a collagen I substrate. Monocytes were subject to mechanical stretch for a duration of twenty to seventy hours. FcγRIIIa expression was compared to cultured monocytes not subject to mechanical stretch for the same period of time. Results from experiment 8a are shown in table and figure 3.8a.

Table 3.8a: The Mean Percentage Change of the Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes Adherent to a Collagen I Substrate +/- SEM Undergoing Different Periods of Stretch and No Stretch.

Duration of Culture (Hours)	Mean Percentage Change of the Mean Fluorescence Intensity of FcγRIIIa on Cultured Adherent Monocytes +/- SEM	
	Stretched	No Stretch
20	-0.3 +/- 22 n=2 (T ₀ 7, 9 T ₂₀ 10, 7.7)	-10 +/- 12 n=2 (T ₀ 7, 9; T ₂₀ 8, 7.7)
24	-15 +/- 10 n=3 (T ₀ 17, 6, 6; T ₂₄ 15, 4, 6)	-4 +/- 7 n=3 (T ₀ 17, 6, 6; T ₂₄ 6, 6, 5)
48	146 +/- 10* n=2 (T ₀ 11, 11; T ₄₈ 28, 26)	118 +/- 0* n=2 (T ₀ 11, 11; T ₄₈ 24, 24)
72	-9 n=1 (T ₀ 11; T ₇₂ 10)	64 n=1 (T ₀ 11; T ₇₂ 18)

n=number of experiments performed, samples analysed in duplicate *p= >0.05

Baseline MFI values given in brackets respectively

T=Time with subscript denoting number of hours

Mechanical stretching of cultured monocytes on a collagen I substrate does not result in an increase in the level of FcγRIIIa expression in comparison to non-stretched cultured monocytes over a time course from twenty to seventy two hours.

Experiment 8b was performed to investigate FcγRIIIa expression on cultured stretched and non-stretched monocytes adherent to a fibronectin substrate with different durations of culture. Results from experiment 8b are shown in table and figure 3.8b.

Figure 3.8a: Mean Percentage Change of the Mean Fluorescence Intensity of FcγR11a on Cultured Stretched and Non-stretched Monocytes Adherent to a Collagen I Substrate +/- SEM versus Duration of Culture (Hours).

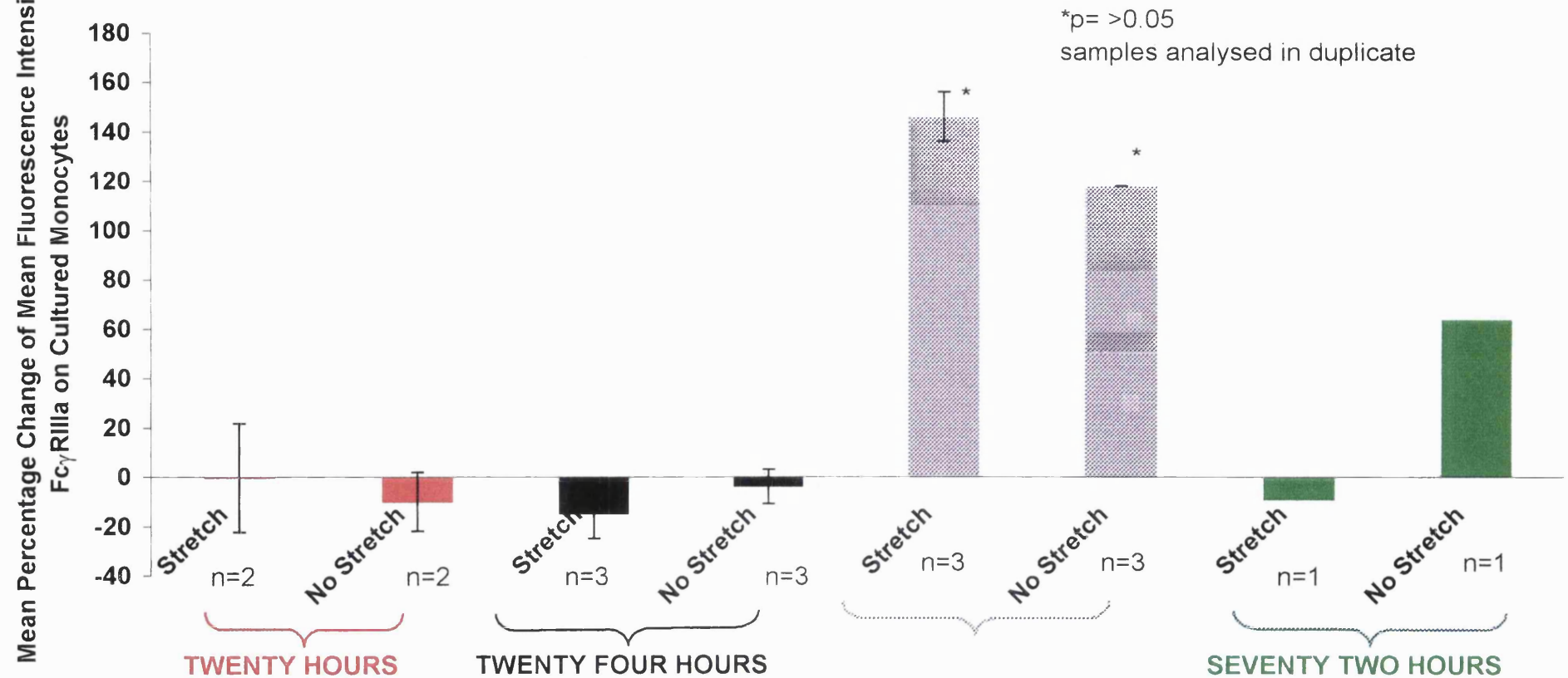


Table 3.8b: The Mean Percentage Change of the Mean Fluorescence Intensity of FcγRIIIa on Cultured Stretched and Non Stretched Monocytes Adherent to a Fibronectin Substrate +/- SEM With Different Durations of Culture

Duration of Culture (Hours)	Mean Percentage Change of the Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM	
	Stretched	No Stretch
48	14 +/- 0 n=2 (T ₀ 7, 7; T ₄₈ 8, 8)	43 +/- 0 n=2 (T ₀ 7, 7; T ₄₈ 10,10)
72	93 +/- 36 n=2 (T ₀ 7, 7; T ₇₂ 11,16)	157 +/- 0 n=2 (T ₀ 7, 7; T ₇₂ 18,18)

n=number of experiments performed, samples analysed in duplicate

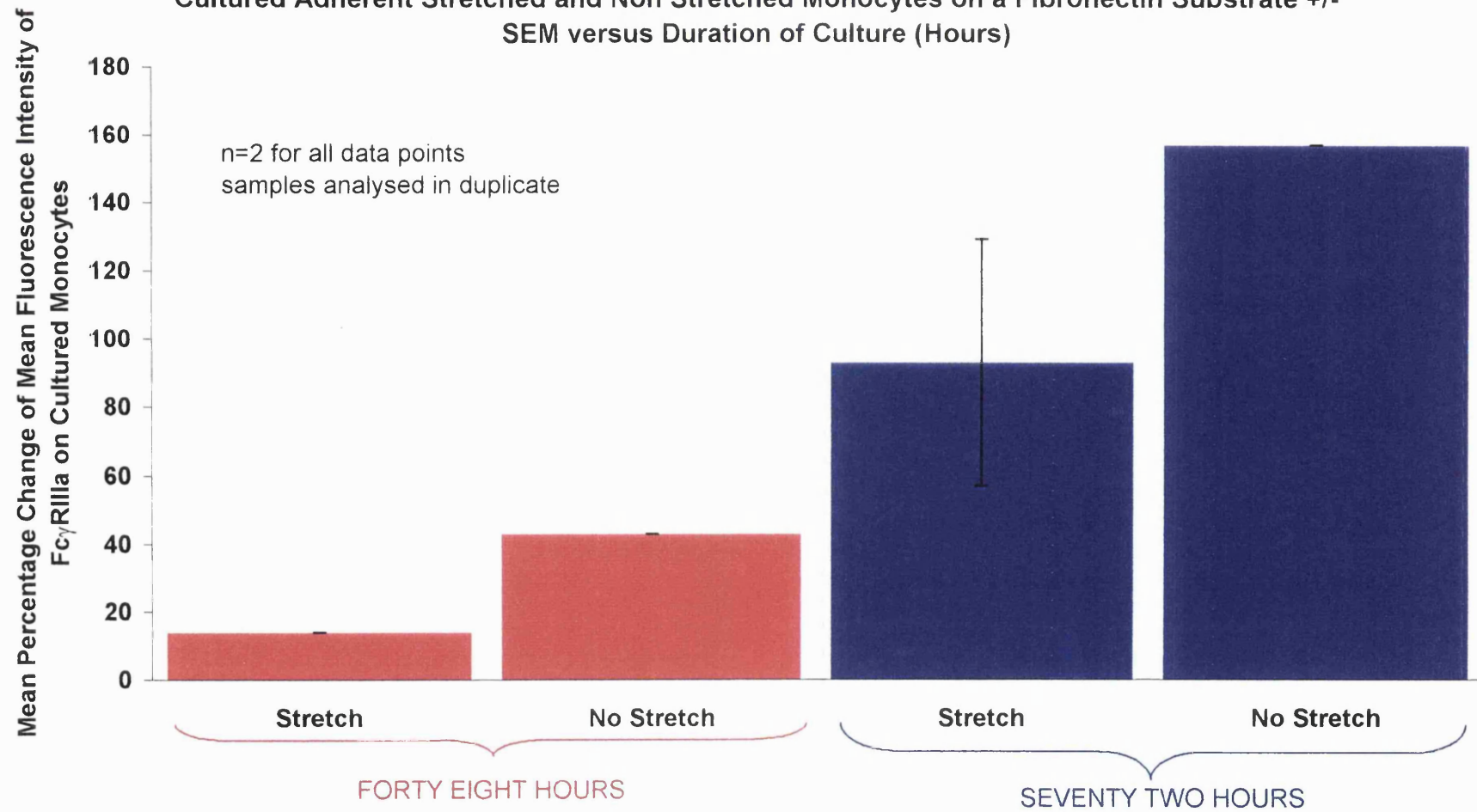
Baseline MFI values given in brackets respectively

T=Time with subscript denoting number of hours

Monocytes subject to mechanical stretch on a fibronectin substrate did not express increased levels of FcγRIIIa compared to non-stretched monocytes for incubation periods of both forty-eight and seventy two hours.

Stretching of monocytes *in vitro* does not result in the up regulation of FcγRIIIa. However clinical observations of rheumatoid disease and the presence of FcγRIIIa on synovium and mechanically stretched dermis suggest a mechanical mechanism in the induction of FcγRIIIa. The mechanical stretch that cultured monocytes were subject to in the above experiments may not accurately mimic physiological stretch. The frequency, degree of elongation and duration of exposure to stretch may be different *in vivo*. These *in vivo* factors are not known. It would be necessary to accurately reproduce *in vivo* conditions of stretch in an *in vitro* system to fully investigate the role of mechanical stretch in the induction of FcγRIIIa. Other forces may be important such as fluid flow or shear forces in the induction of FcγRIIIa.

Figure 3.8b: The Mean Percentage Change of the Mean Fluorescence Intensity of FcγRIIIa on Cultured Adherent Stretched and Non Stretched Monocytes on a Fibronectin Substrate +/- SEM versus Duration of Culture (Hours)



The data from experiments 8a and b suggests that mechanical stretch is not responsible for the induction of FcγRIIIa but the *in vivo* characteristics of stretch may be different from those that are used *in vitro*. Thus mechanical stretch cannot be entirely ruled out as a factor in the induction of FcγRIIIa on monocytes *in vivo*.

EXPERIMENTS 9a-d: THE EFFECT OF SODIUM AUROTHIOMALATE, METHOTREXATE AND HYDROCORTISONE ON FcγRIIIa EXPRESSION ON CULTURED MONOCYTES

Methotrexate

Introduction

Approximately 8 percent of patients commenced on methotrexate for rheumatoid arthritis develop accelerated nodulosis (Kerstens P J S M, Boerbooms A M T *et al*, 1991). Other extra-articular manifestations of rheumatoid arthritis in the form of nailfold lesions, cutaneous ulcers, pleuritis and pericarditis have also been reported with the onset of methotrexate therapy (Abu-Shakra, Nicol P *et al*, 1993). Nodules regress when methotrexate therapy is stopped and recur after rechallange with methotrexate (Segal R, Caspi D *et al*, 1988).

A possible explanation of an increase in appearance of extra-articular manifestations in some patients taking methotrexate might be explained by an up regulation of FcγRIIIa on tissue macrophages. *In vitro* experiments have demonstrated that methotrexate increases the expression of FcγRIIIa on monocytic U937 cells (Seitz M, Zwicker M *et al*, 1998).

The mechanism by which methotrexate may up regulate FcγRIIIa on tissue macrophages may involve IL-10 or adenosine. IL-10 transcription is increased by methotrexate and Calzada-Wack *et al*, (1996) have found IL-10 selectively induces

FcγRIIIa on cultured human monocytes. Methotrexate also increases intra and extracellular adenosine. Adenosine acting through an adenosine receptor may be responsible for inducing FcγRIIIa on monocytes.

The aim of experiment 9a was to investigate whether methotrexate up regulates the expression of FcγRIIIa on human peripheral blood monocytes *in vitro*.

Methods

In experiment 9a peripheral blood monocytes were obtained as described on page 100. Monocytes were cultured on a polystyrene substrate with methotrexate at 10^{-8} and 10^{-7} Molar. After forty hours of culture FcγRIIIa expression was assessed as described on pages 105-107. Results from experiment 9a are shown in table and figure 3.9a.

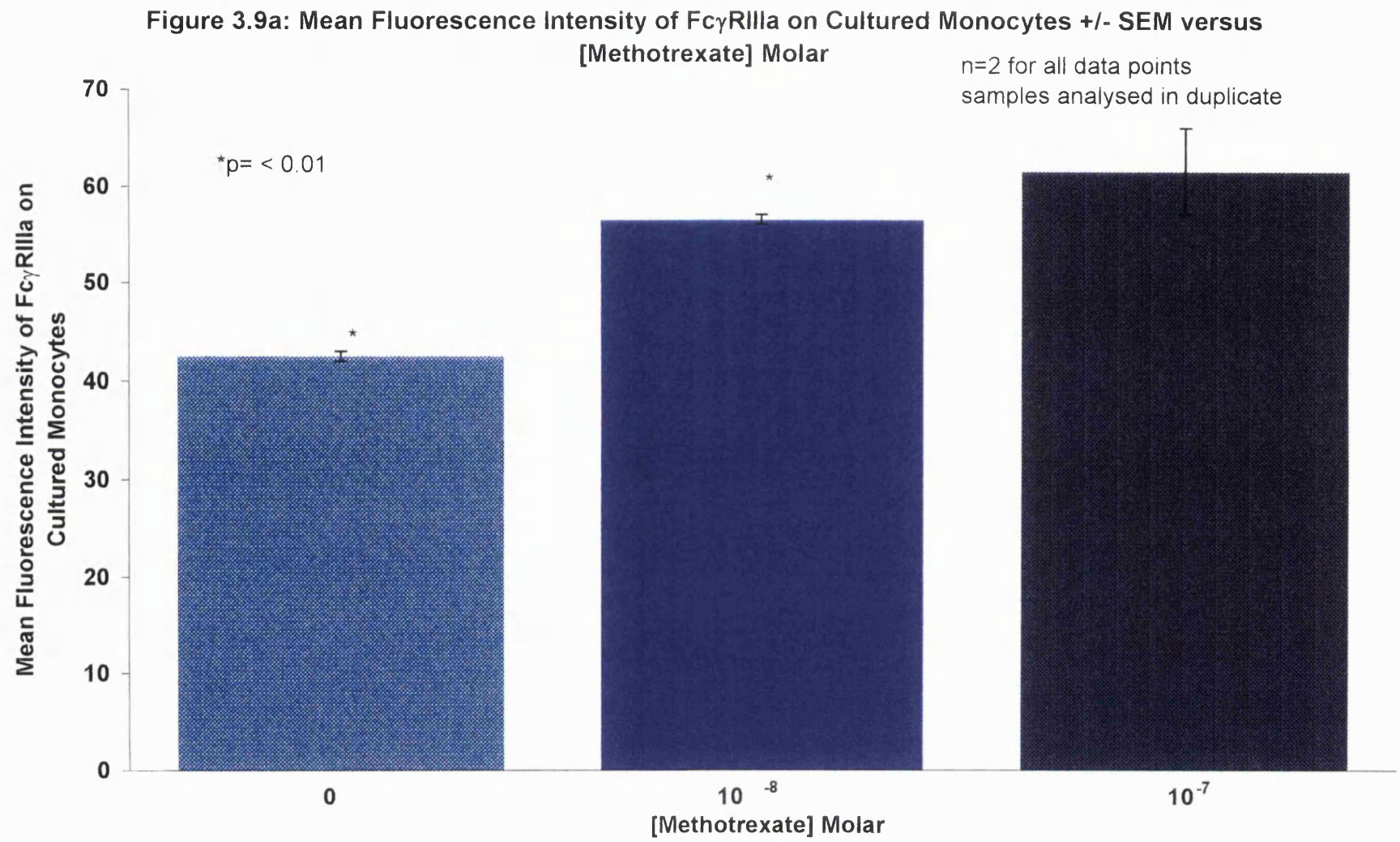
Results

Table 3.9a: The Mean Fluorescence Intensity of FcγRIIIa +/- SEM on Monocytes

Cultured With and Without Methotrexate (10^{-7} M and 10^{-8} M).

Concentration of Methotrexate (Molar)	Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM
0	42.50 +/- 0.5
10^{-8}	56.5 +/- 0.5*
10^{-7}	61.5 +/- 6.4

n=2, samples analysed in duplicate *p= < 0.01



Methotrexate increases the expression of FcγRIIIa on cultured monocytes. This data was not consistently reproducible with different samples of peripheral blood monocytes. This suggests that monocytes from some patients may increase their expression of FcγRIIIa in response to methotrexate. This selective response to methotrexate may suggest why only approximately 8 percent of patients commenced on methotrexate develop accelerated nodulosis.

To investigate the mechanism by which methotrexate increases the expression of FcγRIIIa on cultured monocytes it would be necessary to conduct experiments in the presence of neutralising antibodies to IL-10 and adenosine receptor antagonists. This would identify if the up regulation of FcγRIIIa was secondary to increased levels of IL-10 or adenosine.

Experiment 9b was performed to investigate the effect of adenosine (A) receptor agonists on FcγRIIIa expression on cultured monocytes. Monocytes were obtained as described on page 100 and cultured with A1 and A2 receptor agonists. FcγRIIIa expression was compared to monocytes cultured in the absence of an adenosine receptor agonist. FcγRIIIa expression was assessed as described on pages 105-107. Results from experiment 9b are shown in table and figure 3.9b.

Table 3.9b: The Mean Fluorescence Intensity of FcγRIIIa +/- SEM on Monocytes Cultured With an A1 or A2 receptor agonist (10⁻⁶ M) and with no adenosine agonist.

	Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM
No adenosine agonist	71 +/- 4.3
A1 agonist (10 ⁻⁶) M	75 +/- 2.2
A2 agonist (10 ⁻⁶) M	67 +/- 0.30

n=2, samples analysed in duplicate

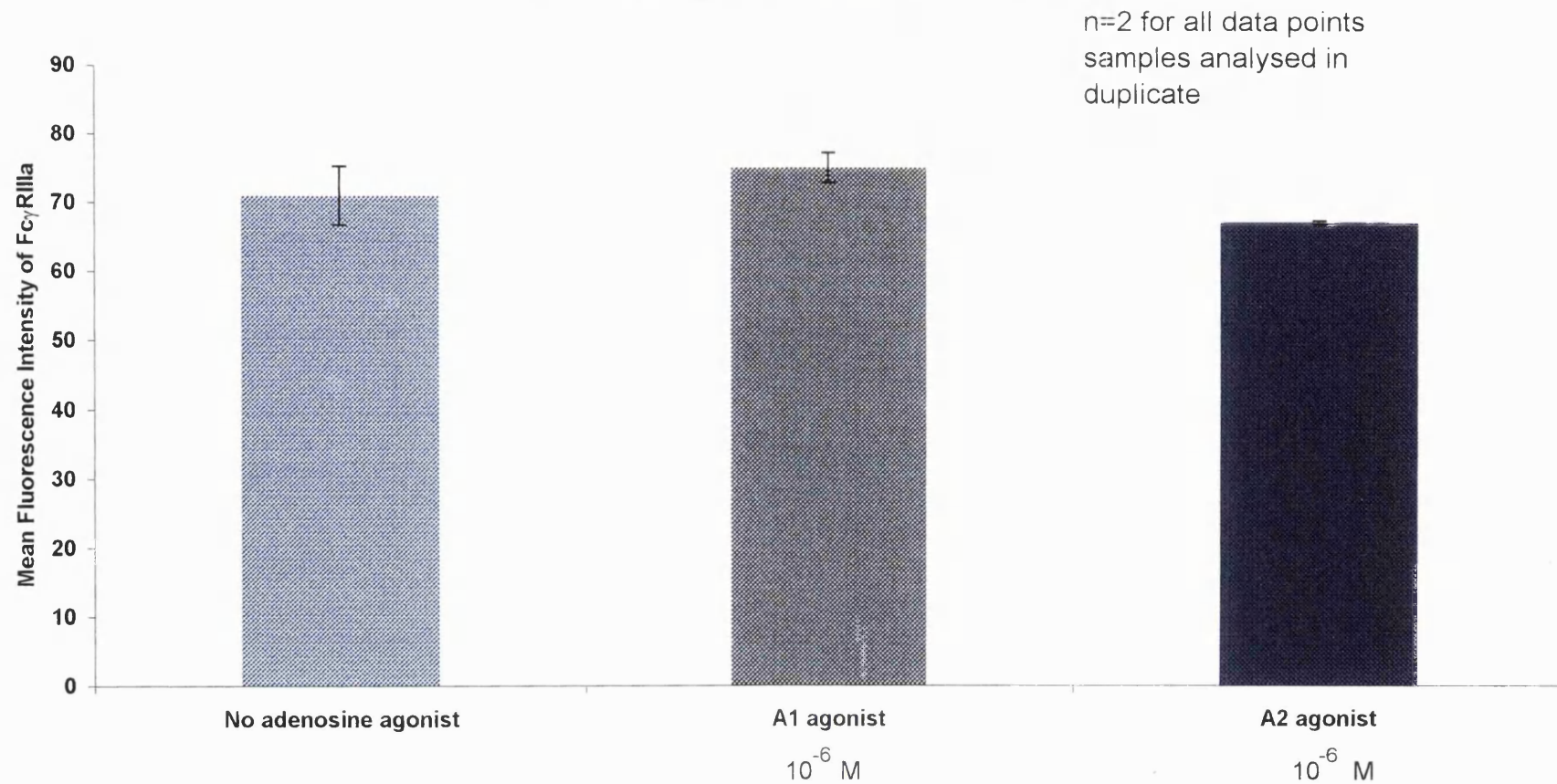
Adenosine receptor agonists did not up regulate the expression of FcγRIIIa on cultured monocytes. This data suggests that IL-10 may be the mechanism by which methotrexate up regulates FcγRIIIa expression on cultured monocytes.

Sodium Aurothiomalate

Introduction

During active chrysotherapy the presence of gold can be demonstrated within tissue macrophages including those in the synovial intima (Vernon-Roberts B, Doré J L *et al*, 1976). Litman B *et al*, (1984) have demonstrated that gold salts impair monocyte-macrophage function (page 78-79). The beneficial effect of gold salts in rheumatoid disease may result from its ability to impair the expression of FcγRIIIa on tissue monocyte-macrophages.

Figure 3.9b: Mean Fluorescence Intensity of Fc γ R11a on Cultured Monocytes +/- SEM versus A1/A2 Agonist Molar and no agonist



Experiment 9c was performed to investigate the effect of sodium aurothiomalate on FcγRIIIa expression on cultured monocytes.

Methods

In experiment 9c peripheral blood monocytes were obtained as described on page 82. Monocytes were cultured on a polystyrene substrate with different concentrations of sodium aurothiomalate. After forty hours of culture FcγRIIIa expression was assessed as described on pages 105-107. Results from experiment 9c are shown in table and figure 3.9c.

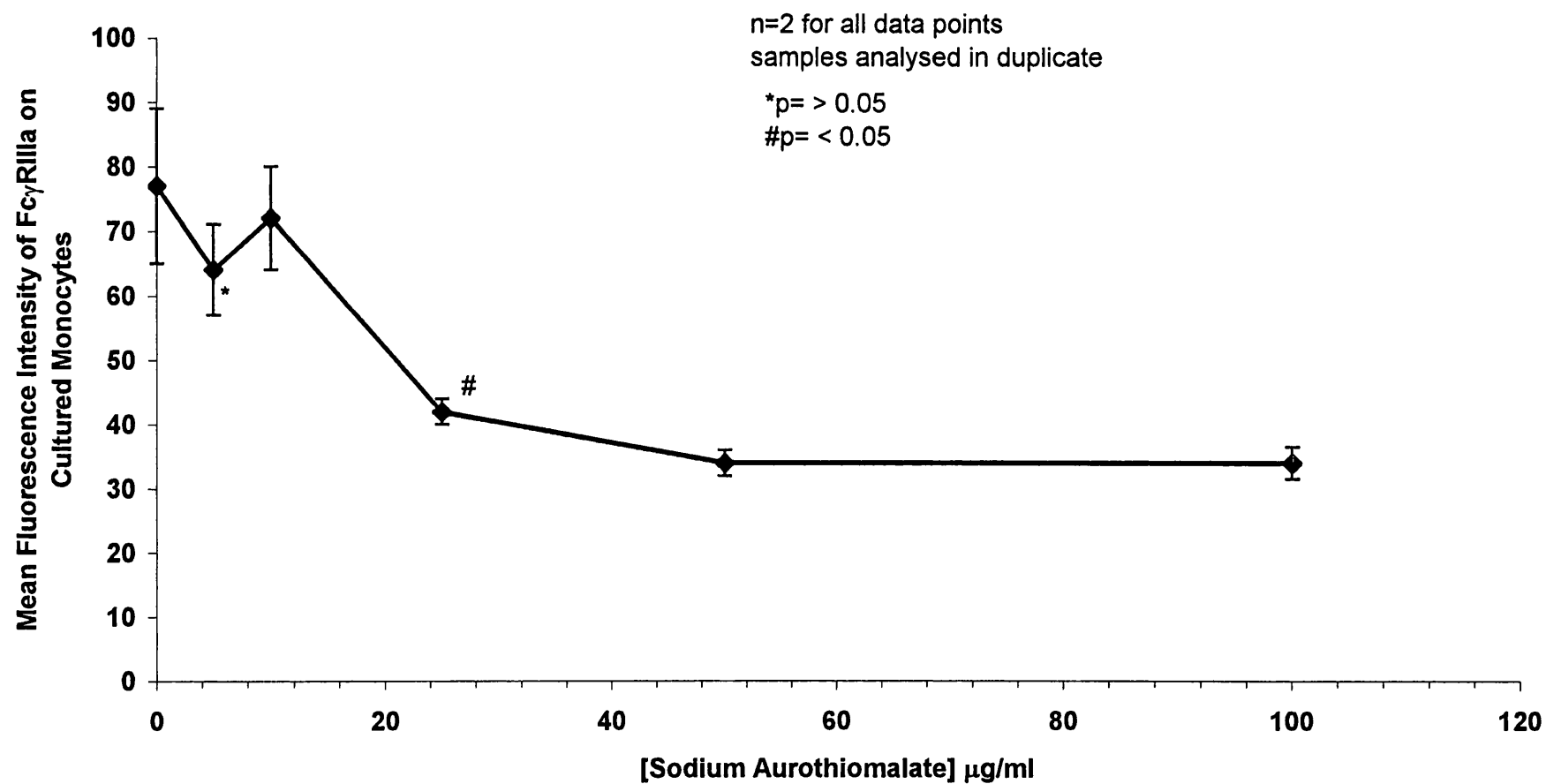
Results

Table 3.9c: Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM With Different Concentrations of Sodium Aurothiomalate

[Sodium Aurothiomalate] µg/ml	Mean Fluorescence Intensity Of FcγRIIIa +/- SEM on Cultured Monocytes
0	77 +/- 12
5	64 +/- 7*
10	72 +/- 8
25	42 +/- 2#
50	34 +/- 2
100	34 +/- 3

n=2, samples analysed in duplicate *p= >0.05 #p= <0.05

Figure 3.9c: Mean Fluorescence Intensity of Fc γ R11a on Cultured Monocytes +/-SEM versus [Sodium Aurothiomalate] μ g/ml



The serum steady state concentration of sodium aurothiomalate in a patient taking 50 mg of sodium aurothiomalate a week via an intramuscular route is approximately 5 µg/ml (personal communication from Rhône-Poulenc Rorer). Five micrograms per millilitre of sodium aurothiomalate results in only a slight reduction in FcγRIIIa expression *in vitro* ($p = > 0.05$). The reduction of FcγRIIIa continues up to a concentration of 25 µg/ml ($p = < 0.05$). Increasing the concentration of sodium aurothiomalate further results in a plateauing of the suppression of FcγRIIIa on cultured monocytes.

The above data demonstrates suppression of FcγRIIIa on cultured monocytes. However at a steady state concentration the suppression of FcγRIIIa is fairly modest. In addition to the reduction of FcγRIIIa gold salts may be able to reduce the release of pro-inflammatory cytokines from tissue macrophages in rheumatoid disease. The above data supports the belief that the efficacy of gold salts in rheumatoid disease may in part be secondary to their ability to reduce FcγRIIIa expression on monocytes.

Further experiments would be needed to investigate a possible reduction in the production of pro-inflammatory cytokines by macrophages treated with gold salts.

Hydrocortisone

Introduction

The effect of glucocorticoids on FcγRIIIa expression by tissue macrophages is not known. Glucorticoids activate latent TGFβ. Welch G R *et al*, (1990) have shown that TGFβ selectively induces FcγRIIIa on cultured monocytes. Glucocorticoids may therefore be expected to induce FcγRIIIa on monocytes *in vitro*.

Experiment 9d was performed to investigate the effect of hydrocortisone on FcγRIIIa expression on monocytes *in vitro*.

Methods

In experiment 9d peripheral blood monocytes were obtained as described on page 100. Monocytes were cultured on a polystyrene substrate with different concentrations of hydrocortisone. After forty hours of culture FcγRIIIa expression was assessed as described on pages 105-107. Results from experiment 9d are shown in table and figure 3.9d.

Results

Table 3.9d: The Mean Percentage Change of the Mean Fluorescence Intensity of FcγRIIIa on Monocytes +/- SEM Cultured With Different Concentrations of Hydrocortisone (Molar).

[Hydrocortisone] Molar	Mean Percentage Change of Mean Fluorescence Intensity of FcγRIIIa +/- SEM on Cultured Monocytes
10^{-10}	25 +/- 7 (H ₀ 63.5, 63.6, 41.9, 43.6; T ₄₀ 76.2, 14.2, 49.2, 15.7)
10^{-9}	34 +/- 20 (H ₀ 63.5, 63.6, 41.9, 43.6; T ₄₀ 70.5, 63.6, 80.9, 57.6)
10^{-8}	19 +/- 14 (H ₀ 63.5, 63.6, 41.9, 43.6; T ₄₀ 65.4, 57.8, 57.6, 63.7)
10^{-7}	-4 +/- 11 (H ₀ 63.5, 63.6, 41.9, 43.6; T ₄₀ 59.3, 43.1, 47.3, 50.2)
10^{-6}	Cytotoxic to monocytes

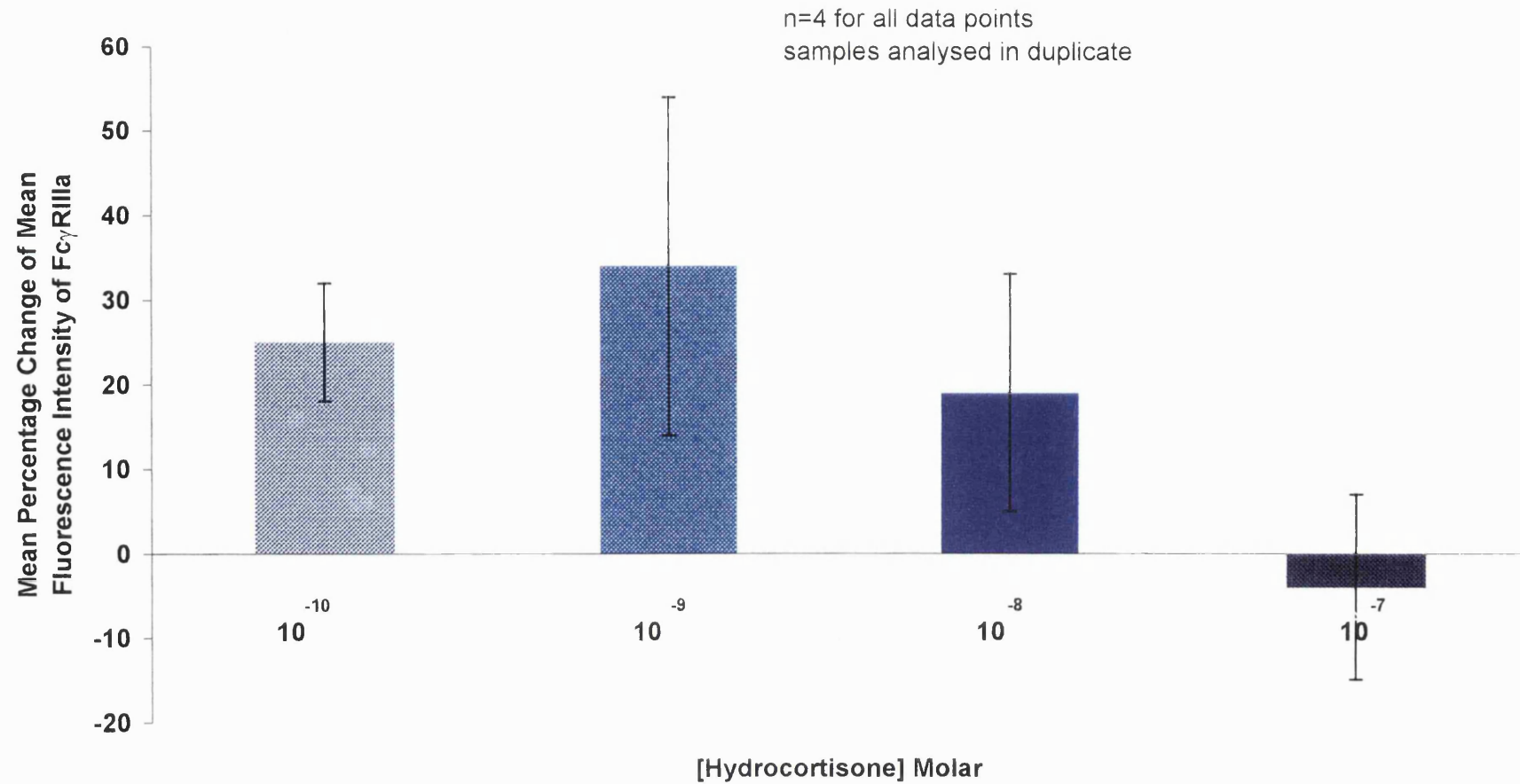
n=4 samples analysed in duplicate

Baseline MFI values given in brackets respectively

T=Time with subscript denoting number of hours H₀= MFI values with no added hydrocortisone

The results from experiment 9d suggest that a concentration of 10^{-10} M hydrocortisone increases the expression of FcγRIIIa. This increased expression peaks at a concentration of 10^{-9} M. The induction of FcγRIIIa declines on increasing the concentration of hydrocortisone further. These changes represent trends and are not statistically significant. At a concentration of 10^{-6} M hydrocortisone appears to be cytotoxic to monocytes. High doses of glucocorticoids interfere with processes that are essential to the maintenance of lymphocytes (Buttgereit F, Wehling M *et al*, 1998). It would appear that monocytes are also affected in the same way. This action of glucocorticoids may in part explain the beneficial effects of steroids in the management of patients with rheumatoid arthritis.

Figure 3.9d: The Mean Percentage Change of the Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM versus [Hydrocortisone] Molar



EXPERIMENTS 10a-c: SYNOVIAL FLUID AND Fc γ RIIIa EXPRESSION

Introduction

Synovial fluid is in close contact with intimal cells of the synovium. Normal intimal macrophages express Fc γ RIIIa on their cell surface (Edwards J C W, Blades S, 1997). It is possible that factors in synovial fluid may be responsible for the induction of Fc γ RIIIa on intimal macrophages. The factors in synovial fluid that may be responsible for the induction of Fc γ RIIIa on intimal macrophages could be:

- Cytokines in particular IL-10 and TGF β (see pages 57-66 for more information)
- Extracellular matrix proteins synthesized and released into the synovial fluid by intimal fibroblasts (see page 39 for more information)
- Hyaluronan (see pages 36-37)
- Lubricin (see page 36)

Experiment 10a was performed to investigate the expression of Fc γ RIIIa on macrophages in non-inflammatory synovial fluid.

Methods

Synovial fluid monocytes were obtained by using the same method as for obtaining peripheral blood monocytes as described on page 100. Fc γ RIIIa expression on

synovial fluid monocytes was assessed as described on pages 105-107. The mean fluorescence intensity of FcγRIIIa on synovial fluid monocytes was compared to freshly isolated peripheral blood monocytes. Results from experiment 10a are shown in table and figure 3.10a.

Results

Table 3.10a: The Mean Fluorescence Intensity of FcγRIIIa on Macrophages in Non-inflammatory Synovial Fluid Compared to FcγRIIIa Expression on Freshly Isolated Peripheral Blood Monocytes +/- SEM.

	Mean Fluorescence Intensity of FcγRIIIa +/- SEM
Synovial Fluid Macrophages	4.5 +/- 0.2
Peripheral Blood Monocytes	3 +/- 0.15

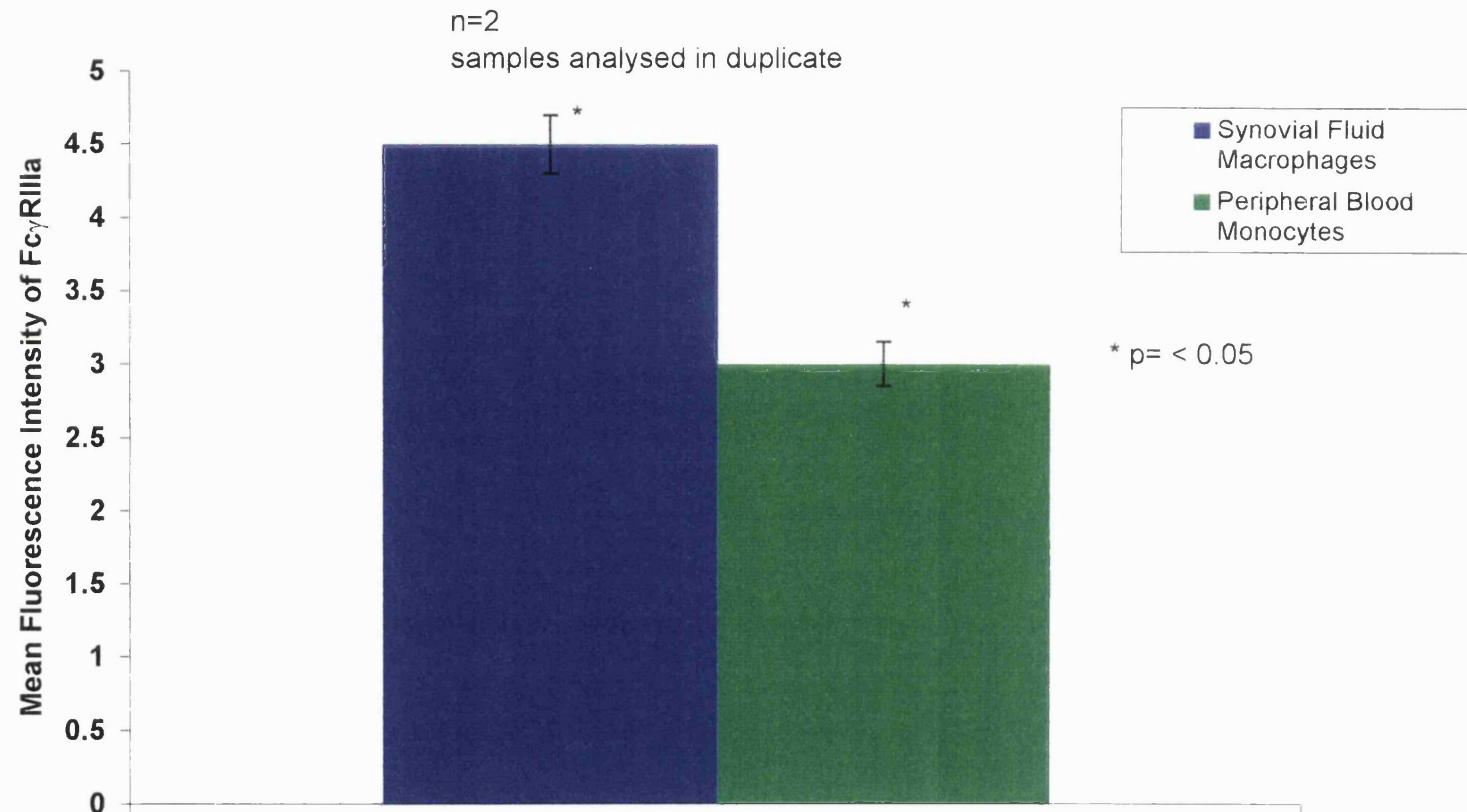
n=2 samples analysed in duplicate

p= < 0.05

The mean fluorescence intensity of FcγRIIIa on peripheral blood monocytes and synovial fluid macrophages (non-inflammatory) is similar. However, the macrophages in synovial fluid may represent intimal macrophages that have become senescent and shed from the synovium and thus express low levels of FcγRIIIa (p= <0.05).

Experiment 10b was performed to investigate the role of synovial fluid being responsible for the induction of FcγRIIIa on synovial intimal macrophages.

Figure 3.10a: The Mean Fluorescence Intensity of Fc γ R11a on Macrophages in Non-inflammatory Synovial Fluid Compared to Fc γ R11a Expression on Freshly Isolated Peripheral Blood Monocytes +/- SEM



Peripheral blood monocytes were obtained as described on page 100. Monocytes were cultured on a polystyrene substrate in normal growth medium and in medium with osteoarthritic synovial fluid (25%). Osteoarthritic synovial fluid from three separate experiments was used. Synovial fluid was centrifuged to remove cells prior to use. FcγRIIIa expression on cultured monocytes was assessed as described on pages 105-107. Results from experiment 10b are shown in table and figure 3.10b.

Table 3.10b: The Mean Fluorescence Intensity of FcγRIIIa on Monocytes Cultured in Growth Medium With No Synovial Fluid and Growth Medium Containing Synovial Fluid (3 Patients).

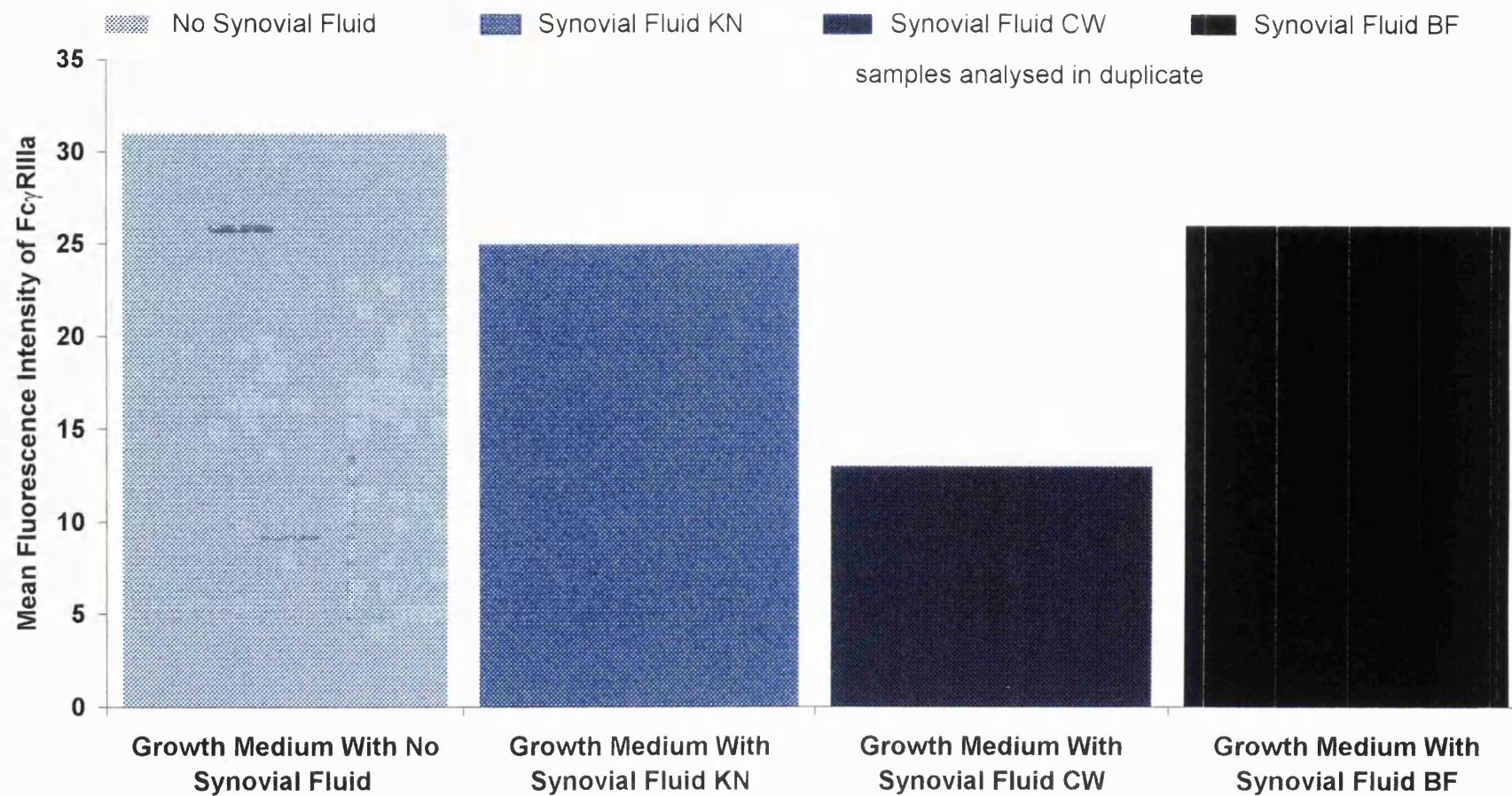
Medium	Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes
Growth Medium With No Synovial Fluid	31
Growth Medium With Synovial Fluid KN	25
Growth Medium With Synovial Fluid CW	13
Growth Medium With Synovial Fluid BF	26

Samples analysed in duplicate

The addition of osteoarthritic synovial fluid to monocyte cultures did not result in the induction of FcγRIIIa. This data suggests that synovial fluid is unlikely to be responsible for the induction of FcγRIIIa on synovial intimal macrophages. The above experiments were conducted with synovial fluid taken from patients with osteoarthritis. Osteoarthritic synovial fluid contains cytokines such as TGFβ, IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α, IL-4, IFN-γ and growth factors (Schlaak J F,

Pfers I *et al*, 1996). These cytokines may account for the lack of induction of FcγRIIIa on monocytes cultured in osteoarthritic synovial fluid. Normal synovial fluid is difficult to obtain. The above experiments would need to be repeated with normal synovial fluid to discount the effect cytokines in modulating FcγRIIIa expression. Experiments with normal synovial fluid would have to be performed before one could exclude a role for synovial fluid in inducing the expression of FcγRIIIa on intimal macrophages *in vivo*.

Figure 10.3b: Mean Fluorescence Intensity of Fc γ R11a on Monocytes Cultured in Medium With (3 Patients) and Without Synovial Fluid



Experiment 10c was performed to investigate the role of hyaluronan in the induction of FcγRIIIa on cultured monocytes. Peripheral blood monocytes were obtained as described on page 100. Hyaluronan was added to monocytes cultured on a polystyrene substrate at 20 mg/ml. FcγRIIIa expression was assessed as described on pages 105-107. Results from experiment 10c are shown in table and figure 3.10c.

Table 3.10c: The Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM With and Without the Addition of Hyaluronan (20 mg/ml).

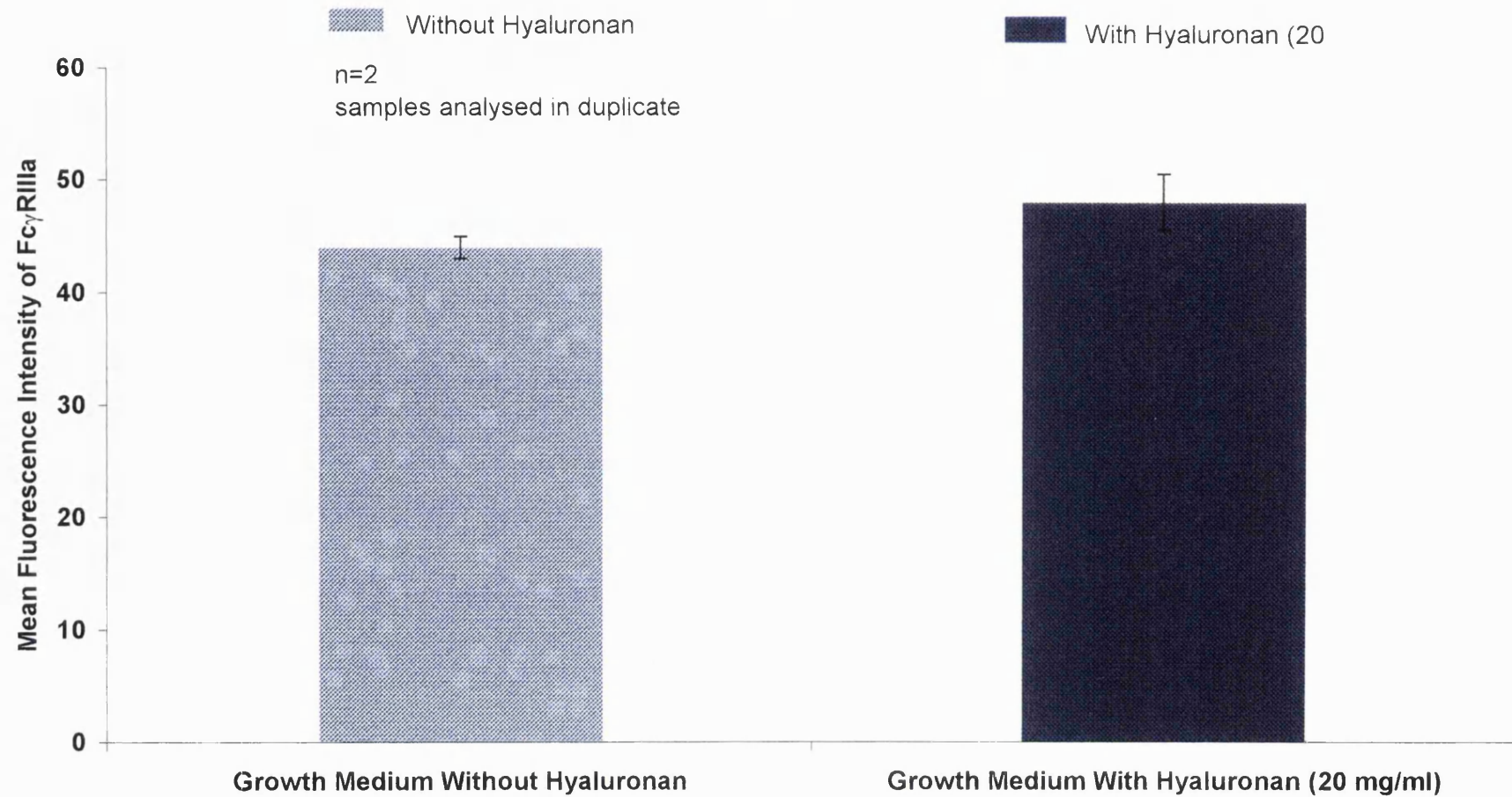
	Mean Fluorescence Intensity of FcγRIIIa +/- SEM on Cultured Monocytes
Growth Medium With No Hyaluronan	44 +/- 1.0
Growth Medium With Hyaluronan (20mg/ml)	48 +/- 2.5

n=2 samples analysed in duplicate

The addition of hyaluronan to monocytes *in vitro* did not result in a greater elevation of FcγRIIIa compared to monocyte cultures without hyaluronan. The molecular weight of the hyaluronan used in the above experiments was approximately 400 kDa. The molecular weight of hyaluronan in synovial fluid is between 2×10^5 and 10×10^6 daltons. The properties of hyaluronan depend on its molecular weight. High molecular weight preparations have greater anti-inflammatory properties than low molecular weight preparations.

To exclude the possibility of hyaluronan being responsible for the induction of FcγRIIIa on tissue macrophages *in vivo* it would be necessary to perform the above experiment with hyaluronan preparations with a spectrum of molecular weights. However the data overleaf suggests hyaluronan is not responsible for the induction of FcγRIIIa on synovial intimal macrophages.

Figure 3.10c: The Mean Fluorescence Intensity of FcγRIIIa on Monocytes Cultured With and Without Hyaluronan (20 mg/ml)



EXPERIMENTS 11a-f: MECHANICAL STRETCH OF FIBROBLASTS AND FcγRIIIa EXPRESSION ON CULTURED MONOCYTES

Introduction

Mechanical stretching of monocytes did not result in an increase in the level of FcγRIIIa expression compared to non-stretched monocytes *in vitro*.

The cellular component of the synovial intima consists of monocyte-macrophages and fibroblasts (Hederson B and Edwards J C W 1987, Revell P A 1989, Athanasou N A, Quinn J 1991 and Wilkinson L S, Pitsillides A A *et al* 1992).

Mechanical stretching of fibroblasts may therefore result in the induction of FcγRIIIa on monocytes. Mechanically stretched fibroblasts in the synovial intima may produce a factor that results in the induction of FcγRIIIa on monocytes in close proximity to synovial fibroblasts. Experiment 11a was performed to investigate whether mechanically stretched synovial fibroblasts release a paracrine factor that induces FcγRIIIa on monocytes.

Methods

In experiment 11a peripheral blood monocytes were obtained as described on page 100. Synovial fibroblasts were isolated from osteoarthritic synovium as described on page 108. Cultured synovial fibroblasts were subject to mechanical stretch in the same way as monocytes were in earlier experiments as described on pages 102-104.

Synovial fibroblasts were stretched by thirty percent of their length for seventy-two hours at a frequency of 0.004 Hz. Conditioned medium was removed from both stretched and non-stretched cultured synovial fibroblasts and used for monocyte cultures to investigate its effect on the expression of FcγRIIIa on cultured monocytes. Expression of FcγRIIIa was assessed as described on page 105-107. Results from experiment 11a are shown in table and figure 3.11a.

Results

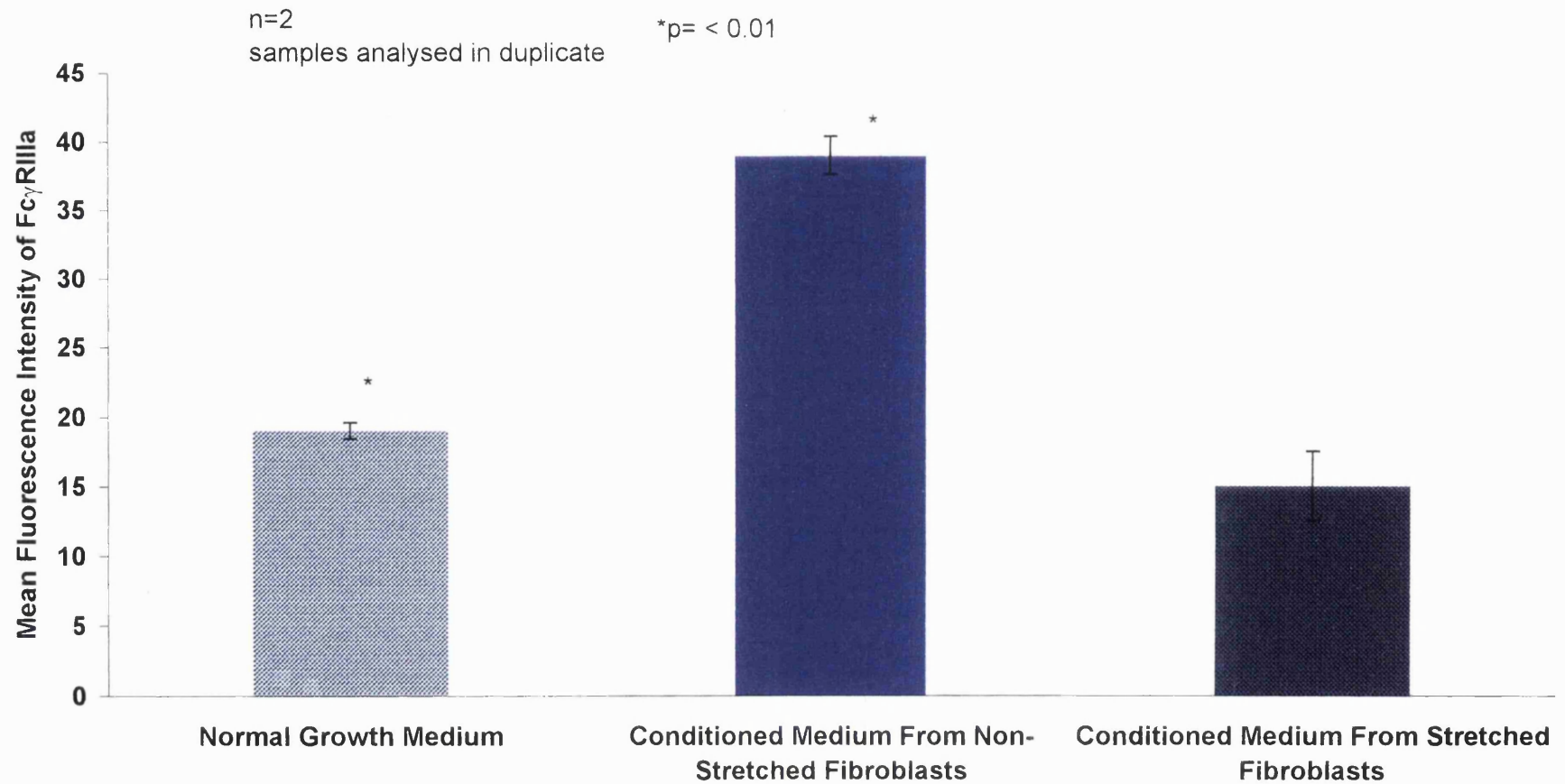
Table 3.11a: The Mean Fluorescence Intensity of FcγRIIIa +/- SEM on Monocytes Cultured in Normal Growth Medium, Conditioned Medium From Non-stretched Fibroblasts and Conditioned Medium From Stretched Fibroblasts.

Culture Medium	Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM
Normal Growth Medium	19 +/- 0.6
Conditioned Medium From Non-Stretched Fibroblasts	39 +/- 1.4*
Conditioned Medium From Stretched Fibroblasts	15 +/- 2.5

n=2 samples analysed in duplicate *p= < 0.01

Conditioned medium from non-stretched fibroblasts increased the level of FcγRIIIa expression on cultured monocytes (p= <0.01). This data suggests that fibroblasts release a soluble factor that induces FcγRIIIa on monocytes. Conditioned medium from stretched fibroblasts did not result in an increase in the level of FcγRIIIa expression

Figure 3.11a: The Mean Fluorescence Intensity of FcγRIIIa +/- SEM on Monocytes Cultured in Normal Growth Medium, Conditioned Medium From Non-stretched Fibroblasts and Conditioned Medium From Stretched Fibroblasts



on monocytes. Fibroblasts that are stretched may therefore produce a factor that inhibits the expression of FcγRIIIa on monocytes. Alternatively, fibroblasts that are subject to mechanical stretch may not produce the FcγRIIIa inducing factor that non-stretched fibroblasts release.

Further experiments were performed to identify the nature of this soluble fibroblast factor responsible for the induction of FcγRIIIa expression on monocytes.

Experiment 11b was performed to determine whether the soluble fibroblast factor was a protein or carbohydrate.

Methods

Peripheral blood monocytes were obtained as described on page 100. Conditioned fibroblast medium was pre-treated with a proteinase before being placed on monocyte cultures as described on page 110. The experiment was performed with conditioned non-stretched fibroblast medium and conditioned non-stretched fibroblast medium that had been pre-treated with a proteinase. FcγRIIIa expression on cultured monocytes was assessed as described on pages 105-107. The results from experiment 11b are shown in table and figure 3.11b.

Results

Table 3.11b: The Mean Fluorescence Intensity of FcγRIIIa +/- SEM on Monocytes Cultured With Normal Growth Medium, Conditioned Medium From Non-Stretched Fibroblasts and Conditioned Medium From Non-Stretched Fibroblasts Pre-treated With Proteinase.

Culture Medium	Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM
Normal Growth Medium	42 +/- 3.8
Conditioned Medium From Non-Stretched Fibroblasts	68 +/- 8.1*
Conditioned Medium From Non-Stretched Fibroblasts Pre-treated With Proteinase	16 +/- 1.7*

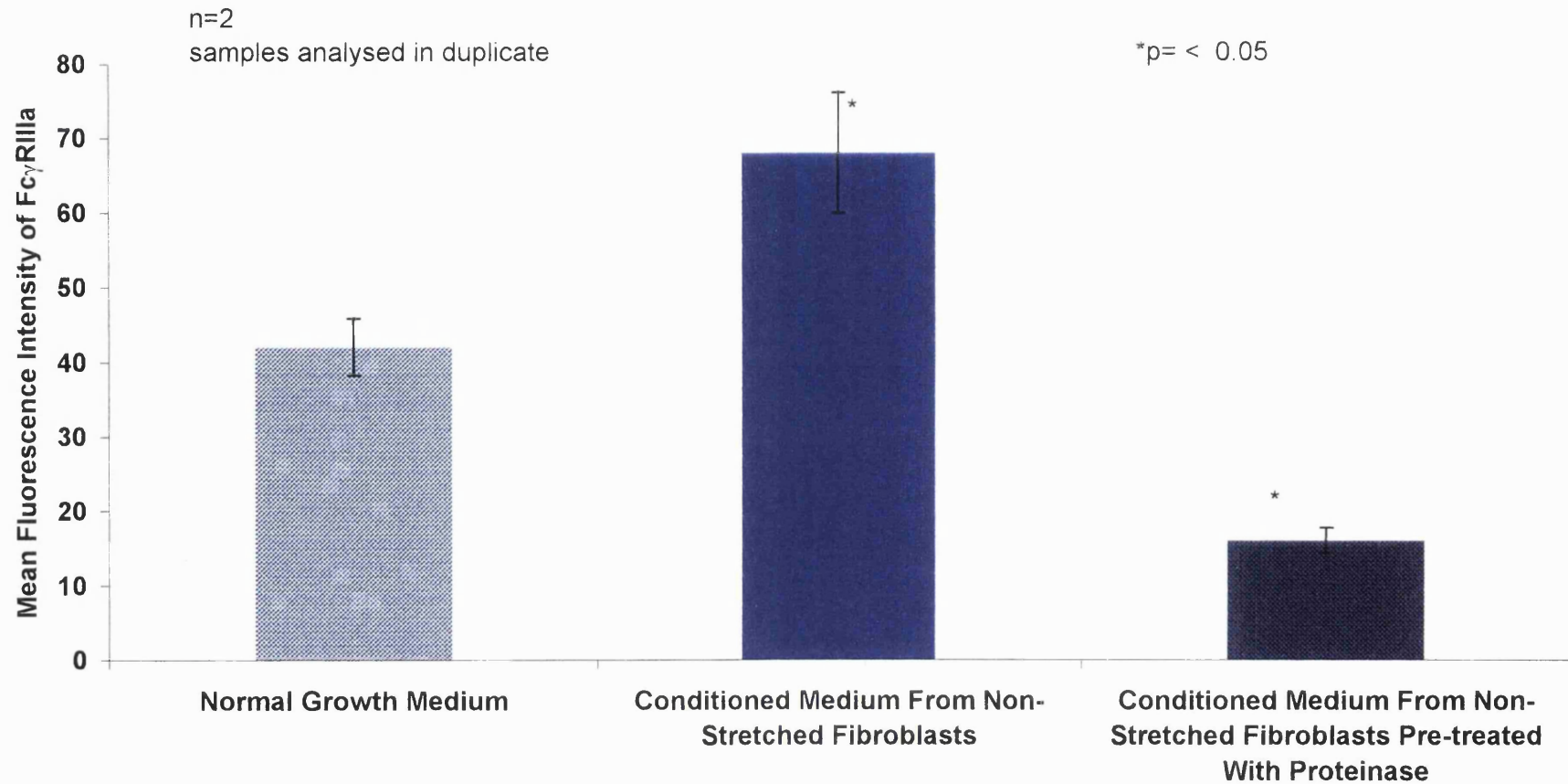
n=2 samples analysed in duplicate

*p= < 0.05

The increased level of FcγRIIIa expression on monocytes cultured with conditioned medium from non-stretched fibroblasts was abolished when monocytes were cultured in conditioned medium from non-stretched fibroblasts that had been pre-treated with proteinase (p= <0.05). This data reveals that the soluble fibroblast factor responsible for inducing FcγRIIIa on monocytes was a protein.

Experiment 11c was performed to investigate the molecular weight of this soluble fibroblast protein.

Figure 3.11b: The Mean Fluorescence Intensity of Fc γ R11a +/- SEM on Monocytes Cultured With Normal Growth Medium, Conditioned Medium From Non-Stretched Fibroblasts and Conditioned Medium From Non-Stretched Fibroblasts Pre-treated With Proteinase



Methods

Peripheral blood monocytes were obtained as described on page 100. Conditioned non-stretched fibroblast medium was separated into fractions more than and less than 100 kDa as described on page 109. These fractions were placed on monocyte cultures to investigate which fraction retained the ability to induce Fc γ RIIIa on cultured monocytes. Unfractionated conditioned medium from non-stretched fibroblasts was also placed on monocyte cultures. Results from experiment 11c are shown in table and figure 11c (4 samples).

Results

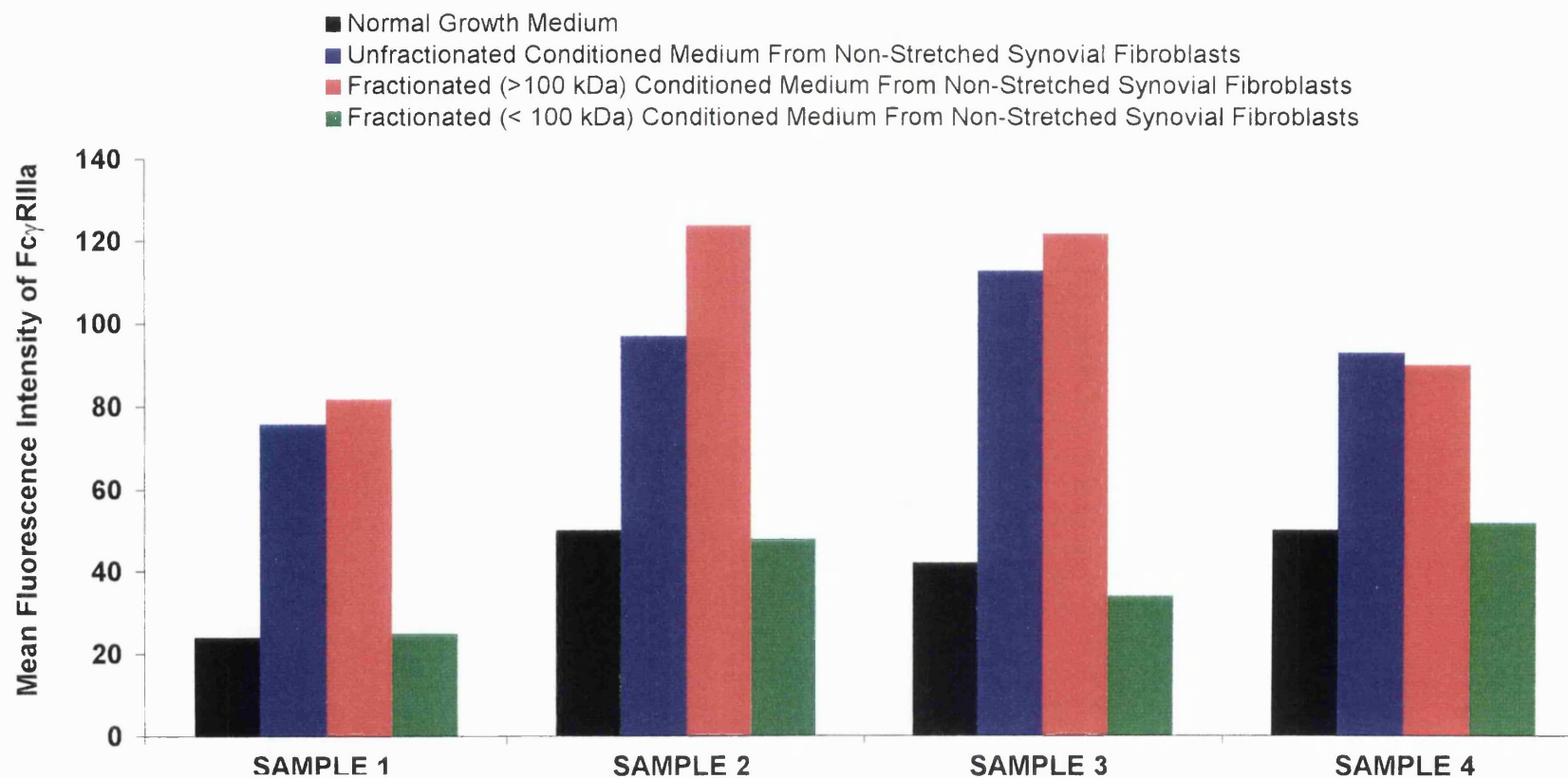
Table 3.11c: The Mean Fluorescence Intensity of Fc γ RIIIa on Monocytes Cultured With Normal Growth Medium, Unfractionated Non-Stretched Fibroblast Medium and Fractions of Non-Stretched Fibroblast Medium of More Than and Less Than 100 kDa.

Culture Medium	Mean Fluorescence Intensity of Fc γ RIIIa on Cultured Monocytes			
	Sample 1	Sample 2	Sample 3	Sample 4
Normal Growth Medium	24	50	42	50
Unfractionated Conditioned Medium From Non-Stretched Fibroblast	76	97	113	93
Fractionated Conditioned Medium From Non-Stretched Fibroblasts > 100 kDa	82	124	122	90
Fractionated Conditioned Medium From Non-Stretched Fibroblast < 100 kDa	25	48	34	52

Samples analysed in duplicate

Figure 3.11c: The Mean Fluorescence Intensity of FcγRIIIa on Monocytes Cultured With Normal Growth Medium, Unfractionated Non-Stretched Fibroblast Medium and Fractions of Non-Stretched Fibroblast Medium of More Than and Less Than 100 kDa (4 Samples)

samples analysed in duplicate



The data from table and figure 3.11c shows that the ability of conditioned fibroblast medium to increase FcγRIIIa expression on monocytes is retained in the fraction of conditioned medium that is more than 100 kDa. This result could be explained by:

- The presence of a protein of more than 100 kDa that induces the expression of FcγRIIIa on monocytes.
- The presence of an inhibitor that is less than 100 kDa in molecular weight.
- The presence of a protein of less than 100 kDa that induces the expression of FcγRIIIa. This protein of less than 100 kDa may be conjugated to another molecule. This molecule may have a total molecular weight of over 100 kDa.

Experiment 11d was performed to investigate whether the fibroblast protein responsible for the induction of FcγRIIIa on monocytes was IL-10 or IFN-β, experiments were performed with neutralising antibody to IL-10 and IFN-β.

Methods

Peripheral blood monocytes were obtained as described on page 100. Monocytes were cultured on a polystyrene substrate with normal growth medium, conditioned medium from non-stretched fibroblasts, conditioned medium from non-stretched fibroblasts pre-treated with neutralising antibodies to IL-10 and conditioned medium from non-stretched fibroblasts pre-treated with neutralising antibodies to IFN-β. After forty hours of culture FcγRIIIa expression was assessed as described on pages 105-107. Results from experiment 11d are shown in table and figure 3.11d.

Results

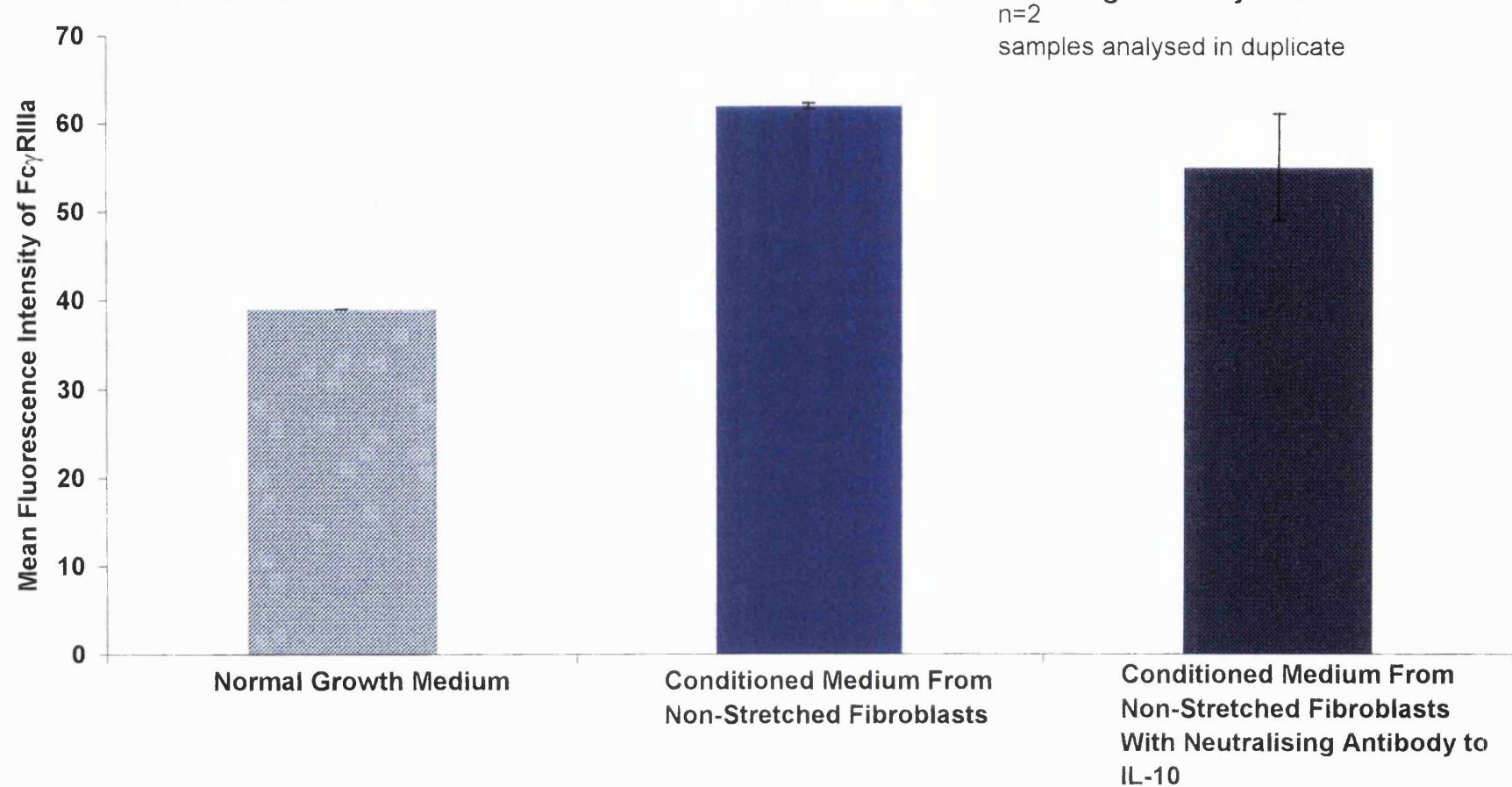
Table 3.11d: The Mean Fluorescence Intensity of FcγRIIIa on Monocytes +/- SEM Cultured With Normal Growth Medium, Conditioned Medium From Non-Stretched Synovial Fibroblasts and Conditioned Medium From Non-Stretched Synovial Fibroblasts With and Without Neutralising antibodies to IL-10 and IFN-β.

Culture Medium	Mean Fluorescence Intensity of FcγRIIIa +/- SEM		
Normal Growth Medium	39*		22*
Conditioned Medium From Non-Stretched Fibroblasts	62 +/- 0.37		62 +/- 0.54
Conditioned Medium From Non-Stretched Fibroblasts With Neutralising Antibodies to IL-10	55 +/- 6.1		
Conditioned Medium From Non-Stretched Fibroblasts With Neutralising Antibodies to IFN-β			74 +/- 0.9

n=2 samples analysed in duplicate *one value only

The results from experiment 11d show that the protein in conditioned fibroblast medium responsible for the induction of FcγRIIIa on cultured monocytes may in part be IL-10. However the effect of neutralising antibodies to IL-10 on reducing the induction of FcγRIIIa on cultured monocytes is small (not statistically significant). This suggests that IL-10 is not the dominant protein in conditioned fibroblast medium causing the induction of

Figure 3.11d: The Mean Fluorescence Intensity of Fc γ R11a +/- SEM on Monocytes Cultured in Normal Growth Medium, Conditioned Medium From Non-Stretched Fibroblasts and Conditioned Medium From Non-Stretched Fibroblasts With Neutralising Antibody to IL-10



FcγRIIIa on monocytes. Neutralising antibodies to IFN-β had no effect in abrogating the induction of FcγRIIIa on cultured monocytes by conditioned fibroblast medium.

Further experiments were performed to identify the characteristics of the protein in conditioned fibroblast medium that induced FcγRIIIa expression on cultured monocytes.

Experiment 11e was performed to ascertain if the fibroblast protein responsible for the induction of FcγRIIIa on monocytes was acid labile or acid dissociable to a molecular weight of less than 100 kDa.

Methods

Peripheral blood monocytes were obtained as described on page 82. Conditioned medium from non-stretched fibroblasts was acidified to pH 2.5 by the addition of 4 mM hydrochloric acid for one hour before being returned to pH 7.6 by the addition of 1 M sodium hydroxide. This medium was then fractionated by molecular weight to portions less than and more than 100 kDa as described on page 109. Fractionated medium was then used for culturing monocytes. After forty hours of culture FcγRIIIa expression was assessed as described on pages 105-107. The results from experiment 11e is shown in table and figure 3.11e.

Results

Table 3.11e: The Mean Fluorescence Intensity of FcγRIIIa on Monocytes +/- SEM Cultured in Normal Growth Medium, Unfractionated Conditioned Medium From Non-Stretched Fibroblasts, Fractionated Conditioned Medium From Non-Stretched Fibroblasts and Fractionated Conditioned Medium From Non-Stretched Fibroblasts (pre-acidified to pH 2.5 for one hour).

Culture Medium	Mean Fluorescence Intensity of FcγRIIIa on Cultured Monocytes +/- SEM
Normal Growth Medium	51*
Unfractionated Conditioned Medium From Non-Stretched Fibroblasts	136*
Conditioned Medium From Non-Stretched Fibroblasts < 100 kDa	29*
Conditioned Medium From Non-Stretched Fibroblasts > 100 kDa	99*
Conditioned Medium From Non-Stretched Fibroblasts < 100 kDa (Pre-acidified)	23 +/- 3 [#] (n=2)
Conditioned Medium From Non-Stretched Fibroblasts > 100 kDa (Pre-acidified)	60 +/- 1 [#] (n=2)

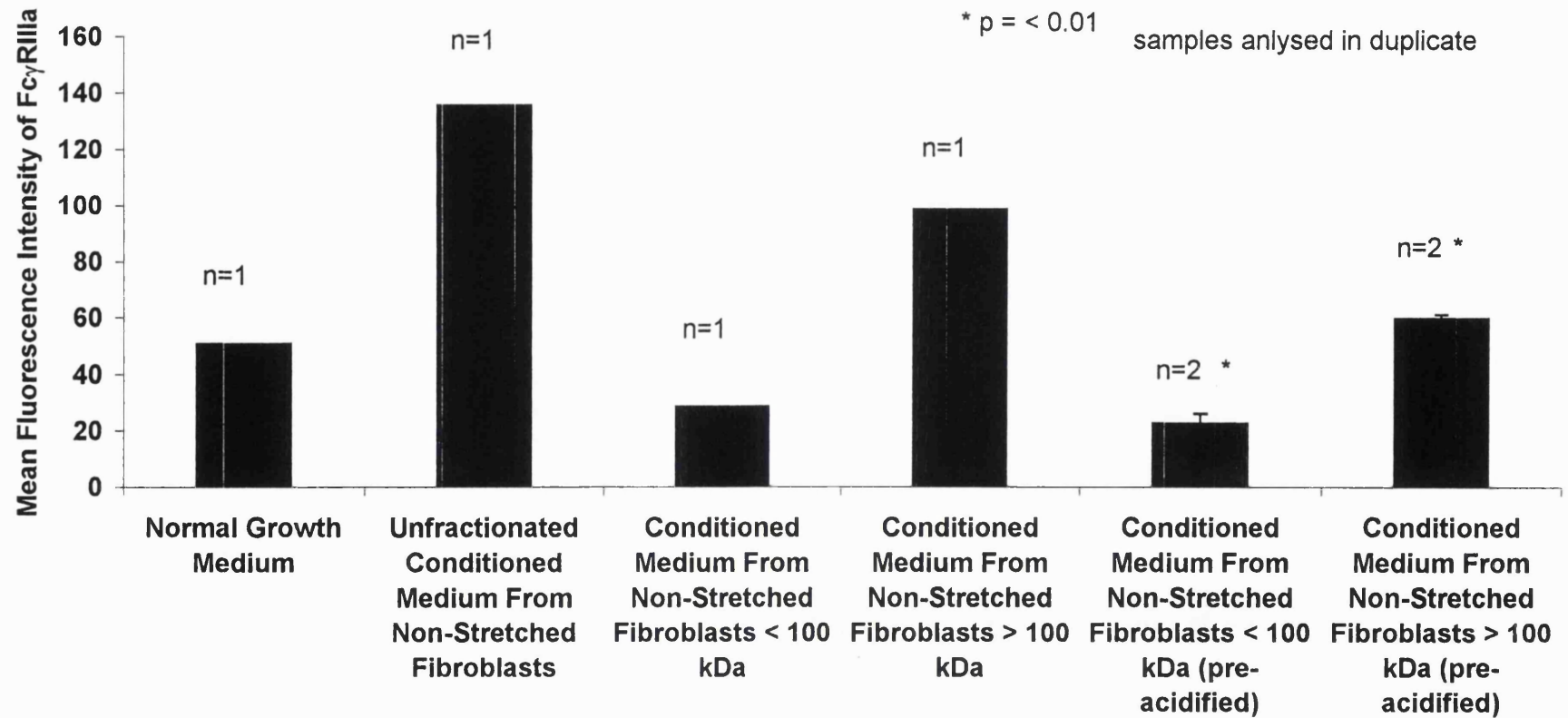
* one value only, samples analysed in duplicate

p= < 0.01

The data in table and figure 3.11e shows that the protein in conditioned fibroblast medium responsible for the induction of FcγRIIIa on cultured monocytes is acid labile. The data also suggests that this protein is not dissociated by acid to the fraction of conditioned medium that is less than 100 kDa.

Experiment 11f was performed to investigate whether the protein responsible for the induction of FcγRIIIa on cultured monocytes was heat stable.

Figure 3.11e: Mean Fluorescence Intensity of Fc γ R1IIa on Monocytes +/- SEM Cultured in Normal Growth Medium, Unfractionated Conditioned Medium From Non-Stretched Fibroblasts, Conditioned Medium From Non-Stretched Fibroblasts less than and more than 100 kDa and Conditioned Medium From Non-Stretched Fibroblasts less than and more than 100 kDa (pre-acidified)



Methods

Peripheral blood monocytes were obtained as described on page 100. Conditioned medium from non-stretched fibroblasts was heated to 56 °C for one hour. Once this medium had cooled to 36° C it was placed on monocyte cultures. After forty hours of culture FcγRIIIa expression was assessed as described on pages 105-107. The results from experiment 11f are shown in table and figure 3.11f.

Results

Table 3.11f: The Mean Fluorescence Intensity of FcγRIIIa +/- SEM on Monocytes Cultured in Normal Growth Medium, Unfractionated Conditioned Medium From Non-Stretched Fibroblasts and Unfractionated Conditioned Medium From Non-Stretched Fibroblasts (pre-heated to 56°C for one hour).

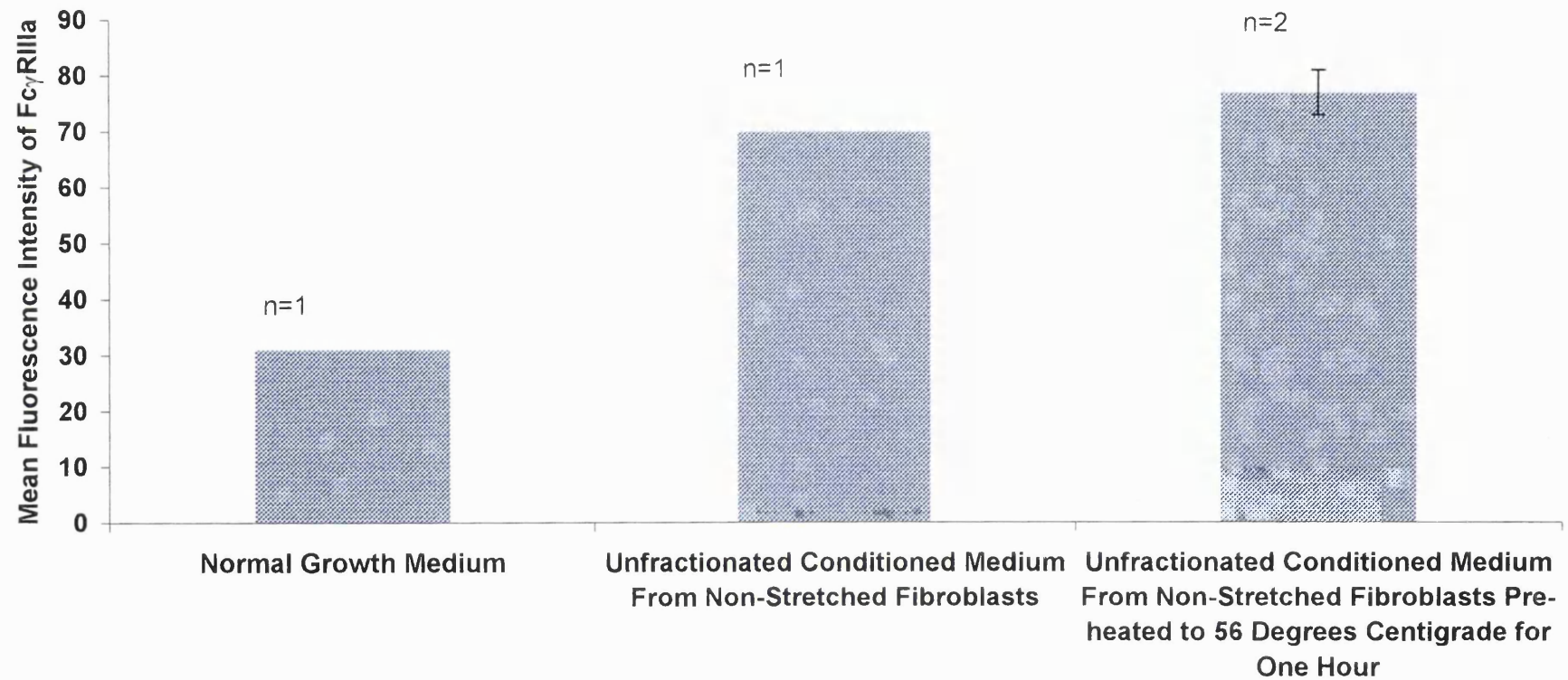
Culture Medium	Mean Fluorescence Intensity of FcγRIIIa +/- SEM
Normal Growth Medium	31*
Unfractionated Conditioned Medium From Non-Stretched Fibroblasts	70*
Unfractionated Conditioned Medium From Non-Stretched Fibroblasts (pre- heated to 56°C for one hour)	77 +/- 4 (n=2)

* one value only samples analysed in duplicate

The data in table and figure 3.11f shows that the protein in conditioned medium from non-stretched fibroblasts responsible for the induction of FcγRIIIa on cultured monocytes is heat stable.

Figure 3.11f: The Mean Fluorescence Intensity of FcγRIIIa on Monocytes +/- SEM Cultured in Normal Growth Medium, Unfractionated Conditioned Medium From Non-Stretched Fibroblasts and Unfractionated Conditioned Medium From Non-Stretched Fibroblasts Pre-heated to 56 Degrees Centigrade for One Hour

samples analysed in duplicate



The factor in conditioned medium from non-stretched synovial fibroblasts responsible for the induction of FcγRIIIa on cultured monocytes is a heat stable acid labile protein with a molecular weight in excess of 100 kDa. Possible approaches to characterize this protein further would involve:

- Separation of conditioned fibroblast medium by gel electrophoresis with followed by staining with coomassie blue or silver nitrate. The pattern of staining could be compared with known molecular markers. Any proteins that are present in conditioned medium which are absent in normal growth medium could be extracted, sequenced and identified.
- High-performance liquid chromatography (HPLC) could be used to separate different proteins in conditioned fibroblast medium. Mass spectrometers can also be interfaced with liquid chromatography to provide structural information and help identify the separated analytes.

Once the protein has been identified it could be introduced to monocyte cultures to assess its ability to induce the expression of FcγRIIIa on monocytes *in vitro*. Immunohistochemical analysis of normal synovium could then be undertaken to investigate whether this protein is present in tissues and therefore likely to be responsible for the induction of FcγRIIIa on tissue macrophages *in vivo*.

DISCUSSION

DISTRIBUTION OF Fc γ RIIIa IN HUMAN TISSUES

Cellular Distribution

Mean maximal microdensitometric readings of macrophage staining for Fc γ RIIIa are presented in table 3.1 (experiment 1). The results from experiment 1 show that Fc γ RIIIa is found on macrophages only in tissues targeted by rheumatoid disease. This supports the suggested role for an interaction between small IgG rheumatoid factor complexes and Fc γ RIIIa on tissue macrophages in the pathogenesis of rheumatoid arthritis.

The cross-linking of 2 or three Fc γ RIIIa receptors with an anti-Fc γ RIII monoclonal antibody is sufficient to trigger macrophages to release TNF- α , IL-1 α and reactive oxygen species (Abrahams V M, Cambridge G *et al*, 2000). In rheumatoid arthritis, small immune complexes (IgG rheumatoid factor dimers) are found in the circulation, and their ligation of Fc γ RIIIa on macrophages at sites where Fc γ RIIIa is expressed may result in TNF- α release. TNF- α causes inflammation in the tissues where it is expressed through a number of mechanisms (page 72), which may contribute in different degrees to the genesis of inflammation in tissues affected by rheumatoid disease, and thus give rise to the clinical features of rheumatoid arthritis (page 19-24).

In the synovium small immune complex ligation of Fc γ RIIIa on intimal macrophages may result in activation and TNF- α release specifically in the intimal

layer. This would explain the early increase in intimal macrophage size and number characteristic of the development of synovitis.

As well as causing inflammation, TNF- α also induces VCAM-1, decay accelerating factor and complement receptor 2 on synovial fibroblasts. These molecules are involved in sustaining B lymphocyte survival and differentiation into plasma cells and therefore will facilitate rheumatoid factor production in the synovium (Edwards J C W, Leigh R D *et al*, 1997).

Macrophages in the lung, pericardium and mechanically stretched dermis also express Fc γ RIIIa. Small immune complex ligation of Fc γ RIIIa on macrophages at these sites may result in TNF- α release. The production of TNF- α at these sites will result in local tissue inflammation, consistent with the clinical features of rheumatoid arthritis in these tissues as shown in figure 1.1. However, these tissues may be less susceptible to secondary colonisation by B lymphocytes and plasma cells.

The predicted result of immune complex ligation with Fc γ RIIIa on Kupffer cells in the liver would be the local production of TNF- α . TNF- α induces the synthesis of IL-6. IL-6 and TNF- α stimulate hepatocytes. This would provide an explanation for the increased circulatory concentration of C-reactive protein in patients with rheumatoid arthritis. It may also account for the common mild elevation of liver enzymes and the nodular hyperplasia seen in association with Kupffer cell activation in Felty's syndrome.

Large FcγRIIIa positive mononuclear cells consistent with macrophages were seen in bone marrow. However, owing to the large number of cells in bone marrow it was not possible to be sure of the true identity of these cells. To identify cell types expressing FcγRIIIa in bone marrow further immunohistochemical experiments would have to be conducted with a variety of cell specific markers. TNF-α production in bone marrow as a consequence of FcγRIIIa ligation by immune complexes would be expected to suppress erythropoiesis (Johnson R A, Waddell T A *et al*, 1989b). This mechanism may well contribute to the anaemia of chronic disease in rheumatoid arthritis. It also explains the reversal of anaemia that occurs in patients receiving monoclonal antibodies against TNF-α (Elliot M J *et al*, 1994).

The pattern of macrophage FcγRIIIa expression is a very reasonable map of pathological changes in rheumatoid arthritis: synovitis, subcutaneous nodule at sites of mechanical stretch, a prominent acute phase response, suppressed haematopoiesis, lymphadenopathy, pericarditis and alveolitis.

Differential levels of macrophage FcγRIIIa expression in different tissues may contribute to the relative susceptibility of these tissues to rheumatoid disease. Factors responsible for the induction of FcγRIIIa on macrophages are therefore likely to be relatively more abundant in tissues where FcγRIIIa is expressed.

Extracellular Distribution of FcγRIIIa in Human Tissues

Extracellular staining for FcγRIII was found on skeletal and cardiac perimysium, synovial intima, mesothelia, in the dermis at sites of mechanical stretch and the internal elastic lamina of arteries in the uterus. Staining patterns of both FcγRIII and DAF at many of these sites followed the pattern of fibrillin-1 based microfibrils.

The role of microfibril-associated FcγRIII is uncertain. TNF-α production will not be possible from FcγRIII bound to microfibrils as the intracellular signalling mechanisms required for this are absent. Microfibril-associated FcγRIII may allow immobilisation and aggregation of small immune complexes. This might lead, in the presence of DAF on fibrils and CD59 on muscle cells, to controlled generation of C3a through either classical or alternative pathways. Muscle biopsies taken from patients with polymyalgia rheumatica show IgG deposits in the perimysium (Shintani S, Tsurruoka S *et al*, 1995). Binding of immune complexes with C3a generation in muscle may contribute to viral myalgia and may also be of relevance to muscle pain and stiffness in patients with polymyalgia rheumatica.

Polymyalgia rheumatica and rheumatoid arthritis show much overlap, but most cases fall into one or other category. Immune complexes in polymyalgia rheumatica differ from those found in rheumatoid arthritis in their content of alternative pathway components (Smith A J, Kyle V *et al*, 1987). The characteristics of immune complexes in terms of size, interaction with complement pathways (as in polymyalgia rheumatica) and the local synthesis of both antigen and antibody (as for

IgG rheumatoid factors in rheumatoid arthritis) may be important in determining whether immune complexes act at cell-associated or matrix-associated Fc γ RIIIa.

FcγRIIIa POLYMORPHISMS

Ravetch and Perussia (1989) have described a polymorphism in the extracellular domain of FcγRIIIa. A nucleotide substitution at position 559 of FcγRIIIa predicts either a valine (V) or a phenylalanine (F) at amino-acid position 158 of FcγRIIIa. The gene frequency for FcγRIIIa-158F and FcγRIIIa-158V in 87 healthy volunteers was found to be 0.57 and 0.43 respectively. IgG binding experiments on natural killer cells from genotyped donors demonstrate that FcγRIIIa^{NK}-158F binds significantly less IgG1, IgG3 and IgG4 than FcγRIIIa^{NK}-158V. Also an anti-FcγRIII monoclonal antibody (MEM154) was found to bind more strongly to FcγRIIIa-158V, compared to FcγRIIIa-158F (Koene H R, Kleijer M *et al*, 1997).

The FcγRIIIa-158V/F polymorphism has been found to be associated with rheumatoid arthritis and in particular, seropositive nodular rheumatoid arthritis. In patients with rheumatoid arthritis homozygosity for the FcγRIIIa-158V allele is associated with the development of nodules (Morgan A W, Griffiths B *et al*, 2000). FcγRIIIa-158V appears to be a marker of severity of disease in patients with rheumatoid arthritis.

This observation can be explained. The FcγRIIIa-158V polymorphism will bind IgG1 rheumatoid factor dimers more strongly than FcγRIIIa-158F. This may result in a significant increase in signalling and thus a greater release of pro-inflammatory cytokines than with IgG1 binding to FcγRIIIa-158F. This data supports the role of

an interaction between small self-associated dimeric complexes of IgG rheumatoid factor and FcγRIIIa in the pathogenesis of rheumatoid arthritis.

FcγRIIIa-158V appears to be a marker of severity of disease in patients with rheumatoid arthritis. It would therefore be predicted that the addition of monoclonal antibodies to macrophages expressing FcγRIIIa-158V in vitro would result in a greater release of TNF-α than with macrophages expressing FcγRIIIa-158F. Functional studies such as these have not been performed.

THE FcγRIII PROMOTER

FcγRIII occurs as two isoforms; FcγRIIIa and FcγRIIIb, the product of two different genes, FcγRIII-A and FcγRIII-B respectively (Fleit H B, Wright S D *et al*, 1982, Ravetch J V, Perussia, 1989; Scanlon B J, Scigliano E *et al*, 1989). FcγRIIIa is found on natural killer cells, a small proportion of peripheral blood monocytes (≈5%), a subset of T-lymphocytes and on tissue monocyte-macrophages only at sites targeted by rheumatoid arthritis (page 117). FcγRIIIb is present on neutrophils and eosinophils. By locating the transcription initiation sites on the promoter of the genes that codes for FcγRIIIa and FcγRIIIb it should be possible to identify potential transcription factors responsible for the regulation of FcγRIIIa and FcγRIIIb. A total of 599 potential transcription factor binding sites for FcγRIIIa and 583 sites for FcγRIIIb have been identified (Gessner J E, Grussenmeyer T *et al*, 1995). The sequences of the two FcγRIII promoters have been identified to differ in ten positions. These nucleotide differences might contribute to cell type-specific transcription of FcγRIII genes.

Therefore, with a detailed knowledge of cytokine-transcription factors and the differences in transcription binding sites on FcγRIII-A and FcγRIII-B promoters, it may in the future be possible to postulate which cytokines are responsible for the induction of FcγRIIIa expression monocyte-macrophages *in vivo*. These cytokines could then be added to monocyte cultures to examine their effect on the induction of FcγRIIIa. Immunohistochemistry could then be undertaken to investigate whether these cytokines are present in tissues where FcγRIIIa is expressed.

REGULATION OF FcγRIIIa

FcγRIIIa expression on macrophages in normal human tissues mirrors those sites where rheumatoid disease occurs. The embryological, immunological or mechanical characteristics of these tissues may be responsible for the selective induction of FcγRIIIa on macrophages at these sites.

Experiments 3a-d did not confirm the results of Welch G *et al* (1990) who found that TGFβ₁ selectively induces the expression of FcγRIIIa on cultured monocytes. Two batches of TGFβ₁ were used to ensure that the results obtained in experiment 3a-d were not due to an inactive or contaminated batch of TGFβ₁. TGFβ is released from cells following mechanical stretch. Tissues where FcγRIIIa is seen are subject to mechanical stretch and thus TGFβ or mechanical stretch itself seemed a plausible candidate for the induction of FcγRIIIa on tissue macrophages *in vivo*. However, experiments have not demonstrated an up regulation of FcγRIIIa on monocytes cultured with TGFβ₁. Experiment 3.3c demonstrates that TGFβ₁ suppresses the induction of FcγRIIIa compared to monocytes cultured in the absence of TGFβ₁ ($p < 0.01$). The *in vivo* effect of TGFβ may ultimately be dependant on the interaction of extracellular proteins, other cells and additional cytokines in the local milieu. It is possible that TGFβ has paradoxical effects dependant on experimental conditions. This might explain the conflicting data. Nevertheless, attempts to confirm this failed.

Experiment 3.8a demonstrated a slight increase of FcγRIIIa expression on cultured monocytes that had been subject to mechanical stretch compared to those not stretched. This increase was however not statistically significant ($p = > 0.05$).

Mechanical stretch cannot be excluded as a factor that may result in the induction of FcγRIIIa on cultured monocytes. Mechanical stretch reproduced *in vitro* may not accurately mimic physiological stretch that occurs *in vivo*. The type of force applied to cells may be important in determining their response in terms of FcγRIIIa expression. More elaborate experiments would need to be conducted where the type and frequency and modality of stretch could be varied to exclude mechanical stretch as a factor responsible for the induction of FcγRIIIa on macrophages. It is interesting to note that the liver is not a site of mechanical stretch. Kupffer cells however are exposed to fluid flow. Shear forces may therefore be a factor responsible for the induction of FcγRIIIa on these cells. It would be necessary to reproduce these type of forces *in vitro* to investigate their effect on FcγRIIIa expression.

The effect of TGFβ₁ in suppressing FcγRIIIa on cultured macrophages was independent of the substrate. However it was not possible to obtain culture plates coated with fibrillin-1 to investigate the effect of this matrix protein in isolation and with TGFβ₁ on the regulation of FcγRIIIa on cultured monocytes. Fibrillin-1 binds a range of immunologically active ligands (Bashir M M, Han M D *et al*, 1996). Fibrillin-1 or molecules bound to its surface may be responsible for the induction of FcγRIIIa on tissue macrophages. To investigate this it would be necessary to perform further experiments with monocytes cultured on a fibrillin-1 substrate.

Experiment 3.4a demonstrates that IL-10 induces the expression of FcγRIIIa on cultured monocytes. This data was not statistically significant ($p = > 0.05$). However the results are consistent with those of Calzada-Wack *et al.*

Interleukin 10 remains the only known candidate for up regulation of FcγRIIIa. However, there are currently no data to suggest that IL-10 is present around tissue macrophages expressing FcγRIIIa casting doubt on IL-10 as a factor responsible for FcγRIIIa expression on tissue macrophages *in vivo*. A key experiment to evaluate whether IL-10 is responsible for the induction of FcγRIIIa would be to use anti IL-10 *in vivo* and look at the effect of this on tissue FcγRIIIa expression.

Clinical trials with IL-10 in patients with rheumatoid arthritis (St Clair E W, 1999) have failed to show any efficacy. A possible explanation for this may be as a result of IL-10 inducing the expression of FcγRIIIa on tissue macrophages.

Conditioned medium from stretched fibroblasts did not induce FcγRIIIa on cultured monocytes. This result may be due to the presence of an inhibitor or absence of the FcγRIIIa inducing protein released from non-stretched fibroblasts in conditioned medium. The inhibitor may be TGFβ. To investigate this further it would be necessary to assess FcγRIIIa expression on monocytes cultured with conditioned medium from stretched fibroblasts pre-treated with neutralising antibody to TGFβ and non pre-treated conditioned medium.

The ability of methotrexate to increase the expression of FcγRIIIa on cultured monocytes ($p = < 0.01$) may explain why approximately 8 percent of patients commenced on methotrexate develop accelerated nodulosis. However sodium aurothiomalate at a concentration of 5 µg/ml did not result in a significant reduction of FcγRIIIa expression on cultured monocytes. A higher concentration of 25 µg/ml did result in a significant reduction ($p = < 0.05$) of FcγRIIIa expression on cultured monocytes. The steady state concentration of sodium aurothiomalate in patients taking 50 mg a week by the intramuscular route is 5 µg/ml. It seems unlikely that the efficacy of sodium aurothiomalate is due to reduced FcγRIIIa expression on macrophages *in vivo*. However the ability of macrophages to synthesize and release pro-inflammatory cytokines may be reduced. Functional experiments would need to be conducted to investigate this.

The most likely remaining factor to explain the restricted expression of FcγRIIIa on tissue macrophages is the heat stable, acid labile protein (more than 100 kDa) that is found in conditioned medium from non-stretched fibroblasts. Further experiments would need to be carried out to characterize this protein further as discussed later on.

FUTURE THERAPIES FOR RHEUMATOID ARTHRITIS

An understanding of the pathogenesis of rheumatoid arthritis and of the source of TNF- α in rheumatoid joints allows one to discuss the merits of differing therapeutic interventions in the treatment of patients with rheumatoid arthritis. It also highlights novel therapeutic strategies that may be worth pursuing.

An ideal drug for the treatment of patients with rheumatoid arthritis would be one that is easy to administer, cheap and free from serious side effects or interactions. It would also have to demonstrate sustained clinical efficacy.

The source of TNF- α in rheumatoid joints as discussed on pages 86-90 highlights a number of areas where therapies might be used to treat patients with rheumatoid arthritis.

There are two anti-TNF agents at present that have been evaluated in clinical trials in patients with rheumatoid arthritis and are licensed for use. The first is a chimeric monoclonal anti-TNF- α antibody called infliximab (Remicaide[®]) and the second a recombinant p75-TNF-receptor fusion protein called etanercept (Enbrel[®]) that acts as a form of soluble receptor that binds TNF and blocks its interaction with receptors on cells thereby interfering with the biological activity of TNF.

A two year phase III, double blind, randomised, placebo-controlled trial assessing infliximab (in 4 different dosing regimes, all with methotrexate) in patients with

active rheumatoid arthritis showed that at 30 weeks approximately 60% of patients receiving infliximab showed a significant clinical response (ACR20) (Maini R N, St Clair *et al*, 1999).

A phase III placebo-controlled study over 6 months with entanercept alone showed a similar significant clinical response (ACR20) at 6 months, when entanercept was combined with methotrexate, a slightly higher proportion of patients, approximately 70% achieved a 20% ACR response (Moreland L W, Schiff M H *et al*, 1999; Weinblatt M E, Kremer J M *et al*, 1999).

Efficacy of these anti-TNF therapies however appears to be transient with disease activity rising if treatment is discontinued.

The blockade of Fc γ RIIIa might lead to a reduction in TNF- α production in patients with rheumatoid arthritis. This may not be a good long-term strategy, as the physiological function of Fc γ RIIIa would also be affected. Fc γ RIIIa is involved in antibody dependant cytotoxicity to tumour cells, antigen presentation, phagocytosis of opsonized antigen and in the release of lysosomal enzymes, TNF- α , IL-1 and IL-6. Long-term blockade of Fc γ RIIIa might therefore be expected to have unwanted side effects.

A drug that reduced the synthesis of the soluble fibroblast factor responsible for the induction of Fc γ RIIIa or blocked its action might be useful in the treatment of patients with rheumatoid arthritis. However this agent would also interfere with the

physiological role of Fc γ RIIIa and therefore give rise to unwanted long-term side effects.

In spite of intensive efforts, T cells responsive to a single antigen, or restricted populations of T cells, have not been identified in rheumatoid synovium. T cells in rheumatoid synovium are hyporesponsive (Zvaifler N J, Firestein G S *et al*, 1998; Breedveld F C, Verweij C L *et al*, 1997). Clinical trials with various anti-T cell monoclonal antibodies have failed to lead to a sustained suppression of disease activity. Although T cells can produce TNF- α and have recently been implicated in the genesis of inflammation, the potential role of immune complexes and Fc γ RIIIa suggest a possible reason for the failure of the T cell approach.

A logical approach to the treatment of patients with rheumatoid arthritis would be to remove immune complexes that bind to Fc γ RIIIa. This could be achieved by the removal of rheumatoid factor producing B-cell clones.

CD20 is a B-cell specific cell surface molecule. The structure of CD20 suggests it functions as a membrane transporter or ion channel as it has multiple membrane-spanning domains and phosphorylation sites a hallmark of channel-forming molecules (Tedder T F, Engel P, 1994). Transfection studies of CD20 into ectopic cell types and electrophysiological data show that CD20 forms a calcium channel that is necessary for B cell activity (Bubien J K, Zhou L J *et al*, 1993). A chimeric anti-CD20 antibody has successfully been used as a treatment in patients with non-Hodgkin's lymphoma.

Five Patients with rheumatoid arthritis have undergone B lymphocyte depletion using a monoclonal anti-CD20 antibody (Mabthera 2.1g), prednisolone (60 mg reducing) and cyclophosphamide (750 mg intravenous x2) in an open study. At approximately one year follow up 3 patients have achieved substantial improvement (ACR70). Two patients relapsed after seven months and ten months respectively. Remission and relapse correlated well with rheumatoid factor levels. Minor adverse events were seen. B lymphocyte depletion appears a safe and effective therapy for rheumatoid arthritis (Edwards J C W, Cambridge G, 2000).

A larger double blind multicentre trial of B lymphocyte depletion in patients with rheumatoid arthritis is being conducted to further test the hypothesis that B lymphocytes may be essential to the perpetuation of rheumatoid arthritis.

FUTURE RESEARCH

It appears, from the experiments described, that a soluble, heat stable, acid labile protein released from synovial fibroblasts with a molecular weight of more than 100 kDa is capable of the induction of FcγRIIIa on monocyte-macrophages *in vitro*. This protein is unlikely to be a cytokine as cytokines generally have a molecular weight of less than 50 kDa (see molecular weight appendix).

Proteomics, the large-scale analysis of proteins would help characterize this protein further. This would involve a number of different steps as outlined below: (Pandey A and Mann M, 2000).

- Biological samples may contain as many as 10^6 proteins. It would therefore be useful to reduce the number of proteins in the conditioned fibroblast medium. Repeated fractionation of the conditioned fibroblast medium would need to be performed. The ability of these fractions to induce FcγRIIIa on cultured monocytes could then be studied. This process would greatly reduce the number of potential proteins in the medium that would need to be considered.
- The proteins in the final fractionate of conditioned fibroblast medium that retains the ability to induce FcγRIIIa on monocytes *in vitro* would need further characterisation. This would involve separation of the proteins by one-dimensional gel electrophoresis. The gel is silver or Coomassie blue

stained and protein bands are excised and digested into peptides by a sequence-specific protease such as trypsin. This results in peptides with arginine or lysine at their C termini as a result of the cleavage specificity of trypsin. The reason for doing this is that gel-separated proteins are difficult to elute and to analyse by mass spectrometry and that the molecular weight of proteins is not usually sufficient for database identification. In contrast, peptides are easily eluted from gels and even a small set of peptides from a protein provides sufficient information for identification.

- Protein identification then involves two further steps. The first step is to obtain the mass spectrum of the eluted peptide mixture. This results in a “peptide-mass fingerprint” of the protein being studied. This mass spectrum is obtained by a simple mass spectrometric method called matrix-assisted laser desorption/ionisation (MALDI). This is an automated process whereby hundreds of proteins can be excised, digested enzymatically, their mass spectra obtained and automatically searched against databases.
- For unambiguous protein identification a second step is employed. This second step relies on fragmentation of individual peptides in the mixture to gain sequence information. In this method, the peptides are ionised by “electrospray ionisation” from the liquid phase. The peptide ions sprayed into a “tandem mass spectrometer” which has the ability to resolve peptides in a mixture, isolate one species at a time and dissociate it into amino- or carboxy- terminal containing fragments. The advantage of this second step is

that sequence information derived from several peptides is much more specific for the identification of a protein than a list of peptide masses that is obtained from the MALDI technique alone. The fragmentation data can be used to search protein sequence databases to identify the protein. As a control proteins in non-conditioned medium could be identified to demonstrate that the soluble protein responsible for the induction of FcγRIIIa is differentially present in conditioned fibroblast medium.

Once this protein has been identified it could be added to monocyte cultures to ensure that it induces FcγRIIIa on monocyte-macrophages *in vitro*. Immunohistochemical analysis of normal synovium could then be undertaken to investigate whether this protein is present in tissues and therefore likely to be responsible for the induction of FcγRIIIa on tissue macrophages *in vivo*.

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APPENDIX 1

MATERIALS

Acetone, BDH Laboratories Supplies, Poole UK

Antibodies: Chicken IgY anti-TGF- β neutralising antibody, Research and Diagnostics, Abingdon, UK

Fluorescein and rhodamine conjugated goat antibodies to mouse IgM, mouse IgG1 and/or rabbit immunoglobulins, Sera-lab, Crawley Down, UK

Fluorescein conjugated monoclonal mouse anti-human monocyte CD14, TÜK4 clone, Dako, High Wycombe, UK

Fluorescein conjugated monoclonal mouse IgG2a anti-*Aspergillus niger* glucose oxidase (negative control), DAK-GO5 clone, Dako, High Wycombe, UK

Fluorescein conjugated F(ab')₂ fragment of goat anti-mouse immunoglobulins, Dako, High Wycombe, UK

Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins, Dako, High Wycombe, UK

Mouse monoclonal anti-human IL-10 antibody, 23738.11 clone, Research and Diagnostics, Abingdon, UK

Mouse IgG1, κ MOPC-21 clone, isotype control, Sigma Chemical Company, St. Louis, MO, USA

Mouse monoclonal IgG1 anti-human fibrillin-1, 11C1.3 clone, Neomarkers, CA

Mouse monoclonal IgG1 anti-collagen IV, Dako, High Wycombe, UK

Mouse monoclonal IgG1 anti-human pan-Fc γ RIII, 3G8 clone, Cambridge Bioscience, Cambridge UK

Mouse monoclonal IgM anti-human DAF, BRIC 128 clone, NBGRL Research products, Bristol, UK

Mouse monoclonal IgG1 anti-human CD68, EBM11 clone, Dako, High Wycombe, UK

Mouse monoclonal IgG1 anti-human Fc γ RI, 10.1 clone, gift from Dr. Hogg, Imperial Cancer Research Fund, London UK

Mouse monoclonal IgG2b anti-human FcRII γ , IV.3 clone, gift of Dr. M. Fanger, Mederex Inc., Lebanon, NH

Rabbit polyclonal anti-collagen laminin, Dako, High Wycombe, UK

Sheep anti-human interferon beta, Serotec, Oxford, UK

Sheep anti-human hepatocytes growth factor, Serotec, Oxford, UK

Bovine Serum Albumin, Sigma Chemicals, St.Louis, MO, USA

Cannula 45 mm luer-lock, Ohmeda, Helsingborg, Sweden

Centricon[®], Centrifugal Filter Devices, Millipore, Bedford, MA

Collagenase, Lorne laboratories Ltd., Reading, Berkshire, UK

Cytokines: Human recombinant interleukin 10, Research and Diagnostics, Abingdon, UK

Human recombinant transforming growth factor beta 1, Research and Diagnostics, Abingdon, UK

Human recombinant granulocyte-colony stimulating factor (Filgrastim[®]), Amgen-Roche, Cambridge, UK

Human recombinant macrophage-colony stimulating factor, Research and Diagnostics, Abingdon, UK

Dextran T500, Pharmacia Biotech, Uppsala, Sweden

Diaminobenzidine hydrochloride, Sigma-Aldrich, Poole, UK

DePex – BDH Laboratories Supplies, Poole UK

Deoxyribonuclease I, Sigma, St. Louis, Missouri, USA

Dulbecco's modified Eagles medium, GibcoBRL, Paisley, Scotland

EDTA, Sigma Chemical Company, St. Louis, Missouri, USA

Ethanol, BDH Laboratories Supplies, Poole, UK

Fetal calf serum, Sigma Cell Culture, St. Louis, Missouri, USA

Gentamicin, GibcoBRL, Paisley, Scotland

Glutamine, GibcoBRL, Paisley, Scotland

Histoclear – National Diagnostics, Alanta GA

Histopaque®-1077, Sigma Diagnostics, St. Louis, MO, USA

Hyaluronic acid (Hyalgan®), Shire Pharmaceuticals Ltd. Andover, UK

Hydrochloric acid, BDH Laboratories Supplies, Poole, UK

Hydrocortisone sodium succinate (Solu-Cortel®), Pharmacia & Upjohn, Milton Keynes, UK

Methotrexate Injection British Pharmacopia, David Bull Laboratories, Warwick, UK

Microdensitometer, M85 Vickers Medical Instruments, Sidcup, Kent, UK

N⁶ Cyclopentyladenosine A₁ (adeosine) agonist, Sigma Chemical Company, St. Louis, MO, USA

N-Ethylcarboxamidoadenosine (NECA) A₂ (adenosine) agonist, Sigma Chemical Company, St. Louis, MO, USA

Non-essential amino acids, GibcoBRL, Paisley, Scotland

N-Hexane – BDH Laboratories Supplies, Poole UK

OCT Compound – Tissue Tek, Elkhart, IN

Paraformaldehyde, BDH Laboratories Supplies, Poole, UK

Penicillin, GibcoBRL, Paisley, Scotland

Petroleum jelly (Vaseline®), London, UK

Polypropylene 3 mm tubing, Codan, Germany

Protease, Sigma Chemical Company, St. Loius, MO, USA

Rothwell Park Memorial Institute essential media 1640 (RPMI), GibcoBRL, Paisley, Scotland

Six well plates (polystyrene; Collagen IV, collagen I, fibronectin and laminin coated), Becton Dickinson Labware, Bedford, MA

Six well plates (Flex I®) flexible-bottomed culture plates coated with collagen I and fibronectin, Flexcell International Corporation, Mckeesport, PA

Sodium Aurothiomalate (Myocrisin[®]), Rhône-Poulenc Rorer, West Malling, UK

Sodium Azide, Sigma Chemical Company, St. Louis, MO, USA

Sodium Chloride, BDH Laboratories Supplies, Poole, UK

Sodium Hydroxide, BDH Laboratories Supplies, Poole, UK

Sodium Pyruvate, GibcoBRL, Paisley, Scotland

Streptomycin, GibcoBRL, Paisley, Scotland

Substance P, Sigma Chemical Company, St. Louis, MO, USA

Syringe filters (Acrodisc[®]), 0.2 µm, Gelman Sciences, Ann Arbor, MI

Syringe filters (Dynagard[®]-ME), 0.2 µm, Microgon Inc., CA, USA

Trypsin-EDTA, GibcoBRL, Paisley, Scotland

APPENDIX 2

MOLECULAR WEIGHTS

	Molecular Weight (kDa)
Interleukin-1	30.6 processed to 17.5
Interleukin-1 β	30.7 processed to 17.5
Interleukin-2	15.4
Interleukin-3	14.6
Interleukin-4	15
Interleukin-5	13.2
Interleukin-6	20.7
Interleukin-7	17.4
Interleukin-8	8
Interleukin-9	15.1
Interleukin-10	18.7
Interleukin-11	23
Interleukin-12	35
Interleukin-13	10
Interleukin-14	53
Interleukin-15	15
Interleukin-16	56
TGF β	12.5
TNF α	3x17
TNF β	18.6
M-CSF	45-60

GM-CSF 14-35

Sustance P 1.36

APPENDIX 3

Abbreviations

ACR	American College of Rheumatology
BSA	Bovine serum albumin
CD	Cluster differentiation
COX	Cyclooxygenase
CR	Complement receptor
CRGP	Calcitonin gene related peptide
Da	Daltons
DMEM	Dulbeco's modified Eagles medium
ELAM	Endothelial adhesion molecule
EDTA	Ethylenediaminetetraacetic acid
GM-CSF	Granulocyte macrophage colony stimulating factor
HPLC	High-performance liquid chromatography
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LTBP	Latent TGF β binding protein
M-CSF	Monocytes colony stimulating factor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NK	Neurokinin
NK	Natural killer cells

PBS	Phosphate buffered saline
RPMI	Rothwell Park Memorial Institute essential media
TGF β	Transforming growth factor beta
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule

APPENDIX 4

Differential distribution of FcγRIIIa in normal human tissues and co-localization with DAF and fibrillin-1: implications for immunological microenvironments

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SUMMARY

FcγRIIIa is a cytokine-inducible IgG Fc receptor implicated in the activation of macrophages by immune complexes. Differential expression of FcγRIIIa by macrophages in different tissues may therefore modulate local immune responsiveness. FcγRIIIa expression in normal human tissues was assessed semiquantitatively using microdensitometry. Synovial intimal, serosal, alveolar, salivary gland and placental macrophages, Kupffer cells, and macrophages in mechanically stressed dermis expressed high levels of FcγRIIIa. Less consistent expression was seen in skeletal muscle and lymphoid organs. No significant expression was observed in brain, thyroid, spine, intestine, myocardium, prostate, uterus, flexor forearm dermis, uterus, or kidney. Staining for FcγRIII was also observed on extracellular matrix, and co-localized with both complement decay-accelerating factor and fibrillin-1. It is proposed that differential levels of both cellular and extracellular FcγRIIIa, by modulating the response to immune complexes, may contribute to relative tissue susceptibility to infection and autoimmune disease.

INTRODUCTION

Specialized immunological microenvironments, based on features such as vascular permeability, adhesion molecule expression and local growth factor levels are well recognized in organs such as the central nervous system, eye, skin, mucosae and gut.^{1–3} This specialization is likely to be important in tissue susceptibility to infection and may also, directly or indirectly, determine tissue susceptibility to autoimmune disease.^{4–6}

An important feature of an immunological microenvironment is the regulation of interactions involving immunoglobulin, complement and associated receptors and regulatory proteins. An example of such specialization is the differential expression of complement decay-accelerating factor (DAF, CD55) on resident cells in tissues such as amnion, synovium and pericardium.⁷ A recent study has also indicated differential expression of the immunoglobulin receptor, FcγRIIIa (CD16a) within synovium.⁶

FcγRIIIa is an IgG Fc receptor expressed by macrophages, certain, chiefly cytotoxic, T-cell subsets and natural killer cells.⁸ The FcγRIIIa α-chain is encoded by the FcγRIIIA gene.⁹ The homologous FcγRIIIB gene encodes FcγRIIIB, expressed on granulocytes. The extracellular domains of the two receptors are near-identical. However, whereas FcγRIIIa has a cytoplasmic domain linked to two γ- or ζ-chains,

FcγRIIIB is glycosylphosphatidylinositol-linked.⁸ Studies on granulocytes suggest that FcγRIII-class receptors are particularly essential to the binding of dimeric immune complexes.¹⁰

Previous information on FcγRIIIa expression in normal tissues is difficult to interpret.^{11–14} *In vitro*, FcγRIIIa is expressed by macrophages cultured for several days.¹² *In vivo*, FcγRIIIa has been consistently described on Kupffer cells, but data on other tissue macrophages are conflicting.^{11–14} One study suggests that, in the absence of lesions such as tumours, expression is minimal.¹⁴ The problem is compounded by the existence of FcγRIIIa⁺ lymphoid cells and the failure of some studies to distinguish granulocytes (carrying FcγRIIIB). Our own study suggests that, at least in fetal tissues, FcγRIIIa expression by tissue macrophages may be highly restricted.⁶

Previous studies of FcγRIIIa expression have been qualitative. It is theoretically feasible to make quantitative observations, using scanning and integrating microdensitometry^{15,16} (digital image analysis systems are less satisfactory). However, there are problems of standardization, which, in the context of biological variation, make semiquantitative analysis more realistic. Mean readings cannot be derived for populations if unstained cells cannot be identified. For this reason mean maximal microdensitometric readings of macrophage staining for FcγRIIIa for 10 randomly sampled high-power fields have been obtained.

In our previous study, it was noted that pericellular staining for FcγRIIIa in synovium frequently co-localized with staining for DAF, in a way difficult to reconcile with evidence that the two molecules were predominantly synthesized by macrophages and fibroblasts, respectively.⁶ DAF is an inhibitor of complement-mediated cell lysis¹⁷ and also of killing by natural

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killer cells.¹⁸ DAF is expressed at high levels on cells lining body cavities.⁷ DAF has also been observed in association with fibrillar matrix components, variously identified as collagens, elastin, or fibrillins.^{7,19,20} The major fibrillar components of synovial intima are fibrillin-1 and collagen VI-based microfibrils.²¹ The objective of the present study was to compare expression of FcγRIIIa in a wide range of normal tissues in a semiquantitative fashion and to analyse the relationship between FcγRIII, DAF and specific matrix elements.

MATERIALS AND METHODS

Tissues

Normal human tissues were obtained from surgical procedures performed at University College London Hospitals, not relating to pathology of the tissue obtained, and post-mortem (Tissue Bank, Addenbrookes Hospital, Cambridge). Normality was confirmed histologically. Tissue samples were embedded in OCT compound (Tissue Tek, Elkhart, IN), snap frozen in *n*-hexane (BDH Laboratories Supplies, Poole, UK) and stored at -80° . Two specimens were examined of each of the following tissues except where numbers are indicated in brackets: synovium (5), lung, intestine, spleen, breast, myocardium, pericardium, brain, prostate, kidney, uterus, thyroid, liver, spinal disc and entheses, placenta, umbilical cord, flexor forearm skin, skin exposed to mechanical stress; at the elbow (1) and over a toe interphalangeal joint (1), fingernail (1), salivary gland (1), gall bladder (1), tonsil (4), bone marrow aspirate smear (1) and skeletal muscle (5).

Immunohistochemistry

Tissues were sectioned to a thickness of 5 μ m at -25° , taken up onto slides, air dried for 1 hr and fixed with cold acetone (BDH Laboratories Supplies) for 10 min. Between all further steps tissues were washed in two changes of phosphate-buffered saline (PBS) for 5 min each.

Serial sections of each tissue were incubated with either a mouse monoclonal IgG1 anti-human pan-FcγRIII (3G8 clone; Cambridge Bioscience, Cambridge, UK) at 25 μ g/ml (in duplicate), a mouse monoclonal IgM anti-human DAF (BRIC 128 clone; NBGRL Research products, Bristol, UK) at 14 μ g/ml or a mouse monoclonal IgG1 anti-human CD68 at 10 μ g/ml (EBM11 clone; Dako, High Wycombe, UK). Synovial sections were also incubated with a purified mouse monoclonal IgG1 anti-human FcγRI at 15 μ g/ml (10.1 clone; gift of Dr N. Hogg, Imperial Cancer Research Fund, London, UK) and a purified mouse monoclonal IgG2b anti-human FcγRII at 12 μ g/ml (IV.3 clone; gift of Dr M. Fanger, Medarex Inc, Lebanon, NH). All antibodies were diluted in PBS and incubated for 60 min at room temperature. Controls were incubated in PBS. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide in methanol. Sections were then incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako) at 40 μ g/ml for 30 min followed by diaminobenzidine hydrochloride (Sigma-Aldrich, Poole, UK) for 10 min, washed in tap water, counterstained in Harris's haematoxylin (except for one of each pair stained for FcγRIII) dehydrated in alcohol, cleared in Histoclear (National Diagnostics, Atlanta, GA) and mounted in DePeX

(BDH Laboratories Supplies, Poole, UK). Uncounterstained sections stained for FcγRIII were analysed by microdensitometry.

Double staining

Serial sections of each tissue were co-incubated in relevant pairs of the following antibodies: BRIC 128 at 14 μ g/ml, 3G8 at 25 μ g/ml, a mouse monoclonal IgG1 anti-human fibrillin-1 (11C1.3 clone; Neomarkers, Fremont, CA) at 40 μ g/ml, a rabbit polyclonal anti-collagen VI antibody (gift of Dr C. Kiely, Manchester University, UK) at a dilution of 1:10, a mouse monoclonal IgG1 anti-collagen IV (Dako) at 10 μ g/ml and a mouse monoclonal IgG1 anti-collagen laminin (Dako) at 12 μ g/ml. Tissues were then incubated in a mixture of fluorescein- and rhodamine-conjugated goat antibodies to mouse IgM, mouse IgG1 and/or rabbit immunoglobulins (Sera-Lab, Crawley Down, UK), at final dilutions of 1:20; for 30 min. Tissues were washed and mounted in diamino bicyclo-octane. All double-stained sections were compared with single-stained and second-layer control sections.

Cell identification

In the absence of an available antibody with high specificity for FcγRIIIa versus FcγRIIIb, measures were taken to ensure discrimination of FcγRIII⁺ cell populations. Comparison of counterstained and uncounterstained immunoperoxidase staining for FcγRIII, and immunoperoxidase staining for the macrophage marker CD68 indicated that the shape of the unstained nuclei of macrophages, granulocytes, or lymphocytes, with FcγRIII⁺ cytoplasm, on uncounterstained sections, together with nuclear to cytoplasmic ratio allowed reliable discrimination of cell types in normal tissues except for the mononuclear cell types in densely packed lymphoid tissue and bone marrow.

Microdensitometry

Semiquantitative analysis of FcγRIII expression was performed on sections stained with the immunoperoxidase technique. As far as possible two specimens for each tissue were analysed microdensitometrically (Table 1). Sections were stained using supraoptimal concentrations of primary antibody and rigidly standardized incubation times. Analysis was performed using an M85 Vickers scanning microdensitometer (Vickers Medical Instruments, Sidcup, Kent, UK).²² Absorption was measured at 550 nm with a mask diameter corresponding to a 5- μ m section. Absorption was measured as relative extinction, in arbitrary units, using unstained areas of the section as reference. Measurements were taken over the cytoplasm of macrophages showing maximum staining in each of 10 randomly selected high-power fields. Mean readings and standard errors were obtained for each sample. Measurements from each staining batch were normalized by defining 1 unit as the value obtained for macrophages in a normal synovial intima included in each batch.

RESULTS

Cellular staining

Measurable levels of staining for FcγRIII were present on synovial intimal, and to a lesser degree subintimal, macrophages, alveolar macrophages, pericardial macrophages,

Table 1. Semiquantitative assessment of macrophage FcγRIII expression in normal tissues

Tissue	Macrophage population	FcγRIII Index*	Source
Synovium	Intimal macrophages	1	Biopsy
		1.28 ± 0.09*	Biopsy
		0.72 ± 0.04*	Biopsy
	Subintimal macrophages	0.38 ± 0.02	Biopsy
		0.35 ± 0.04	Biopsy
Lung	Alveolar macrophages	0.99 ± 0.10	Autopsy
		0.99 ± 0.08	Autopsy
Pericardium	Macrophages	1.03 ± 0.07	Biopsy
		0.92 ± 0.08	Biopsy
Liver	Kupffer cells	1.50 ± 0.09	Autopsy
		0.42 ± 0.04	Biopsy
Stressed dermis (elbow)	Macrophages	0.68 ± 0.07	Biopsy
Stressed dermis (toe)		0.27 ± 0.07	Biopsy
Salivary gland	Macrophages	0.43 ± 0.05	Biopsy
Placenta	Hofbauer cells	0.39 ± 0.04	Biopsy
		0.39 ± 0.03	Biopsy
Umbilical cord	Intravascular monocytes	0.86 ± 0.04	Biopsy
Bone marrow	Macrophages/precursors	Not measured†	Biopsy
Skeletal muscle	Macrophages	Not measured	Biopsy
Spleen/tonsil	Macrophage subsets	Not measured	Biopsy
Breast		Undetectable	Autopsy
		Undetectable	Biopsy
Brain	Microglia	Undetectable	Biopsy
		Undetectable	Biopsy
Thyroid	Macrophages	Undetectable	Autopsy
		Undetectable	Autopsy
Spinal disc/enthesitis	Macrophages	Undetectable	Biopsy
		Undetectable	Biopsy
Intestine	Macrophages	Undetectable	Autopsy
		Undetectable	Biopsy
Myocardium	Macrophages	Undetectable	Biopsy
		Undetectable	Biopsy
Prostate	Macrophages	Undetectable	Biopsy
		Undetectable	Biopsy
Flexor forearm dermis	Macrophages	Undetectable	Biopsy
		Undetectable	Biopsy
Uterus	Macrophages	Undetectable	Biopsy
		Undetectable	Autopsy
Kidney	Macrophages/ Mesangial cells	Undetectable	Autopsy
		Undetectable	Autopsy
Gallbladder	Macrophages	Undetectable	Autopsy
Nailbed	Macrophages	Undetectable	Biopsy
Unstressed dermis	Macrophages	Undetectable	Biopsy
		Undetectable	Biopsy

*Mean relative extinction for immunoperoxidase staining for FcγRIII normalized to synovial intima = 1, ± standard error. Comparative values for two synovial samples given in parentheses.

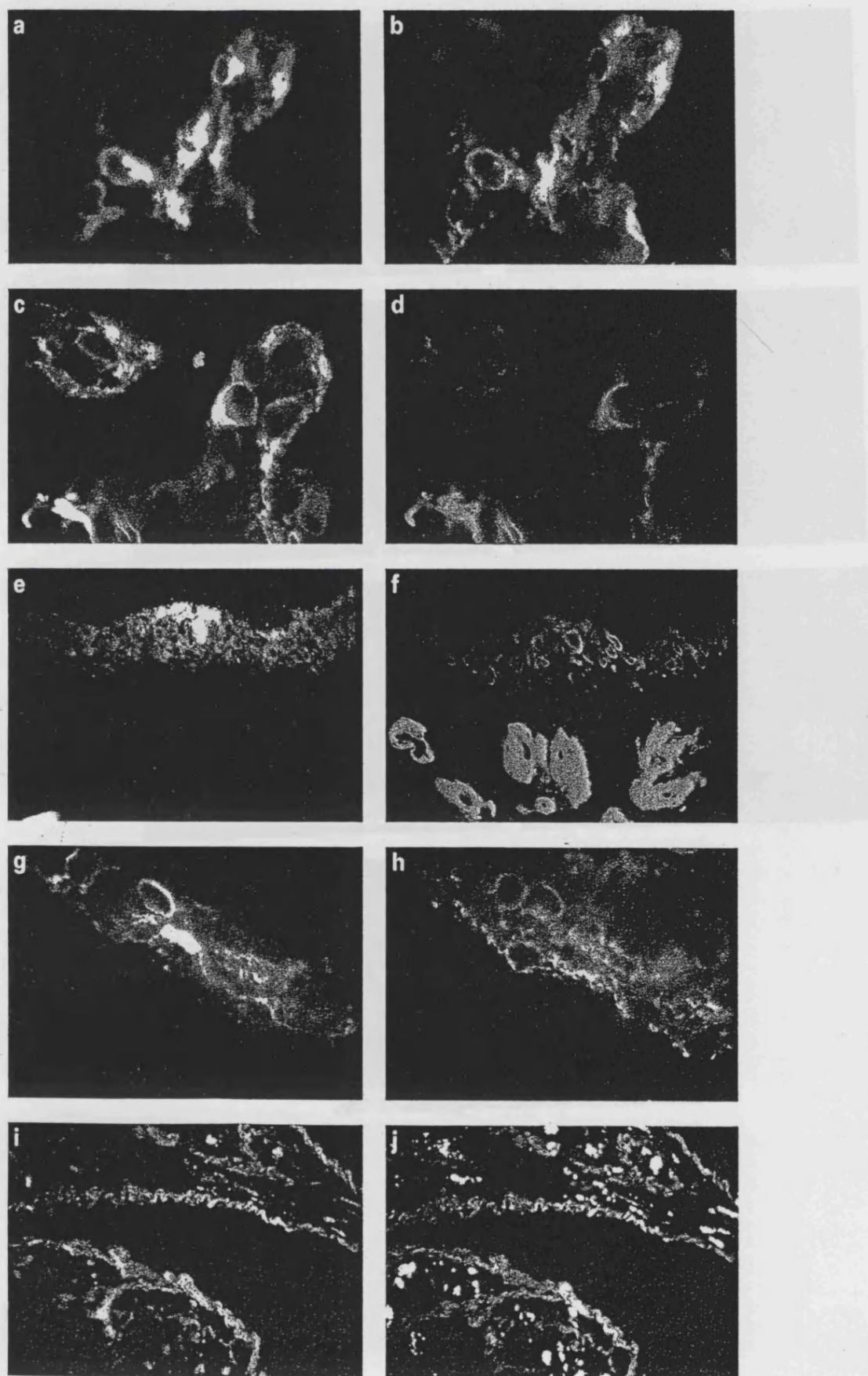
†See text, index not derived for technical reasons.

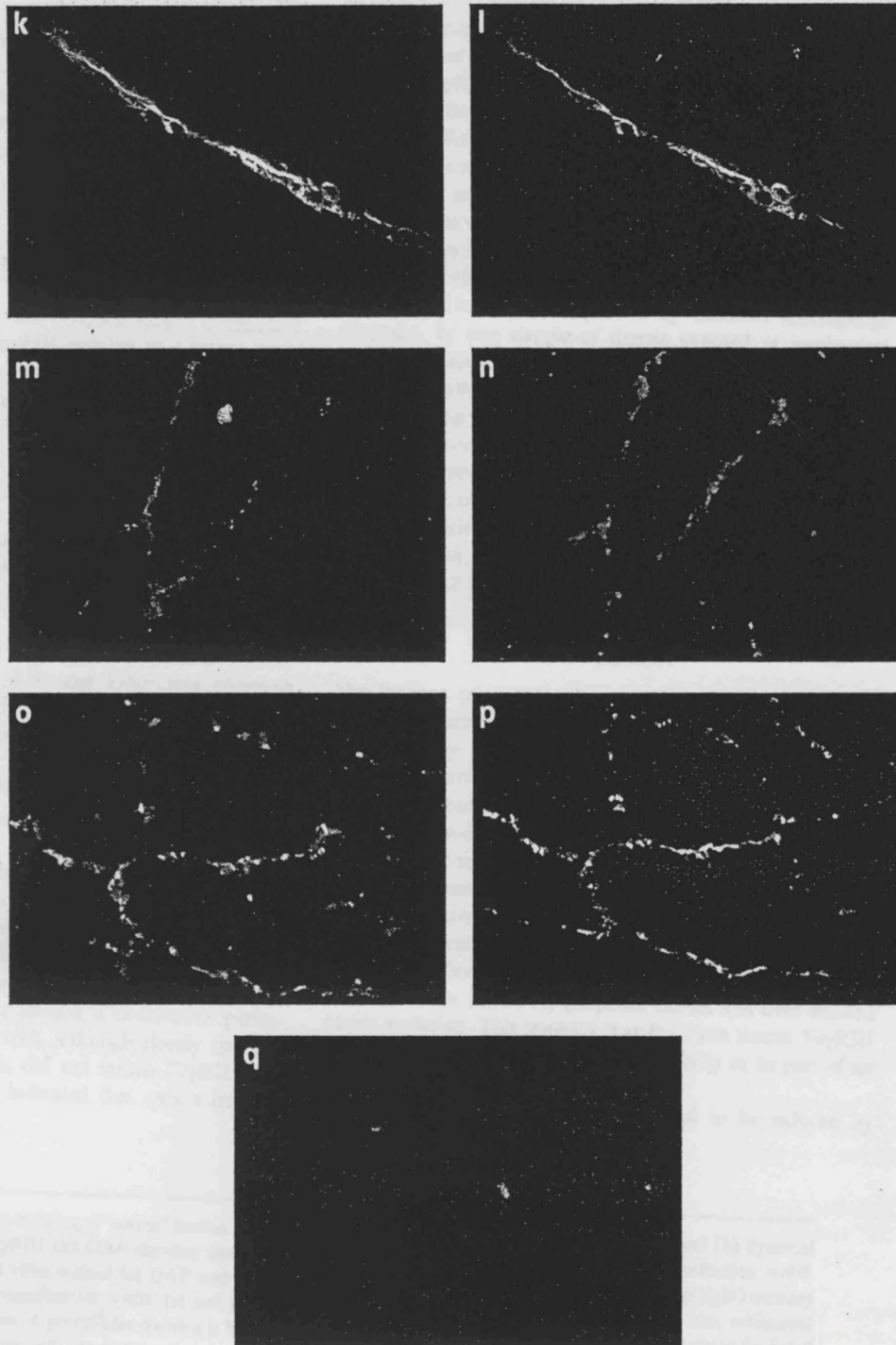
Kupffer cells, macrophages in mechanically stressed dermis, salivary gland macrophages, placental Hofbauer cells (macrophages), and monocytes in umbilical cord vessels (Table 1). Macrophage FcγRIII staining was also observed in muscle, bone marrow and lymphoid tissue, but was not measured for technical reasons. In other tissues, FcγRIII staining was not seen on macrophages despite their presence, as indicated by CD68 staining.

In muscle very few FcγRIII⁺ cells were found in comparison to the number of CD68⁺ cells present. The level of FcγRIII staining on this minority of macrophages was signifi-

cant, but the etiolated profile of the cells precluded reliable measurement using the standard mask. Large FcγRIII⁺ mononuclear cells consistent with macrophages were seen in bone marrow and some samples of tonsil and splenic white pulp. However, it was not possible to be certain of the identity and state of maturation of these cells. Moreover, in other samples of tonsil virtually no staining was seen other than on scattered granulocytes.

Staining with antibodies to FcγRI and FcγRII confirmed the specificity of differential staining obtained for FcγRIII in synovium. As reported in diseased tissue,²³ all macrophages





stained for FcγRII and FcγRI staining was limited to sub-intima (not shown).

FcγRIII⁺ granulocytes were seen sparsely scattered in most tissues and in large numbers in splenic white pulp. Comparison of both granulocyte and macrophage staining in post-mortem and biopsy samples indicated that the source of tissue had no significant effect on staining levels.

Extracellular staining

Extracellular staining for FcγRIII was restricted to synovial intima, mesothelia, skeletal and cardiac perimysium, uterine arterial internal elastic lamina and dermis at sites of mechanical stress. In synovial intima FcγRIII staining was chiefly cellular and pericellular, and less often on isolated fibrillar structures. Pericellular FcγRIII and DAF (Fig. 1a,b) frequently co-localized around the same cells in a pattern which matched closely to that of fibrillin-1 (Fig. 1c,d). Extracellular DAF staining was not confined to sites of fibrillin-1 but showed some apparent overlap with pericellular staining for laminin (Fig. 1e,f). Neither DAF nor FcγRIII staining co-localized with collagen VI-containing fibrillar structures (Fig. 1g,h) or with collagen IV, which did not show a pericellular or fibrillar pattern (not shown).

In pericardium, and on the peritoneal surface of spleen, linear staining for both FcγRIII and DAF was observed (Fig. 1i,j). For pericardium, FcγRIII and DAF staining was discontinuous with both brightly stained and unstained areas (Fig. 1k). Linear staining co-localized with fibrillin-1 (Fig. 1k,l) and not collagen VI (not shown). A similar pattern of staining was seen on scattered fibrous septae between cardiac myocytes (Fig. 1i,j).

Linear staining for both FcγRIII and DAF was observed on skeletal muscle epimysium (Fig. 1m,n). Staining patterns for the two molecules demonstrated major overlap and followed the pattern of fibrillin-1-based microfibrils, which formed a discontinuous perimyseal network (Fig. 1o,p). Collagen-VI-based microfibrils formed a continuous perimyseal envelope (not shown), which, although closely apposed to fibrillin-1-based microfibrils, did not match FcγRIII and DAF staining. CD68 staining indicated that only a fraction

of epimysial staining for FcγRIII and DAF could be accounted for by macrophage cytoplasm/membranes (Fig. 1q).

A proportion of small to medium-sized uterine arteries showed FcγRIII staining of the internal elastic lamina, but vessels in other tissues did not stain.

Extracellular matrix-associated FcγRIII was assumed to represent FcγRIIIa, being associated with FcγRIII⁺ macrophages and not granulocytes. In muscle, although FcγRIII⁺ macrophages were scarce, CD68 staining occasionally revealed macrophages with a cytoplasmic extension of similar extent to a stretch of microfibril-associated FcγRIII staining suggesting that FcγRIIIa may be deposited by transient macrophage filopodia. In one sample of dermis exposed to mechanical stress granulocytes were present, raising the possibility that fibrillar staining was due to FcγRIIIb in this case.

In keeping with previous reports,⁷ extracellular DAF staining was observed in many tissues, but with major variation in intensity. A proportion of DAF staining precisely co-localized with a subset of fibrillin-1-containing fibrillar elements, including large elastic fibres in tissue stroma but not vascular internal elastic lamina. DAF was also present in epithelial basal laminae. DAF did not co-localize with collagen VI.

DISCUSSION

The findings presented indicate that macrophage FcγRIIIa expression varies widely between different tissues. FcγRIIIa expression by monocytes following 'maturation' *in vitro*²⁴ appears to mirror macrophage behaviour in synovial intima, hepatic sinusoids and alveoli, but not, for instance, gut, skin, or brain. Low-level expression of FcγRIIIa by macrophages in these latter tissues is not ruled out and might be detectable with more sensitive methods, but the differential in FcγRIIIa expression between tissues demonstrated is likely to be of major functional significance.

Although levels of staining for FcγRIII were reproducible in most tissues (Table 1), lymphoid tissues and liver showed major variation. This suggests that for these tissues FcγRIII expression may vary with immune reactivity or as part of an acute phase response.

FcγRIIIa expression has been found to be induced by

Figure 1. Immunofluorescent staining of normal human tissues for FcγRIII, DAF and matrix components. (a) and (b) Synovial intimal villus stained for FcγRIII and DAF showing unexpected co-localization of pericellular staining; final magnification × 400. (c) and (d) Synovial intimal villus stained for DAF and fibrillin-1, both pericellular (middle left) and fibrillar (top right) staining patterns co-localize; final magnification × 400. (e) and (f) Synovium stained for DAF and laminin (intima at top, subintimal vessels below). Co-localization of pericellular staining is less consistent than for DAF and fibrillin-1. Even where staining for DAF and laminin outlines the same cell, the pattern appears concentric rather than identical; final magnification × 200. (g) and (h) Synovial intima stained for DAF and collagen VI. The images are totally discordant, with the exception of one elliptical cell outline (top centre). On precise registration the outlines of this cell are partially concentric, and not coincident; final magnification × 400. (i) and (j) Pericardium stained for FcγRIII and DAF showing an area of bright continuous curvilinear staining of the mesothelium and some associated fibrous septa. Macrophages are sparse at this site and account for only a small fraction of FcγRIII staining. Autofluorescent glycogen granules are also present in the underlying tissue, seen more brightly on the rhodamine channel (j); final magnification × 200. (k) and (l) Pericardium stained for DAF and fibrillin-1. Pericellular and extracellular linear staining for DAF co-localizes almost entirely with fibrillin-1. This area of bright staining merges with an area of minimal staining at bottom left; final magnification × 200. (m) and (n) Muscle stained for FcγRIII and DAF. The images share many common elements but are not identical; final magnification × 200. (o) and (p) Muscle stained for DAF and fibrillin-1. The images show close correspondence, on fibrillar structures outlining individual muscle cells; final magnification × 200. (q) Muscle stained for CD68. This field is of a serial section of the field shown in (o) and (p), demonstrating that the FcγRIII staining in (o) is not accounted for by cellular staining of macrophages; final magnification × 200.

transforming growth factor- β (TGF- β) and interleukin-10 and down-regulated by interleukin-4.^{25,26} It is likely that the relative availability of these cytokines in liver, lung and synovium favours Fc γ RIIIa expression. The appearance of Fc γ RIIIa on synovial intima at the time of embryonic joint cavity formation⁶ and in mechanically stressed dermis suggests that at these sites TGF- β may be produced locally in response to mechanical stimuli.²⁷ Moreover, fibrillin-1-based microfibrils are known to bind a wide range of immunologically active ligands, including the latent TGF- β -binding proteins.^{28–32} Fibrillin-1-based microfibrils may therefore provide a reservoir of latent TGF- β .

The techniques used in this study permit the conclusion that both extracellular Fc γ RIII and DAF associate predominantly with fibrillin-1-based structures and it is tempting to suggest a direct interaction with fibrillin-1 itself. However, such an interaction will require demonstration by further analytical and ultrastructural studies.

The role of Fc γ RIIIa⁺ macrophages may vary from tissue to tissue. Clearance of small immune complexes by liver and spleen provides a means of preventing dissemination of toxins via the circulation. Alveolar macrophages are at an interface with the environment where recognition of small amounts of antigen may be important. The role of Fc γ RIIIa in synovium and mesothelia is less clear. However, the close association of Fc γ RIIIa with DAF and fibrillin-1 in these tissues suggests some co-ordinated function in the protection of internal body interfaces. These tissues are unlikely to encounter foreign antigen often, but a rapid response to antigen may help prevent the development of sequestered infection within a cavity. Anecdotal accounts of intra-articular antigen administration suggest that it is a potent route for immunization (R. R. Coombs; personal communication).

Synovium is a major target for autoimmune disease, particularly in the form of rheumatoid arthritis (RA). Moreover, the pattern of Fc γ RIIIa expression observed fits closely with the pattern of extra-articular involvement in RA. Fc γ RIIIa signals via two common FcR γ -chains,³³ which in macrophages gives rise to production of mediators such as tumour necrosis factor- α (TNF- α). In RA subjects, events occurring in synovium, liver, lung, pericardium and bone marrow are consistent with the local production of TNF- α by macrophages in response to Fc γ RIIIa ligation by small immune complexes, such as IgG rheumatoid factor dimers, found in the circulation in RA.³⁴

The significance of microfibril-associated Fc γ RIII, particularly in muscle, is puzzling. Binding to extracellular Fc γ RIII should not lead to macrophage cytokine production, but may allow aggregation of small complexes. This may lead, in the presence of DAF on fibrils and CD59 on muscle cells, to controlled generation of C3a. Perhaps perimysium acts as an extravascular sump for complexes in the way that red cells, through complement receptor 1,³⁵ do for circulating complexes. Binding of complexes in muscle may contribute to viral myalgia and may also be of relevance to muscle pain and stiffness in polymyalgia rheumatica (PMR). Circulating immune complexes occur in PMR, but differ from those found in RA in their content of alternative pathway components.³⁶ Muscle biopsies show IgG and fibrin deposits in the perimysium.³⁷ RA and PMR show major overlap, but most cases fall into one or other category. This suggests that whether the impact of extravascular complexes is predominantly at sites of

cell-associated or matrix-associated Fc γ RIIIa depends on detailed characteristics of the complexes such as size, interactions with complement pathways (as in PMR) and local synthesis of both antigen and antibody (as for IgG rheumatoid factors in RA synovium).^{6,38}

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