A STUDY ON ANTIBODIES TO CYTOKERATIN-18 IN RHEUMATOID ARTHRITIS

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To the memory of my Father

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or other institute of learning.

CHAPTER 6: Appendix

CHAPTER 7: REFERENCES

INTRODUCTION

1.1

ABSTRACT

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder which predominantly affects the joints. The aetiology and pathogenesis of this heterogenous disease is not known, but, as is discussed in this thesis, there is strong evidence implicating an important role for autoantibody formation against host structures in response to as yet unidentified antigens.

Cytokeratins are present throughout the cytoskeleton of many eukaryotic cells and damage to these structures during inflammation may well result in autoantibody formation. Cytokeratin-18 (CK-18) represents one member of the cytokeratin family and is unusual in being present in endothelial cells within synovial blood vessels. In this thesis, five aspects of the antibody responses to cytokeratins were investigated.

In the first part of the study, a sensitive ELISA for the binding of antibodies to CK-18 was developed. This system was used to measure the responses of three immunoglobulin classes to CK-18 and was designed to improve the sensitivity and specificity over previous immunofluorescence assays. Increased levels of IgA antibodies to CK-18 were found in serum and synovial fluid in RA compared to normal controls.There were no differences in IgG or IgM antibody responses.

In the second study the specificity of this response was tested by measuring antibody responses to other cytokeratins in RA as well as in other disease states. Increased IgA responses were also found in Psoriasis and in Psoriatic arthritis (PsA). In the third study attempts were made to characterise the antigen against which the antibody response was directed using Western Blotting with different sera on purified cytokeratins. An IgG response was mainly shown.The mechanisms and implications of this are discussed in the thesis.

In the fourth part of this study attempts were made to determine the specificity of the IgA antibody response in RA given the common finding of elevated serum IgA in these patients. The lack of correlation with serum IgA levels suggests that the antibody response to CK-18 in RA is a specific antibody response.

In the final part of the study attempts were made to identify disease subsets within RA according to elevated IgA antibody levels to CK-18. None were identified. In patients with PsA, a significant difference was found in the levels of IgA antibodies to CK-18 in patients with peripheral arthritis compared to those with spondylitis. This is the first study to show an increased IgA response to CK-18 in Rheumatoid Arthritis, Psoriasis and Psoriatic Arthritis.

1.2

Hypothesis

Serum and synovial fluid levels of antibodies to cytokeratin-18 (CK-18) in inflammatory rheumatic diseases reflect disease activity in specific subsets of the broadly defined disease rheumatoid arthritis. Within these subsets effective treatment may be associated with a change in levels of antibodies to CK-18 so, recognition of these subsets is important for accurate selection of therapy. Recognition of subsets based on abnormal levels of CK-18 antibodies may provide insight into the underlying aetiology of inflammatory joint disease.

1.3

OBJECTIVES

The objectives of the thesis are:-

1. To develop an Enzyme Linked Immunosorbent Assay (ELISA) for measuring binding of sera and synovial fluid antibodies to Cytokeratin-18 and Epidermal Keratin in patients with chronic arthritis.

2. To define which particular cytokeratins are recognised by autoantibodies to cytokeratin-18 and epidermal keratin.

3. To investigate whether antibodies to cytokeratin-18 cross-react with other antigens such as epidermal keratins.

4. To characterise the antigens involved by performing immunoblotting experiments.

5. To explore the relationship between antibody levels to cytokeratin-18 and epidermal keratin and measures of disease activity in RA.

6. To explore any relationship between abnormal serum or synovial fluid levels of cytokeratin-18 autoantibodies and features of disease which may enable recognition of RA disease subsets.

7. To investigate the antibody response to cytokeratin-18 and epidermal keratin in other inflammatory disorders.

The thesis will attempt to answer the following questions:-

1. Are autoantibodies to CK-18 raised in RA ?.
2. Do levels of autoantibodies to CK-18 correlate with autoantibody levels to EpK ?.
3. Are elevated levels of CK-18 antibodies disease specific ?.
4. Do antibodies to CK-18 recognise different epitopes from epidermal keratin ?.
5. Are all classes of antibodies to CK-18 raised or is the rise limited to particular immunoglobulin classes ?.
6. Is an abnormal serum or synovial fluid level of antibody to CK-18 just an epiphenomenon or does it change in a different way from other measures of disease activity ?; if so, is it important and relevant to patient management ?.

1.4

ABBREVIATIONS USED

AKA	: Anti-keratin Antibodies
APF	: Antiperinuclear Factor
AS	: Ankylosing Spondylitis
AU	: Arbitrary Units
BBS	: Borate Buffered Saline
BSA	: Bovine Serum Albumin
CK-8	: Cytokeratin-8
CK-18	: Cytokeratin-18
CRP	: C-Reactive Protein
EBV	: Epstein-Barr Virus
ELISA	: Enzyme Linked Immunosorbent Assay
EpK	: Epidermal keratin
ESR	: Erythrocyte Sedimentation Rate
FITC	: Fluorescin Isothiocyanate Conjugate
HLA	: Human Leucocyte-associated Antigen
IF	: Intermediate Filaments
IgA	: Immunoglobulin A
IgG	: Immunoglobulin G
IgM	: Immunoglobulin M
IL-1	: Interleukin-1

JCA	: Juvenile Chronic Arthritis
JRA	: Juvenile Rheumatoid Arthritis
MDCK	: Madin Darby Canine Kidney
MHC	: Major Histocompatibility Complex
OA	: Osteoarthritis
OD	: Optical Density
PBS	: Phosphate Buffered Saline
RA	: Rheumatoid Arthritis
RANA	: Rheumatoid Arthritis Nuclear Antigen
RF	: Rheumatoid Factor
SDS	: Sodium Dodecyl Sulphate
TNF	: Tumour Necrosis Factor

1.5

Original Publications relevant to this Thesis

1. **Borg A A**, Dawes P T, Mattey D L.
   Increased IgA antibodies to Cytokeratins in Rheumatoid Arthritis.
   Arthritis Rheum 1993 (In Press).

2. **Borg A A**, Dawes P T, Hothersall T E.
   Seronegative Spondyloarthropathies : review of the Genetics and Pathogenesis.
   Malt Med J (In Press).

3. **Borg A A**, Fitzgerald D, Heagerty A M H, Smith A G, Dawes P T, Mattey D L.
   Antibodies to Cytokeratins in the Seronegative Spondyloarthropathies and in Psoriasis.
   Manuscript in Preparation.

1.6

Published Abstracts

1. **Borg A A**, Dawes P T, Mattey D L.
   Antibodies to Cytokeratin-18 in Rheumatoid Arthritis.
   Br J Rheumatol 1992;31:14.

2. **Borg A A**, Wilkinson M, Dawes P T, Mattey D L.
   Antibodies to Cytokeratins in Psoriatic arthritis
   Clin Exp Rheum (In Press)

3. **Borg A A**, Fitzgerald D A, Heagerty A M H, Mattey D L.
   Increased IgA Antibodies to Cytokeratins in psoriasis.
   Br J Dermatol (In Press)

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CHAPTER 2

LITERATURE REVIEW

2.1

RHEUMATOID ARTHRITIS

2.1.1

Definition

Rheumatoid Arthritis (RA) can be defined as a heterogenous, chronic systemic inflammatory disorder of unknown aetiology which commonly affects the joints throughout the body, often leading to severe functional disability.

In keeping with other autoimmune diseases, RA predominates in females with the ratio of female to male patients being about 4:1. By definition, the age of onset is 16 or older, and the commonest ages affected are in the 20-55 year range, although no age is exempt.

2.1.2

Clinical Features

Initially joint involvment is polyarticular in about 75% of cases and is characteristically bilateral and symmetrical. Small joints of the hands or feet are predominantly affected in about 60% of cases at onset, while in about 30% large joint involvment is the initial manifestation of RA.

Ultimately, most joints are affected, especially the metacarpophalangeal (MCP) and the proximal interphalangeal (PIP) joints of the hands, the wrists, small joints of the feet and the knees (70-80% of patients). The ankles, shoulders, elbows, hips and cervical spine are involved in about 50-60% of patients.

The onset of joint pains and stiffness is insiduous in the majority of cases, with only about 15% of patients describing an acute onset. Stiffness is characteristically generalised, and most noticeable after rest and on waking up. Typically it is improved by exercise.

Affected joints are usually swollen and tender with limitation of movement. Swelling may be due to an effusion or synovial thickening (synovitis). In advanced cases, it may even be due to bony overgrowth. Commonly, there is quite marked muscle wasting around affected joints.

RA follows a persisent course punctuated by partial remissions and exacerbations in 75% of cases. Individual joints once involved, usually remain involved although the activity within them may fluctuate.

Episodic or Palindromic rheumatism occurs in up to 25% of patients and is characterised by recurrent, acute but self-limiting attacks of arthritis. This form of arthritis attacks males and females equally and tends to affect younger adults. A number of patients eventually develop chronic, persistent RA.

RA is a multisystem disease and non-articular manifestations are well recognised and few sites are exempt. These include: periarticular soft tissues (nodules, synovial cysts, muscle wasting and ligamentous laxity). Nodules are the hallmark of RA and can occur in any part of the body but are most common at sites of pressure such as below the elbow. Other sites commonly affected by the rheumatoid process include skin, eyes, heart and lungs. Vasculitis is an uncommon but potentially life-threatning manifestation. The involvement can vary from deep skin ulcers, severe nail fold thrombi, digital gangrene, palpable purpura, neuropathy, visceral infarction and cerebral inflammation. Neuropathy is more commonly due to entrapment by synovitis or bony overgrowth at the wrist, elbow or ankle, rather than in association with vasculitis. Infections are probably the commonest cause of death in RA.

Other less common extra-articular manifestations of RA include non-pitting oedema of a limb, Sjogren's syndrome, osteoporosis and fractures, myositis and amyloidosis.

Radiological features of RA include periarticular osteoporosis and erosions in the joints of the hands and feet in early stages. In advanced RA, loss of joint space and bone destruction occurs with secondary osteoarthritis. Rarely, this is followed by ankylosis of the affected joint.

2.1.3

Investigation and Assessment

Laboratory features of RA include anaemia and a raised acute phase response reflected by an elevated erythrocyte sedimentation rate (ESR) and C-Reactive protein (CRP) in active disease. Up to 75% of RA patients are positive for IgM rheumatoid factor. Anaemia is common and tends to mirror disease activity. Less commonly it is due to occult gastrointestinal blood loss or iron deficiency.

2.1.4

Immunological Features

Cellular immunity, humoral immunity and nonimmunologic pathways all contribute to the pathogenesis of RA.

The role of Cell-mediated Immunity revolves around the association between disease susceptibility in RA and the inheritance of certain Major Histocompatibility Complex (MHC) and encoded elements which are restricted to T cells.

The role of a disturbance of humoral immunity in RA pathogenesis is suggested by a number of features [Ball and Koopman,1992]. These include the presence of :

1. Rheumatoid factors (see below) not only as markers of RA but also as potential contributors to immune complex formation.

2. Autoantibodies against a wide range of intracellular as well as extracellular antigens including collagen, keratin, heat shock proteins, proteoglycans, nucleoproteins and IgG degredation products.

3. Immune complex formation within the joint. These complexes can initiate activation of the complement cascade. Complement activation in turn can result in increased vascular permeability locally and further uptake of immune complexes by macrophages thus perpetuating the cycle of inflammation and tissue damage.

4. Activated phagocytic cells at the site of inflammation release a number of products including proteolytic enzymes (collagenase, elastase), arachidonic acid metabolites (leukotrienes, prostaglandins) and oxygen free radicals. These cells can be activated by the ingestion of immune complexes as well as by the presence of complement products, Transforming growth factor Beta, Interleukin-8 and leukotriene B_4 within the synovial fluid in RA.

Other immunologic pathways are also postulated to play an important role in the pathogenesis of RA. These include the production of a number of cytokines including Interleukin-1 (IL-1), Interleukin-6, Interferons (alpha, beta and gamma), Tumour necrosis factors alpha and beta, colony stimulating factors and Transforming growth factor [Isenberg,1992].

IL-1 is the prototype of the interleukins and is produced by a number of different cells. It has a number of actions including fibroblast proliferation and collagen synthesis and upregulation of the expression of adhesion molecules on vascular endothelial cells [Kahaleh,1990]. Increased levels of IL-1 are thought to be important in the pathogenesis of tissue damage in RA, by a direct effect on cartilage degredation, via proteoglycan synthesis [Brennan et al,1992].

2.1.5

Rheumatoid Factors

Rheumatoid factors are immunoglobulins (G,A,M,D,E) directed against antigenic determinants on the Fc portion of IgG. Rheumatoid factors are found in a wide range of disorders where there is chronic stimulation of the immune response, including infections such as Tuberculosis. They also occur in the serum of a small proportion of the normal population (<5%). The most commonly measured RF is IgM-RF, found in about 70% of RA patients.

IgM-RF (and possibly also IgG-RF) has been shown to be capable of activating the complement cascade via the classical pathway, suggesting that Rheumatoid factors contribute to tissue injury in RA. Further evidence for this capability relates to the occurence of RF in immune complexes present in the sera, synovial fluids and synovial phagocytes of RA patients; the local production of RF in rheumatoid synovium, pleura, and pericardium.

Further evidence for the pathogenic potential of rheumatoid factors relates to the local consumption of complement in the joint space of RA patients and the correlation of factor B (properdin) and C4 (as markers of the alternative and classical complement pathways respectively) turnover rates with levels of IgM-RF in the sera of RA patients [Koopman et al,1985].

RA patients with high levels of RF, irrespective of class, have been shown to be associated with increased radiological damage, clinical disease activity, extra-articular features, worse functional ability and the need for more disease-modifying agents than patients with persistently negative or variably positive and negative results [Van Zeben et al,1992].

2.1.6

IgA Rheumatoid Factor

Of all the RF classes, IgA-RF positivity correlates most closely with the development of erosions in RA. The reason for this is unclear. IgA has been shown to possess complement-independent opsonic effects on phagocytosis by monocytes but not on neutrophils. Brik et al,[1990], further postulated that the presence of IgA complexes could activate macrophages which in turn could cause bone resorption secondary to production of prostaglandins and Interleukin-1.

Increased levels of IgA Rheumatoid Factors (IgA-RF) can often be found in serum from patients with RA [Schrohenloher et al, 1986]. Otten et al [1991], using an ELISA assay demonstrated elevated levels of IgA-, IgA1-, and IgA2-RF in 69%, 73% and 36% respectively of patients with RA compared to patients with SLE, AS, Polyarteritis Nodosa and in normal controls.

Significant positive correlations were found between levels of IgA-RF subclasses in synovial fluid and serum, but not between serum and saliva nor synovial fluid and saliva. This suggested that although both IgA-RF subclasses are produced locally in salivary glands and synovial tissues, the production of both IgA-RF subclasses at mucosal and nonmucosal sites are independent.

Koopman et al [1985], studying rheumatoid factor synthesis by dissociated synovial B cells from patients with RA, showed that local synthesis of IgA-RF frequently occurs in the synovia of RA patients and is only weakly correlated with IgM-RF production. Patterns of local production of both IgA-RF and IgM-RF in the synovium bore little relationship to those exhibited by peripheral blood B cells. The locally synthesised IgA-RF was predominantly of the IgA1 subclass and existed in both polymeric and monomeric forms.

2.1.7

Serum IgA.

Elevated levels of serum IgA were found in 23% of patients with RA and have been implicated in the pathogenesis of RA [Pillemer et al,1987]. Surprisingly, the authors pointed out that the elevation in the serum IgA was polymeric in nature rather than monomeric (see above). Jorgensen et al [1992], demonstrated increased levels of serum IgA by nephelometry in 15% of RA patients and correlated elevated serum IgA levels with an increased frequency of distal interphalangeal arthritis (41.4%), unilateral sacroiliitis (34.5%) and an increase in recurrent microscopic haematuria (20.7%).

In healthy adults, the daily production of serum and secretory IgA exceeds that of all the other immunoglobulin classes combined. Its rapid catabolism, however, results in lower serum levels than IgG [Mestecky et al,1986].

Serum IgA molecules are essentially 160 kilodalton monomers (mIgA), produced by the central immune system. The polymeric forms (pIgA) are mainly covalent dimers (330 kilodaltons) produced by the central immune system and the mucosa associated lymphoid tissue. A minor subset of polymers (400 kilodaltons) is similar to secretory IgA (SIgA).

Two subclasses of IgA have been described: IgA1 and IgA2. IgA1 predominates in serum where it makes up about 90% of the total IgA. Mean serum levels of IgA2 vary between 11-23% [Mestecky, 1986]. IgA2 is found predominantly in mucosa associated lymphoid tissue and in secretions (saliva, intestinal, milk, tears, bronchial, hepatic) with the highest proportions being found in tears, saliva and milk [Delacroix et al,1982]. Secretory IgA has an equal distribution of IgA1 and IgA2 subclasses.

In humans, the bone marrow and spleen are the major sources of serum IgA whereas IgA in external secretions is mainly produced locally by cells distributed in the tissues of secretory glands. The polymeric IgA is then transported into the external secretions by a secretory component-dependent mechanism [Mestecky and McGhee,1987].

The production of specific antibodies secreted in mucosal fluids is either the result of a local antigenic stimulus or of antigenic stimulation of the Peyer's patches, a specialised organ in the small intestine regulating the mucosal immune responses [Kagnoff,1975]. A local antigenic stimulus results in local production of antigen-specific IgA antibodies. Antigen presentation in the Peyer's patches may result in priming of antigen-specific B cells which subsequently home via the lymph nodes and circulation to distant mucosal sites [Craig and Cebra,1971]. This mechanism, which leads to the presence of antibodies with identical specificity at different mucosal sites, is often referred to as the common mucosal immune system [Mestecky and McGhee,1987]. However, although both external secretions and serum contain IgA isotypes, the secretory and serum IgA systems appear to be independent with respect to the molecular properties of IgA produced, antigen specificities and cellular origins. As a consequence, antigen-specific IgA antibodies can be found in mucosal fluids and not in the circulation, and vice versa [Mestecky et al,1986].

2.2

Seronegative Spondyloarthropathies

The seronegative spondyloarthropathies consist of a group of non-rheumatoid disorders with similar clinical, laboratory and genetic features. Several of these disorders have salient characteristics that allow recognition of the individual syndromes.

The clinical entities included in the seronegative spondyloarthropathy group are [Moll et al,1986]:

1. **Ankylosing Spondylitis**
2. **Psoriatic Arthropathy**
3. **Reiter's Disease/Reactive Arthritis**
4. **Inflammatory Bowel Disease**
   (Ulcerative colitis, Crohn's disease, Whipple's disease)
5. **Juvenile Chronic Arthritis (JCA).**

However, it is not invariably possible to make a clear distinction between the various clinical entities, lending support to the notion that they should be considered as part of a clinical spectrum [Espinoza et al,1989].
These disorders (Table 1) are characterised [Calin,1984] by:

1. Absence of high-titre IgM rheumatoid factor.
High titres of rheumatoid factor have been correlated with severe progressive rheumatoid arthritis (RA), but low titres are found in many connective tissue diseases, chronic

Table 1

Characteristics of the Spondyloarthropathies.

1.Absence of high-titre IgM Rheumatoid Factor.

2.Sacroiliitis/Spinal involvment.

3.Peripheral inflammatory arthropathy.

4.Familial aggregation.

5.Extra-articular involvment.

6.Enthesopathy.

Table 2

Classification of joint involvment patterns in Psoriatic Arthritis.

1. Asymmetric Oligoarticular Arthritis.

2. Predominant Distal Interphalangeal Joint Involvement.

3. Arthritis Mutilans.

4. Symmetric Polyarthritis.

5. Spinal Involvement.

infections and 5% of normal individuals. The frequency of rheumatoid factor increases with age [Wolfe et al,1991].

2. Sacroiliitis and/or spinal involvment.

The hallmark of ankylosing spondylitis (AS) is bilateral, symmetric sacroiliitis. An asymmetric or unilateral distribution may be evident in early disease. In patients with psoriatic arthritis or Reiter's disease, the pattern of sacroiliitis is most often bilateral and symmetric, but asymmetric or unilateral involvment is seen more often than in AS.

3. Peripheral inflammatory arthropathy

Involvment of peripheral joints in AS is often mild and transient. The most common extra-axial joints involved are the hip and the knee. Prominent erosive and destructive changes may occur and the hip, in particular, may be an important source of clinical disability. Radiographic findings include osteophytosis, subchondral cysts and concentric joint space narrowing [Moll et al,1973].

The peripheral arthritis of Reiter's disease/Reactive arthritis is characterised by asymmetric involvment of the axial or large weight-bearing joints. The knees and ankles are most often involved. Prominent enthesitis is often a feature. Radiographic findings are similar to those of psoriatic arthritis, however, significant destruction or deformity and bony ankylosis is less than in psoriatic arthritis.

4. Familial aggregation

The occurence of AS has been recognised in twins, brothers, fathers, mothers, and other relatives of affected individuals [Graham et al,1957]. The different disease expressions of relatives of patients with AS, Psoriatic arthritis or Reiter's disease is also well recognised.

5. Extra-articular involvment:

Iritis and Conjunctivitis

Urethritis

Mucous membrane ulcers

Skin rash

Cardiovascular involvment in the form of myocardial dysfunction, cardiac valvular lesions and conduction disturbances is being increasingly recognised in AS and the related spondyloarthropathies [Bergfeldt et al,1988; Nagyhegyi et al,1988; Shetty et al,1988].

6. Enthesopathy

This is defined as inflammation at the ligament-bone junction [Moll,1983]. Favoured sites of involvment include the ischial tuberosities, iliac crests, femoral trochanters and plantar aspect of the calcaneus. Inflammation results in subtendinous or subligamentous erosion, which is followed by reactive sclerosis and heterotopic ossification [Resnick,1981].

The term "Reactive Arthritis" was introduced by Ahvonen in 1969, and can be defined as a "Syndrome characterised by non-purulent joint inflammation following an infection elsewhere in the body" [Ahvonen et al,1969].

Epidemiological evidence accumulated from different sources strongly favours a causal relationship between gastro-intestinal infections with Shigella, Salmonella, Yersinia, Campylobacter and genito-urinary infection with Chlamydia and the subsequent development of arthritis.

The time lag between infection and the onset of arthritis is the main difficulty in identifying the aetiology of the triggering infection. By the time the diagnosis is suspected, microbiological culture, whether in urine, blood or stool is usually negative. Thus, the diagnosis is usually serological [Borg et al,1992].

2.3

PSORIATIC ARTHRITIS

2.3.1

Psoriasis

Psoriasis is a chronically relapsing inflammatory skin disorder that can be physically and emotionally debilitating. It is variable in both appearance and incidence. Racial, geographic and environmental factors seem to play a large role [Krueger et al, 1985].

Males and females are equally affected. Most patients develop symptoms in the third decade of life. Onset before the age of 15 predicts more severe disease, both with respect to the percentage of body area involved and being refractory to treatment [Farber et al,1974].

Psoriasis is characterised histologically by hyper-proliferation of the epidermis, accumulation of inflammatory cells (especially T lymphocytes), and elongation and increased tortuosity of dermal papillary blood vessels [Barker,1991b].

It has been postulated that inappropriate activation of keratinocytes may account for the cascade of events leading to inflammation and hyperproliferation in psoriasis [Barker et al, 1991a]. Monoclonal antibodies raised to group A streptococci have been shown to cross-react with products of keratinocytes in psoriatic human skin [Swerlick et al,1986].

2.3.2 Classification

Psoriatic arthritis demonstrates a wide variety of patterns of peripheral joint disease. These include an asymmetric oligoarthritis (70% of cases), a symmetrical polyarthritis similar to RA (15%), predominant distal interphalangeal involvment (<5%) and a mutilating arthritis (<5%). In less than 5% of patients with psoriatic arthritis, AS is the predominant feature (Table 2).

2.3.3 Epidemiology

Precise figures for the incidence and prevalence of psoriatic arthritis are not known. Difficulties arise from variable criteria being used for diagnosis, the unpredictable temporal relationship between onset of skin lesions and arthritis and because mild psoriasis may be easily missed especially if localised to the natal cleft or scalp.

It is generally accepted however, that between 5-8% of patients with psoriasis have an inflammatory arthritis [Espinoza,1985]. The sex ratio in psoriatic arthritis is nearly equal, although in distal joint arthritis and spinal arthritis males predominate.

2.3.5 Clinical Features

Psoriasis usually precedes the onset of arthritis in 75% of cases. In 15% the onset is synchronous, while in the remaining 10% arthritis precedes the development of psoriasis [Wright et al,1992]. A severe form of psoriasis may occur in association with HIV [Johnson et al,1985]. This is often a poor prognostic factor and a rapidly progressive arthropathy may supervene.

2.3.6 Immunological Features

There is a potential role of the immune system in the pathogenesis of psoriasis because of an increased number of activated T lymphocytes in psoriatic skin and the response of psoriasis to cyclosporin A [Baadsgard et al,1990].

Also, in contrast to normal keratinocytes, psoriatic keratinocytes express HLA-DR antigens and intracellular adhesion molecule-1 (ICAM-1) suggesting the potential to regulate antigen presentation, lymphocyte adherence, lymphocyte trafficking and migration of neutrophils into the epidermis [Griffiths et al,1989].

Rheumatoid Factors (RF)

As described earlier, by definition, IgM RF (Latex) is absent. In an ELISA study of IgA RF in 19 patients with psoriatic arthritis, Brik et al [1990] only managed to identify one patient (7%) who had a high IgA RF and concluded that measurement of rheumatoid factor isotypes in psoriatic arthritis was not needed.

Immune complexes

Laurent et al [1981], demonstrated the presence of IgG and IgM circulating immune complexes and increased levels of serum IgA and IgG in both psoriasis and psoriatic arthritis and postulated that the increased IgA response may be due to defective function of a T Suppressor cell subset allowing increased production of IgA.

Similarly, Hall et al [1984], using a radioimmunoassay method analysed the sera of 35 patients with Psoriatic arthritis for the presence of IgA immune complexes. 80% of their cohort of patients were found to have IgA - containing circulating immune complexes while 37% had IgG - containing immune complexes. Furthermore, they found a significant correlation ($p<0.05$) between the level of IgA - containing circulating immune complexes and the severity of the arthritis.

Immunoglobulins

Immunoglobulins localised to the dermo-epidermal junction in psoriasis have been demonstrated using a direct fluorescent antibody technique [Burnham et al,1963]. Using a technique of mixed agglutination with tissue sections, the immunoglobulins in the epidermis have been demonstrated to be of the IgG and IgM classes [Krogh and Tonder,1972].

Krogh and Tonder [1973] demonstrated that scales from patients with psoriasis contained IgG, IgM, IgA and C3. They postulated that a passive transepidermal diffusion of serum proteins occurs into psoriatic lesions and specific antibodies are then bound in the parakeratotic stratum corneum. The antigen-antibody complexes were shown to fix anti-IgG factors and complement.

2.4

ANTIGENS, ANTIBODIES AND AUTOIMMUNITY

2.4.1

Mechanisms of Autoimmunity

Autoimmune diseases arise as a result of an interaction between a variety of features, including inherited, hormonal and environmental [Isenberg, 1992]. Whereas most of the factors associated with autoimmune diseases seem now to be defined, much remains to be determined about precisely how they interact and give rise to disease. Potential targets for autoimmune attack can be found all over the body (Table 3).

A key issue for the immune system of any organism is to be able to distinguish between self components and foreign material. The ability to tolerate or distinguish self antigens is not inherent to the immune system, and is the result of a very sophisticated mechanism which is acquired and continuously maintained.

Table 3

Some Potential Targets for Autoimmune Attack

Organ Specific Autoimmune Diseases

Endocrine

Autoimmune (Hashimoto's) Thyroiditis
Hyperthyroidism (Graves' disease, Thyrotoxicosis)
Diabetes Mellitus (Type 1 - insulin requiring)
Addison's disease

Haematological

Autoimmune thrombocytopenia
Autoimmune neutropenia
Pernicious anaemia

Dermatological

Pemphigus vulgaris
Pemphigoid

Neuromuscular

Myasthenia Gravis
Multiple Sclerosis
Autoimmune polyneuritis

Overlapping Autoimmune Diseases

Dermatomyositis	(skin and muscle)
Goodpasture's syndrome	(lung and kidney)

Systemic Autoimmune Diseases

Systemic Lupus Erythematosus
Sjogren's syndrome
Scleroderma
Rheumatoid arthritis
Wegener's Granulomatosis

Modified from Isenberg,1992.

The basis of this mechanism is not fully elucidated, and a breakdown in tolerance to self antigens (in the presence of other factors), appears to allow the development of a self-destructive autoimmune disease [Isenberg, 1992]. In animal models, T lymphocytes specific for mycobacterial antigens can produce diseases resembling RA, SLE and progressive Systemic Sclerosis and it is likely that T cells also participate in human autoimmune diseases (Richardson, 1992).

2.4.2

Antigen Presentation

Antigen-specific, receptor-bearing T lymphocytes recognise antigenic determinants presented by cells expressing class I or II Major Histocompatibility Complex (MHC) molecules on their outer membranes. The T cell antigen receptor recognises a complex formed between the MHC determinants and antigen fragments. However, for proper recognition, antigen-presenting cells must also be able to stimulate the activation of T cells by releasing appropriate cytokines (Geppert and Jasin,1991). CD8+ (suppressor/ cytotoxic) T lymphocytes recognise antigen presented in conjunction with class I MHC molecules present on most cells, while CD4+ (helper) T lymphocytes recognise antigen presented with class II MHC molecules present on specialised antigen presenting cells (Richardson,1992).

Endothelial cells and psoriatic keratinocytes (but not keratinocytes elsewhere) are known to express class II MHC molecules in areas of inflammation (Scheynius et al,1982;Volc-Platzer et al,1984). Interferon-gamma (IFN-gamma) is thought to be the inflammatory mediator responsible for inducing class II molecule expression at these sites (Geppert and Lipsky, 1989).

Human chondrocytes can also be induced to express class II MHC molecules when incubated with IFN-gamma (Alsalameh et al,1991). Articular chondrocytes behave as fibroblasts or keratinocytes in that they are weak stimulators of allogeneic and autologous resting T cells, they present exogenous antigen poorly or not at all to resting T cells, and they are able to present antigen efficiently to activated antigen specific T cell lines.

2.4.3

MHC Class I and II Expression

Genes encoding MHC determinants, complement components, antibody variable regions, as well as the T cell receptors may all predispose to autoimmunity. The MHC genes in particular play an important role in most autoimmune diseases [Isenberg, 1992].

The MHC linked genes are subdivided into three groups: class I, class II and class III, which are structurally and functionally distinct. The class I genes, HLA-A,B and C code for the classical transplantation antigens expressed on the surface of all nucleated cells.

The class II molecules are recognised in conjunction with antigens on the surface of macrophages/monocytes by the T-cell receptor of helper-inducer T-cells and presumably also of suppressor cells. The HLA-DRB1 alleles, especially the Dw14 (DRB1*0404), Dw15 (DRB1*0405) and Dw4 (DRB1*0401) subtypes of DR4 and the Dw1 subtype of DR1 (DRB1*0101) are, in particular, associated with RA.

The class I genes are recognised in conjunction with antigen by the T-cell receptor of cytotoxic T-lymphocytes and they therefore form the target for self-recognition. The action of cytotoxic T-cells against autologous cells which are either infected or chemically modulated or which show malignant transformation, is considered to be one of the most important functions of immunological surveillance [Scholz et al,1983]. Particular class I antigens are associated with the spondylo-arthropathies.

The process of "presentation" in the initiation and in the regulation of an immune response, is one of the most important functions of the immune system. It can also be presumed that allelic variations of class II molecules (as well as the possibility of formation of hybrid antigens), could provide the genetic basis for an effective immunologic function in the vast majority of the circumstances confronting a given individual and for an occasional dysfunction triggered by a set of environmental circumstances.

The mechanism by which the MHC complex confers disease susceptibilty is not definitely known. One such mechanism is known as the T cell interaction hypothesis whereby the polymorphic genes of the MHC encode cell surface glycoproteins that bind peptides to form unique structures recognised by clonally expressed T-cell receptors. Upon cross-linking, these initiate a cascade of biochemical reactions resulting in activation of the T-cell [Schwartz,1986].

Both class I and class II MHC-encoded molecules have distinct but related, functional requirements in immune recognition. The class I molecules primarily bind antigenic peptides available to them in their biosynthetic journey from the rough endoplasmic reticulum to the plasma membrane, while class II molecules bind proteolytic fragments derived from endocytosed extracellular proteins generated in acidic cellular compartments [Germain,1986].

This functional distinction can be illustrated further by the mutually exclusive expression of the CD8 and CD4 'coreceptor' molecules on mature T-cells that are committed to the class I - and class II-peptide complexes respectively [Marguiles,1992].

Certain class I or class II genotypes might provide for a defect in the T-cell repertoire, which could lead to the type of autoimmunity seen in HLA-associated diseases [Scholz et al,1987].

The class III region of the HLA chromosome contains the genes for the complement components C4 and C2 as well as the genes for the C21-hydroxylase. C2 and C4 are important factors in the classical pathway, while Bf is the proactivator of C3 in the alternate pathway of complement activation. Their immunological importance is best documented by the observation of severe lupus-like syndromes in the rare cases of genetic deficiency of C2 or C4 [Tappeiner et al,1982].

Hypotheses linking the spondyloarthropathies with HLA-B27 include [Khan et al,1990; Benjamin et al,1990; Gaston,1990]:

1. B27 acting as a receptor site for an infective agent.
2. B27 being a marker for a gene close by on chromosome 6 that determines susceptibility to an unknown trigger.
3. B27 inducing tolerance to cross-reactive foreign antigens- Molecular Mimicry [Yu et al,1989] eg.Klebsiella [Avakian et al,1980], Shigella, Chlamydia,and Yersinia [Thomas et al, 1983].

A great deal of evidence both clinical and immunological has been accumulated to support the role of infectious triggers through an indirect action on the pathogenesis of AS [Khan,1989], possibly via the mucosal immune system. In AS and other seronegative spondylo-arthropathies there are increased serum levels of IgA (particularly of the secretory type) and circulating immune complexes [Calin,1988; Inman,1987].

Inflammatory gut lesions have been found in 65% of patients with reactive arthritis and in 57% of AS patients. A good correlation was also shown between the presence of gut inflammation in biopsy specimens and the persistence of peripheral joint inflammation [Mielants et al,1988].

These findings suggest that environmental factors lead to an increase in the permeability of the intestinal wall or disturb local immunological defence mechanism allowing the entry of bacterial antigens into the circulation and induction of joint inflammation [Taurog,1989].

The association of HLA-DR4 with RA strongly implicates genes of the MHC as contributing to disease susceptibility and expression. DR4 is found in 30% of the normal population compared to 60% of RA patients and 95% of patients with Felty's syndrome.

Molecular analysis of MHC genes expressed as haplotypes in association with HLA-DR4 reveals that at least six different alleles of the DRB1 locus and at least three different alleles of the DQB locus occur on different DR4+ haplotypes. Some of these allelic differences are quite substantial, while others are rather subtle, involving as little as two amino acids. Some known HLA associations with the Spondyloarthropathies and with other Rheumatic conditions are shown in Table 4.

2.4.4

Autoantibodies

A common feature of autoimmune diseases is the presence of autoantibodies targeted against cellular proteins and nucleic acids that play the role of antigens. Some autoantigens are extracellular, such as the keratinocyte cell surface glycoprotein of the cadherin family of cell adhesion proteins, against which autoantibodies in pemphigus vulgaris react.

Other antigens are intracellular, such as HuD, a nuclear protein found in human brain cells and in small cell lung cancer which is reactive with antibodies produced in paraneoplastic encephalomyelitis syndromes (Tan,1991).

Table 4

Some HLA associations in the Spondyloarthropathies and other rheumatic conditions.

Disease	HLA antigen	Frequency in Patients (%)	Frequency in Controls (%)
SPONDYLOARTHROPATHIES			
Ankylosing spondylitis	B27	80-100	6-8
Reiter's disease	B27	60-85	6-8
Psoriatic arthritis	B27	20-50	6-8
	B38	20-45	2-8
	B39	20-30	2-6
	DR7	30-45	15-20
	DR4	40-50	20-30
Reactive arthritis			
Yersinia	B27	50-75	6-8
Salmonella	B27	50-70	6-8
OTHER RHEUMATIC CONDITIONS			
Behcet's disease	B5	20-85	10-25
Rheumatoid arthritis	DR4	45-75	20-30
Systemic lupus erythematosus	DR2	45-55	20-30
	DR3	40-50	15-25
Scleroderma	DR5	35-45	20-25
Sjogren's syndrome (Primary)	B8	35-60	15-25
	DR3	50-65	15-25
Giant cell arteritis	DR3	30	15-25
	DR4	40-50	20-30

after Moll, 1987.

Antibodies directed towards intracellular antigens are frequently found in the rheumatic diseases. Some of these autoantibodies correlate with the presence of certain clinical features or syndromes and may be of a diagnostic or prognostic value. According to Tan (1989), anti-Sm antibodies are diagnostic markers of SLE, while anti-DNA antibodies additionally identify a subset of SLE patients predisposed to develop renal impairment. Similarly, anti-SSA antibodies have been shown to identify a subset of SLE patients characterised by photosensitivity and skin changes (Tan, 1989).

Other autoantibodies, however, are not so specific. Thus, anti-ribonuclear protein (anti-RNP) antibodies were initially used as serological markers for a syndrome called mixed connective tissue disease (MCTD). However, anti-RNP antibodies have also been found in a number of other rheumatic disorders including SLE, myositis and Sicca syndrome [Lundberg et al,1992].

Most individuals are able to produce a range of "natural" autoantibodies. The binding specificities of such auto-antibodies are usually directed against highly conserved molecules including cytochrome C, actin, transferrin, nucleic acids and cytoplasmic filaments. Hypotheses have been put forward as to their possible function including suggestions that they may play a role in disposing of degraded autoantigens, as well as prevention of autoimmunisation by blinding the immune system to environmental epitopes that cross-react with auto-antigens

The relationship between natural and pathogenic autoantibodies however, is not clear. Whereas the former tend to be of the IgM isotype, the latter are usually IgG, but whether pathogenic autoantibodies develop from previously natural autoantibodies by isotype switching or are separately derived is uncertain (Isenberg, 1992).

The appearance of certain autoantibodies is usually closely associated with the development of the autoimmune disease. These autoantibodies may be identified in pathogenic lesions associated with the disease, such as antibodies to Deoxy Ribonucleic Acid (DNA) in Systemic Lupus Erythematosus (SLE).

A major issue in the past few years has been the demonstration of germline genes that may encode for autoantibodies. However, there is a distinct tendency for somatic mutation to occur (carrying the implication of antigen drive) and that this is an important mechanism in the production of more pathogenic autoantibodies [Isenberg, 1992].

The mechanisms by which autoantibodies may induce disease include formation of immune complexes, complement dependent destruction of a target cell, opsonisation, blockade of receptor sites for their physiological ligands, and stimulation of cell surface receptors.

2.4.5

Hormones

The increased prevalence of most autoimmune disorders in women suggests that hormones play a major modulating role in the pathogenesis of these disorders.

Sex hormones, via surface receptors on lymphocytes, can act directly on T cells especially those carrying the CD8+ phenotype.

Oestrogen tends to inhibit these cells allowing increased antibody production by B cells (Isenberg,1992).

Oral contraceptives with a higher oestrogen content can exacerbate SLE, and the disease can be reactivated in postmenopausal women treated with hormone replacement therapy. In RA, however, although the evidence appears to be somewhat conflicting, on the balance, it appears that oral contraceptives may protect against the development of RA (Spector et al,1990) but do not affect the disease process or the treatment (Hazes et al,1989). Similarly, Carette et al (1989) failed to demonstrate any benefit from the use of hormones (oestrogen and progesterone) to prevent RA in postmenopausal women suggesting that the protective effect was related to the age of the women.

Further evidence for the role of sex hormones in autoimmunity relates to the different effects on pregnancy in RA and SLE. In RA, 75% of patients go into remission. Improvement starts as early as the first trimester and is usually maximal in the third trimester. However, 90% of RA patients experience a relapse or flare in the weeks or months post-partum . Similarly, as for the protective effect of the contraceptive pill, the younger the women were at their first pregnancy the greater was the likelihood of protection against the development of RA (Hazes, 1991).

Proposed biological mechanisms for the protective effect of pregnancy in RA include:

1. Immunomodulating properties of female sex hormones.
2. Immunosuppression by various pregnancy-associated proteins such as alpha 2 glycoprotein.
3. Pregnancy induced suppression of cell-mediated immunity.
4. Alteration (Increase) in the glycosylation of IgG (Stanworth, 1988).

In SLE, however, not only are disease flares common, but the first presentation of the illness may occur during pregnancy itself (Petri et al,1991). However, most SLE patients go through pregnancy without any untoward event, and on balance pregnancy in SLE poses a greater threat to the foetus than to the mother.

2.4.6

CD5 Positive B Cells

A subset of B cells carry the marker CD5 which is usually associated with T lymphocytes. It has been suggested that these cells constantly recognise and stimulate each other through their idiotypes and demonstrate a limited response to external antigens. However, CD5 positive B cells seem to be associated with the increased production of certain IgM auto-antibodies, notably Rheumatoid factor and single-stranded DNA antibodies. These cells are increased in number in patients with RA and Sjogren's syndrome [Watts and Isenberg, 1990].

2.4.7

Age and Diet

The immune response is maximal during puberty, and the efficiency of the cellular and humoral mechanisms declines progressively with age. Autoantibody production is known to increase with age and is accompanied by a general decrease in immune resposiveness (Isenberg,1992).

The role of diet in the pathogenesis of autoimmunity is unclear. Short-term reduction of dietary fat and/or fish oil supplementation have been shown to give short-term improvement in the symptoms of patients with RA and SLE (Kremer et al,1990).

2.5

CYTOSKELETON, INTERMEDIATE FILAMENTS AND CYTOKERATINS

Cells require organisation and structure as well as a degree of plasticity to perform various cellular functions. The cytoskeleton possesses the required characteristics for both these properties [Rogers et al,1992].

The cytoskeleton in nucleated cells is made up of a complex three dimensional lattice radiating throughout the cytoplasm and nucleus and is made up of three distinct types of filament - microfilaments, microtubules and intermediate filaments (Table 5) [Schliwa et al,1986].

The protein composition of the cytoskeleton is characteristic for a specific cell or tissue type, and provides the machinery required for cellular or tissue function. Within the cytosol these provide a structural as well as an organising role. Many organelles have a close association with the various types of filaments. These include the mitochondria, Golgi apparatus, and the endoplasmic reticulum.

An intact cytoskeleton is essential for vital cellular functions (including vesicle or organelle transport), cell-cell interaction, cell extracellular matrix interactions through association with surface receptors, cell motility and cell cycle [Rogers et al,1992].

Table 5

Composition of the Eukaryotic Cytoskeleton

Filament	Size(nm)	Subunit protein	Functions
Microfilaments	7	Actin	Structure Cell Motility
Microtubules	25-39	Tubulin	Transport(IC) Cell Division
Intermediate Filaments	10	20-30 different types	

after Williams,1992.

Table 6

<u>Intermediate Filaments</u>

Type I	Acidic Cytokeratins
Type II	Basic Cytokeratins
Type III	Vimentin Desmin GFAP Peripherin
Type IV	Neurofilaments
Type V	Nuclear Lamins

Microfilaments are made up of actin monomers (43 kilodaltons) and are essential for cell structure and morphology [Cooper,1987]. Microtubules are hollow cylinders with internal and external diameters of 15 and 25nm respectively [Tucker,1986].

Microtubules radiate throughout the cytoplasm from the nucleus to the surface membrane and are extensively associated with the endoplasmic reticulum and Golgi apparatus. Thus, they play an important part in vesicle transport and endo/exocytosis [Matter,1989].

Five major types of intermediate filaments (IF) have been identified (Table 6) based on the sequence homology of the proteins from which they are constructed [Lazarides,1982]. Type I and Type II are cytokeratins. Type III IF proteins include vimentin, desmin, glial fibrillary acid protein (GFAP) and peripherin. Vimentin is restricted largely to mesenchyme-derived cells such as fibroblasts, chondrocytes and endothelial cells as well as in vascular smooth muscle cells and in many cultured cell lines. Type IV (Neurofilament) IF proteins are found in neural cells. Type V IF proteins are the nuclear lamins which are ubiquitously expressed in eukaryotic cells.

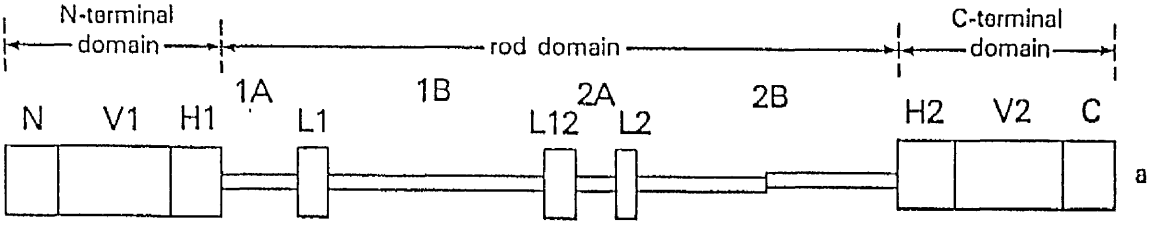
During cell transformation and tumour development, this cell type specificity of intermediate filaments is largely conserved and classification of tumours by their specific type of intermediate filaments has become very valuable in clinical histodiagnosis [Moll et al,1982].

The intermediate filaments of the vimentin, desmin or glial types all consist usually of only one type of subunit protein. In contrast, the cytokeratins are a complex family of many different polypeptides. These cytokeratins, which show biochemical and immunological relationships of various degrees, are expressed, in different epithelia, in different combinations of polypeptides ranging in their isoelectric pH values from 5 to 8 and in their apparent molecular weights from 40 to 60 kilodaltons. A given epithelium or epithelial cell can therefore be characterised by the specific pattern of its cytokeratin components [Moll et al,1982].

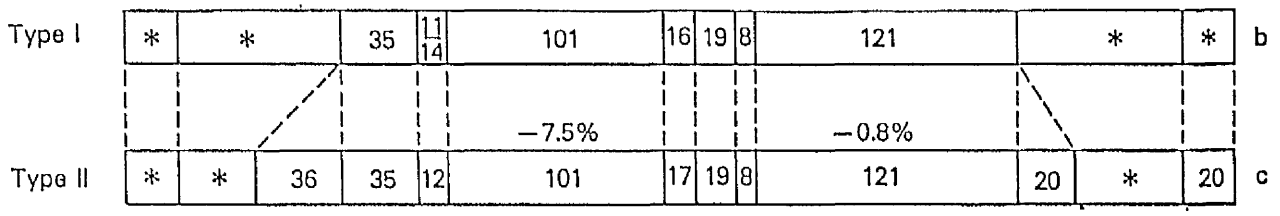
All IF subunits possess a central highly conserved 38 Kd region flanked by end domains (Figure 1). The rod domain consists of four segments (horizontal rods) of constant size. These are composed of a seven residue (heptad) repeating peptide which participates in the formation of a coiled-coil with another subunit.

Figure 1

Subunit structure of Cytokeratin Intermediate Filaments



(a) Universal model. All IF subunits possess a central alpha-helical rod domain flanked by end(terminal) domains. The segments(1A/B and 2A/B) are separated by short regions (linkers - L1, L12, L2). Numbers show length of linkers. Each sequence type of rod domain is coupled with a specific set of end domains, which themselves may be further subdivided into subdomains based on their basic charge (N or C), sequence homology (H), or sequence variability (V).



Type I Cytokeratins (b) and in Type II Cytokeratins (c). At the middle of segment 2B, the polarity of the progression of heptads is abruptly reversed causing irregularity of the coil at that point. In the cytokeratin subunits, the N and C subdomains vary in size as do the highly variable V1 and V2 subdomains.

Modified from Steinert and Parry, 1985.

The segments are separated by short regions (linkers) that do not possess the heptad repeat and therefore cannot form a coiled-coil. Despite the conserved structural features, the exact sequences of the heptads in the segments and of the linkers vary. These sequences allow the classification of the various classes of IF subunits. Each sequence type of rod domain is coupled with a specific set of end domains, which themselves may be further subdivided into subdomains based on their basic charge (N [amino] or C [carboxyl]), sequence homology (H), or sequence variability (V). In the cytokeratin subunits, the N and C subdomains vary in size as do the highly variable V1 and V2 subdomains [Steinert and Parry, 1985].

Cytokeratins are characteristically found in epithelial cells. The stratified squamous epithelium is the most common covering or lining surface of the animal body. It may be of ectodermal origin (the epidermis) or of endodermal origin (the oesophageal epithelium).

The dominant cell type of these epithelia (the keratinocyte) contains abundant 100-A filaments composed of keratin proteins. These are intermediate in diameter between actin micro-filaments (60A) and the larger myosin filaments (150A) and microtubules (250A) [Lazarides,1980]. Cells of this type cultivated from different statified squamous epithelia are rather similar, with cytokeratins accounting for about 30% of the cellular protein [Sun et al,1977].

Cytokeratins are a family consisting of about 20 intermediate filament polypeptides of molecular weight 40,000 - 67,000. These cytokeratins can be fractionated by the use of electrophoresis which allows them to be distinguished chemically and immunologically [Fuchs et al,1978].

It is now apparent that the different epithelia do not contain identical keratins, even though they may all be stained by antisera to single purified keratins. Thus, cytokeratin 13 and 16 is found in non-keratinising squamous epithelia (oesophagus, tongue), basal layer of pseudo-stratified epithelia (bronchus) and transitional epithelium. They are not present in either the epidermis or simple epithelia.

Cytokeratin 18 is a low molecular weight cytokeratin (MW 45,000) which can be found in simple epithelia (intestine, liver, respiratory, urinary). It is not found in stratified squamous epithelia or non-epithelial cells (Table 7).

Table 7

Distribution of Cytokeratins- 8 and 18 in different human epithelia

	CK-8	CK-18
Normal Epithelia		
Epidermis(various locations)	-	-
Sebaceous Glands	-	-
Oesophagus Epithelium	-	-
Tracheal Epithelium	+	(+)
Eccrine sweat glands(total)	+	+
Transitional Ep. (Bladder)	+	+
Gallbladder epithelium	+	+
Small intestine (mucosa)	+	+
Colon (mucosa)	+	+
Hepatocytes	+	+

(modified from Moll et al,1982).

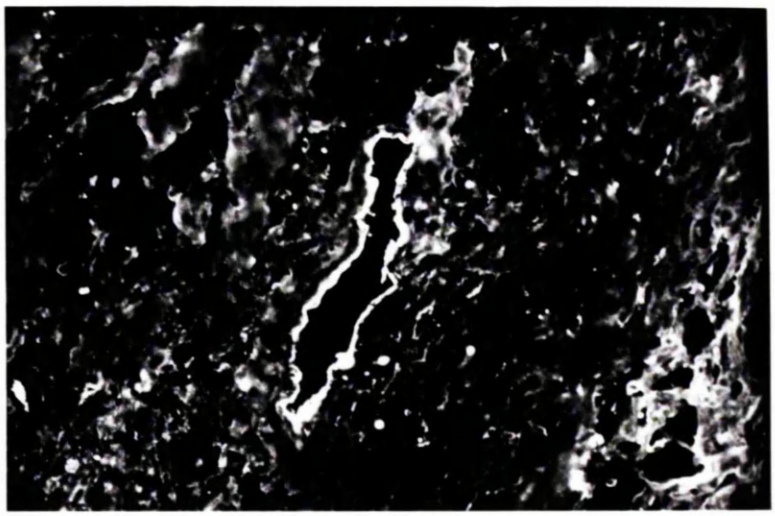
Most other epithelia, such as those of the intestinal, respiratory, genital and urinary tracts seem quite different. Although the cells of these epithelia frequently contain 80- to 100-A filaments, they are not as abundant as those of the keratinocyte and may not show their typical lateral aggregation.

The cells of epithelia other than stratified squamous are often secretory and have morphology and specialisation very different from those of the keratinocytes.

Using antiserum against human epidermal keratin and indirect immunofluorescence, cytokeratins have been detected in many human tissues [Sun et al,1979]. Strong staining has been observed in all stratified squamous epithelia (epidermis, cornea, conjunctiva, tongue, oesophagus, vagina and anus), in epidermal appendages (hair follicle, sebaceous glands, ductal and myoepithelial cells of sweat glands) as well as in Hassall's corpuscles of the thymus, indicating that all contain abundant cytokeratin. No staining was observed in fibroblasts, muscle (striated or smooth), cartilage, nerve tissue, iris or lens epithelium, or the glomerular or tubular cells of the kidney.

Figure 2A: Photomicrograph showing CK-18 lining endothelium of synovial blood vessels in a patient with RA.

Figure 2B: Photomicrograph of cytokeratin-18 filamentous structure in cultured synovial sarcoma cell line.



In contrast, the antiserum stained the cells of most epithelia of the intestinal tract, urinary tract (urethra, bladder, collecting ducts of kidney), female genital tract (cervix, cervical glands, uterus and oviduct), respiratory tract (trachea and bronchi) and the fine ductal system in the pancreas and submaxillary gland [Sun et al,1977].

Cytokeratin-18 (CK-18) has been identified within the blood vessels of synovium in normal controls and patients suffering from rheumatoid arthritis (Figure 2). These findings have been confirmed by the use of anti-CK-18 antibodies, immunofluorescence and Western Blotting and has additionally led to the identification of the presence of CK-18-containing vessels in different joints and in other rheumatic conditions [Mattey et al, In Press].

Thus, there is evidence to suggest that endothelia from synovial blood vessels may differ from most endothelial cells in that they express intermediate filament proteins of the cytokeratin as well as the vimentin type [Jahn et al,1987; Kasper et al,1988]. In general, endothelial cells in mammals only possess vimentin IFs.

Geiger [1987] suggested that IFs play a role in the transmission of information within the cell. Vimentin has been shown to possess binding sites for both the nuclear envelope and the plasma membrane [Georgatos et al,1987], thus forming a basis for transport and signal transduction mechanisms between the cell surface and nucleus.

It is unknown whether cytokeratin IFs play a similar role to that postulated for vimentin IFs although attachment of cytokeratin to both the nucleus and the plasma membrane is likely [Goldman et al,1986].

In epithelial cells, cytokeratin filaments insert into desmosomes at certain plasma membrane regions and may be involved in maintaining the structural integrity of epithelial tissues [Garrod,1985]. However, synovial endothelial cells do not possess desmosomes so there are no obvious membrane-associated sites for the attachment of cytokeratin filaments in these cells. Thus, the expression of cytokeratin in endothelial cells may be associated with some different or additional function.

2.6

AUTOANTIBODIES TO CYTOSKELETAL COMPONENTS IN RA

2.6.1

Antibodies To Cytokeratins

IgG (mainly) and IgM autoantibodies to cytokeratins have been identified by indirect immunofluorescence in normal human sera [Serre et al,1987] as well as in the sera of patients with RA by a number of workers [Young et al,1979; Johnson et al,1981; Scott et al,1981; Miossec et al,1982; Mallya et al,1983; Quismorio et al,1983; Ordeig and Guardia,1984; Hajiroussou et al,1985; Kataaha et al,1985; Youinou et al,1985; Kirstein and Mathiesen,1987; Kirstein et al,1989; Vincent et al,1989; Paimela et al,1992].

The first identification of naturally occuring anti-keratin antibodies (AKA) in human sera using rat oesophageal tissue and fluorescent antibody techniques was reported in 1979 [Young et al].

In the RA studies mentioned above, the positivity of AKA varied between 36% to 69%. The wide variation in positivity reflects one of the major practical difficulties of immunofluorescence and is due to different interpretations by various workers as to what constituted characteristic patterns of AKA reactivity as well as the arbitrary designation of the intensity of the immunofluorescence staining.

Miossec et al [1982], examined sera of 336 patients, 96 of whom had RA and a further 37 with psoriasis. Less than 2% of the patients with psoriasis had AKA compared to 40% of the RA sera tested. Additionally, they also showed a significant correlation between the presence of AKA and the anti-perinuclear factor but not with disease activity in RA.

However, they were unable to characterise the antigen recognised by these antibodies and suggested that AKA may be cross-reacting with antigens in the stratum corneum given the presence of keratins in the epidermis. This conclusion, however, is not clear given the common occurence of antibodies to stratum corneum which are found in psoriasis [Jablonska et al,1979].

The question of cross-reactivity was addressed by Quismorio et al [1983] who demonstrated that in patients with Psoriatic arthritis, none had AKA, while half of them had circulating antibodies to human stratum corneum. Youinou et al [1985], however, demonstrated cross-reactivity between AKA positive human sera and rabbit stratum corneum.

In contrast, Mallya et al [1983], demonstrated a significant correlation between the intensity of the AKA reaction and articular indices, grip strength, ESR, CRP, serum amyloid A protein concentration, antibodies to single stranded DNA and IgM RF (Latex) as well as with extra-articular manifestations of RA. These results contrasted with those of previous workers who had only been able to show a correlation with IgM RF [Johnson et al,1981; Scott et al,1981].

Quismorio et al [1983], failed to identify AKA in sera of patients with Psoriatic arthritis, SLE, Reiter's syndrome or polymyositis. Most of the AKA were of the IgG class, with IgA AKA being identified in only 1.3% of RA sera tested. They also failed to show any significant association between IgG AKA and HLA-DR4 or other autoantibodies including antibodies to intermediate filaments (? vimentin), smooth muscle or nuclear antigens suggesting that factors involved in the induction of AKA could be different from those involved in the induction of autoimmunity to other antigens. Using a 3-stage indirect immunofluorescence method with human serum as a source of complement and fluorescin labelled anti-human C3 as conjugate, they additionally demonstrated that AKA could fix complement.

Also, Quismorio et al [1983], examined for the presence of IgG AKA in the paired samples (serum and synovial fluid) of patients with RA and failed to demonstrate any difference in the antibody titre suggesting that there was no preferential concentration of AKA in synovial fluid of RA patients.

Kirstein and Mathiesen [1987], demonstrated a significant correlation between AKA and hand deformities in RA and suggested that in Latex negative patients AKA were specific to and of diagnostic value in RA. In a subsequent study on synovial fluids [Kirstein et al,1989], it was further suggested that AKA positivity in synovial fluid was 80% sensitive for a diagnosis of RA, despite previous results from other workers [Quismorio et al,1983 and Youinou et al,1985] showing a sensitivity of only 48%.

Recently, Paimela et al [1992] again using indirect immunofluorescence on unfixed cryostat sections of rat oesophagus prospectivey studied a cohort of patients with early RA and suggested that the patients with increased IgG AKA at the time of diagnosis (27/71, 38%) had more active disease at the end of two years as measured by clinical, laboratory and radiological criteria.

Additionally, they suggested that identification of IgG AKA in early RA may have prognostic significance since they are present at the time of initial diagnosis. This suggestion was also made by Kurki et al [1992] who examined pre-illness serum specimens in 39 individuals who subsequently developed RA and demonstrated the presence of AKA in 20.4% suggesting that the antibodies may precede the clinical disease.

However, measurement of "antikeratin" antibodies in RA has produced conflicting results. Most studies have reported an increased incidence and/or titre of these autoantibodies in RA compared with controls, although no significant difference was found between these groups using an ELISA assay [Birkenfeld et al,1990].

This may be explained by a major difference in the methods used, as human epidermal keratin was used in the ELISA assay as the antigen, while previous studies were based on indirect immunofluorescence microscopy on tissue sections of rat oesophagus a method which is less sensitive and only semiquantitative.

Antibodies to cytokeratins have also been described in a number of other conditions including Sjogren's syndrome [Saku et al,1990], in Scleroderma [Scott et al,1981], in Ankylosing Spondylitis [Quismorio et al,1983], in various cutaneous lesions [Grubauer et al,1986; Diaz et al,1987], in liver diseases [Youinou et al,1983] as well as in lympho-proliferative disorders [Vainio et al,1983].

It is open to question whether indirect immunofluorescence is actually measuring "antikeratin" antibodies, and it has been suggested that the antigen recognised is more likely to be a protein associated with cytokeratin in rat oesophageal cells [Hoet et al,1991]. Cytokeratin-18 is not found in human epidermis or rat oesophagus [Moll et al,1982].

Antibodies to both native type II collagen and denatured collagen have been shown to be increased in the sera and synovial fluid of patients with RA [Clague et al,1984]. In contrast to AKA, these antibodies characteristically appear after disease onset, suggesting that the response is elicited by the liberation of articular breakdown products.

2.6.2

Antibodies to other Intermediate Filaments

Autantibodies to intermediate filament antigens other than cytokeratins have been detected by a number of workers and in a number of different conditions. Thus, Senecal et al [1985] using indirect immunofluorescence have identified IgG as well as IgM autoantibodies to vimentin in polymyositis and dermatomyositis, in systemic sclerosis, RA and in normal sera. IgM autoantibodies have also been identified in haematologic malignancies including Angioimmunoblastic lymphadenopathy [Dellagi et al,1982] and in Waldenstrom's macroglobulinaemia [Dellagi et al,1984].

Using an ELISA, Mayet et al [1990] have detected autoantibodies to vimentin, keratin and desmin in patients undergoing long-term haemodialysis.

Hansson et al [1984], using permeabilised vascular endothelial cells which had vimentin-enriched cytoskeletons, demonstrated intracellular binding of IgG and Fc fragments and correlated this binding with evidence for cell death. They also suggested that some of the reports on autoantibodies to vimentin detected by immunofluorescence may have positive reactions due to Fc binding. However, they were unable to exclude the possibility of the IgG binding to cell components other than intermediate filaments.

Linder [1981], demonstrated that in addition to IgG, complement factor C1q also bound to intermediate filaments. C1q also binds to mitochondrial membranes, and its function in the complement cascade can be mimicked by nerve growth factor which in turn is able to bind to actin microfilaments and microtubules. These findings support the concept of the importance of interactions between cytoskeletal components and the humoral immune system [Hansson et al,1984].

Autoantibodies to nuclear lamins have also been identified by a number of workers. Lassoued et al [1988] demonstrated that high titres of autoantibodies to lamin B1 or to lamins A and C were associated with a novel connective tissue disorder similar to SLE characterised by vasculitis, thrombocytopenias, anti-phospholipid antibodies and autoimmune hepatitis and suggested that they may be markers for this disorder. However, autoantibodies to nuclear lamins have also been demonstrated in RA [Lassoued et al,1990], in SLE [Reeves et al,1987] and in autoimmune liver disease as well as in normal controls [Wesierska-Gadek et al,1988].

Mechanisms for the removal of damaged or dead cells are not entirely clear. One such mechanism involves apoptosis which can be viewed as a gene regulated programmed cell death.

Apoptotic keratinocyte death may be associated with generation of "keratin bodies" which consist largely of keratin intermediate filaments. Keratin bodies covered with IgM have been shown to be a characteristic finding in skin lesions of patients with various dermatoses such as Lichen Planus or chronic discoid Lupus Erythematosus.

These findings led to the suggestion that physiological apoptotic keratinocyte death may cause the liberation of autoantigenic material and production of autoantibodies [Grubauer et al,1986].

2.6.3

Antibodies to Anti-Perinuclear Factor

The anti-perinuclear factor (APF) is an antibody that binds to spherical "keratohyaline granules" 0.5-4 micrometers in diameter surrounding the nuclei of human buccal mucosal cells [Neinhuis et al,1964;Marmont et al,1967]. The biochemical nature of the antigen is unknown [Smit et al,1980]. It does not contain DNA or RNA and stains positively with methylene blue and periodic-acid-Schiff [Vivino et al,1990], suggesting that it might be a glycoprotein or proteoglycan, although an earlier study [Smit et al,1980] did not find PAS positive staining of the granules.

In RA, it has been suggested that the presence of APF correlates both with disease severity [Marmont et al,1967; Wesgeest et al,1987; Janssens et al,1988], with HLA-DR4 [Boerboom et al,1990] as well as with IgM rheumatoid factor seropositivity [Neinhuis R L F,1964; Marmont et al,1967; Youinou et al,1984c; Westgeest et al,1987].

Nesher et al [1991] have suggested that the APF may be polyclonal antibodies directed against an exogenous or endogenous antigen which may share epitopes with the perinuclear antigen, such as glycoproteins, proteoglycans or streptococcal cell-wall peptidoglycan-polysaccharide [Nesher et al,1991].

An increased prevalence of APF has been found in a number of autoimmune disorders other than RA (59% seropositive, 36% seronegative) namely, juvenile chronic arthritis (34%) [Nesher et al,1992], SLE (46%), Scleroderma (26%) and Sjogren's syndrome (35%), although it was significantly lower than in RA. A surprising finding has been the high frequency of APF in patients with lung cancer [Youinou et al,1984b].

Further investigation may also be warranted to determine whether the high prevalence of APF among various autoimmune disease patients denotes recognition of a common autoantigen or a common link among connective tissue diseases.

2.6.4

Cytokeratins and the Epstein-Barr virus

It has recently been shown that IgG antibodies to P62 (corresponding to the glycine/alanine repeat sequence of Epstein-Barr virus nuclear antigen) cross-react with epidermal keratin, as well as with collagen and actin [Baboonian et al,1991].

Using an ELISA assay, Birkenfeld et al [1990], showed that p107, the major epitope of the EBV-encoded EBNA-1 antigen, cross-reacts with denatured collagen and with human keratin (? epidermal). However, they failed to demonstrate any significant difference in antibody titres to p107 (also known as P62) or keratin in normal controls and RA patients. Only antibodies to denatured collagen were present in higher titres in RA sera.

The cross-reactivity of the anti-p107 antibodies with denatured collagen and keratin, led them to suggest that such antibodies, produced by RA patients following persistent stimulation with EBV, might react IN VIVO with collagen and keratin exposed in previously damaged areas and thus reinforce the disease process.

A further possibility was suggested by the demonstation of a host protein in the cytoplasm of cells in the lining layer of rheumatoid synovial membranes, which reacted with a monoclonal antibody to EBNA-1, suggesting a cross-reaction related to an antigen within the synovial membrane [Fox et al,1986].

CHAPTER 3: MATERIALS AND METHODS

3.1

ELISA Assays for measurement of Epidermal keratin and CK-18 in serum and synovial fluid.

3.1.1

Materials

For materials used in assays please see Appendix I.

3.1.2

Assay Methodology

Flat 96-well microtiter plates (Greiner high affinity, Denley) were incubated overnight at 4^{o}C with 100 microlitres per well of purified CK-18 from bovine liver (Progen,1.0 micrograms/ml) in carbonate buffer, pH 9.6. After every incubation step, the plates were washed with Phosphate Buffered Saline-0.05% Tween 20 (PBS/Tween). Test plates were first coated with 1% bovine serum albumin (BSA) in PBS/Tween for one hour at 37^{o}C. The plates were incubated with the test samples diluted 1:80 in PBS/Tween for one hour at 37^{o}C.

The plates were then incubated at 37C for 1 hour with 100microlitres per well of anti-human immunoglobulin (IgA, IgG or IgM) conjugated with alkaline phosphatase (Sigma) at a final dilution of 1:1000. 1% goat serum was also added. After washing, the plates were incubated with 100microl. per well of 1mg/ml p-nitrophenyl phosphate in diethanolamine buffer at 37C for two hours. The reaction was stopped by the addition of 50 microl. 3M sodium hydroxide (NaOH) to each well.

The absorbance of the reaction mixtures was then read at 405nm in a Titertek Multiskan Plus MKII Microplate Reader. Concentrations of IgA anti-CK-18 antibodies were expressed as arbitrary units as defined by 1 serum sample from a patient with arthritis and high levels of IgA anti-CK-18 antibodies.

Standard series of dilutions of this serum sample were assayed with every Enzyme Linked Immunosorbent Assay (ELISA) in order to construct a standard curve. A similar procedure was used for estimation of IgG and IgM anti- CK-18 antibodies, but using a different standard serum. Blank (background) wells were used in each assay together with sera from previous assays with both high and low results to ensure inter- and intra-well reliability. The background values were subtracted from the O.D. readings prior to calibration.

Assays were also carried out on microtiter plates coated with human epidermal keratin (Sigma, 1.0 micrograms/ml) as described for CK-18. Synovial fluids were also examined for antibodies to epidermal keratin and CK-18 using the same assay as described above without any modifications.

3.1.3

Validity and Reliability of ELISA Assay

3.1.3.1

Factors affecting sensitivity of Assay :

A number of experiments were performed on each part of the ELISA assay as described below.

1. ANTIGEN - both the concentration of antigen as well as the binding method were examined. Experiments were performed using a range of antigen concentration from 0.5 to 100 micrograms per ml. Three sets of 96-well plates were set up. In the first set the antigen was made up in distilled water, while in the second set the antigen was made up in bicarbonate coupling-buffer. In the third set the antigen was dried onto the Denley IA plates. One plate from each set was incubated overnight at 37^{o}C or at 4^{o}C, while the other plates were incubated for a week at 37^{o}C or at 4^{o}C. Erratic results

were obtained with the plates where the antigen was dried or made up in distilled water. Consistent and reproducible results were obtained with antigen made up in bicarbonate coupling-buffer. Similar results were obtained for plates incubated at $4^{o}C$ or $37^{o}C$. Overnight incubation at $4^{o}C$ was chosen for all future assays. The optimal concentration of antigen was found to be 1 micro-gram per ml.

2. BLOCKING AGENT - Bovine serum albumin (BSA) in PBS/Tween was used to block non-specific binding sites on the wells which might interfere with the results. Plates were set up with 1% and 5% BSA. No difference was found between both dilutions, so the lower one was adopted throughout the assays.

3. PRIMARY ANTIBODY - Sera from normal controls, patients with inflammatory arthritis and mouse anti-cytokeratin 8.13 monoclonal antibody (Sigma) as a control were initially used to determine the optimal dilution. Sera were diluted as follows in PBS/Tweem 1:20. 1:40, 1:80, 1:160. The anti-cytokeratin antibody was diluted 1:5, 1:10, 1:20, 1:40.

4. SECONDARY ANTIBODY - Various dilutions of monoclonal Anti-human IgG, IgA and IgM and polyclonal IgG conjugated to alkaline phosphatase (Sigma) were used. The affinity isolated antigen-specific antibodies (antibody developed in goat) were diluted 1:1000, 1:2000, 1:4000, 1:8000 in PBS/Tween. Due to the different enzymatic activities of the antibodies, the

optimal dilutions of antibody were as follows, monoclonal IgG 1:1000, polyclonal IgG 1:1000, IgM 1:2000 and IgA 1:2000.

5. INCUBATION TIMES AND TEMPERATURES - The effect of incubating the primary antibody for one versus two hours was examined. Similarly different incubation times were investigated for the binding of the secondary antibody as well as the substrate buffer. All incubation times were found to be optimal at one hour rather than two. The possible effect of incubating the antigen at $4^{o}C$ and at $37^{o}C$ was also investigated. Binding of the antigen was found to be more reproducible at $4^{o}C$ while the remainder of the incubations used in the assay (primary antibody, secondary antibody and substrate buffer) were found to be more reproducible at $37^{o}C$.

3.1.3.2

Factors affecting Reproducibility of Assay :

Experiments were performed to determine the degree of inter- and intra-plate error. All samples were tested in duplicate and similar specimens were used on different plates both on the day of the assay and on different assays days. The possibility of any inter-well difference was also investigated to exclude the possibility of any edge effects by placing samples in different parts of the plate.

The viability of the sera was also tested with respect to the effects of temperature and time. Assays were performed with

sera stored at -20°C and at -70°C. The effect of repeated freezing and thawing of a particular serum specimen was also tested. Attempts were made to determine whether the antibody titre fell with time due to natural breakdown and thus whether results were influenced by the age of the stored serum. No difference was noted between sera which had been stored at
-20°C against -70°C even in sera which had been collected up to twelve months previously. Similarly, no drop in levels was noted in sera which had been used repeatedly (up to five times) and were thus subject to repeated freezing and thawing.

Experiments were also performed to examine whether a diurnal variation existed in the levels of antibodies to cytokeratins, whether levels were affected by blood being collected with the donor standing/supine and whether levels were affected by specimens being collected with the use of a tourniquet. No difference in the results was noted in any of these experiments in the two normal volunteers used for the assays.

3.2

Western Blotting

3.2.1

Materials

See Appendix II.

3.2.2

Methodology

The SDS-polyacrylamide gel electrophoresis (SDS-Page) was carried out as follows: keratin specimens were separated by SDS-Page. The separate keratin antigens were transferred electrophoretically onto nitrocellulose which was cut into vertical strips and reacted with 1:100 dilution of patient sera. The strips were then incubated with either Protein G conjugated to horseradish peroxidase (HRP) at a 1:3000 dilution, or mouse anti-human IgA conjugated to HRP at 1:1000 dilution. Protein bands were detected using an enhanced chemi-luminescence technique (ECL- Amersham International).

Further details of the methodology used can be found in Appendix III.

3.3

ELISA measurement of anti-P62 antibodies.

3.3.1

Preparation of P62

The 20 amino-acid long P62 peptide was prepared by Ian Lewin in the Rheumatology and Allergy Research Unit, University of Birmingham using a peptide maker.

3.3.2

Materials

See Appendix IV.

3.3.3

Methodology

An ELISA measuring system was set up in a similar way as for measurement of antibodies to cytokeratins (see above). Certain modifications were however necessary to ensure proper binding of the antigen (P62) to the 96-well plate.

1. 96 well plates were coated with 100ul/well P62 at 20ug/ml in Borate Buffered Saline (BBS). The plates were incubated overnight at 4°C.

2. Plates were then washed in PBS/Tween 20 (0.2%).

3. Blocking of non-specific binding sites was performed with PBS/TWEEN + 1% BSA.

4. Test sera were diluted in PBS/Tween (1:1000)
   Solution A: 10 microlitres of test serum + 990 microlitres of BBS.
   100 microlitres of solution A were then added to 900 microlitres. of BBS and labelled as solution B. 100 microlitres of solution B were added to each well.

5. The plates were then incubated at 37°C for 2 hours followed by further washing in PBS/Tween (0.2%).

6. The samples were then incubated with secondary antibody linked to alkaline phosphatase (1:1000 in PBS/Tween + 1% goat serum) for 2 hours at 37°C.

7. The plates were then washed three times with PBS/Tween and three times with distilled water.

8. The substrate was made up in diethanolamine buffer. Three tablets per 15ml of buffer were used. 100 microlitres of solution were added to each well followed by incubation for 1 hour at 37°C.

9. The reaction was stopped by the addition of 50 microlitres per well of 3M NaOH.

3.4

Determination of Homology between P62 and Cytokeratin-18

The complete amino acid sequence for cytokeratin-18 was obtained from the GenBank/EMBL Database in order to compare it with that of the P62 peptide. Comparison was made by visual examination of the sequences.

3.5

Measurement of serum IgA

Serum IgA was estimated by nephelometry according to the "Beckman Array Immunochemistry System" which measures the rate of increase in light scattered from particles suspended in solution as a result of complexes formed during an antigen-antibody reaction.

The assays were performed by laboratory staff in the Biochemistry Department of the Central Pathology Laboratory, North Staffordshire Royal Infirmary.

3.6

ELISA for Determination of IgA Rheumatoid Factor

3.6.1

Methodology

The IgA rheumatoid factors were measured using an ELISA IgA Rheumatoid Factor Kit purchased from Walker Diagnostics (Cambridge Life Sciences PLC Code: S9196).

See Appendix V for full details of the Methodology.

3.7

Immunofluorescent antibody staining of cells grown on glass coverslips

Methodology

1. Glass coverslips (13mm diameter) were sterilised by dipping in absolute ethanol and passing rapidly through a bunsen flame until the alcohol was burnt off.

2. The coverslips were then placed in sterile tissue culture petri dishes (4 x 13mm coverslips can be placed in one 35mm diameter petri dish).

3. The cells were trypsinized, washed in complete medium with FCS, and seeded onto coverslips in the petri dish.

4. Cells were cultured at 37^{o}C until the required confluence was achieved.

5. Cells were then fixed in 3.5% formaldehyde in phosphate buffered saline (PBS) for 30 minutes at room temperature.

6. To stain intracellular or cytoskeletal antigens cells needed to be permeablised first. This was done by placing coverslips in 95% ethanol at -20^{o}C for 2 minutes (after previous formaldehyde fixation).

7. Cells were then washed three times in PBS.

8. Primary antisera (15 microlitres per coverslip) were then added for 30 minutes at room temperature.

9. Washing was then repeated in PBS.

10. Fluorescein conjugated second antibody, e.g. FITC anti-human IgG, IgM or anti-human IgA was then added for 30 minutes at room temperature.

11. Washing was then repeated in PBS.

12. Rinse was then performed in distilled water. (Important to prevent salt crystals on coverslip).

13. The specimens were then mounted in PBS/glycerol (1:9) + DABCO (114-diazobicyclo-(2,2,2,-octane) to prevent fading of the fluorescence. 25mg/ml in 9:1 glycerol/PBS were used. The DABCO was dissolved in glycerol in a warm water bath before adding PBS and the pH brought to 8.6.

14. The edge of coverslip was then sealed with nail varnish.

3.8

Keratin Blocking Experiment

3.8.1

Materials

The materials used were as for the previous ELISA assays (see Appendix I).

3.8.2

Methodology

Sera from 40 patients (20 with psoriasis and 20 with psoriatic arthritis) were chosen. Each specimen was divided into 2 aliquots. One aliquot was diluted 1:80 in PBS/Tween as per the usual ELISA (see Section 3.1), while the other was diluted 1:80 in PBS/Tween containing 50 micrograms/ml of human epidermal keratin. The aliquots were incubated for one hour at 37^{o}C or overnight at 37^{o}C. The ELISA assay was then continued as usual.

3.9

Sequential ELISA in RA

17 patients with RA of disease duration less than twelve months had sera collected sequentially over a six month period.

Two metrologists (Mrs.S.Clarke and Mrs.J.Fisher) examined the patients at each visit and recorded a number of measures of disease activity including : Ritchie Articular Index, Stoke Index, CRP, ESR, Visual Analogue Scale for Pain, Number of Painful joints and duration of Early Morning Stiffness.

3.10

Patient Selection and Clinical Measures

Patients. Serum specimens were obtained from 106 patients with classical or definite RA as defined by the ARA criteria, and 23 patients with osteoarthritis. Sera was also obtained from 70 healthy control subjects consisting of department staff and normal blood donors. 16 of the RA patients had subcutaneous nodules, while 9 had evidence of vasculitis. Vasculitis was judged present when one or more of the following features were found: deep ulcers, nailfold lesions, digital gangrene, purpuric eruptions or neuropathy.

Serum specimens were also obtained from 42 patients with psoriatic arthritis, 21 patients with ankylosing spondylitis and 11 patients with reactive arthritis/Reiter's syndrome.

Since cytokeratin-18 has been identified in endothelial blood vessels in both normal and inflamed synovium, it was felt necessary to include a further disease group consisting of patients with an inflammatory condition but without synovial inflammation (synovitis). Thus serum specimens were also collected from 33 patients with psoriasis alone.

Skin specimens were also collected from patients with psoriatic arthritis and patients with psoriasis. All skin specimens as well as the serum samples from the patients with psoriasis were kindly collected by Dr.David Fitzgerald and Dr.Mark Wilkinson.

Ethical Committee approval was received for the collection of specimens.

3.11

Statistical Methods

Data was analysed with the Mann-Whitney U test for results between groups, the Wilcoxon Signed Rank test for analysis of intra-group results and Spearman rank test for correlations. A value of $p<0.05$ was considered significant. Group values are given as means with 95% Confidence Intervals throughout. All statistics were calculated using the Number Cruncher Statistical System.

RESULTS

4.1

ELISA study of antibody response to CK-18 and epidermal keratin in RA

Introduction

The presence of CK-18 within blood vessels of human synovium is unusual for this particular cytoskeletal protein, as it is normally confined to epithelial cells. A number of reports have been published claiming both specificity and sensitivity of IgG and IgM cytokeratin autoantibodies in RA.

However, no one has yet identified which particular cytokeratins are recognised by these autoantibodies. Most of the reports have used indirect immunofluorescence on the middle third of rat oesophagus as their chief identification method for "anti-keratin" antibodies. Many of these studies have been cast into doubt by the suggestion that the antigen recognised in the indirect immunofluorescence is a protein associated with cytokeratins in rat oesophageal cells (Hoet et al,1991), rather than cytokeratin itself.

A sensitive ELISA assay for measuring binding of patients' sera and synovial fluid was thus developed. Purified CK-18 and human epidermal keratin were used as the antigens to investigate whether keratin autoantibodies recognise the particular keratins found in the endothelium lining the synovial blood vessels.

4.1.2

Results

The demographic data of the patients used in the study are shown in Table 9.

A significant difference ($p<0.0001$) in IgA antibody levels to both CK-18 and epidermal Keratin was detected in the serum samples from patients with rheumatoid arthritis compared to the samples from patients with osteoarthritis or normal controls (Table 10 and Figure 3).

The upper limit of the normal values for serum IgA anti-CK-18 levels was defined by the upper 95% Confidence Interval of the value (10.2 arbitrary units) in the sera from the 70 healthy controls. By this criterion, 4.5% of the sera from the healthy controls and 17% of the sera from the OA group were elevated. Conversely, elevated IgA anti-CK-18 levels were found in 76% of the sera from the patients with RA (Figure 4).

TABLE 9

DEMOGRAPHIC DATA

	RA	OA	NORMALS
NUMBER	106	23	70
AGE	55(17-81)	70(54-92)	41(26-73)
SEX	40M:66F	9M:14F	26M:44F
DISEASE DUR (Years + Range)	2.5(0.3-16)	2.4(0.8-5)	--------

Values for age and disease duration are means with ranges in parentheses.

Table 10

ELISA Results

	CONTROLS (n=70)	RA (n=106)	OA (n=23)
CK-18 IgA	9.4(8.5-10.3)	15.8(14.0-17.5)*	8.9(5.2-12.7)
CK-18 IgG	12.5(6.4-18.6)	9.8(7.9-11.7)	10.4(8.4-12.4)
CK-18 IgM	15.8(10.9-20.8)	16.2(5.1-34.1)	15.2(10.3-21.8)
EPK IgA	6.9(5.3-9.2)	12.1(10.8-13.4)*	8.6(7.5-9.7)
EPK IgG	15.4(10.2-20.6)	20.7(16.4-25.2)	11.9(8.1-15.6)
EPK IgM	19.4(12.7-26.1)	20.6(13.2-27.9)	21.2(12.1-28.6)

* = P<0.0001

Mean values with 95% confidence intervals (in parentheses) of IgG, IgA, IgM- anti- CK-18 and anti- EPK antibodies in normal controls, RA and OA sera.
Values indicate arbitrary units (see text for details).

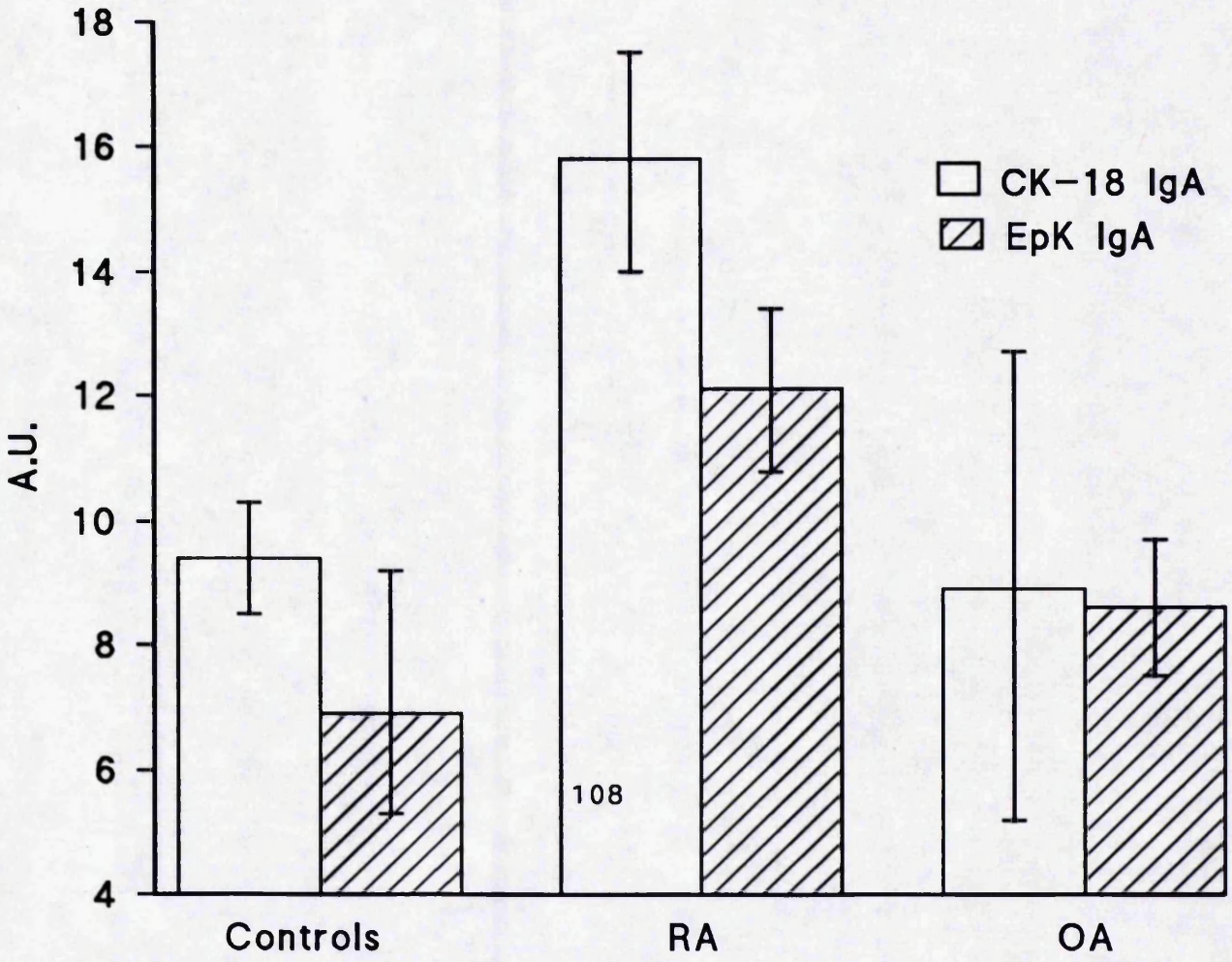
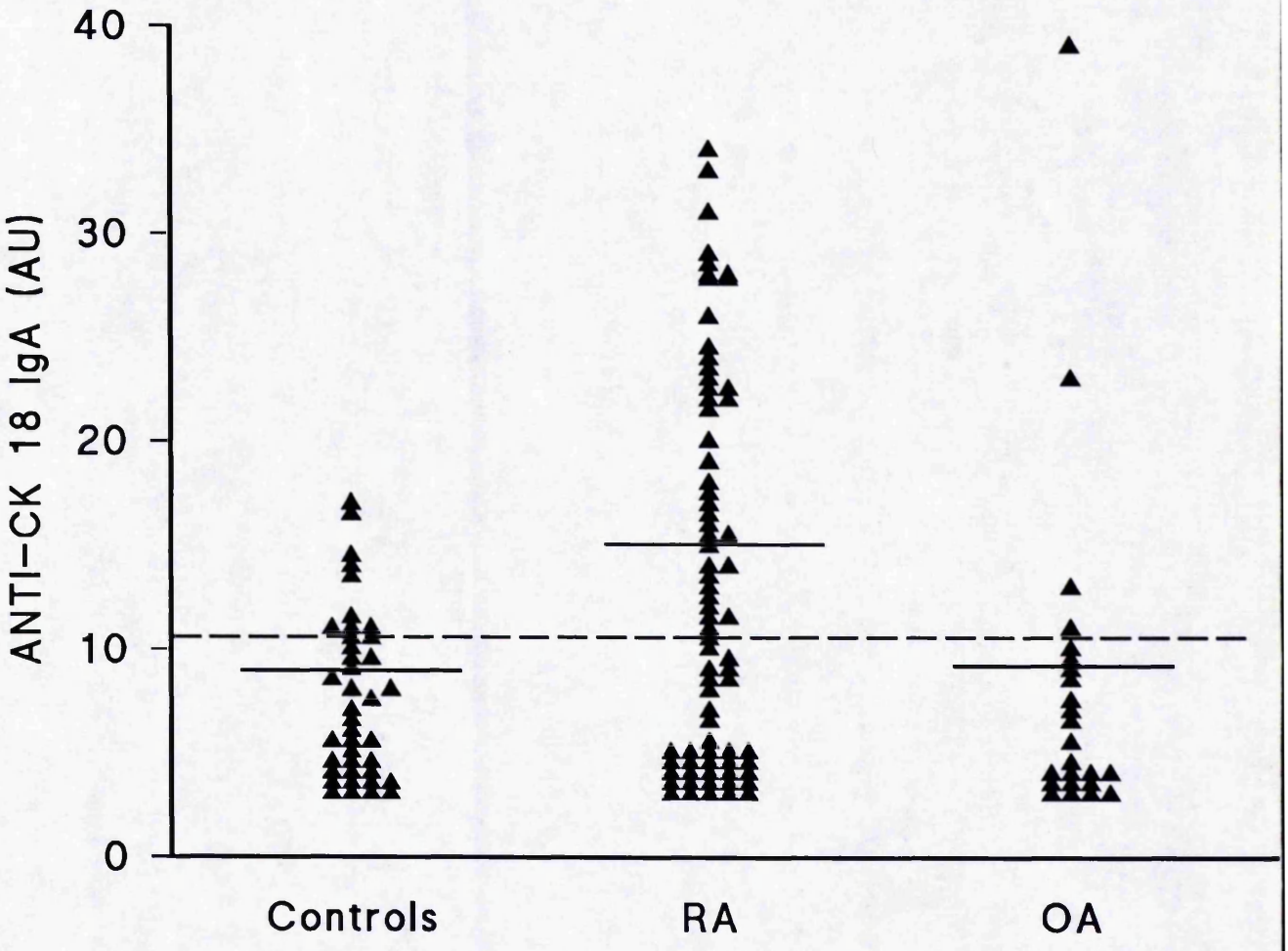


Figure 3. Chart showing IgA anti-CK-18 and anti-EpK levels as determined by ELISA in standardised arbitrary units in sera from normal controls, and in patients with RA and OA.

Values indicate group means (A.U.) and 95% C.I.


40
30
20
10
0
ANTI-CK 18 IgA (AU)
Controls
RA
OA

Similarly, the upper limit of the normal values for serum IgA antibodies to epidermal keratin was defined by the upper 95% Confidence Interval of the value (9.2 arbitrary units) in the sera from the healthy controls. 3.3% of the normal sera and 30.4% of the sera from the OA group were elevated compared with 67.8% of the sera from RA patients. Two of the sera tested from the OA group had very high levels of IgA antibodies to CK-18 raising the possibility that these in fact were clinically misdiagnosed and more likely to be RA than OA.

There was no significant difference in levels of IgG or IgM antibodies to CK-18 or epidermal Keratin between the RA sera and the control or OA sera (Table 10 and Figures 5 and 6). There was no significant difference between the levels of IgA antibodies to CK-18 or epidermal Keratin in the sera from patients with early rheumatoid disease (defined as a disease duration of less than twelve months) compared to sera from patients with established rheumatoid disease (disease duration greater than twelve months) (Table 11). A weak correlation (Figure 7) between IgA autoantibodies to epidermal Keratin and CK-18 was found in the RA sera ($r=0.39$, $p<0.02$, Spearman).

Age (patients classified as < or > than the mean for the whole group), sex or seropositivity (defined as the presence of IgM-RF (Latex) in serum at a titre greater than 1 in 40) do not appear to have any influence on the levels of IgA antibodies to CK-18 or epidermal Keratin.

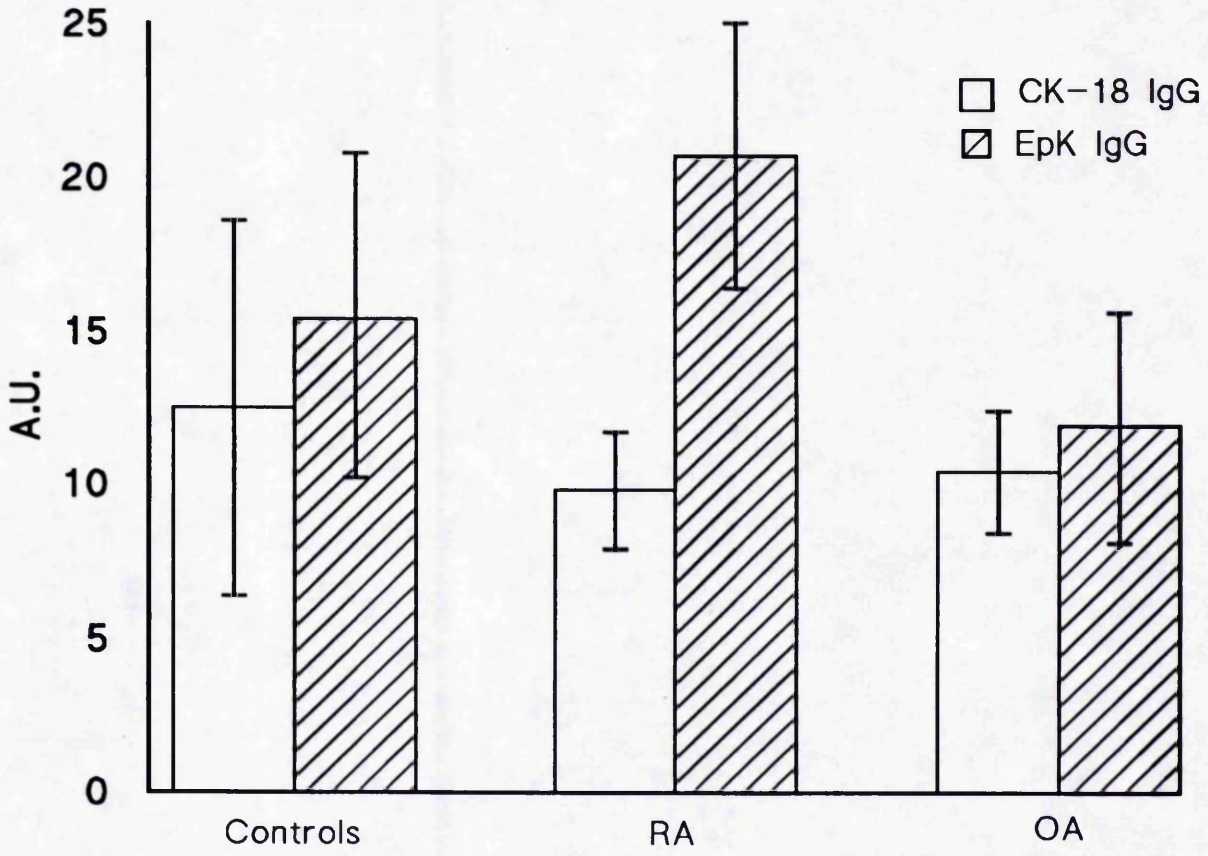
TABLE 11

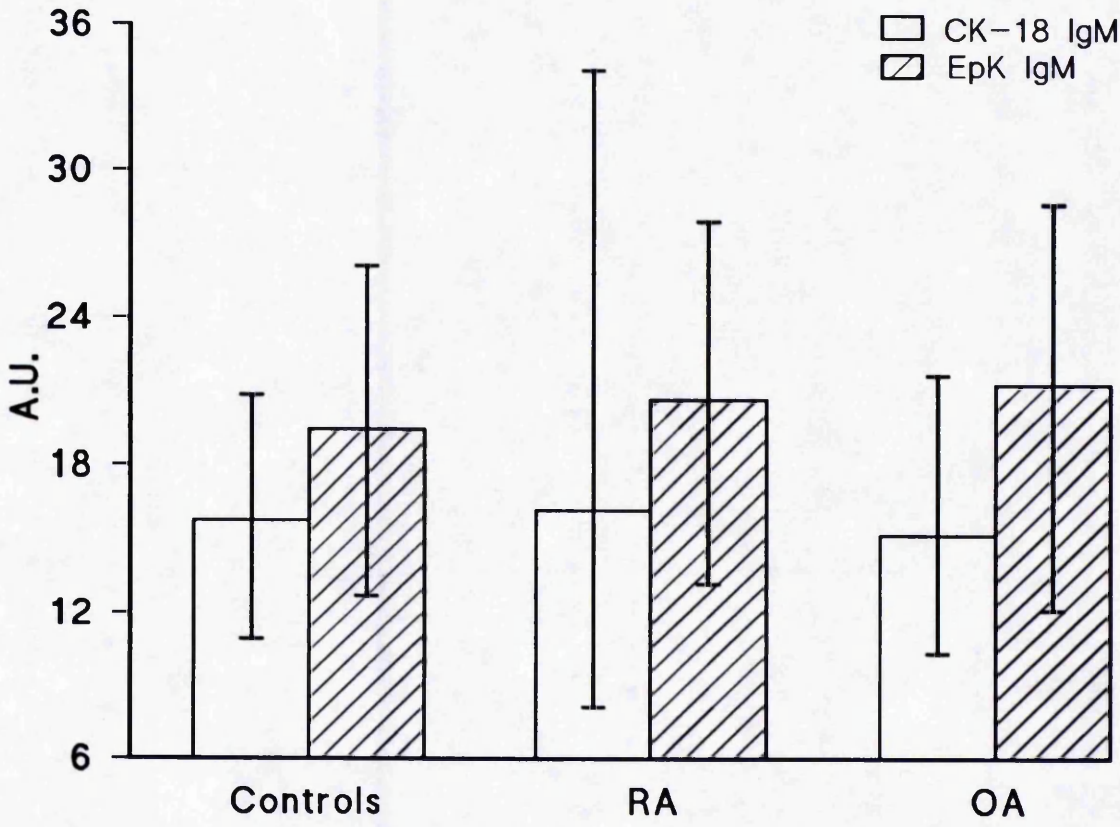
	Early RA	Established RA
No.of Patients	38	68
Dis.Duration(months)	<12	>12
% Positive Latex(>1:40)	49	57
CK-18 IgA	18.0(15.7-20.2)	14.9(12.5-17.4)
EPK IgA	13.3(11.7-14.7)	11.5(9.5-13.6)

Means with 95% Confidence Intervals of levels of IgA antibodies to CK-18 and EPK in patients with early and established RA.

Figure 5. Chart showing levels of IgG anti-CK-18 and anti-EpK antibodies as determined by ELISA in sera of normal controls, RA and OA. Values indicate group means (A.U.) and 95% C.I.

Figure 6. Chart showing levels of IgM anti-CK-18 and anti-Epk antibodies as determined by ELISA in sera of normal controls and in RA and OA. Values indicate group means (A.U.) and 95% C.I.


25
20
15
10
5
0
A.U.
CK-18 IgG
EpK IgG
Controls
RA
OA


36
30
24
18
12
6
A.U.
CK-18 IgM
EpK IgM
Controls
RA
OA

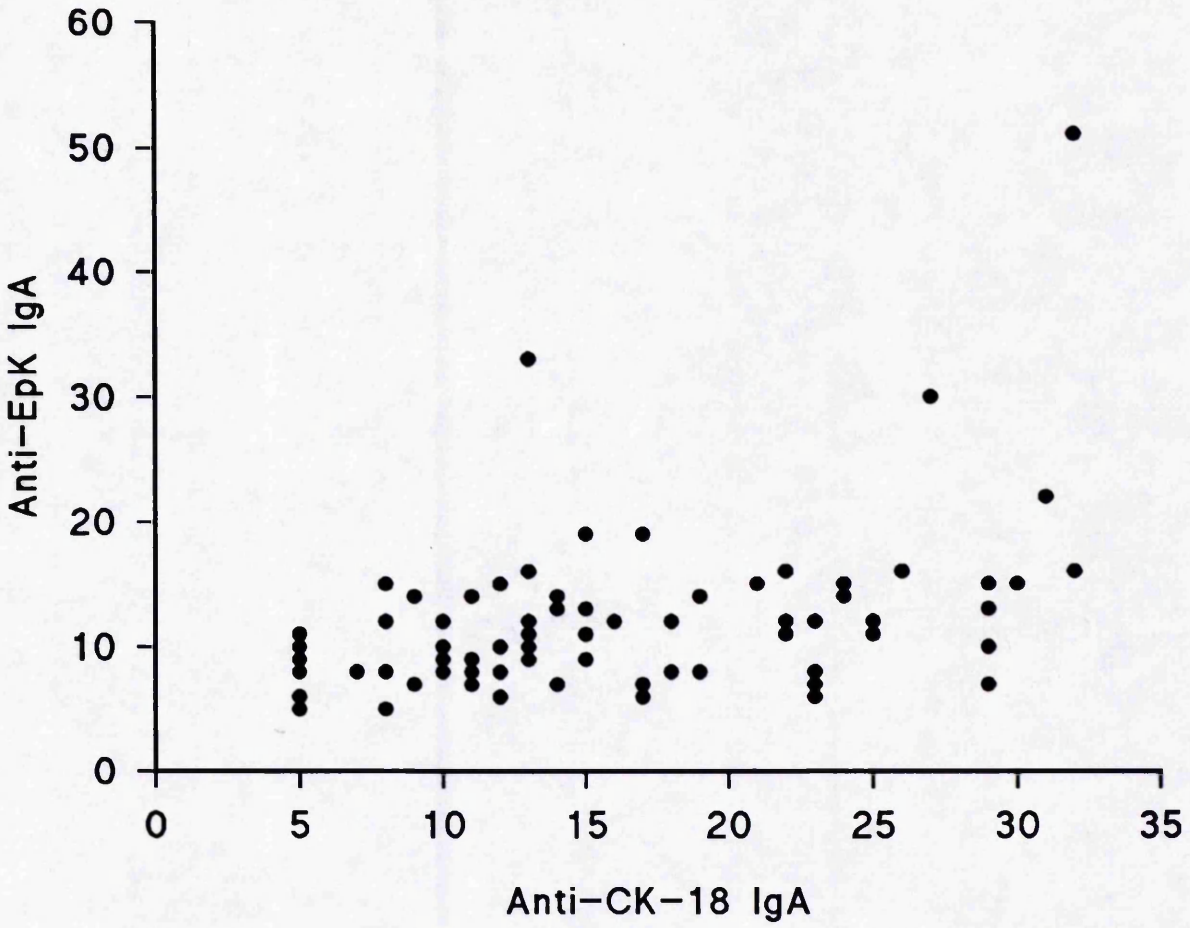


Figure 7. Scatterplot showing correlation between IgA antibody levels to CK-18 and EpK as determined by ELISA in serum from patients with RA ($r = 0.39$, $P<0.02$, Spearman).

No correlation was found between the levels of the CK-18 IgA autoantibodies and ESR (r = 0.10, Spearman) or CRP (r = 0.04, Spearman) (Figures 8 and 9). Similarly no difference was noted between the levels of ESR or CRP between patients whose antibody level was higher than 10.3 A.U. and those whose levels were within the normal range.

Of the nine RA patients with vasculitis, 5 had elevated levels of IgA autoantibodies to CK-18, and of these 4 had elevated levels of IgA autoantibodies to epidermal Keratin. Among the 16 patients with subcutaneous nodules, no difference was noted in IgA antibody levels to either CK-18 or epidermal Keratin.

40 sets of paired samples (sera and synovial fluid) from patients with RA were tested for the levels of IgG, IgA and IgM antibodies to CK-18 (Figure 10) and epidermal keratin (Figure 11). No statistical difference was demonstrated although generally serum levels of anti-cytokeratin antibodies tended to be slightly higher than the synovial fluid levels

4.1.3

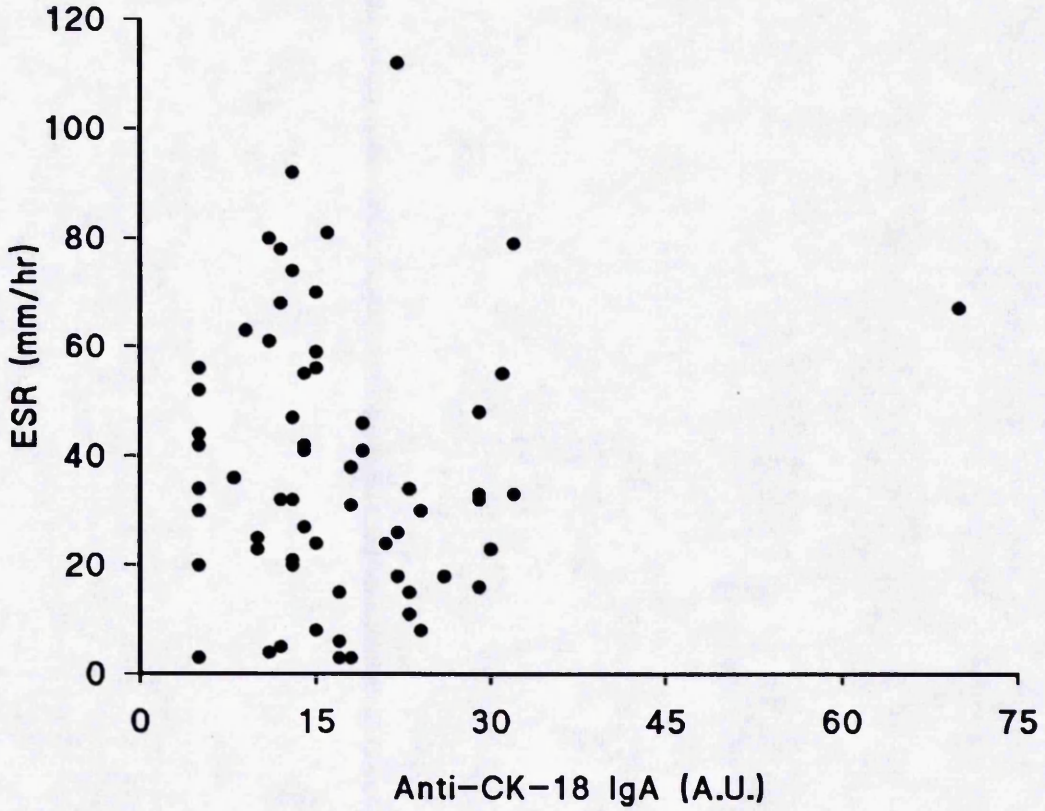
DISCUSSION

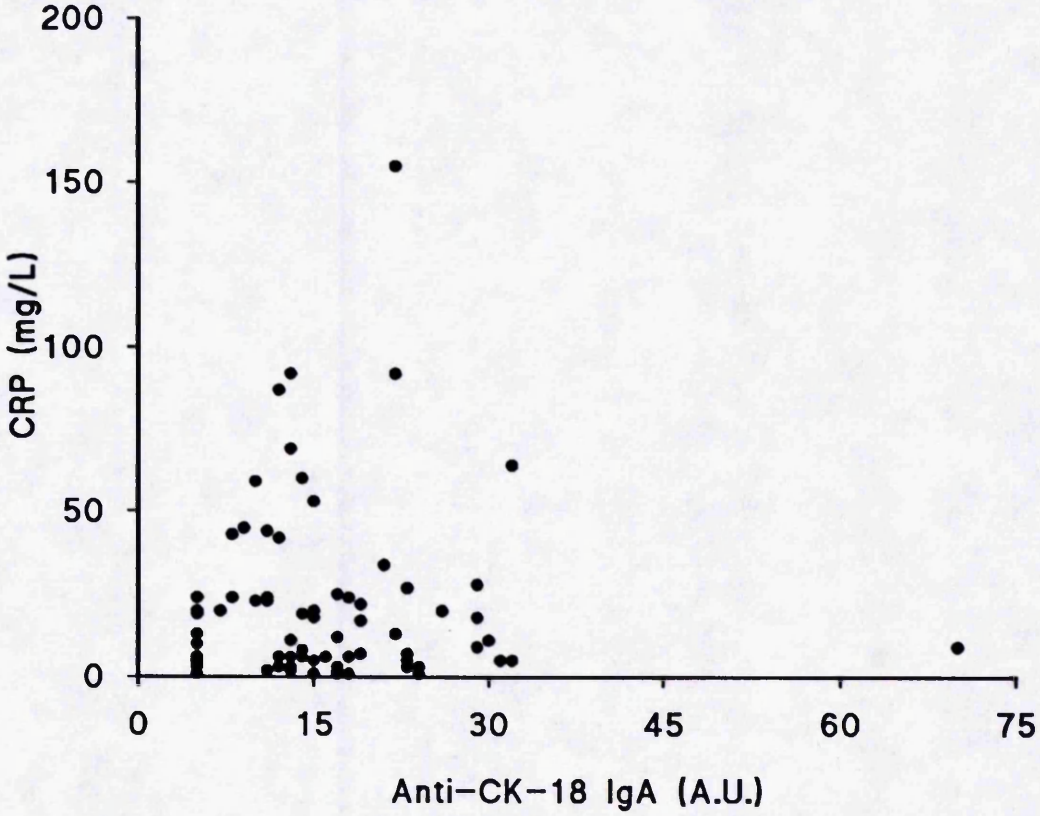
Our results demonstrate that levels of IgA autoantibodies to CK-18 and epidermal Keratin are significantly higher in RA than in patients with OA or in normal controls.

No specific disease subsets based on abnormal levels of IgA CK-18 have so far been identified in RA. Measures analysed

Figure 8. Scatterplot showing lack of correlation between the Erythrocyte Sedimentation Rate (ESR) and IgA anti-CK-18 levels in sera from patients with RA ($r = 0.10$, Spearman).

Figure 9. Scatterplot showing lack of correlation between C-Reactive Protein (CRP) levels and levels of IgA anti-CK-18 antibody levels in sera from patients with RA. ($r = 0.04$, Spearman).


120
100
80
60
40
20
0
ESR (mm/hr)
0
15
30
45
60
75
Anti-CK-18 IgA (A.U.)


200
150
100
50
0
CRP (mg/L)
0
15
30
45
60
75
Anti-CK-18 IgA (A.U.)

included disease activity (ESR and CRP), as well as the presence of vasculitis, subcutaneous nodules or leg ulcers.

Our ELISA results are quite different from previous studies on antikeratin antibodies which were mainly based on indirect immunofluorescence microscopy on tissue sections of the middle third of the rat oesophagus. It is questionable whether these assays are actually measuring "anti-keratin" antibodies, and it has been suggested that the antigen recognised is more likely to be a protein associated with cytokeratins in rat oesophageal cells [Hoet et al,1991].

CK-18 is not found in rat oesophagus although it is found in secretory cells of sweat glands and Merkel cells of human epidermis [Moll et al,1982]. Surprisingly, it has been shown to be a component of the intermediate filament cytoskeleton of synovial endothelial cells [Jahn et al,1987]. Most other endothelia lack cytokeratin, although it is found in some blood vessels in lymph nodes and mucosal sites [Franke and Moll,1987].

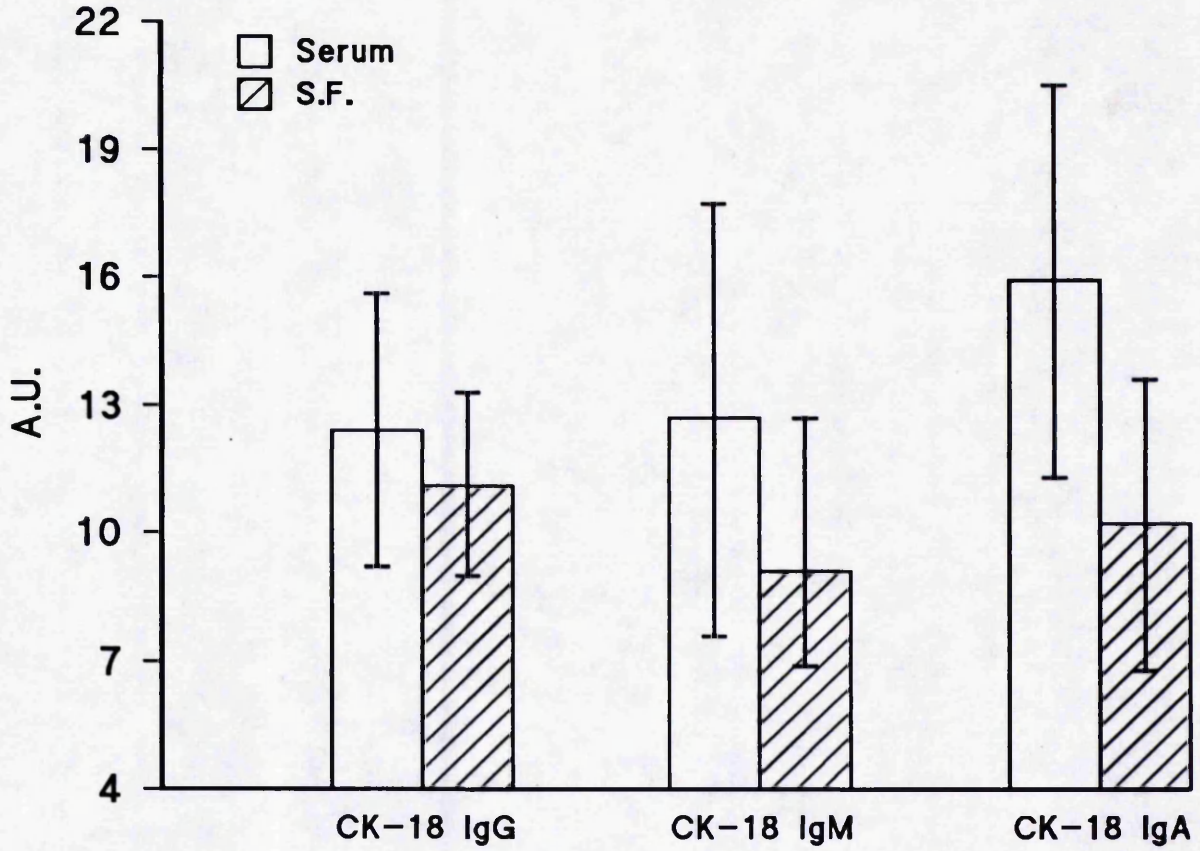
The role of the CK-18 filament system in synovial endothelial cells is unclear. Generally, an intact cytoskeleton is essential for vital cellular functions (including vesicle or organelle transport), cell-cell interaction, cell extracellular matrix interactions through association with surface receptors, cell motility and cell cycle [Rogers et al,1992]. The cytoskeleton may be disrupted by various inflammatory mediators including reactive oxygen species

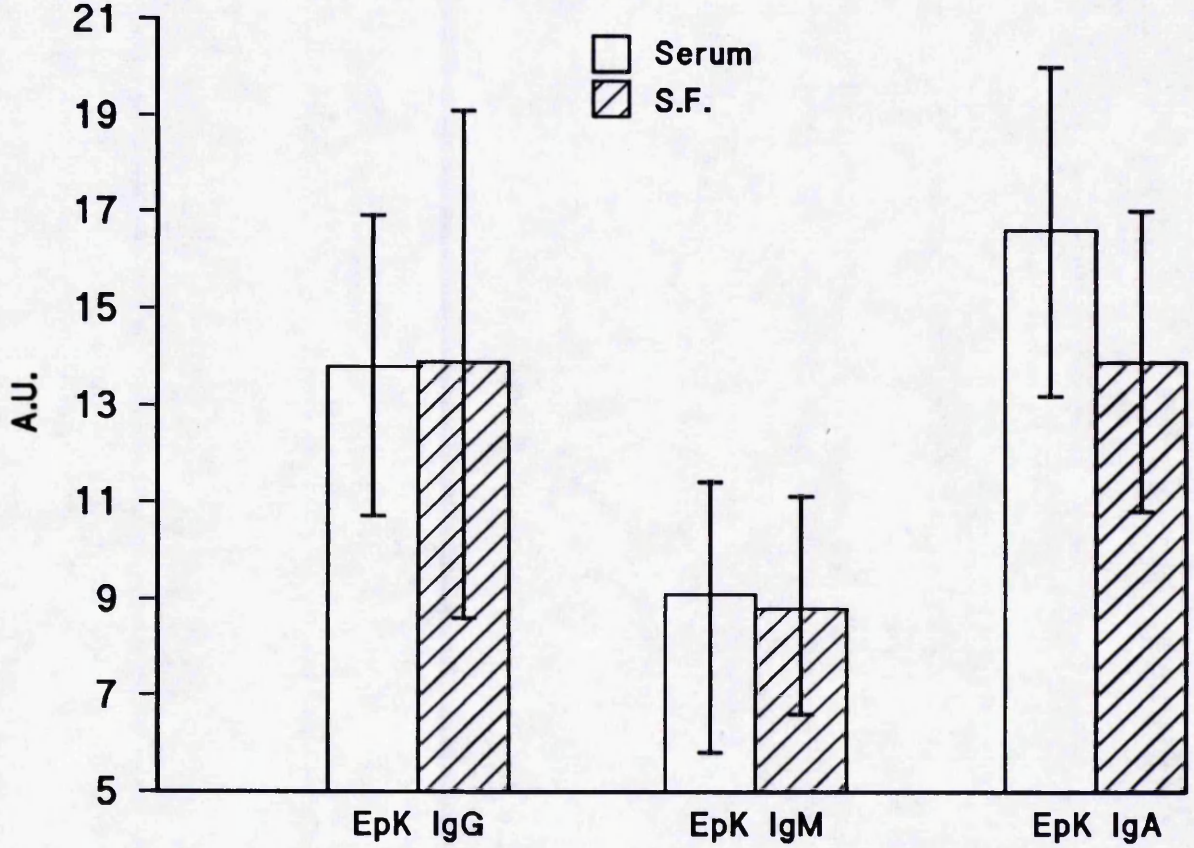
Figure 10 Chart showing antibody levels to CK-18 in serum and synovial fluid (Paired samples) in RA (A.U.).

Values are group means with 95% C.I.

Figure 11 Chart showing antibody levels to CK-18 in serum and synovial fluid (Paired samples) in RA (A.U.).

Values are group means with 95% C.I.





[Rogers et al,1989]. Such disruption may lead to changes in cell shape and alterations in microvascular integrity due to loss of endothelial barrier function.
It is possible that the increased IgA antibody response to CK-18 in RA patients is a consequence of damage to endothelial cells during inflammatory synovitis. Degenerating endothelial cells and cell debris have been observed in the thickened basement membrane region of post-capillary venules and capillaries in transitional areas of RA synovium containing macrophages, lymphocytes and plasma cells [Matsubara and Ziff,1987].

However, a number of alternative explanations are possible. For example, damage to epithelial tissues in other regions of the body (e.g. skin, gut, kidney) may lead to production of keratin autoantibodies which recognise one or more epitopes common to all cytokeratins, including CK-18.

The concept of "subclinical" skin inflammation in RA is also well recognised. Fitzgerald et al [1985], examined skin biopsies from 48 patients with RA for the presence of immunoglobulins and complement in the vessel walls. Deposits of C3, IgM or IgG were detected in 10 patients and they concluded that the presence of vessel immunofluorescence identified a subgroup of patients who had evidence of more severe immunological disturbance.

These findings were in keeping with previous work [Rapoport et al,1980] showing that the presence of vascular immuno-fluorescence in the skin from patients with RA correlated with the presence of circulating immune complexes and vasculitis. Similarly, epidermal phospholipase A2 has been shown to be elevated in patients with RA or psoriasis compared to normal controls suggesting the potential to cause clinical inflammation [Taylor et al,1991].

The correlation between IgA antibody levels to epidermal Keratin and IgA antibodies to CK-18, although weak, may point to cross-reactive epitopes as a possible mechanism for autoantibody production. There was no evidence of increased extra-articular disease in the RA patients with elevated antibodies to cytokeratin. The reason for an elevated IgA anti-cytokeratin response rather than an IgG or IgM response is not clear at present. It cannot be explained by a general increase in serum IgA levels as these showed no correlation with IgA anti-CK-18 levels ($r = 0.15$, Spearman).
The lack of correlation between IgA antibody levels to CK18 or epidermal Keratin with ESR or CRP suggests that the findings probably do not reflect part of the non-specific globulin response which is commonly found in RA. Presently, results do not indicate any disease subsets within the RA group related to IgA antibodies to either CK-18 or epidermal Keratin.

4.2

ELISA Study of Antibody Responses to CK-18 and Epidermal keratin in Psoriasis and in the Seronegative Spondyloarthropathies

4.2.1

Introduction

Following the demonstration of elevated IgA antibodies to CK-18 and epidermal Keratin in RA it was postulated that these antibodies may also be present in other inflammatory disorders with synovitis such as the Seronegative Spondyloarthropathies. Following preliminary results, an investigation was carried out to see whether these antibodies were elevated in an inflammatory skin disorder without synovitis.

Antibodies to CK-18 and epidermal Keratin were therefore measured in Psoriatic Arthropathy, Ankylosing Spondylitis and Reiter's syndrome/ Reactive Arthritis. These antibodies were also measured in the serum of patients with Psoriasis without arthritis.

4.2.2

Results

The demographic details of the patients studied are shown in Table 12.

IgA antibodies to both epidermal Keratin and CK-18 were raised compared to normal controls in both psoriatic arthritis and psoriasis but not in the patients with AS or Reiter's Syndrome (Figure 12). There was no significant difference in the levels of IgG or IgM antibodies to CK-18 or epidermal Keratin in any of the disease groups (Table 13).

Elevated levels of IgA antibodies to CK-18 were present in 75% of the patients with psoriasis, 58% of the patients with psoriatic arthritis. In the case of IgA antibodies to epidermal Keratin, elevated levels were present in 83% of the patients with Psoriasis, 50% of the patients with Psoriatic Arthritis.

A significant correlation between IgA antibody levels to CK-18 and epidermal Keratin was found in Psoriasis ($r = 0.47$, $p<0.03$ Spearman correlation) and in Psoriatic Arthritis ($r = 0.59$, $p<0.05$, Spearman correlation).

TABLE 12

DEMOGRAPHIC DATA

	PSORIASIS	PSOR.ARTHR.	ANK.SPOND.	REACTIVE	CONTROLS
NUMBER	33	42	20	11	58
AGE	47(16-83)	46(14-76)	43(31-60)	31(18-45)	41(26-73)
SEX (M:F)	15:18	28:14	16:4	8:3	20:38
DISEASE DUR. (Months)	195(2-480)	53(2-168)	71.4(5-138)	2(1.1-2.7)	---------

Values for age are means with ranges in parentheses.

Values for disease duration are means with 95% Confidence Intervals in Parentheses.

TABLE 13

ELISA RESULTS

	PSORIASIS	PSOR.ARTHR.	ANK.SPOND.	REACTIVE	CONTROLS
CK-18 IgA	24.2(18.1-31.2)*	24.1(16.3-32.2)*	9.0(7.6-10.4)	8.2(3.4-17.9)	9.4(8.2-10.1)
CK-18 IgG	14.4(5.1-22.2)	11.3(8.1-14.3)	15.2(12.6-17.8)	19.1(13.7-24.5)	13.0(7.1-19.3)
CK-18 IgM	12.2(8.3-15.1)	12.2(9.1-14.4)	13.1(10.9-15.1)	13.2(10.0-16.3)	16.3(11.1-21)
EK IgA	19.3(15.1-22.4)*	17.2(14.1-20.3)*	7.1(6.4-7.8)	6.1(5.2-7.0)	7.2(5.1-9.3)
EK IgG	11.9(4.9-19.8)	12.1(5.9-18.1)	14.1(6.0-34.0)	15.1(8.4-21.7)	14.9(9.9-19.3)
EK IgM	16.3(11.8-20.2)	13.4(8.1-17.8)	21.2(2.2-40.1)	15.5(13.2-17.7)	19.2(13.4-25.9)

* = P<0.0001

Mean values with 95% confidence intervals (in brackets) of IgA, IgG, IgM- antibodies to CK-18 and EPK in sera from patients with psoriasis, psoriatic arthropathy, Ankylosing Spondylitis, Reactive Arthritis and normal controls.

Analysis of the various clinical subsets of psoriatic arthritis according to the pattern of joint involvment (Table 14) revealed that in psoriatic patients with peripheral joint arthritis the levels of IgA autoantibodies to CK-18 were significantly higher than in the patients whose arthritis was limited to axial involvment alone (i.e. psoriatic spondylitis). Within the group of patients with peripheral joint disease however, no significant difference was found between patients who had pauciarticular, classical or symmetrical joint involvment (Figure 13).

4.2.3

Discussion

These results demonstrate that levels of IgA autoantibodies to CK-18 and epidermal keratin are significantly higher in patients with psoriasis or psoriatic arthropathy than in normal controls or patients with AS or Reiter's Syndrome.

The inability to demonstrate any difference in levels of IgA antibodies to CK-18 between the sera from the patients with psoriasis and psoriatic arthritis may well relate to the extent of skin involvement since the patients with psoriasis all had severe skin disease while the patients with arthritis had skin involvement varying from minimal to severe.

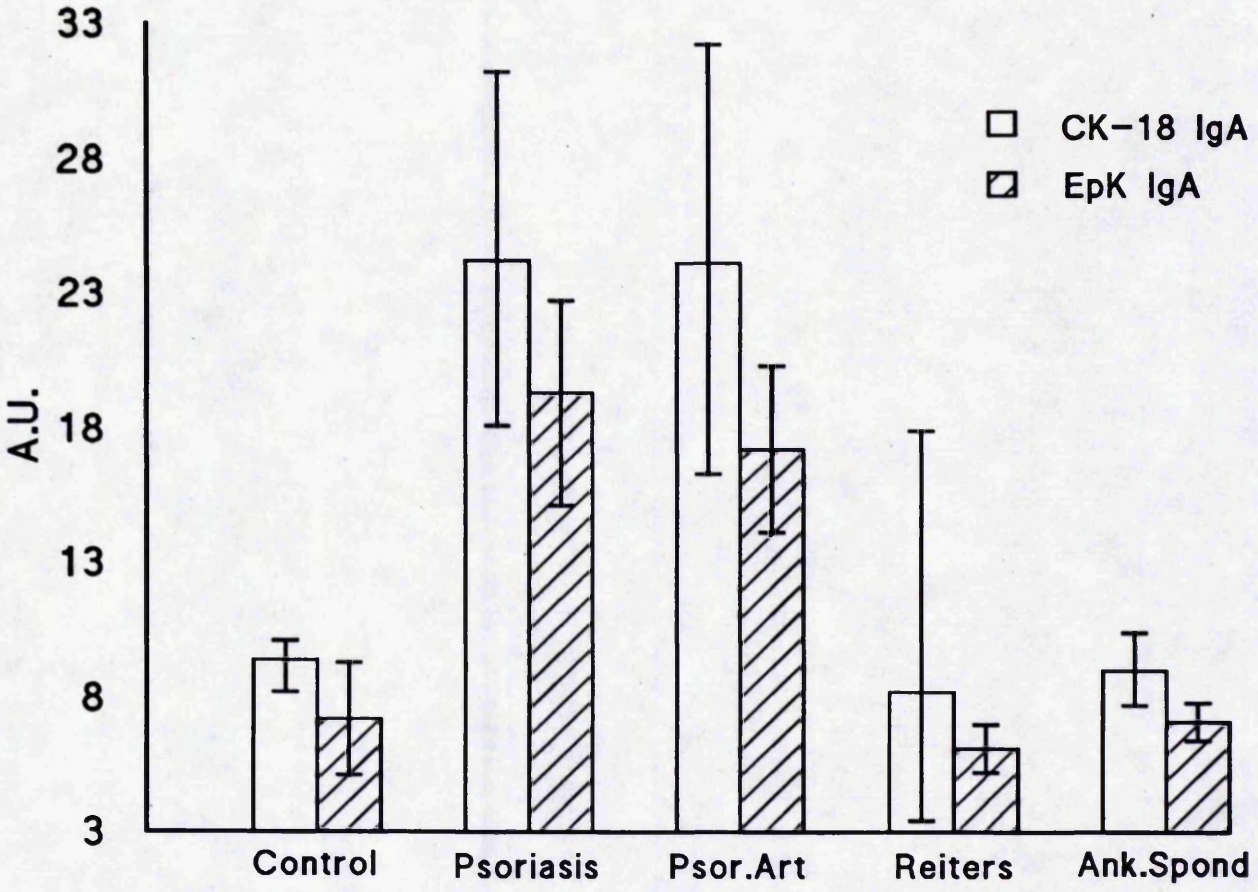


Figure 12. Chart showing levels of IgA anti-CK-18 and anti-EpK antibodies as determined by ELISA in sera from patients with Psoriasis, Psoriatic arthritis, Reiter's syndrome, AS and in normal controls.

Values indicate group means (A.U.) and 95% C.I.

Using a similar technique, Miossec et al [1982], could only detect IgG and/or IgM antikeratin antibodies in less than 2% of patients with psoriasis. This is hardly surprising, given the low sensitivity of the technique and the suggestion by Hoet et al [1991], that the antigen used is more likely to be a protein associated with cytokeratin in rat oesophageal cells and was not CK-18.

Damage or dysfunction of the cytoskeleton or of endothelial cells is recognised in response to various environmental stimuli that may arise in the setting of inflammatory arthritis, including hypoxia, acidosis, free radical formation, inflammatory mediators, increased blood flow or fibrin release [Rogers et al,1992].

Psoriasis is an inflammatory skin disorder characterised histopathologically by hyperproliferation of the epidermis, accumulation of inflammatory cells (particularly T lymphocytes, monocytes and neutrophils), and elongation and increased tortuosity of dermal papillary blood vessels [Barker,1991a].

It has been postulated that inappropriate activation of keratinocytes may acccount for the cascade of events leading to inflammation and hyperproliferation in psoriasis [Barker,1991b].

TABLE 14

Pattern	No.	IgA EpK	IgA CK-18
1. Classical	11	18.2 (16.5-19.8)	24.0 (14.8-33.2)
2. Pauciarticular	14	16.9 (12.0-21.9)	21.2 (13.2-29.1)
3. Symmetrical	11	26.7 (9.91-43.4)	29.2 (12.5-45.8)
4. Spondylitis	6	* 10.5 (5.03-15.9)	* 9.9 (1.51-18.3)
5. Psoriasis	33	19.3 (15.1-22.4)	24.2 (18.1-31.2)

* = p <0.001

Correlation between clinical patterns of Psoriatic Arthritis and IgA antibodies to cytokeratins.
Values are means with 95% Confidence Intervals in parentheses.
See text for definition of clinical patterns.

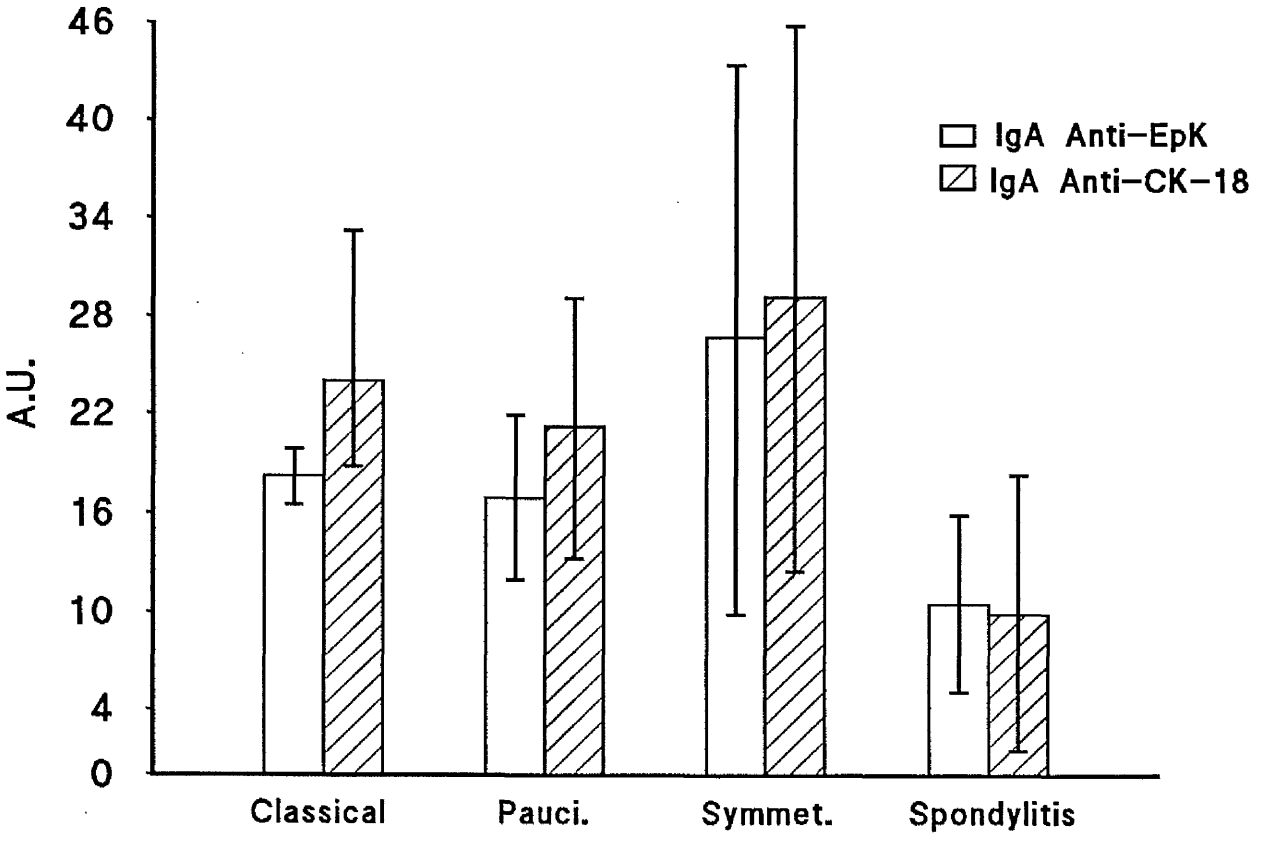


Figure 13. Chart showing relationship between clinical patterns of PsA and cytokeratin IgA antibody levels.
Values are group means.

The potential role of the immune system in the pathogenesis of psoriasis can be noted from the increased numbers of activated T lymphocytes in psoriatic skin and the response of psoriasis to cyclosporin A [Baadsgard et al,1991]. Also, in contrast to normal keratinocytes, psoriatic keratinocytes express HLA-DR antigens and intracellular adhesion molecule-1 (ICAM-1) suggesting the possibility of regulating antigen presentation, lymphocyte adherence, lymphocyte trafficking and migration of neutrophils into the epidermis [Griffiths et al,1989]. The antigenic nature of keratinocytes has been shown by the use of monoclonal antibodies raised to group A streptococci which have been shown to cross-react with products of keratinocytes in psoriatic human skin [Swerlick et al,1986].

Immunoglobulins localised to the dermo-epidermal junction in psoriasis have been demonstrated using a direct fluorescent antibody technique [Burnham et al,1963]. Using a technique of mixed agglutination with tissue sections, the immunoglobulins in the epidermis have been demonstrated to be of the IgG and IgM classes [Krogh and Tonder,1972].

Krogh and Tonder [1973] demonstrated that scales from patients with psoriasis contained IgG, IgM, IgA and C3. They postulated that a passive transepidermal diffusion of serum proteins occurs into psoriatic lesions and specific antibodies are then bound in the parakeratotic stratum corneum. The antigen-antibody complexes were shown to fix anti-IgG factors and complement.

Laurent et al [1981], demonstrated The presence of IgG and IgM circulating immune complexes and increased levels of serum IgA and IgG in both psoriasis and psoriatic arthritis and postulated that the increased IgA response may be due to defective function of a T Suppressor cell subset allowing increased production of IgA.

Mechanisms for the removal of damaged or dead cells can be difficult for mesenchymal tissues such as cytokeratins except where they are in contact with the external environment. One such mechanism involves apoptosis which can be considered as a form of gene-regulated programmed cell death involving the production of keratin bodies. Keratin bodies are generated by apoptotic keratinocyte death and consist largely of keratin intermediate filaments. Keratin bodies covered with IgM have been shown to be a characteristic finding in skin lesions of patients with various dermatoses such as Lichen Planus or chronic discoid Lupus Erythematosus.

These findings led to the suggestion that physiological apoptotic keratinocyte death may cause the liberation of autoantigenic material and production of autoantibodies [Grubauer et al,1986].

The concept of "subclinical" bowel inflammation presenting with arthritis is well recognised [Mielants et al,1985]. Simenon et al [1990] with a combination of ileocolonoscopy and biopsy revealed subclinical inflammatory gut lesions in 67% of patients with unclassified seronegative spondyloarthropathies.

The reason for an IgA antibody response to CK-18 in psoriasis and is not entirely clear, given the lack of apparent damage to endothelial blood vessels in this condition. Certainly, the possibility of a gut-related antigenic source is an intriguing hypothesis.

The concept of dermatogenic enteropathy to link skin with gut disorders was introduced by Shuster and Marks [1965] as an entity in which the skin lesion itself produced a small intestinal disorder and malabsorption. This definition was eventually modified by the same authors following their finding that small bowel biopsies from patients with psoriasis and eczema were non-specific and similar in controls [Shuster and Marks, 1970].

However, Barry et al [1971], demonstrated that in patients with severe psoriasis, the jejunal mucosal architecture differed significantly from that in controls and suggested that the mucosal absorptive surface of the small bowel may be decreased.

A genetic component in both psoriasis and psoriatic arthritis is well recognised, not only from the increased prevalence among relatives of patients with psoriasis, but also from the associations with MHC antigens. However, psoriatic arthritis only occurs in about 7% of patients with psoriasis.

Many studies have confirmed an increased prevalence of HLA-B27 in psoriatic arthritis [McClusky et al,1974; Metzger et al,1975], reflecting the frequent occurence of sacroiliitis and spondylitis in these patients. However, various differences in other HLA antigens between psoriasis and psoriatic arthritis are also recognised.

In psoriasis with and without arthritis, there is an increase in frequency of A1, B13, B17 and Cw6. Additionally, in patients with arthritis there is also an increased frequency of A26, B38 and DR4, while in patients with psoriasis there is an increased frequency of DR7 [Armstrong et al,1983].

The Seronegative Spondyloarthropathies are grouped together on the basis of their negativity for IgM rheumatoid factor, the increased prevalence of HLA-B27 and the spectrum of clinical presentations. This clearly masks any differences in their pathogenesis. The absence of a significant cytokeratin response in AS and Reiter's syndrome certainly suggests a different pathogenetic mechanism for psoriasis and psoriatic arthritis via antibodies to cytokeratin.

This work suggests that the antibodies to cytokeratin-18 and epidermal keratin are, however, an epiphenomenon rather than pathogenic antibodies per se, given their presence in a number of different disorders.

As far as is known this is the first study to demonstrate an increased IgA antibody response to a particular cytokeratin in psoriasis or psoriatic arthropathy.

4.3

Investigation of Antibody Response using SDS Page electrophoresis and Western Blotting

Introduction

4.3.1

Following on from the ELISA results in the patients with RA as well as the patients with Psoriatic arthritis, a number of questions were raised. The questions we attempted to answer by the SDS Page Blotting were the following:

1. Do antisera which recognise CK-18 and epidermal keratin in ELISA also recognise purified antigen on Western blots ?.
2. Do antisera with high anti-keratin antibodies recognise EBNA-1 antigen in Raji cells ?.
3. Do antisera recognise CK-18 in Madin Darby Canine Kidney (MDCK) cells ?.
4. Do the antisera recognise CK-18 in synovium ?.
5. Do antibodies which recognise CK-18 and/or epidermal keratin also recognise EBNA-1 antigen (P62) ?.

4.3.2

Results

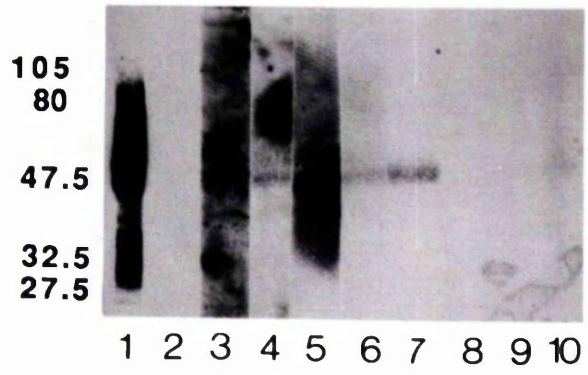
Despite using sera from patients with known elevated levels of antibodies to both epidermal keratin and CK-18 it proved impossible to detect any IgA Antibodies to CK-18 while only 1/20 of the sera reacted to epidermal keratin as the antigenic source. This was the serum sample with the highest level of anti-epidermal keratin antibodies as measured by ELISA.

Similarly, using sera with known elevated levels of IgG antibodies to CK-18 and/or epidermal keratin, no IgG reaction was seen with CK-18 as the antigen although 6/9 (67%) of the antisera gave a positive IgG reaction with epidermal keratin (Figure 14).

A number of skin specimens were then blotted and a skin specimen from a patient with Psoriatic arthritis was used for subsequent study since the pattern of bands obtained was clear and discrete. 3 out of 9 sera tested (with known elevated IgG anti-cytokeratin levels showed a reaction (Figure 15) while only one of the eight sera with known high levels of IgA antibodies to CK-18 and epidermal keratin showed a reaction (Figure 16).

Figure 14. Immunoblot showing human epidermal keratin blotted with different patient antisera (IgG Antibodies).
Lane 1: Monoclonal anti-Epk (Sigma)
Lane 2-10: Patient antisera.

Figure 15. Immunoblot of skin extract from a patient with Psoriatic arthritis blotted with different patient antisera (IgG Antibodies).
Lanes 1-9: Patient antisera.


105
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47.5
32.5
27.5
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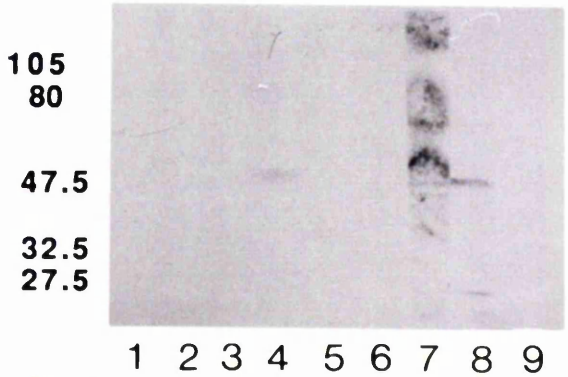
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1 2 3 4 5 6 7 8 9

Skin samples from patients with both Psoriasis and Psoriatic Arthritis were then blotted with polyclonal anti-keratin antibody (IgG) and with monoclonal antibody to CK-18 (Figure 17). Results confirm that CK-18 is not present in skin while both the skin specimens show a range of keratin bands with overexpression of different bands in the two samples.

Sera used to address the above questions included specimens with a range of antibody levels to epidermal keratin as well as CK-18 together with various normal control sera.

Immunoblots were also performed on Raji cell extracts which were blotted with different patient antisera with high levels of IgG anti-CK-18 antibodies and a secondary monoclonal IgG anti-keratin antibody. Raji cells are cell lines established from Burkitt's lymphoma lymphoblasts which carry EBV markers. A number of different bands were recognised by the antisera (Figure 18) including a band with a similar molecular weight (approximately 70KD) to that of EBNA-1 [Baboonian et al,1991]. This band was seen in 8/15 of the sera tested.

Figure 16. Immunoblot showing extract of skin from patient with Psoriatic arthritis blotted with different patient antisera (IgA Antibodies).

Figure 17. Immunoblot showing extracts of different skin preparations and purified epidermal keratin and CK-18 blotted with antibodies to polyclonal IgG group anti-keratin (a) or monoclonal anti-CK-18 (b).

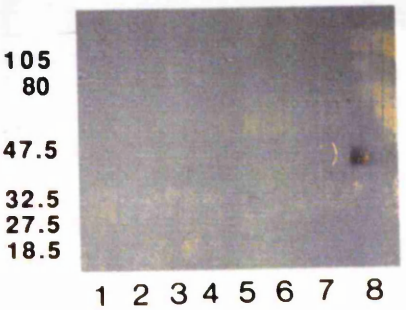
Lane 1: Epidermal keratin (Sigma).

Lane 2: Normal Skin

Lane 3: Psoriasis

Lane 4: Psoriatic Arthritis

Lane 5: Cytokeratin-18.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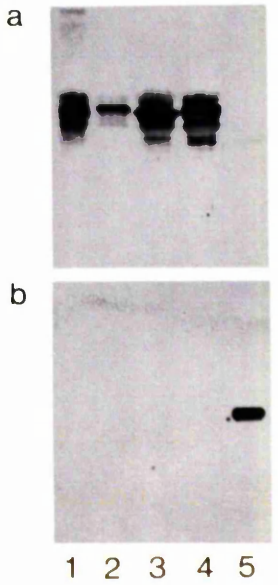

a
b
1 2 3 4 5

4.5.3

Discussion

The reason for the apparent failure to detect IgA anti-Ck-18 antibodies on Western Blotting may well be multifactorial and of a technical nature. Possibilities include failure of Western blotting to detect very low concentrations of auto-antibodies given the decreased sensitivity of detection of this system compared to ELISA assays.

Other possibilities include denaturation of the antigenic protein or antibody recognition being dependent on the secondary or tertiary configuration of the antigen rather than a linear epitope.

In the Raji cell blots, despite the apparent band at approximately 70KD it is uncertain whether this band is actually recognising EBNA-1 as a monoclonal antibody to EBNA-1 was not available as a control. However, a similar band was detected by Baboonian et al [1991] using anti-P62 antibodies on extracts of Raji cells.

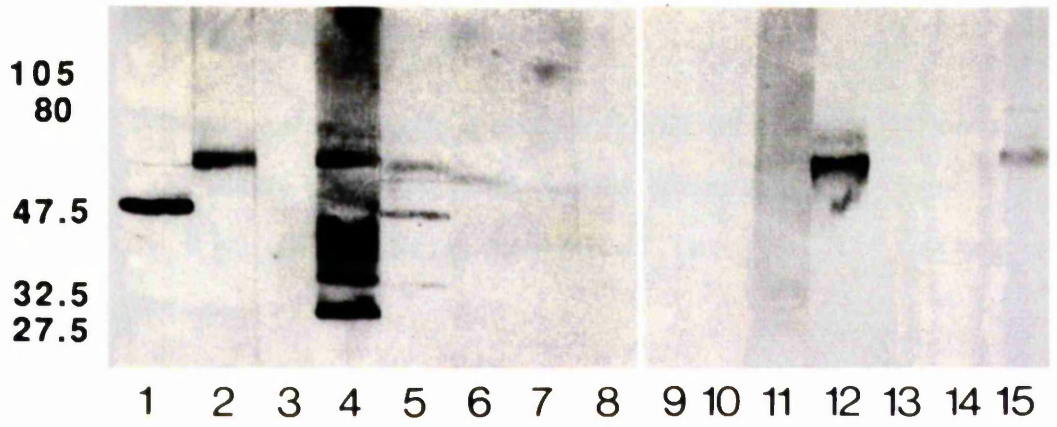


Figure 18. Immunoblot on Raji cell extract blotted with different patient antisera (IgG Antibodies) . Lanes 1-15 : different patient antisera .

Normal human epidermis is made up of two different layers - basal and suprabasal. The basal keratinocytes contain the primary keratins 5 and 14 [Skerrow and Skerrow,1983] while the suprabasal layer contains keratins 1 and 10 [Fuchs and Green,1980]. This keratin pair is characteristic of cornifying, or skin-type differentiation [Cooper et al,1985]. Keratins 8 and 18 are expressed by simple epithelial cells that in the skin are represented by cells of the sweat gland (secretory cells), but, with the exception of Merkel cells, are not present in the epidermis. CK-18 has been identified in the endothelial cells lining the blood vessels of synovium in patients with psoriatic arthropathy but not in the skin of these or normal patients [Mattey et al,1993].

The cytokeratin profiles in psoriasis are complex, with the presence of differentiation markers (keratins 1,2 and 10), basal cell keratins (14 and 5) as well as hyperproliferative markers (keratins 16 and 6) [Holland et al,1989].

The Western blotting results confirm the work of Markey et al [1991] that CK-18 is not expressed in normal human epidermis or in the epidermis of patients with psoriasis.

The reason for the failure of detection of IgA antibodies to CK-18 on the Western blots in synovium as well as the low percentage of samples positive for epidermal keratin may well relate to a sensitivity problem. The IgA antibodies may be present in too low a titre in the serum to be detectable by the blotting experiment. Conversly, failure of antibody recognition may relate to the altered configuration of the antigen on the Western Blots.

4.4

Investigation of cross-reactivity between IgA antibodies to CK-18 and Epidermal Keratin

4.4.1

Introduction

Following the finding of raised IgA autoantibodies to both CK-18 and epidermal keratin, the possibility of cross-reacting IgA autoantibodies was investigated by repeating the IgA ELISA assay with a slight modification i.e. preincubation of all sera with EpK to see if binding to CK-18 was inhibited. The experiment was performed on both RA sera as well as sera from patients with psoriasis and psoriatic arthritis since it was possible that the IgA autoantibodies were recognising different epitopes in the different diseases.

4.4.2

Results

The experiment involved incubation of different sera with 50 micrograms/ml of epidermal keratin for one hour at 37^{O}C. No difference was noted between the two sets of samples i.e. preincubation of CK-18 with epidermal keratin did not block or diminish the reaction of naturally occuring antikeratin antibodies in serum.

This was felt to be due, if anything, to the length of incubation rather than the concentration of EpK added to the sera tested. It was considered that 50 micrograms/ml of epidermal keratin would be enough to fully saturate anti-epidermal keratin binding sites.

4.4.3

Discussion

The retention of anti-CK-18 antibody activity despite previous incubation with epidermal keratin would suggest that the IgA autoantibodies to CK-18 and to epidermal keratin actually recognise different epitopes and are probably not cross-reactive, but a confirmatory experiment using overnight incubation would be needed.

Additionally, given the different blotting patterns of normal skin, psoriatic skin (both involved and uninvolved) and psoriatic arthritis skin it is conceivable that IgA antibodies to Cytokeratins in these two conditions recognise different epitopes. To address this question would necessitate preincubation of purified anti-CK-18 antibodies with skin extracts from the different pathological conditions.

4.5

Investigation of cross-reactivity between Epstein-Barr virus and CK-18

4.5.1

Introduction

Currently there is no data on the source of the IgA antibodies (i.e. mucosal or central immune system) although it is possible that they are produced in response to stimulation of the mucosal compartment. IgA is the major isotype produced by mucosal associated lymphoid tissue and antigenic stimulation of the mucosal immune system has been shown to elicit the appearance of specific IgA antibodies in peripheral blood [Czerkinsky et al,1987]. One possibility is that antibodies directed against another antigen(s) (e.g.from a micro-organism), cross react with CK-18. IgG antibodies to P62 (corresponding to the glycine/alanine repeat sequence of Epstein-Barr virus nuclear antigen), have been shown to cross-react with collagen, actin and epidermal keratin [Baboonian et al,1991].

Furthermore, the Epstein-Barr virus (EBV) has been implicated in the pathogenesis of RA following the finding that RA sera reacted with rheumatoid arthritis nuclear antigen (RANA) which is found in EBV-transformed B cells [Venables,1988a]. The synthetic peptide P62 has been shown to be a major epitope of RANA [Venables et al,1988b]. Also, amino acid sequences

similar to P62 have been found in human collagens and keratins [Sulitzeanu and Anafi,1989].

Thus, we set out to address this question in two ways:

1. By determining whether any homologous amino acid sequences existed between P62 and CK-18.
2. Developing a sensitive ELISA assay to measure binding of antibodies to P62 in serum.

4.5.2

Results

1. Homology between P62 and CK-18

We have compared the amino acid sequence of CK-18 (obtained through the GenEMBL database) with P62 and found 2 peptides of 13 and 15 amino acids long (nos 54-68, and 62-74) with 53.8% and 53.3% homologies respectively (Figure 19).

2. ELISA investigation of cross-reactivity between EBNA-1 and CK-18.

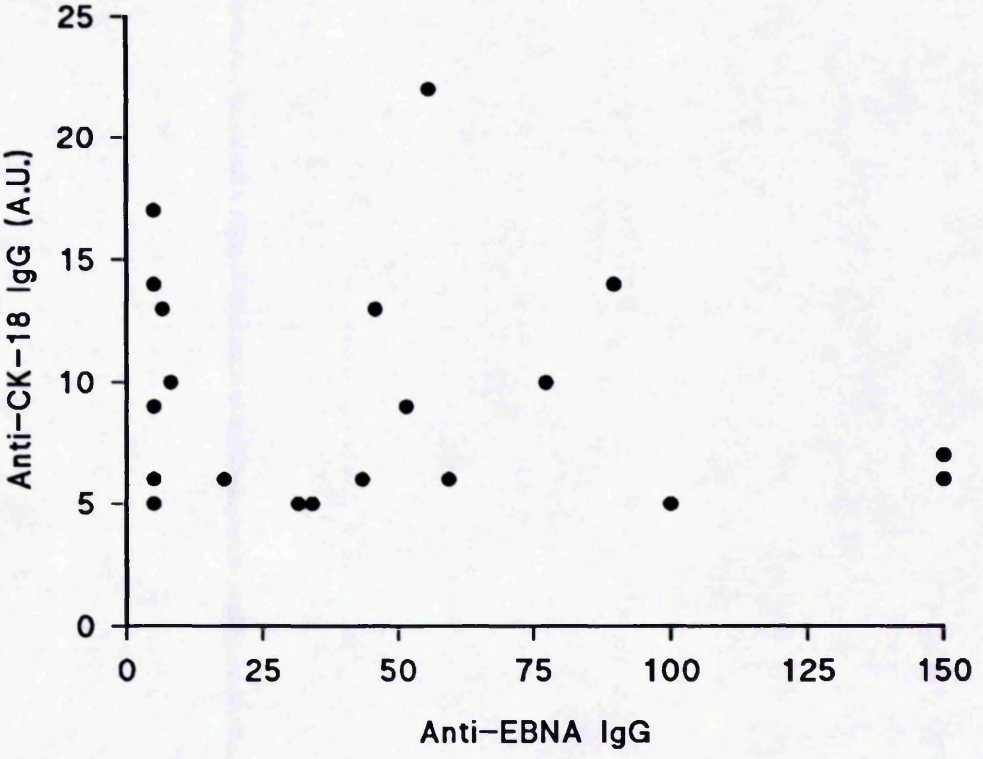
An ELISA system to measure IgG, IgA and IgM antibodies was successfully established in a similar way to the ELISA for measuring CK-18 antibodies.

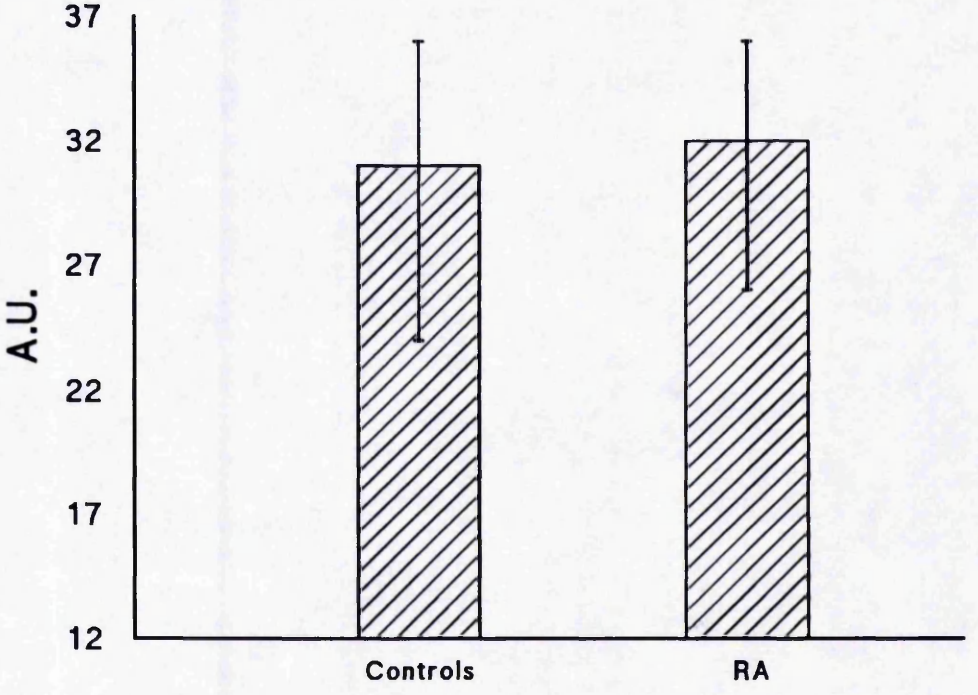
Figure 19. Homologous amino acids sequences in CK-18 and P62.
See text for details.

Figure 20A Scatterplot showing lack of correlation between IgG antibodies to CK-18 (A.U.) and IgG antibodies to EBNA (A.U.).
(r = 0.02, Spearman).

Figure 20B Chart showing levels of IgG Anti-EBNA-1 antibodies in normal controls and in RA.
Values indicate group means (A.U.) and 95% C.I.

P62	1	A G A G G G A G G A G A G G G A G G A G	20
CK-18	54	G G M G S G G L A T G I A G G	68
	62	A T G I A G G L A G M G G	74





However, only the IgG ELISA gave a sufficient spread of values to allow standardisation of the assay. Only a few sera (including normal controls) recognised IgM antibodies to P62 and only one (1/40) of the sera tested recognised IgA antibodies to P62. No correlation was demonstrated between levels of IgG anti-CK-18 antibodies and IgG anti-EBNA antibodies (Figure 20A). Similarly, no difference was demonstrated between levels of IgG antibodies to P62 in RA or the normal controls (Figure 20B).

4.5.3

Discussion

The significant amino acid sequence homologies between P62 and CK-18 suggest that the possibility of a cross-reaction between antibodies to CK-18 and EBNA-1 cannot be ruled out. Further studies are needed to address this question further.

The lack of any difference between the levels of IgG antibodies to EBNA in controls and RA sera are not surprising given the ubiquitous nature of the EBV and the probability that most people at some time or other in their life have been infected with the EBV. A possible way to address the problem further would be by measuring the EBV viral capsid antigen (VCA) in these sera. VCA positivity is associated with ongoing EBV infection.

4.6

Cell Staining

4.6.1

Introduction

To further ascertain the specificity of the antibody response in the patient antisera tested, cultured Madin-Darby Canine Kidney (MDCK) cells and synovial sarcoma cells were used as both of these cell lines express CK-18.

4.6.2

Results

Most of the antisera used stained the cytoskeletal network of both the synovial sarcoma cells and the MDCK cells but to a different extent and with different but not exclusive staining patterns (Table 15). Patterns recognised were arbitrarily classified as cell-membrane associated, cytoskeletal or nuclear (Figure 21). No difference in the staining patterns was noted when different FITC conjugates (i.e. IgG, IgA or IgM) were used.

Table 15

	MDCK Cells			Synovial Sarcoma Cells	
	Membrane	Filament	Nuclear	Filament	Nuclear
Patient No.					
1	+	+	+	+	+-
2	+	+	-	+	+-
3	+	+-	+	+	+-
4	+-	+-	-	-	-
5	-	+-	+	-	-
6	-	+-	-	-	-
7	+	+	+	+	+-
8	+	+	+-	-	+
9	+	+	+	+	+
10	+	+-	+	+	+

+ = Strong immunofluorescence

+- = Weak immunofluorescence

- = Negative immunofluorescence

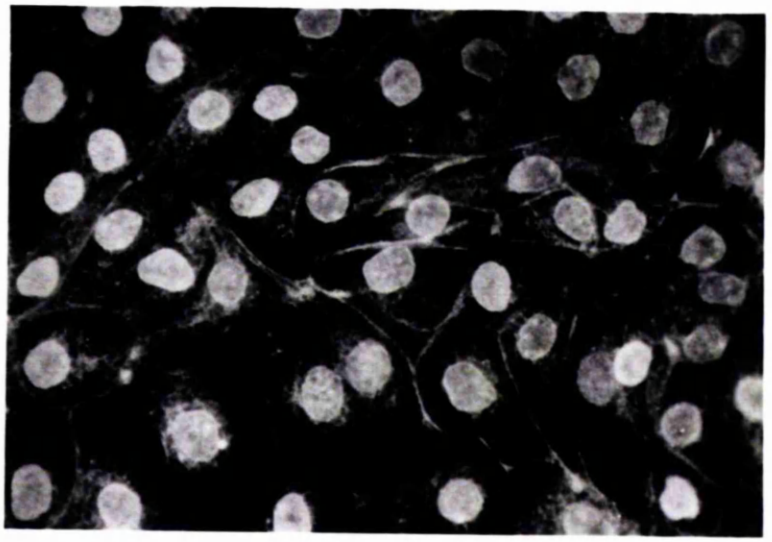
Table showing staining patterns on cell lines expressing CK-18 with antisera from patients with RA (IgG Antibodies).

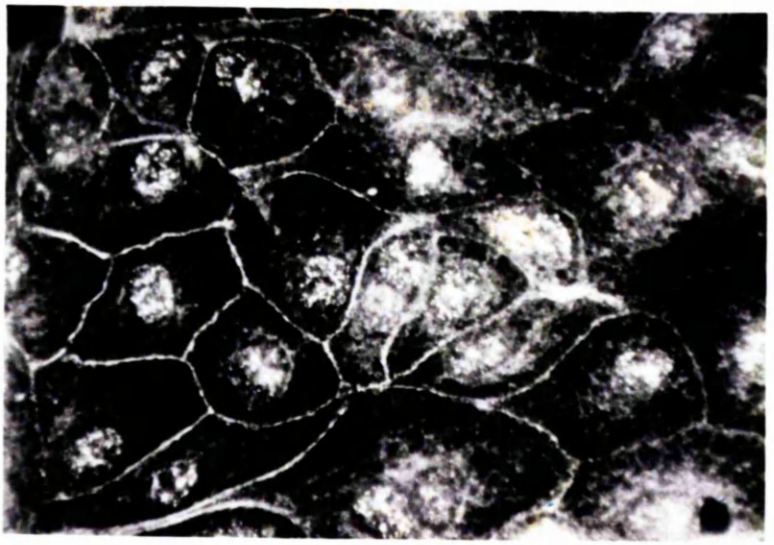
Figure 21. Photomicrographs of different staining patterns of MDCK cells with patient antisera (IgA Antibodies).

A: Filamentous + Nuclear pattern.

B: Nuclear + Membranous.

Similar Patterns were observed using IgG and IgM Antibodies.





4.6.3

Discussion

The wide range of structures stained as well as the different staining patterns suggest that the antigens recognised are heterogenous and the antibody respose is probably polyclonal in nature.

4.7

Study on Serum IgA and Rheumatoid Factor Isotypes in Rheumatoid Arthritis

4.7.1

Introduction

Serum IgA and IgA Rheumatoid Factor are commonly elevated in patients with RA. Following our finding of an increased IgA antibody response to CK-18 and epidermal keratin it was important to determine whether this was merely a feature of a non-specific increase in immunoglobulins as seen in RA.

This question was also addressed with regards to the increased IgA antibody response to CK-18 and epidermal keratin which was demonstrated in psoriasis and psoriatic arthritis. (See section 4.7.2).

Results

Although a number of the RA sera in the cohort tested had increased levels of serum IgA and IgA Rheumatoid factor, no correlation was found with the levels of IgA anti-CK-18 antibodies (Figures 22 and 23).

IgA Rheumatoid Factor was elevated in 30% of the patients with psoriasis and 40% of the patients with psoriatic arthritis respectively. As in the case of RA, no correlation was found with IgA antibody levels to CK-18 in Psoriasis or in Psoriatic Arthritis.

4.7.3

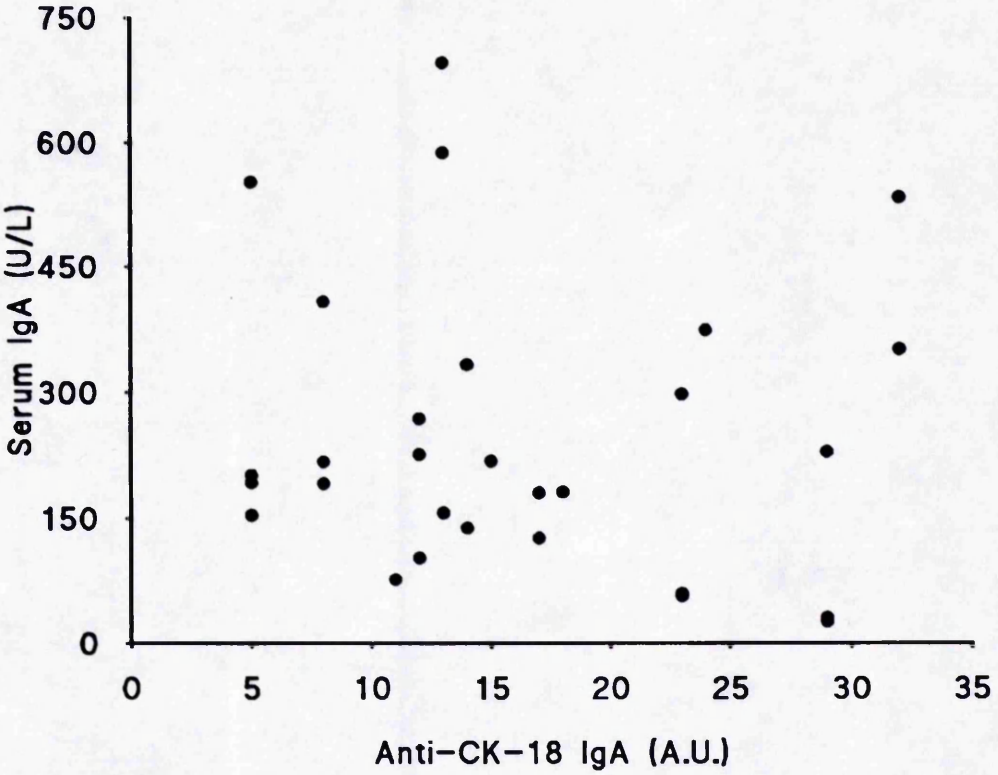
Discussion

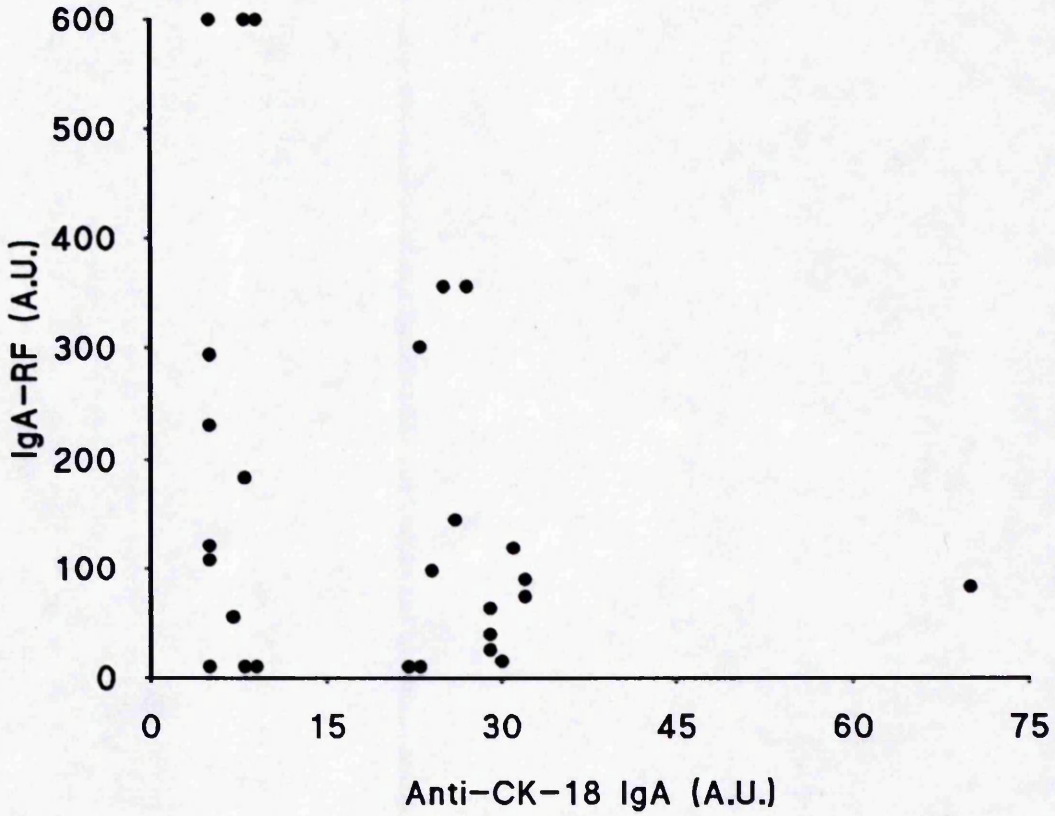
No relationship was demonstrated between levels of IgA CK-18 and total serum IgA or IgA-RF, suggesting that the increased IgA response to CK-18 in RA is not part of the non-specific immunoglobulin elevation which commonly accompanies active RA.

The high percentage of seropositive patients for IgA-RF in Psoriasis (30%) and Psoriatic Arthritis (40%) is unexpected and in contrast to previous work by Brik et al [1991].

Figure 22. Scatterplot showing lack of correlation between serum IgA and IgA anti-CK-18 levels in RA.
(r = 0.15, Spearman).

Figure 23. Scatterplot showing lack of correlation between IgA-RF and IgA anti-CK-18 levels in RA.
(r = 0.23, Spearman).


750
600
450
300
150
0
Serum IgA (U/L)
0
5
10
15
20
25
30
35
Anti-CK-18 IgA (A.U.)


600
500
400
300
200
100
0
IgA-RF (A.U.)
0
15
30
45
60
75
Anti-CK-18 IgA (A.U.)

Only 1 patient out of 19 (7%) in Brik's cohort was positive for IgA-RF. However, in Brik's study, although the methodology used was similar, the cohort of patients studied was different and was not clearly defined. As in the case of the RA sera tested, the lack of correlation between IgA-RF and levels of IgA autoantibodies to CK-18 in the patients with psoriatic arthritis suggests that the antibody response is likely to be a specific response to an antigen or antigens rather than a nonspecific immunoglobulin response.

4.8

Sequential ELISA IN RA

4.8.1

Introduction

From the point of view of clinical application of elevated IgA antibody levels to CK-18 in RA, it would be useful to demonstrate whether:

1. Levels of IgA antibodies to CK-18 fluctuate in RA.
2. Fluctuating levels correlate with other recognised measures of disease activity in RA.

Sera from 20 patients with RA of less than 12 months duration were collected monthly for 6 months and stored at -70^{o}C. Two metrologists also recorded a number of measures of disease activity. These included: Ritchie Index, Stoke Index, Visual Analogue Scale for pain, ESR, CRP, and duration of Early Morning Stiffness.

4.8.2

Results

A fluctuation in the levels of IgA antibodies to CK-18 was demonstrated (Figure 24). However, no correlation was demonstrated between change in level of IgA antibodies to CK-18 and change in any of the measures of disease activity except duration of early morning stiffness ($p<0.0001$, Mann-Whitney U Test).

4.8.3

Discussion

Measurement of IgA antibodies to CK-18 in RA would be very useful clinically if they could identify a subset of RA patients whose outcome was different or if the fluctuating levels correlated with changes in objective measurements of disease activity.

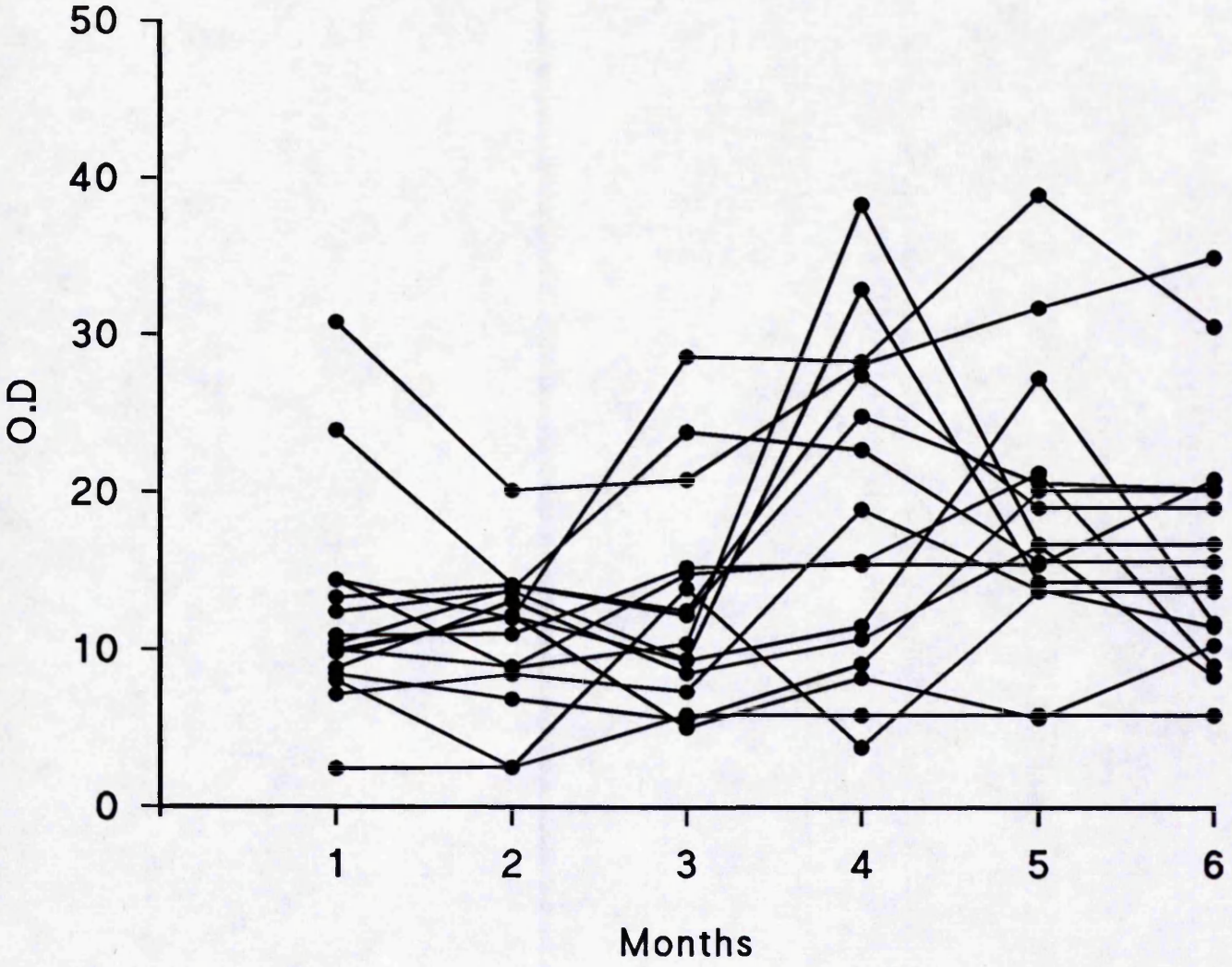


Figure 24 Plot showing sequential ELISA results in RA.

However, although levels do fluctuate, the extent of variation is limited in most of the sera tested, and there was no correlation with changes in objective disease activity measures. A significant correlation was only found with change in duration of early morning stiffness, but this is only a subjective measure of disease activity.

Chapter 7

DISCUSSION

Chronic inflammatory synovitis in RA is an immunologically mediated phenomenon in which an as yet unidentified antigen stimulates reactive T and B lymphocytes. Rheumatoid synovial T cells have been shown to proliferate in response to a number of autoantigens including type II collagen and heat-shock protein. It has even been suggested that these antigens which are present locally within the joints may play a role in the amplification and persistance of RA [Ziff,1991].

Cytokeratin-18 has been demonstrated within the blood vessels of synovium. This is an unusual site for this particular cytoskeletal protein which is normally confined to epithelial cells. Furthermore, a number of previous reports suggested that autoantibodies to keratins were highly specific for RA. However, not only were the diagnostic methods used nonspecific and insensitive, but also, no attempt had been made to define which particular cytokeratins were recognised by these particular antibodies.

This study has demonstrated that patients with RA have significantly higher levels of IgA autoantibodies to both Cytokeratin-18 and epidermal keratin compared to normal controls and patients with OA. So far we have been unable to define any disease subsets based on duration of disease, sex or age. As far as is known, this is the first study to demonstrate an increased IgA autoantibody response to Cytokeratin-18 or human epidermal keratin in RA.

The increased IgA antibody response however, does not appear to be specific for RA since it is also present in Psoriasis and Psoriatic arthritis. In the patients with Psoriatic arthritis there is a significant difference between patients who have peripheral joint disease and those with spondylitis alone thus allowing for the identification of a subset of patients within this disorder based on abnormal levels of IgA antibodies to Cytokeratin-18.

The lack of correlation between IgA antibody levels to Cytokeratin-18 or epidermal keratin with ESR or CRP suggests that the findings probably do not reflect part of the non-specific globulin response which is commonly found in RA.

Further studies need to be performed to determine whether any relationship exists between the elevated levels of IgA antibodies to these cytokeratins in RA and a number of clinical and laboratory based measures of disease activity such as synovitis scores, pain scores, haemoglobin, platelet count, alkaline phosphatase, and radiological damage and also whether their levels change with therapy.

The detection method used (ELISA) has a number of advantages over the semi-quantitative method of indirect immunofluorescence which was previously used to detect antikeratin antibodies. ELISAs are more specific (antibody for antigen and enzyme for substrate) and sensitive and are fully quantitative once standardised. However, even ELISAs have their functional limitations. A certain amount of non-specific absorbance in which substances other than the desired antigen or antibody attach, cannot be avoided. Secondly, each specific capture antibody has a limiting absorbance, beyond which a surface coated by these antibodies cannot capture more specific antigen. Thirdly, detection may be impaired by washing, because reactants that are not firmly anchored may be removed. ELISAs take a long time to perform, are complex, and since the specificity of the assay depends on the avoidance of antibody cross-reactions, the more antibodies there are in a system the greater is the opportunity for cross-reactivity [Meier and Hill,1985].

There is clearly an intimate link between infection, the immune system and autoimmunity. The immune system may have developed primarily to defend the organism against infectious diseases and the autoimmune process may be a deviation of this. Infectious agents are often thought of as triggers of any disorders. Furthermore, the onset of disease in many patients with myositis, diabetes mellitus, and to a much lesser extent RA, seems to follow an infectious disorder [Isenberg, 1992].

Furthermore, the Epstein-Barr virus (EBV) has been implicated in the pathogenesis of RA following the finding that RA sera reacted with rheumatoid arthritis nuclear antigen (RANA) which is found in EBV-transformed B cells [Venables,1988a]. The synthetic peptide P62 has been shown to be a major epitope of RANA [Venables et al,1988b]. Also, amino acid sequences similar to P62 have been found in human collagens and keratins [Sulitzeanu and Anafi,1989].

Thus, in summary, it has been shown that Cytokeratin-18 is present within endothelial cells lining synovial blood vessels in patients with various rheumatic conditions including Rheumatoid Arthritis (RA), as well as in normal controls [Mattey et al, In Press; Jahn et al,1987]. Since damage to synovial endothelial cells has been demonstrated in RA [Matsubara and Ziff,1987], it was considered that such damage may lead to increased production of autoantibodies to Cytokeratin-18. A sensitive ELISA was therefore developed to

measure antibodies to Cytokeratin-18 in serum and synovial fluid. The data demonstrated significantly increased levels of IgA but not IgG or IgM antibodies to Cytokeratin-18 and human epidermal keratin in RA compared to patients with Osteoarthritis and normal controls [Borg et al,1992; Borg et al, In Press].

These findings thus contrast with those of Senecal and co-workers who found increased IgG and IgM but not IgA antibodies to CK-8, given that Cytokeratin-18 is commonly found in association with CK-8 [Moll and Franke,1982]. However, it was found in general that levels of IgG antibodies to Cytokeratin-18 were elevated in RA, but this did not reach statistical significance when compared to normal controls.

It was additionally demonstrated that the cytokeratin autoantibody response is independent of the polyclonal immunoglobulin expansion typical of RA, and is not a specific finding to RA since an increased IgA response to Cytokeratin-18 was also detected in Psoriasis and in Psoriatic arthritis [Borg et al,1993; Borg et al, manuscript in preparation]. Within the latter group however, the elevation was limited to patients with peripheral joint disease rather than to patients with psoriatic spondylitis alone.

Although damage to synovial endothelial cells may lead to an increased anti-Cytokeratin-18 response in RA, it probably does not explain the increased autoantibody production in other conditions such as Psoriasis. In this condition, damage to

epithelial tissues in other regions of the body (e.g. skin, gut, kidney) may lead to production of keratin autoantibodies which recognise one or more epitopes common to all cytokeratins, including Cytokeratin-18.

The reason for an elevated IgA anti-cytokeratin response rather than an IgG or IgM response is not clear at present. It cannot be explained by a general increase in serum IgA levels as these showed no correlation with anti-Cytokeratin-18 levels. It is interesting to note that most of the conditions where raised levels of these antibodies were found have been associated to different degrees with abnormalities of the gut mucosa and/or mucosal immune system. As pointed out by Senecal et al [1993], Cytokeratin-18 is a major cytoskeletal polypeptide of gastrointestinal epithelia, so it is possible that damage to such epithelia may result in increased exposure of CK filaments to cells of the immune system. If the normal role of natural anti-Cytokeratin antibodies is indeed to eliminate cell breakdown products, then increased production of such antibodies might be expected in response to pathological cell damage and/or increased cell turnover. In some conditions the tissue damage may be accompanied by a defect in immune function (e.g. polyclonal B cell activation) leading to further enhancement of autoantibody production.

It appears that the nature of autoantibodies to Cytokeratin-18 is probably natural rather than pathogenic. Certainly, they fulfil a number of features which Senecal et al [1993] list as being characteristic of natural (as against pathogenic)

autoantibodies including detection in all normal adults (albeit in lower concentrations), off all isotypes, low in affinity, polyreactive and in low concentration in serum.

Currently there is no data on the source of the IgA antibodies to cytokeratin (i.e. mucosal or central immune system). Indeed it may depend on the disease condition. It is hoped that further studies will reveal the source of IgA anti-CK antibodies and whether there is a common mechanism in their production in different conditions.

IDEAS FOR FUTURE WORK

5.1

Lymphocyte Proliferation Assays

This experiment would test the hypothesis that cell-mediated autoimmunity in RA develops as a result of cellular immune responses to normal cellular components of the cytoskeleton and/or extracellular matrix and that these may arise from responses to infective agents such as the Epstein-Barr virus or Cytomegalovirus, both of which possess polypeptides sharing amino-acid sequence homologies with normal cellular proteins.

Lymphocytes obtained from blood and synovial fluid from patients with high levels of autoantibodies to cytokeratins, would be incubated with purified cytokeratins or synthetic peptides and the proliferative response measured. Increased lymphocyte proliferation would indicate that the humoral response to cytokeratins is accompanied by a cell-mediated autoimmune response to these proteins.

5.2

Identification of radiological subsets in RA

Increased levels of IgA-RF have been associated with a subset of RA characterised by a predilection for radiological wrist damage and a worse outcome.

Other radiological patterns have not been explored, and it may be useful to try and identify subsets of RA based on abnormal levels of cytokeratin antibodies. This would involve prospective studies of patients with RA of not less than three years duration but not more than ten years. It is very difficult to identify distinct radiological patterns in early or advanced RA.

5.3

Indirect Immunofluorescence on Rat Epithelia

5.3.1 Introduction

Previous workers have identified antikeratin antibodies by using indirect immunofluorescence on the middle third of the rat oesophagus. However, there is no real rationale for this given the absence of cytokeratins from this site [Hoet et al, 1991].

It would be interesting to determine what epitopes in rat oesophagus or in other rat epithelia are actually recognised by antibodies to CK-18 or epidermal keratin.

5.3.2 Methodology

A similar technique to that employed by Jurik et al (1987) will be used. Briefly, unfixed 5 micrometre thick cryostat sections of middle-third rat oesophagus, stomach and large bowel will be used as antigenic substrate.

All sera screened will be prediluted 1:40 in phosphate-buffered saline (PBS), pH 7.2. Sera containing high levels of IgA, IgG and IgM autoantibodies to CK-18 (as determined by ELISA) will be applied to the tissue and incubated at 37^{o}C for 30 minutes.

The slides are then washed three times in PBS. Fluorescin-conjugated rabbit anti-human immunoglobulin (IgA, IgG, IgM;FITC, fluorescin isothiocyanate) is then added at a dilution of 1:80 following which the slides will be incubated in darkness at 20^{o}C for 30 minutes followed by further washing in PBS. FITC-conjugated rabbit anti-human chain-specific antisera will be used to determine the Ig class of antibodies in positive sera and to determine antibody titres.

Positive sera will be defined as those giving a fluorescent staining of the superficial layer of the oesophagus epithelium. Only distinct laminar or speckled fluorescence of the keratin layer will be considered positive.

Mouse Monoclonal antibody to CK-18 at a dilution of 1:20 will be used as a control. The same procedure will then be repeated using sera with known high levels of IgG, A and M autoantibodies to epidermal keratin.

5.4

Epstein-Barr Virus and Cytokeratins

It has been shown that antibodies to a particular sequence of the Epstein-Barr virus nuclear antigen cross-react with epidermal keratin, as well as with collagen and actin [Baboonian et al,1991].

The Epstein-Barr virus (EBV) has been implicated in the pathogenesis of RA because it is persistent, ubiquitous and has profound effects on the immune system [Tosato et al,1985]. Also, patients with RA have increased amounts of EBV infected B lymphocytes in their peripheral blood [Tosato et al,1984]. Persistence of the EB virus relates to its ability to remain latent in two cell types - the B lymphocyte and the salivary gland epithelium [Wolf et al,1984] - both of which could be envisaged as possible sources for some of the immunological abnormalities in RA.

In naturally occuring EBV infection antibodies to three major classes of antigen are seen - the viral capsid antigens (VCAs), the early antigens (EAs), and Epstein-Barr nuclear antigens (EBNAs) [Henley et al,1979]. All three antigens are encoded by EBV DNA but only VCA is present on the virus itself. The function of the EAs and EBNAs is unknown, though it is thought they are involved in cell transformation and replication of the virus.

The above studies suggest that the EB virus, although not necessarily of aetiological importance, may be an important probe for the analysis of the finer specificities of autoimmunity in RA.

Further investigation of this hypothesis with regard to cytokeratins would involve the development of an ELISA assay to measure antibodies (IgG, IgM and IgA) to EBNA-1 in RA and normal control sera with known cytokeratin antibody values which would allow correlations to be determined. In those sera measured, VCA status will then be determined (commercial kits available) to determine whether ANTI-EBNA-1 antibodies correlate with VCA positivity.

5.5

ELISA Study of Antibody Responses to CK-18 and epidermal keratin in Inflammatory bowel disease and in IgA Nephropathy

5.5.1 Introduction

Following on from the finding of an increased IgA response to CK-18 and epidermal keratin in RA, psoriasis and psoriatic arthritis it would be interesting to investigate whether a cytokeratin antibody response could also be present in disorders where :

1. CK-18 is present in abundance (gut epithelium, kidney mesangium).

2. IgA is related intimately to the pathogenic lesions (Inflammatory bowel disease, IgA Nephropathy).

The sera of the patients with active inflammatory bowel disease are stored within the department and consist of a cohort of 40 patients with Coeliac disease, Crohn's disease and Ulcerative colitis. No evidence of synovitis or arthritis is present in any of the patients.

Preliminary results using an ELISA as for the measurement of autoantibodies to cytokeratins in RA (Section 3.1) has demonstrated an increased IgA response to CK-18 but not an IgG or IgM response in both active inflammatory bowel disease and in IgA nephropathy compared to patients with other forms of bowel disorder/renal disease or normal controls.

APPENDIX I

ELISA Assays for measurement of Epidermal keratin and CK-18 in serum and synovial fluid.

Materials

0.1 M Bicarbonate coating buffer - pH 9.6

4.24g Na_2CO_3 (0.04 M) (BDH)

5.04g $NaHCO_3$ (0.06 M) (BDH)

Make up to 1 litre with distilled water and check the pH (approx 9.6). Store at 4^oC.

0.1 M Diethanolamine buffer - pH 9.8 (for alkaline phosphatase)

101mg $MgCL_2.5H_2O$ (50mM) (BDH)

97 ml diethanolamine (BDH)

Concentrated HCL (BDH)

Dissolve $MgCL_2$ in 800ml of distilled water. When dissolved add diethanolamine and mix thoroughly, then adjust the pH to 9.8 with concentrated HCL. Finally make up to 1 litre with distilled water and store in the dark at 4^oC.

APPENDIX II

Materials for SDS PAGE Slab Gels

II.1

Separating Gel Preparation

	15%	12%	7.5%
Distilled Water	2.35ml	3.35ml	4.85
1.5MTris-HCl pH 8.8	2.5ml	2.5	2.5
10% SDS	100ml	100ml	100ml
Acrylamide/BIS (30% stock)	5.0ml	4.0ml	2.5ml
10% APS (fresh daily)	50ml	50ml	50ml
TEMED	5ml	5ml	5ml
TOTAL MONOMER	10mls	10mls	10ml

Stacking Gel Preparation

	4% gel
Distilled Water	6.1ml
0.5M Tris-HCL pH6.8	2.5ml
10% (W/V) SDS	100ml
Acrylamide/BIS	1.3
10% APS (fresh daily)	50ml
TEMED	10ml
TOTAL	10ml

SAMPLE BUFFER

Dithiothreitol (D.T.T.)	0.77g
SDS (2%) (10ml of 10%)	1.0g
1M Tris-HCL pH6.8	4ml
Glycerol	5ml
Bromophenol Blue (1.01%)	0.3ml

Make up to 50ml with water and store at -20^{o}C in small aliquots.

II.2

Solutions for Immunoblot Assay

1. ANTIBODY BUFFERS - (1% gelatin in TTBS) - add 1g gelatin to 100 mls Tris Buffered Saline with Tween (TTBS). Heat with stirring to 50^{o}C until dissolved. For protein G Horse Radish Peroxidase (HRP) also prepare 100 mls of 1% gelatin in Tris Buffered Saline with Citrate (TCBS).

2. FIRST ANTIBODY SOLUTION - Dilute first antibody to the appropriate litre in 25ml of antibody buffer (1% gelatin in TTBS).

3. SECOND ANTIBODY SOLUTION - (1:3,000 dilution) - add 16.5 ul of protein G HRP to 50 ml antibody buffer (1% gelatin in TCBS).

RENATURATION BUFFER

10 mM Tris	0.24g	
50 mM NaCL	0.58g	pH 7.0
4 M Urea	48.05g	
5 mM DiTrioThreitol	0.15g	

Make up to 200 ml in distilled water and soak for 1 hour followed by a 20 minute soak in Transfer buffer.

BLOCKING SOLUTION

0.9% NaCL pH 8.0

10 mM Tris/HCL pH 8.0

3% BSA

0.1% Azide

Make up to 100 ml in distilled water.

Appendix III

Methodology

III.1

1. Casting Polyacrylamide Gel.

The separating gel monomer solution was prepared by combining all reagents except ammonium persulphate (APS) and TEMED (see materials). The stacking gel monomer solution was prepared by combining all reagents except APS and TEMED. Both solutions were deaerated under vacuum for at least 15 minutes. APS and TEMED were then added to the gel solutions just before pouring into a Bio Rad Mini-Protein II electrophoresis cell.The gel was allowed to polymerize for 45 minutes before addition of the stacking gel which was polymerised for a further 15 minutes.

2. Loading Sample Wells.

i) 500mls of electrode buffer were prepared (see materials).

ii) The inner cooling core was lowered into the lower buffer chamber of the Mini-Protean II cell. Approximately 115ml of buffer were added to the upper buffer chamber.

iii) The remainder of the buffer was poured into the lower chamber so that at least 1cm of the bottom of the gel was covered.

iv) Samples were loaded into the wells under the electrode buffer with a Hamilton syringe and the gel was then run.

III.2

MINI TRANS-BLOT ASSEMBLY

III.2.1

Preparation for Blotting

1. Following electrophoresis the gels were placed in renaturation buffer (see Appendix II) for an hour.

2. The gels were then soaked in transfer buffer to allow equilibration for approximately 20 minutes prior to blotting .

3. The nitrocellulose was cut to the dimensions of the gel. Labelling with a soft pencil was used to identify the gel and the orientation of the membrane. The membrane was wet and allowed to soak for 15-30 minutes.

4. The pre-cut filter paper and fibre pads were then soaked completely in deaerated transfer buffer for 30 minutes.

5. The Mini Trans-Blot electrode was then installed in the buffer chamber.

6. The frozen cooling unit was installed in the buffer chamber, next to the electrode, a few minutes before starting the transfer.

The surface of the gel was flooded with transfer buffer and the pre-wetted blotting media was lowered on top of the gel. The surface of the membrane was flooded with buffer. The sandwich was completed by placing a piece of saturated filter paper on top of the membrane and placing a saturated fibre pad on top of the filter paper.

III.2.2

IMMUN-BLOT ASSAY PROCEDURE

1. Antigen Transfer - The antigen was transfered onto the nitrocellulose membrane by electrophoretic transfer.

2. Blocking - The membrane was immersed into the blocking solution (see Appendix II) and incubated at 37^{o}C overnight.

3. Wash - The blocking solution was decanted and TTBS was added. The membrane was washed twice at room temperature for 5 - 10 minutes with gentle agitation.

4. First Antibody Incubation - the TTBS was decanted and the first antibody solution was then added followed by incubation for 2 hours.

5. Washes - The unbound first antibody was removed (poured into a universal and saved at -20^{o}C) and the remainder washed off with TCBS.

6. Conjugate Incubation - The membrane was incubated in the second antibody, Protein G-HRP conjugate solution for 30 minutes to 2 hours at room temperature.

7. Final Washes - The membrane was washed (in PBS-Tween 0.2%) for 10 minutes at room temperature.

APPENDIX IV

IV.1

Materials for P62 ELISA Assays

Borate Buffered Saline

Boric Acid	6.18 g/L	
Sodium Tetraborate	9.54 g/L	pH 8.3
Sodium Chloride	29.22 g/L)	

APPENDIX V

Estimation of IgA Rheumatoid Factor

Methodology

1. 100 microlitres of standards and diluted sera (1 in 100) were added to a 96 well plate (Greiner, Denley) and incubated for 30 minutes.

2. After washing the plates in wash buffer, 100 microlitres of enzyme conjugate was added to each well and incubated for a further 30 minutes.

3. After a further wash, 100 microlitres of enzyme substrate was added to each well and incubated for 10 minutes.

4. The reaction was then stopped by the addition of 50 microlitres of sulphuric acid to each well.

5. The optical density was then read at 492nm on a Titertek Multiscan Plus MKII Microplate reader. The optical density of each standard was then plotted against the concentration of IgA rheumatoid factor on the microplate reader to give a standardised value (as per ELISA assay for determination of cytokeratin antibodies - vide supra).

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