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AN EARLY SYNOVITIS CLINIC:

DIFFERENTIATING PERSISTENT FROM SELF-LIMITING SYNOVITIS

By

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LIST OF ABBREVIATIONS

ARA	American Rheumatism Association
B19	Human Parvovirus B19
C(n)	Complement (component number)
CAEV	Caprine Arthritis Encephalitis Virus
CHIK	Chikungunya virus
CPE	Cytopathic Effect
DIP	Distal Inter-Phalangeal (joints)
ELISA	Enzyme Linked Immunosorbent Assay
HAI	Haemagglutination Inhibition
HLA	Human Leukocyte Antigen
IC	Immune Complexes
Ig	Immunoglobulin
IgG,M,A	Immunoglobulin G or M or A
IgMRF	IgM Rheumatoid Factor
Mc	Monoclonal
MCP	Metacarpo-Phalangeal (joints)
MTP	Metatarso-Phalangeal (joints)
NSAID	Non-Steroidal Anti-Inflammatory Drug(s)
PBL	Peripheral Blood Lymphocytes
PBM	Peripheral Blood Mononuclear cells
PIP	Proximal Inter-Phalangeal (joints)
PS	Persistent Synovitis
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor(s)
RIA	Radio-immuno Assay
RNA	Ribonucleic Acid
SF	Synovial Fluid
SLS	Self Limiting Synovitis

TFR	Transferrin Receptor
TMJ	Temporo-Mandibular Joint(s)

DEDICATION

I dedicate this thesis to

Lizzie

her love, patience and strength

made this work possible

and to our children Joseph and Ruth

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By design the work for this thesis was collaborative, the intention being to study a particular group of patients in detail. The patients were being studied as early in their disease as possible and therefore presented a precious opportunity to examine a number of questions covering the fields of clinical medicine and of immunology. The skills and labour of several colleagues were necessary to prepare the information assembled for this thesis and I am pleased to be able to acknowledge and define the considerable extent of their help.

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SYNOPSIS

An Early Synovitis Clinic - Differentiating Persistent from Self-Limiting Synovitis

Background

My interest in differentiating between persistent and self-limiting synovitis (PS and SLS) developed from the Bath experience, where Professor Bacon and Dr Blake ran an early synovitis clinic. A proportion of the patients were noted (in an epidemic year) to have had Human Parvovirus B19 (B19) infection coinciding with the start of their synovitis (White DG et al. Lancet 1985; 1: 419-22). A minority of the B19 patients had persisting symptoms a year later adding to speculation on the possible role of viruses in the aetiology of chronic rheumatic diseases.

The other cornerstone of this work is the difficulty in diagnosing rheumatoid arthritis (RA) in very early disease. This is revealed in the requirement of the standard diagnostic criteria for symptoms to be persistent and was hinted at in the Bath study where the pattern of joint involvement in B19 patients was very similar to RA. Thus viral arthritis might offer itself as a (usually) self-limiting model of RA, permitting a search for variables predictive of persistence which might also be relevant in understanding the pathogenetic mechanisms of persistence.

Aims

An early synovitis clinic was established with a view to:

- identifying clinical or laboratory variables which might predictively distinguish at an early stage those with RA from those

with self limiting disease.

- considering the pathogenetic relevance of any such variable, with particular reference to the role of virus in early synovitis.

Methods

Target patients were those with less than six months of synovitis, recruited by means of circular letter to General Practitioners. Clinical and laboratory variables were recorded on a standard proforma designed for computer analysis. Selected variables were monitored at subsequent visits, patients being followed until either symptoms disappeared or until a definite diagnosis declared itself. The proforma includes an itemised clinical history, examination and routinely available laboratory tests. In addition, serum was banked at each visit and, where appropriate, lymphocytes were stored on freeze dried slides for surface marker studies. Analysis was by applying non-parametric tests to single variables, comparing PS with SLS. Analysis was extended to Stepwise Variable Selection, a form of multiple logistic regression, applied to the clinical variables in the search for the most predictive combination of the least number of variables.

Results:

These refer to data obtained at first visit only, analysed on the basis of subsequent outcome.

Clinical

Analysis of 114 patients reveals three approximately equal sized groups: Persistent Synovitis (PS), and self-limiting synovitis (SLS), both with patients having symmetrical, peripheral, polyarthritis similar to RA, and "Others" (having nothing by history or examination

to suggest RA, and including patients with psoriatic arthritis, ankylosing spondylitis, osteoarthritis and the like). Clinically the PS and SLS were found to be similar (by onset, pattern of joint involvement, duration of symptoms, even ARA criteria) at first visit to the clinic but by around three months symptom duration the SLS patients had all undergone spontaneous remission. Although differences (severity, height of acute phase response, rheumatoid factor et al) can be observed retrospectively between these two groups, they would be difficult to apply to a new cohort of patients because of overlap between the groups. The main conclusion is that although traditional variables eventually separate the groups, in the early stages the similarities are greater than the differences.

T-Lymphocytes

I collaborated with colleagues in this department to study T-lymphocyte subsets in SLS and PS. We measured CD4:CD8 ratios both in the whole blood and within the activated (transferrin receptor bearing, TFR+) cell population. Within the whole peripheral blood, the ratio did not differ between the two groups but the number of TFR+ cells was higher in PS than in normals or in SLS. Within the activated, TFR+ population the ratio in PS was higher than in SLS. This increase in the ratio was due to higher numbers of CD4+, TFR+ cells in PS with no overlap with SLS. There was no difference in the numbers of CD8+, TFR+ cells between the two groups.

Rheumatoid factor: the antibody and its antigen.

Rheumatoid factors (RF) are seen in RA but also during the course of many infections. I have collaborated with colleagues in the Department of Immunology to define quantitative and qualitative variation in RF between SLS and PS. An overall quantitative

difference was found with PS patients as a group having higher RF levels, irrespective of the IgG subclass used in the assay. In these patients with very early disease, however, quantitation of RF is of limited predictive value because of the overlap between the groups In PS we also found greater diversity of idiotypic expression, inviting speculation on the role and control of gene rearrangement and somatic mutation. These qualitative observations may enhance our understanding of the pathogenesis of persistence of synovitis.

We also looked at fluorescent IgG as a possible marker of damaged IgG but the results showed no difference between SLS, PS and normal subjects.

Virology

Simple viral serology was performed, to be evaluated in relation to disease duration at first visit. Interestingly, in a non-epidemic year, Human Parvovirus B19 (B19) accounts for very few cases of acute arthritis. Six patients were identified as having had B19 near the start of their arthritis, of whom five were allocated to the SLS group and one to PS. In single patients it has been possible to send tissue to colleagues for molecular probing, seeking the persistence of virus but none was found.

Discussion

The similarity in symptoms between the two groups indicates elements of inflammation in common, suggesting that the SLS group of patients might offer themselves as a model for the study of PS and hence for the study of RA. There are also differences between the groups, particularly in the numbers of activated T-helper lymphoctyes and in

the apparent somatic mutation underlying the IgMRF results: the possible underlying mechanisms are discussed. I have shown that in turn B19 arthritis is representative of the SLS group of patients and thus B19 offers itself as a human model for the study of RA. I have discussed the implications for further work based on the use of B19, with its defined protein structure, to study the responses of the immune system to specific antigens, in individuals of defined Human Leukocyte Antigen phenotype.

CHAPTER 1

INTRODUCTION AND HISTORICAL REVIEW

CHAPTER 1

INTRODUCTION AND HISTORICAL REVIEW

1.1 Aims

The aim of this thesis is to study patients afflicted with symmetrical peripheral polyarthritis within the first 6 months of their disease. The hope is to find ways of predicting those who will recover spontaneously (Self Limiting Synovitis, SLS) and those whose disease will become persistent (Persistent Synovitis, PS).

1.2 Introduction: SLS versus PS

Rheumatoid Arthritis (RA) is a disease of unknown aetiology and is probably heterogeneous in its manifestation. At its simplest it is a disease defined by its persistence and recognized by its crippling sequelae. There is much interest in seeing RA early because it may be during the onset of the disease that definable pathogenetic mechanisms will be separable from phenomena which, although well recognized in established disease, may be a consequence of the disease rather than its cause. This however leads to a potentially circular difficulty because the interest is in the initiation of a disease whose definition requires persistence. One way out of this is to study during initiation all of those diseases which may potentially evolve into RA. Depending on how widely one initially casts the net this approach is likely to reveal three groups of patients. The first is eventually recognisable as RA: to avoid pedantic debate about the timing of applying the label of RA, I shall in this study refer to patients in this evolving group as having "Persistent Synovitis" (PS). The second group includes diseases which also persist but evolve into recognisable diagnoses distinct from RA

"Others". Previous studies have shown that a third group of patients evolves from those presenting with symptoms initially suggestive of RA but which resolve spontaneously within a sufficiently short time to traditionally preclude them from being defined as having RA. I will refer to these patients as having "Self-limiting Synovitis" (SLS). While many of the latter group never have a definitive diagnosis made, recent studies have revealed a significant proportion of them have suffered a recent viral infection. Consequently, much interest has been shown in trying to confirm the association of specific viruses with definable patterns of arthritis. As discussed below, most arthritis convincingly associated with viral infection is both of the self-limiting type and initially mimics the earliest stages of RA. Thus virus induced SLS might offer a human disease model of some aspects of RA but with the crucial absence of persistence. A comparative study of SLS and PS in their earliest stages offers the potential for finding variables which will discriminate between the two groups. Such discriminant variables would offer the opportunity to predict persistence and might even offer insights into the pathogenesis of persistence.

Since it is crucial to the concept of comparing two groups of patients, I will now briefly review the way in which the definition of RA has evolved in an effort to define a reasonably homogeneous entity. I will also review the justification for associating viruses with arthritis, particularly SLS.

1.3 Historical review

1.3.1 Rheumatoid arthritis

Over the last 30 years we have gradually come to accept that it is easy to make a diagnosis of RA in a patient suffering a symmetrical peripheral polyarthritis in association with subcutaneous nodules and erosive joint damage. This confidence has been due mainly to the international acceptance of two sets of diagnostic criteria for the diagnosis of RA, that from the American Rheumatism Association (ARA)[1] and that named after a symposium held in New York[2]. However those publications are but recent highlights at point time in the still evolving understanding and definition of RA and its close mimics.

There is debate about how far back in time can be traced the origins of the disease which would meet our current definition of RA[3]. Bearing in mind that failure to find written or skeletal evidence does not prove that it did not previously exist, the earliest descriptions of RA are believed to have begun in the 17th century[3a]. It was more than a century later that the disease resurfaced in the literature in a detailed case history[3c]. Perhaps the gap in time reflects the relatively poor communications and indeed the priorities of physicians before the advent of sanitation, antiseptics and antibiotics. It was later in that 19th century that the term "Rheumatoid Arthritis" was introduced[4] but even then this may have arisen mainly because of its importance in the differential diagnosis of Rheumatic Fever: in that context RA could be regarded as having a relatively benign prognosis.

There is, of course, an important distinction to be drawn between the strength of descriptions of individual cases and the reliability of

prognostication based on observations of wider populations of patients with arthritis. In order to understand a single disease, it is necessary to try to describe a population of patients with a homogeneous pattern of clinical features. The passage of time tends to increasingly refine a diagnostic label and earlier populations are seen to include more than one disease. Thus gout diluted early descriptions of RA[3a]. Ankylosing Spondylitis was, as recently as 1957, regarded as a variant but nevertheless real part of RA[5]. The acceptance a year later of the ARA criteria for the diagnosis of RA[1] set the currently recognized picture of RA, with the symmetrical and peripheral characteristics being emphasised. Although pre-dating our understanding of the human leukocyte antigen (HLA) system the seronegative, asymmetrical, axial and oligo-arthritides (eg ankylosing spondylitis, psoriatic arthritis) were able to be excluded on clinical grounds. Those exclusions were later justified when their association with the HLA B27 phenotype was recognized[6] and then the association of RA with HLA DR4 was noted[7]. In an attempt to improve the still emerging picture of RA, the New York criteria were introduced[2].

The relative strengths of the ARA and New York criteria are sensitivity and specificity respectively. One effect of this polarisation is to lead to the prevalence of RA differing according to which criteria are used to define the afflicted within the study population. In a proper population study (as opposed to a report on hospital attenders) both the ARA and New York criteria were used to define the prevalence of RA in the community of Sudbury, Massachusetts[8]. Both sets of criteria were applied at the start of the study and again at follow-up examination 3 to 5 years later. Of the 4552 subjects (77% of the possible subjects aged between 15 and

75 years) RA was initially diagnosed in 3.8% of the women and 1.3% of the men by ARA but in only 0.5% and 0.1% respectively by the New York criteria. The follow-up concentrated on those in whom RA had been diagnosed by either system. For those initially with RA by ARA, some 70% no longer satisfied the criteria. In contrast, of those initially diagnosed RA by New York criteria, nearly 70% still had the disease by the same criteria at the later assessment. Those authors concluded that the New York criteria identified a more meaningful disease. Looked at from the opposite viewpoint, that study places in sharp focus those patients with initial symptoms who then recovered: arguably similar to the SLS group in this thesis. A number of interesting questions are raised.

The SLS patients recover: is this not desirable, and why can those with PS not do so? Is there an absolute difference between those with SLS and those with PS, or is it a gradient? What is SLS? Where exactly does RA fit: as well as PS, can RA behave in the SLS manner? When is SLS not RA: only when there is an identified viral infection? Would that exclusion be valid if eventually RA was shown to be caused by a virus?

Most early synovitis clinic reports concentrate on the persistent diseases such as RA. I would now like to review what is known about the aetiology of SLS, in particular the viral arthritides, to see whether any information can be gleaned to help the understanding of RA where the aetiology is unknown. That done I will then review the information about RA which has been gleaned from early synovitis studies.

1.3.2 Viruses and arthritis

In this part of the historical review it is intended to: 1) establish that viral arthritis is a reasonable model for RA with the crucial distinction that viral arthritis is not (usually) persistent.
2) review the evidence for the implication of individual viruses in causing arthritis, with particular attention to the strength of the techniques used.

What clinical patterns of arthritis can viruses produce?

Leaving aside those demonstrably due to intra-synovial bacteria and possibly to crystals, the inflammatory arthritides irrespective of aetiology can initially be allocated to one of two groups: either the symmetrical peripheral polyarthritides, such as sero-positive RA; or the asymmetrical oligo-arthritides, classically sero-negative for rheumatoid factor and associated with an over-representation in sufferers of the HLA B27 positive phenotype[6]. Although the virus-associated arthritides are usually sero-negative, the clinical picture (detailed below) is usually different to the classical sero-negative spondylo-arthropathies. High prevalence of HLA B27 subjects in the post viral arthritides has not been found: one small study hinted at a possible association when 4 out of 5 patients with chronic arthritis after Chikungunya infection were found to be B27 positive[9]. However, this was not confirmed in a larger study of similarly afflicted subjects[10]. In addition the prevalence of arthritis occurring after a viral epidemic or vaccination program is much greater than the prevalence of the B27 phenotype in the population[11] and the B27 phenotype was not over-represented in patients with viral arthritis[12]. Those limited reports of viral identification in the presence of, for example, ankylosing

spondylitis[13], will be evaluated in more detail below. Those arthritides which are known to follow virus infections, typically in epidemics, tend to be symmetrical, peripheral, self-limiting and without enthesitis or spondylitis. An example would be those associated with the Togavirus infections discussed in detail below. Genetic factors in the viral arthritides should not be dismissed since there is emerging evidence that within those who do develop a post-viral arthritis, eg human parvovirus, severity and persistence of the arthritis may be associated with specific HLA DR phenotypes[12] and it may be that studies at or near the DR-locus will prove helpful. Thus the pattern of arthritis associated with viral infection is more akin to that seen in RA than in the B27 asymmetrical oligoarthritides.

Do viruses cause arthritis?

Great caution is required in evaluating the evidence of viral involvement in any arthritis. Simply identifying viral proteins in a joint by immunofluorescence does not prove the presence of intact, viable, functioning and infectious virion. Even identifying virion by electron microscopy and showing its infectivity by co-cultivation experiments, thus demonstrating presence in a joint, does not prove a pathological role for that virus in that joint. The virus may simply be an irrelevant passenger if it is using as host a cell (eg a macrophage) which has been called to the site by other signals. Thus the hypothesis that an arthritis may be the result of virus-host interaction, will require careful evaluation of temporal association, specificity of host response and biology of the implicated virus. Rubella is one of the most studied of the viruses implicated in

arthritis and the evidence for its implication will be reviewed in detail and then the evidence for other viruses will be outlined.

1.3.3 Togaviruses - Rubella, Chikungunya, etc.

The Togaviruses are enveloped, single stranded RNA viruses. The family includes the genera: Alphavirus, Flavivirus, Rubrivirus and Pestivirus. The Alphaviruses (group A arboviruses) include chikungunya (CHIK), O'nyong-nyong and Ross River. The Rubrivirus genus includes Rubella. These named viruses are all implicated in human arthritis syndromes and are now discussed further to illustrate the current state of knowledge of mechanisms of virus induced arthritis. They are of course not the only viral agents described in relation to arthritis.

Rubella - clinical evidence:

Acute arthritic syndromes following wild Rubella infection are well described and are probably common - but because of the frequently mild nature of the musculo-skeletal manifestations and the occurrence of sub-clinical Rubella infection, it is difficult to arrive at a reliable figure for the frequency of this post-viral complication. In one study of those who had clinically apparent Rubella, the incidence of objective arthritis was reported as 30% with a further 30% having arthralgia[14]. Figures for post Rubella-vaccination (women only) are probably more accurate; the same study observed arthritis in 14% and arthralgia in 41% of vaccine recipients. Care should be taken with such figures since post-Rubella joint symptoms are rare in children and increase in frequency in adulthood. However, the mean age in the wild and vaccine groups was similar (19 and 23 years). The study also highlighted a feature of many studies of viral arthritis, that women are more severely affected than men. The wild group contained equal

numbers of men and women but objective arthritis was seen more often in women (52% versus 9% in men). Symptoms were observed to start 9 to 27 days post vaccination and it is suggested that in wild infection the onset of arthritis occurs within one week of the appearance of the rash. The duration of joint symptoms was variable. Although most settled within 2 months, continued symptoms were seen at 18 months in 5% of the (all female) vaccine group and in 30% of the women and 9% of the men in the wild group. Thus for the great majority of those who develop post Rubella arthralgia or arthritis this is short lived, ie SLS.

However, if this thesis is to regard viral arthritis as short lived then it is important to evaluate the very small amount of evidence, derived from a small number of cases, which is used to implicate viruses in more chronic arthritis. Chronic polyarthritis following Rubella or Rubella vaccination has been described, lasting up to 6 years[15]. The severity of symptoms is not easy to assess from these papers. The usual pattern seems to be one of relapse and remission but in two other reported cases[16,17] the clinical picture was sufficiently severe and persistent to meet the ARA definition of RA. Although both of these cases were well documented as having severe persistent disabling symmetrical peripheral polyarthritis and became sero-positive for Rheumatoid Factor, neither had subcutaneous nodules or erosions on X-ray, perhaps suggesting the diagnosis belonged to a sub-group of "RA".

In addition to epidemiologic observations, measurement of host response is widely used to seek evidence of viral causation.

Rubella - antibodies:

The B-lymphocytes may be producing specific anti-viral antibody. The anti-Rubella antibody profiles immediately following an acute Rubella infection are conventional irrespective of whether the subject develops arthritis[18]: as measured by the conventional Haemagglutination Inhibition (HAI) titres the IgM antibody shows a transient rise, falling back to pre-exposure levels by 6 months. The IgG rise is long-lived. The ability of virus to persist despite circulating antibody has been shown in the congenital Rubella syndrome[19]. In the two clinical cases of severe post-Rubella polyarthritis described above[16,17], although persistent virus was not seen (not sought in one, limited search in the other), there were persistently high HAI titres, including high levels of the IgM class. This finding of persistently high titres of anti-Rubella antibody raised two initial questions: (1) might this suggest persistence of the Rubella antigen in post-viral arthritis? (2) Could such high titres be found in other patients with persistent arthritis without any apparent temporal relationship to a recognized Rubella infection? The attempts to answer these have served to increase the confusion.

One serological survey showed no increased Rubella HAI antibody levels in RA compared with other arthritis including osteoarthritis[20]. The significance of the antibody titres was further challenged by another study[15] of 7 patients with post-Rubella (1 wild, 6 vaccination) persistent arthritis. Each had increased Rubella-specific cell-mediated immunity and indeed Rubella virus was isolated from each patient (see below) but despite this the Rubella HAI titres were not elevated in any of the patients. These negative HAI results are in marked contrast to those reported[13] in 6 patients with persistent inflammatory oligo- or polyarthritis (and from each

of whom Rubella virus was isolated, see below), where the HAI titres were elevated (to Rubella but not to other micro-organisms tested) but there was no apparent history of recent Rubella. It may be relevant that in these patients the Rubella specific IgM was usually absent, even at times when virus could be isolated. Perhaps the answer to the relevance of antibody titres to arthritis lies in a prospective immunological study of post-Rubella arthritis[18], where although HAI titres were elevated to levels comparable with those of the previous study, there was no difference between those who did and those who did not develop arthritis. Whilst it may be invidious to compare results from clinically different arthritides, it is clear that for the study of mechanisms (including possible persistence) of arthritis induced by a given virus, the simple study of antibody levels will provide more questions than answers. Thus, in a patient at point time, a high titre of a specific anti-viral IgM antibody may indicate recent infection OR some form of persistence. A low titre may indicate no recent infection with that virus OR that the IgM rise and fall has already been completed.

Of potentially greater importance than observations on post-infection/vaccination serology are the findings in the pre-vaccination serology. In the study mentioned above[18] patients had been selected for vaccination on the traditional basis of HAI negativity. A separate Enzyme Linked Immunosorbent Assay (ELISA) technique for anti-Rubella antibody was also employed pre-vaccination (together with lymphocyte reactivity). The ELISA results suggested that 73% of these HAI negative women had some pre-vaccination immunity to Rubella (or to a similar antigen?). Moreover, such status appeared to confer an increased risk of post vaccination arthritis (although the numbers developing arthritis were small). It is

interesting to note that one of the patients who developed a transient arthralgia post vaccination, did not sero-convert as judged by HAI but did produce high levels of the ELISA detected antibody. It was therefore suggested that the arthritogenic infection in these patients was a second meeting of the virus. Since in the ELISA the antigen was fixed to plastic, concern may be expressed that the quaternary structure of the antigen may be altered in the process: this in turn might question the in vivo relevance or specificity of an antibody detected in this manner. However there is corroborating evidence for the role of second exposure in generating arthritis from the goat model of symmetrical proliferative erosive arthritis produced by a retrovirus, the caprine arthritis encephalitis virus (CAEV). Essentially the production of arthritis in goats given live virus is enhanced in those animals previously exposed to the virus, either naturally (live, wild virus) or by vaccination with inactivated virus[21].

Rubella - Immune Complexes:

If simple antibody levels do not discriminate between SLS and PS (or even select for those who do develop arthritis), is it possible that there are some qualitative differences in the antibodies formed as the pre-immunity ELISA data would hint? For example, do some patients more readily produce immune complexes (IC)? When the Vancouver group examined this question in their acute post-Rubella (wild or vaccination) arthritis subjects[22] they were able to detect IC, using both Clq binding and Raji cell assays, in both those who did and those who did not develop arthritis. Although the levels tended to be higher in the arthritis group (as opposed to arthralgia or no symptoms), this reached statistical significance only for the Raji cell assay at 6 and 12 weeks post immunization. However, "clearly

raised circulating IC levels" were seen in entirely asymptomatic individuals, so the biological relationship of circulating IC to the arthritis is not clear.

Tissue deposition of IC might be more relevant in pathogenesis. This is not easy to study in SLS after viral infection since, by definition, it is short-lived and usually too benign to merit invasive investigation. Immunofluorescent studies of synovial fluid cells and synovial biopsies were performed as part of an investigation of chronic seronegative inflammatory oligo-arthritis[13]. In addition to identifying Rubella antigen (see below), the sections stained positively for IgG and, in some, also for IgM. C3 could also be detected in some sections and the synovial fluid C3 levels were low compared to those in serum, suggesting Complement consumption by IC. It was not exactly clear where (in or around which cells) the antigen and immunoglobulins were found but the authors concluded from the staining pattern that the virus particles were predominantly extracellular; that the immunoglobulin and Complement findings were consistent with local IC deposition; and suggested that these IC might block receptors on phagocytic cells, preventing the uptake of the virus.

Rubella: T-lymphocyte reactivity:

T-cells are also used as tools in the hunt for the virus in chronic arthritis. Several authors have used the technique of challenging peripheral blood lymphocytes (PBL) or synovial fluid (SF) cells with specific antigen and found that in some patients, their lymphocytes respond to the test antigen by proliferation. Most control their studies by showing negative responses to other antigens. The attractive deduction is that the observed responses are antigen

specific. This leads on to models of foreign antigen persistence with chronic immune stimulation; or of molecular mimicry between viral and auto-antigens. How strong are the data on which such discussions are based? The group in Vancouver have produced a series of related publications. These support the existence but not the relevance of pre-vaccination immunity. In contrast to their data on ELISA detected antibodies, no increased lymphocyte reactivity could be demonstrated _pre_-vaccination in recipients who subsequently developed arthritis compared with recipients who did not[18]. Indeed lymphocyte responses were judged to be positive in around half of the patients in each of their three outcome groups: arthritis, arthralgia or no symptoms. _Post_-vaccination, those with arthritis or arthralgia had higher "stimulation indices" than the asymptomatic group at 6 weeks but not at 6 months. That prospective study makes it difficult to interpret the same group's more anecdotal observations on 7 women with post Rubella arthritis who were found to have high lymphocyte responsiveness to Rubella antigen (compared with 5 symptomless vaccine recipients) up to 6 years after infection[15]. In a further detailed case report[23] these authors followed the lymphocyte stimulation index over time in one patient with a sero-negative inflammatory arthritis (which began at least 12 years after a possible episode of Rubella). A correlation was seen between the presence of Rubella virus (blood and synovial fluid), synovial lymphocyte (but not PBL) reactivity to Rubella antigen and the clinical severity of the synovitis. Despite the fascination this begs the question: does the Rubella virus have an aetiopathologic role in this context or is Rubella simply utilising for its replication metabolically active cells - including eg those involved in synovitis? A high stimulation index to Cytomegalovirus was also found in this patient and questions the specificity of the Rubella data, as

does the further work of that group on other viruses[24,25]. In these studies lymphocyte responsiveness, expressed as a stimulation index, was measured over up to three years in patients with RA. Increased responsiveness was reported to a number of viruses including adenovirus, mumps and cytomegalovirus perhaps with some differences between sero-positive and sero-negative RA. Interestingly, these patients did not have increased responsiveness to Rubella! Perhaps these RA results say more about the control of B-cells in sero-positive disease than about viral involvement.

The difficulty in interpreting much of the Vancouver data has been well reviewed recently[26]. The main problem is that the lymphocyte responses reported were not shown to be specific for a given virus, either within a given patient or across a group.

Rubella - Isolation of the virus:

If anti-viral antibody levels and lymphocyte reactivities serve to heighten interest in virus-associated chronic arthritis but fail to prove the association or define the markers or mechanisms of the problem, then is viral identification more specific? Clear distinction must be drawn between isolation of virus as intact infectious virion (as from co-cultivation with permissive cell lines, possibly visualizing by electron microscopy) - and identification of viral antigen (eg by immunofluorescence) which may or may not represent intact virion. In addition distinction should be made between identification sites: ie peripheral blood versus synovial fluid or cells. Even if whole virus is identified distinction has to be drawn between replicative forms indicating synthesis of the virus in that particular cell as against intact virus which may have been made by that cell but could simply have been sequestered, as might

happen with a phagocytic cell. Finally a clinical distinction: some reports refer to acute arthritis after Rubella (acquired naturally or by vaccination) but others refer to patients with chronic arthritis, some apparently unrelated to Rubella by history.

That the virus can persist in the congenital Rubella syndrome has already been mentioned[19]. It tends to be assumed that Rubella virus does not persist in normal subjects after an acute infection: this is supported by the negative isolation attempts in normal subjects quoted as controls by some authors[13,15].

Beginning with acute post Rubella arthritis, there are very few cases of isolation of the virus in the synovial fluid, reflecting the usually mild or transient clinical course. The first report was in a child with naturally acquired disease[27] and this was followed by the report in an adult post vaccination[28]. In a detailed report on three patients with arthritis after naturally acquired Rubella[29] the virus was detected (by cytopathic effect in co-cultivated target cells, CPE) in the synovial fluids at 1 to 5 days after the first Rubella symptoms. It is interesting to note that, in one of these cases, the synovial fluid cell count was elevated: Rubella <u>antigen</u> was identified by immunofluorescence in 45% of the cells but electron microscopy failed to find any Rubella <u>virion</u>.

Turning to chronic arthritis and firstly to that which can be temporally related to Rubella: 7 patients with arthritis subsequent to infection (natural in 1 case, vaccination with the attenuated but live virus in 6 cases) have previously been mentioned in regard to their lymphocyte studies[15]. Rubella virus was isolated, as defined by CPE, from their peripheral blood lymphocytes in 6 of the 7 cases, up

to 6 years post infection/vaccination. (It will be recalled that these were the patients who had normal serum Rubella antibody levels by HAI but increased lymphocyte responsiveness to Rubella). Those authors were unable to isolate Rubella from the peripheral blood lymphocytes from non-arthritic antibody-positive adults or from 3 cases of congenital Rubella. However they did find that in the above 6 patients in whom Rubella virus could be identified, this was possible even during periods when their arthritis was in remission. This finding does not entirely support their conclusion that the ability to identify virus was related to the pathogenesis of the arthritis.

Rubella virus has also been identified in a very small number of patients with chronic arthritis not apparently related to any history of Rubella[13,23]. The former presented 6 cases of seronegative, inflammatory oligoarthritis and apparently identified Rubella virus on at least one occasion in each patient. The viral antigen was detected in synovial fluid cells (mainly lymphocytes) by immunofluorescence. In one of their cases electron microscopy revealed intracellular Rubella-like particles though the phylogeny of the cells was not given. It is interesting that a control group of patients with other rheumatic diseases did not have evidence of Rubella virus persistence; however it was not indicated whether those included patients with active inflammatory synovitis. On the other hand, any dismissive suggestion that Rubella might be found in the synovial fluid of any inflamed joint would have to be tempered by the finding, in the virus-bearing patients, of coexisting high anti-Rubella titres (with negative serology for control viruses). The second paper[23] detecting Rubella virus in chronic arthritis (temporally discrete from any apparent Rubella) was the single case

report previously mentioned as showing a correlation between the appearance of virus, lymphocyte responsiveness and clinical severity of synovitis. Here the virus was identified by co-cultivation of donor cells with target cells; in the targets CPE was not seen but viral antigen was identified by immunoperoxidase method; in addition the culture medium contained "plaque forming virus". This was true for donor cells from both peripheral blood and from the synovial fluid mononuclear cells: it must be difficult to ensure that no blood contaminates synovial fluid.

Rubella - Discussion of the evidence:

There is little doubt that the above publications contain positive observations. There is good evidence to link to Rubella with some cases of acute, self-limiting synovitis (SLS): by clinical association, by acute response antibody levels and by virus isolation. The problem lies in the interpretation of the data relating to chronic arthritis. Rubella virus has been identified in a small number of patients with chronic arthritis, at least in peripheral blood and possibly in synovium. Whether virus is identified in synovial fluid as a result of contamination with blood during sampling remains contentious. Specificity is not a strong virtue of much of the host response data and it is apparent that there is a range of different arthritides encompassed by some of the phenomena described. This might suggest that viral identification or implication could be a secondary phenomenon of inflammation rather than its cause. It would appear that Rubella has a special place in this context even though some of the lymphocyte data would point also at other viruses. This may reflect the position of Rubella as the commonest Togavirus in European or European emigrant populations and

should be viewed in the light of information on arthritis and other Togaviruses in Africa and Asia (see below).

Chikungunya (CHIK)

The Rubella data is enhanced by some remarkable parallels in other Togaviruses such as CHIK.

CHIK - Clinical association:

This arbovirus is mosquito borne and can cause sporadic or more commonly epidemic disease in man. There are close similarities with the diseases caused by other arboviruses of the Togaviridiae including O'nyong-nyong and Ross River. First described in Tanganyika in 1955[30], the acute illness, of about 10 days duration, is characterised by fever, rash and joint pains. In addition there may be lymphadenopathy, headache, conjunctivitis and gastrointestinal symptoms. In the acute phase the joint pains are accompanied by objective symmetrical polyarthritis including swelling. Because of the absence of asymptomatic controls it is not certain what proportion of those who are infected with CHIK will develop arthritis. Of those who have (any) symptoms and have the disease proven by rising titres of specific antibody, 73% have musculoskeletal manifestations[9]. Although "short-lived" in most, the joint symptoms resolve slowly (months) in some patients and in around 5% arthritis including swelling was still present after 3 years[31]. Like Rubella, the symptoms tended to be more severe and persistent in adults than in children, although one of those with symptoms lasting 3 years was aged 16y. These reports drew attention to the similarities between a few patients with severe persisting symptoms and RA: the assertion that they closely mimicked RA is of interest

but not beyond dispute. There are no reports of association of this virus with chronic arthritis without a history of infection.

CHIK - Antibodies and Virus Isolation:

CHIK virus specific antibody is identified by rising HAI titres, with high values persisting in those with persisting arthritis in one small series[31]. This might suggest that the CHIK virus can persist in the human host but those authors were unable to culture the virus from either the serum or synovial fluid of these particular patients. The virus has been cultured from the serum of patients with arthritis in the acute phase after CHIK infection[32] but not from those with chronic arthritis[31]. As yet there are no reports of it being cultured from either circulating lymphocytes or from synovial fluid.

1.3.4 Parvovirus

One can conclude from the above Togavirus studies that the clinical data implicating these viruses in inflammatory arthritis is stronger than the data arising from the laboratory investigations. It will be interesting to see whether the laboratory investigation of newly implicated viruses, such as the human Parvovirus B19 (B19), will provide clearer answers. This small, naked, single stranded DNA virus is becoming quite well characterised with the cloning[33,34] and sequencing[35] of the genome. Its association with the childhood exanthem Erythema Infectiosum (Fifth Disease) and with aplastic crises is now well known[36].

The possibility that the virus may persist in the host long after the initial infection, is currently being explored. Only one case of persistence of B19 has been reported in a child with severe combined

immunodeficiency[37]. One further case report purported to show (by dot-blot DNA hybridisation) B19 persistence in the synovium of a patient with synovitis[38] but the report lacked such details as the duration of the symptoms and whether there was concurrent viraemia. The virus could persist either free in the infected cell (primarily the erythroid stem cell in the case of B19[39] or incorporated into the host genome. Those Parvoviruses whose genomic structure includes palindromic terminal sequences (characterised by formation of "hairpin ends") are theoretically capable of persistence by incorporation into the host genome[40]. The human Parvovirus B19 genome has such a structure[35] but incorporation has not yet been shown.

The association of B19 with arthritis is at the stage of clinical description. An inflammatory polyarthritis is seen post infection and as with Rubella, the arthritis is probably commoner in adults than children[41] - most studies[42,43] are not controlled for this variable and have a reporting bias towards adults. The arthritis is clearly commoner in women than in men[11,42,43] and is usually transient but symptoms may persist for some months in a small and diminishing percentage of patients. There are reports of this virus being associated temporally with the onset of RA based on the finding of a specific IgM antibody response in serum from patients with RA examined retrospectively[44]. However, evidence (detectable levels of specific IgG) of past infection is seen in at least 60% of adults[36] and during epidemics the virus is widespread. Therefore it would be surprising if we did not find a few adults suffering a B19 infection coincident with the notoriously insidious onset of RA. Furthermore, it is difficult to assess from published data whether a B19 infection confers an increased risk of persistent arthritis. Although in one often quoted study[42] incidence of persistent joint symptoms after a

B19 infection was high: 3/19 ie 16%, these were patients already selected for having joint symptoms and furthermore, this percentage was not controlled by a figure for persistent arthritis in those not having recent B19 infection. It is not appropriate to compare the figures with those for RA since in the case of the three patients mentioned above, although they could initially have been diagnosed as RA according to ARA criteria[1], by 3 years "persistence... was not associated with any long term damage or functional incapacity".

1.3.5 Viruses - In Conclusion

It is clear that the most commonly recognized articular manifestation of the above virus infections is a brief episode of symmetrical peripheral poly- arthralgia or arthritis, ie SLS. There is debate about the role of viruses in longer lasting arthritis, ie PS. The most commonly recognized form of RA (almost by definition) is a persistent arthritis, although it can be argued that an unknown percentage of SLS might have been abortive RA. While the data analysis which follows is based on the hypothesis that the groupings (PS/RA versus Viral/SLS) are broadly correct, it will be important during the interpretation of the analysis to consider that some overlap may be present. Notwithstanding the constraints on data interpretation, virus related arthritis may offer a human model for the study of those chronic and crippling arthritides whose aetiology remains elusive.

It is quite clear that the studies cited above, which looked at viruses in arthritis, used assays which had difficulty discriminating between infected patients with and without joint symptoms. It seems unlikely that those same methods would act within the arthritis group

at an early stage to sort those destined to have PS from those with SLS. What methods might be used to distinguish between PS and SLS and thus select for early interventive treatments those with PS?

1.4 Clinical studies in identifying PS (or RA)

Why is it important to predict persistent synovitis at an early stage of the disease?

1.4.1 The therapeutic problem

The available treatments for RA are of limited efficacy and have significant cost, particularly in terms of side-effects.

First Line Drugs:
Non-steroidal anti-inflammatory drugs (NSAIDs) produce a limited reduction in pain and stiffness on a day-to-day basis but do not result in a reduction in serological markers of inflammation - the acute phase proteins[47]. Although the common serious side-effect of gastro-intestinal upset including bleeding is well recognized, their ability to reduce renal blood flow has recently caused additional concern[46].

Second Line Drugs:
These are slow-acting with the onset of any benefit not arising until the patient has been taking the drug for several weeks or months and they are defined by their ability to produce a reduction in the serum levels of acute phase proteins in some patients[47]. By inference the hope is that this in turn means a reduction in the disease severity

and consequences. Certainly there is evidence showing a correlation between reduction in acute phase proteins and clinical well-being[48]. Unfortunately, by the end of one year approximately one third of all patients started on such drugs will have discontinued because of inefficacy and an additional one third will have discontinued the drug because of side effects of which the two most serious are the marrow suppression and the protein losing nephropathy[49].

In addition the best time to start treatment remains uncertain. Traditionally, in view of their side effects, the "second-line" drug treatments are reserved for patients with severe persistent disease. Although there is debate about the correlation between the two main "outcome measures" in RA ie functional capacity and joint damage seen on X-ray[50], there is enthusiasm for attempting to preserve the former by reducing the latter. The evidence supporting the case for a role for Second Line Drugs in reducing the progression of joint damage is scanty indeed[51]. One proffered explanation is that in most patients the drug is started too late: it has been shown that some 45% of patients have already begun to develop erosive joint damage by the end of their first year of symptoms, rising to 90% by the end of the second year[52]. There is some evidence that those in whom the acute phase proteins have been lower (by nature or treatment?) tend to have less joint damage[53]. If joint damage correlates best with the area under the curve of "acute phase protein level versus time" then perhaps even an imperfect treatment applied early enough might reduce the progression of joint damage. The pursuit of this strategy requires the earliest possible distinction between PS and SLS since otherwise, at worst, those with PS will receive, too late, treatment

which will not help and those with SLS will suffer side-effects unnecessarily.

1.4.2 The diagnostic problem

Unlike the mathematically discrete description of dead or not dead, in the rheumatic diseases the diagnosis is a continuous variable. Arguably the most important criterion for the traditional diagnosis of RA is the persistence of synovitis, which in turn depends on the passage of time, this in turn mitigating against optimal early treatment suggested above. There have been a number of studies of relatively early disease but before discussing those it is worth reviewing the two major sets of criteria used in an effort to bring a degree of homogeneity to the classification of patients described in epidemiological and therapeutic publications. It has to be borne in mind that these criteria were established with a view to describing RA and since they directly or implicitly require the passage of time they might not be expected to cope with the subtly different task of predicting persistence in very early disease.

New York Criteria[2]:

In addition to a history and positive examination, the diagnosis of RA requires either erosions or positive serology (Table 1.1). Since only 45% of patients have begun to show erosions by the end of the first year[52] this criterion will fail to find at least 55% (probably more) of patients with RA of, for example, 6 months duration. This then puts pressure on the rheumatoid factor (RF) which is itself only positive in 70% of established RA[54]. Review of all of the above publications on viral arthritis reveals no case of viral arthritis with typical RA erosions except for a very few instances (single

figures) where a virus was thought to have been present at the start of otherwise typical RA. As discussed below (page 60,[54]), while RF are found in infectious diseases these are not usually in significant titres. In short, in the context of synovitis of less than 6 months duration, these criteria will be specific but not sensitive.

1 History:	Pain in three or more joints. (Proximal interphalangeal, metacarpophalangeal or metatarsophalangeal joints of one side counting as single joints).
2 Examination:	Swelling, limitation of movement or subluxation of at least three limb joints, two of which should be symmetrical. (The exclusions do not help in the current context).
3 X-ray:	evidence of erosion of specified severity, in hands or feet.
4 Serology:	positive for rheumatoid factor.

Table 1.1 New York Criteria for the diagnosis of RA (abridged)

RA present if 1 and 2 plus either 3 or 4 are met.

American Rheumatism Association (ARA) criteria[1]:

In the discussion of a report on parvovirus arthritis[42] the authors commented that 3 patients could have fulfilled the diagnosis of "definite RA" by the ARA criteria (Table 1.2). Perusal of that text and that of a simultaneous report[43] suggests that even more of the patients might have done so. Thus these criteria are sensitive at the expense of specificity. The optimistic exclusion criterion "history suggestive of infection.." is itself insensitive[42,43] and fails to help. To improve the specificity it has been necessary for the original criteria to be revised (and indeed simplified)[55]. These will be further examined in the present study.

1 Morning stiffness, more than 60 mins.
2 Pain on motion or tenderness in one joint.
3 Swelling in at least one joint for at least 6 weeks.
4 Swelling of at least one other joint.
5 Symmetrical joint swelling
6 Subcutaneous nodules
7 X-ray changes (at least peri-articular osteoporosis) typical of RA.
8 Positive rheumatoid factor
9 Poor mucin clot
10 Characteristic synovial histology
11 Characteristic nodule histology

Table 1.2 ARA criteria for diagnosis of RA (Abridged)

Number of criteria met define category: classical RA(7); definite RA (5); probable RA (3).
Relevant exclusions: see text

Previous early arthritis studies:

Prognostication in recent onset arthritis has been addressed in previous studies but the aim of those most often cited was generally to predict severity of outcome in cohorts of patients referred to hospital and selected for having RA, as distinct from taking from the wider community any patient with polyarthritis and trying to predict eventual evolution into persistent synovitis (which approximates to RA). Indeed Short[5] specifically stated "No doubtful case was included in the series until, through follow-up, the passage of time had made clear the true nature of the patient's illness" and "....designed to be representative of RA severe enough to warrant hospitalisation". Duthie[56] studied the value of hospitalisation on the course of RA. Ragan in a retrospective study[57] sought prognostic indices in patients presenting to hospital clinic who already had classical or definite RA by the original ARA criteria at the first clinic visit.

Furthermore, each of the above three studies had a definition of

"early" which differed from this thesis. Although some of their cases were within 3 years of onset, they each included significant numbers of patients with disease durations of up to and beyond 10 years. Even those studies of first visit disease durations of less than one year[58-60] refer to patients with sufficiently severe disease to present to hospital and again address prognosis in those with RA.

Two studies of very early disease, while addressing the question of prognostic indicators, have highlighted the concept of short-lived, RA-like polyarthritis, ie SLS. The first[61] deals mainly with disease sufficiently severe to present to hospital but included only patients with less than 6 months disease duration. Despite the severity, around half of the patients had recovered by the end of the first year. The second[62] specifically addressed the question of the outcome of early synovitis: persistent or transient. Interestingly the conclusions are broadly in agreement with those studying established disease. The best predictor of persistence was the presence of a positive RF; the best predictor of transience was a positive diagnosis of the specific viral infection, B19 parvovirus. However the value of these variables is arithmetic and not absolute: it will be recalled that B19 may be involved in persistent synovitis (page 47) and RF is not always detected by routine methods ab initio[61].

Despite the reservations, the prognostic indicators derived from the above studies will be relevant to this study since they will (if detectable early enough) define those with most severe disease, ie define a subset of PS. They may also point the way to more sensitive tests for use in very early disease. There is broad agreement on some

of their suggested prognostic indicators, dispute on others:(Table 1.3)

GOOD	BAD
Acute onset	Joint erosions
Disease of < 1yr	Continuous disease activity
Age < 40yrs	Extra articular manifestations
Male	Rheumatoid nodules
	Rheumatoid factor
	Poor grip strength
	Many joints affected
	Many ARA criteria positive
	Raised ESR
	Poor functional status

Table 1.3 Prognostic features in RA: summary

From the table it is clear that many of these prognostic criteria are interdependent. It would seem that within those patients diagnosed as having RA, a poor prognosis is likely for the those who already have severe, persistent disease. In the context of patients with polyarthritis of very recent (less than 6 months) onset arthritis, the first 4 criteria on the BAD list are likely to be rare, ie more specific than sensitive. The discriminatory value of these listed variables will be considered in this study.

1.5 T-Cells

1.5.1 Introduction

Thymus derived T-lymphocytes have been implicated in the pathogenesis of RA by many authors using a number of techniques. Furthermore the apparent association of severity and persistence of viral arthritis with specific HLA Class II phenotypes[12], suggests involvement of the T-helper lymphocytes to which Class II molecules present antigen[63].

In patients with RA, increased numbers of activated lymphocytes are found in the peripheral blood as shown by the increased numbers of circulating immunoblasts[64]. The circulating lymphocytes in RA patients show increased incorporation of tritiated thymidine (indicating increased nucleic acid synthesis) compared with normal control subjects[65] and increased levels of low-density lymphocytes (with unfolded chromatin and hence activated) are also found[66]. At the local site of inflammation, the synovium, there is infiltration with lymphocytes which are predominantly activated CD4 helper/inducer cells[67]. Removal of recirculating lymphocytes by either thoracic duct drainage[68] or by lymphopheresis[69] produces significant, if transient clinical improvement in patients with RA. A study of the activated lymphocytes in the peripheral blood of patients with very early synovitis might reveal patterns predictive of persistence of disease.

The methods mentioned above all have disadvantages when attempts are made to use them to look at sub-populations of activated cells. It is difficult to use radiolabelled nucleic acid precursors to study individual cells. Low density lymphocytes in the peripheral blood are a heterogeneous group of cells, not all of which are activated[70]. Clearly, neither of the above methods of lymphocyte removal is selective for subpopulations.

1.5.2 Transferrin receptor bearing cells (TFR+)

All cells by virtue of their requirement for iron, express transferrin receptors on their surface during activation[71-73]. Lymphocytes express this activation marker from soon after the activation signal is received and throughout the cycle of division, with peak density of receptor expression being during S-phase, in close temporal association with DNA synthesis[74]. The transferrin

receptor can be labelled either with a monoclonal antibody to the receptor, OKT9[75] or by loading the receptor with transferrin and labelling that carrier protein with an antibody[76]. Thus if smears of circulating lymphocytes are made on glass slides, the numbers of transferrin receptor bearing (TFR+) cells can be enumerated and, using double labelling techniques, can be further characterised.

In keeping with the earlier information about circulating activated lymphocytes, raised levels of TFR+ cells have been shown in the peripheral blood of some patients with RA particularly during the onset of flares in disease activity. High levels of TFR+ cells are also found in patients with viral infections such as acute upper respiratory tract infection[77].

1.5.3 Further characterising TFR+ cells

It it not possible to characterise the TFR+ cells for normal subjects because their number of such cells is too small to allow the double labelling necessary to establish subsets. TFR+ cells (in elevated numbers) in the peripheral blood of patients with RA have been characterised using cell surface markers and the subsets so obtained were compared with those in the whole peripheral blood mononuclear population (ie including resting cells) from the same subjects[77]. In keeping with their activated state, 90% of the TFR+ cells co-expressed HLA DR antigens as compared with only 30% in the total peripheral blood population. The finding of 70% T-cells (T3+) and 9% B-cells (Ig+) was similar for both TFR+ and whole blood. Of the remaining 20% (null cells; T3-,Ig-) some were NK cells (Leu-7+). Thus

T-cells form the bulk of the TFR+ cells and TFR labelling is a useful tool in their study.

1.5.4 Subdividing activated, TFR+ cells

The activated, TFR+ T-cells have been further subdivided and compared with the subsets in the whole blood populations[78]. In patients with RA, a chronic disease, the CD4:CD8 ratios were significantly higher within the TFR+ population due to increased numbers of activated CD4+ cells. Interestingly, in patients with acute self-limiting viral infections the CD4:CD8 ratios have been found to be lowered, both in the whole blood[79] and in the TFR+ population, due to increased numbers of CD8+ cells[78].

Since some of the cases of acute self-limiting synovitis seen in an early synovitis clinic are virus induced, the CD4:CD8 ratio particularly within the TFR+ population may be an early marker of disease persistence. The ratio might be expected to be raised in PS and reduced in SLS.

1.6 B-CELLS

1.6.1 Introduction

The importance of rheumatoid factors has been discussed above (page 51-55) purely in terms of RF as a useful predictive variable. Does pathogenetic relevance underlie its clinical prominence? Here the study of the B-lymphocytes is of peculiar interest since, as a group, these cells have the capacity to produce both of the opposing/complimentary facets of the globulin - antiglobulin system. That is, this cell group can produce both the Rheumatoid Factor (RF) and its antigen, the Fc portion of IgG. If indeed this system is

relevant to the persistence of inflammation, then its closer study in early synovitis, with the opportunity to compare PS with SLS, may not only refine the predictive value of RF but also offer insights into fundamental disease mechanisms.

1.6.2 The antibody (RF)

RF - History:

Serendipity surrounded the discovery of rheumatoid factors on each of the almost independent occasions when the phenomenon came to light. Waaler was in the process of testing sera from patients suspected of having syphilis using a complement consumption assay involving sheep red cells sensitized with a sub-agglutinating concentration of rabbit-anti-sheep IgG. When serum from a patient with RA but not syphilis was tested, instead of haemolysis there occurred haemagglutination[80,81] and he repeated the observations in several patients with RA. He demonstrated the requirement for the sensitizing rabbit antibody in the agglutination: this was to eliminate as candidate explanation the possibility that non-specific agglutinins in the tested sera were directly responsible for the reaction. That complement was not necessary for the reaction and that the "agglutinating activating factor" resided in the globulin fraction of the serum was also shown in the same work. After the Second World War, while testing the serum of a patient with Rickettsial Pox using a haemolysis assay (to confirm the infection) there was observed instead haemagglutination: the patient was known to suffer also with RA[82]. Again the observations were extended to and repeated in other RA patients and were compared with other diseases: they showed that high titres of rheumatoid factors (as they were later designated[83] were found in RA but not in healthy controls or in other inflammatory arthritides. The demonstration that the RF activity resided in the

19S fraction of centrifuged serum[84] revealed it to consist of what are now known to be antibodies of the IgM class and the same paper showed that a 22S fraction could be dissolved to yield RF activity in a 19S fraction leaving a 7S fraction, the IgG. Although immunoglobulins with RF activity from the G and A classes are found in RA[85] their assay presents difficulties[54], the resolution of which was not the primary objective of this thesis. Accordingly, this study will concentrate on RF of the IgM class (IgMRF).

Why cannot IgMRF be used as a simple, reliable predictor of persistence in very early synovitis? The short answer is that any predictive value of RF is arithmetic and not absolute. This is true both when considering titres of RF in serum and when applied across disease groups. That RF is not specific to RA has been well reviewed[54]. Among the many conditions in which RF may appear are included infections and of particular relevance this includes viral diseases. Thus there is the possibility that RF may be detected in some virally induced SLS. Conversely, in PS, even those who become seropositive for RF do so at differing times after disease onset[61] and furthermore, of those with otherwise undoubted RA, only some 70% become seropositive[86]. Interestingly one recent study has helped to quantify the specificity and sensitivity of RF by applying it to synovitis of less than 6 months duration[62]: of those with the eventual classification of "persistent" 50% were seropositive for RF at presentation compared with 11% of "transient" synovitis. So although its value is not absolute, RF does have a limited predictive value in very early disease which reflects its more common application in prognostication once RA has become established. The use of RF in very early disease might be enhanced by considering that the RF - IgG interaction is not a homogeneous phenomenon. The IgMRF

in RA sera have numerous specificities and react differently with IgG according to the isotype[86].

Although the occurrence of RF in high titres in the serum and synovial fluids of patients with RA[87] has been suggested as being of fundamental pathogenetic significance[88], the finding of RF in benign conditions such as short lived infections suggests the possibility that RF have a physiological role[89]. This apparent contradiction would in part be explained if the RF produced in RA were different from the physiological response. Such differences are hinted at by the in vitro observation that the specificity of a positive RF test is enhanced according to the test antigen used: hence the test is more specific for RA if positive with both human and rabbit IgG[54]. RF can be further characterised by considering the structure and antibody specificity of the variable parts of the molecule.

IgMRF - Structure:

The pentameric form of IgM derives from the association of its subunits, each of which has the classic Ig structure: 2 heavy (H) chains of μ subclass and 2 light (L) chains either κ or λ, each chain having a "constant" (C) region and a "variable" (V) region. For IgM the heterogeneity is located in the V regions of the H and L chains ie V_H and V_L. Amino acid sequencing reveals much homology between antibodies of different specificities[90] with some homologies being closer than others permitting those with the closest homologies to be ascribed to the same sub-groups (Table 1.4).

CHAIN	V-REGION	SUBGROUP
L	κ	VκI VκII VκIII
	λ	VλI VλII VλIII VλIV VλV
H	V_H	V_HI V_HII V_HIII

Table 1.4 Subgroups of V-regions of light (V_L) and heavy (V_H) chains

However, the sequence of a protein can only be defined if there is a manipulable quantity. The above classification is based on the analysis of paraproteins, that is monoclonal (Mc) proteins produced in large amounts. The RF occurring in RA is polyclonal[86] and any individual RF will be in quantity to small for this kind of analysis. To study the RF of RA and its close mimics alternative strategies have to be employed.

RF antigen binding site specificity (idiotypy):

Previous studies of RF idiotypy used polyclonal rabbit antisera to examine the IgM in those 10%[91] of patients with Waldenstrom's macroglobulinaemia whose (IgM) paraprotein has RF activity[92]. This and similar work on other naturally occurring Mc RF[93] revealed that there are idiotypic similarities in the proteins produced by unrelated individuals, falling into 2 main cross-reactive groups, Wa and Po. That within such a group there may occur extremely high amino-acid homology[94] argues for these specificities arising from

germ-line coding rather than from gene rearrangement and somatic mutation. The existence of such highly conserved "primitive" specificities invites speculation on their evolutionary benefit but it must be recalled that these refer to "malignant" clonal protein production and the focus is therefore being turned on the polyclonal RF in RA and similar conditions.

To do this the "malignant" monoclonal RF are used to inoculate mice in order to raise and select mouse hybridoma (ie monoclonal) antibodies which have specificity for epitopes in the V-region of the RF. Some of the hybridoma antibodies have specificity for epitopes within the antigen binding site of the V-region of the inoculum RF (anti-idiotopic). Others may have specificity for epitopes at other parts of the V-region but if they bind close to the antigen binding site of the RF they can alter its shape (ie exert a conformational influence). These hybridoma antibodies can then be used to explore the pattern of idiotope expression on RF within human serum in SLS and PS. The source and nature of the hybridoma antibodies used in the present study are outlined in Methods (Chapter 3). Thus there is exciting possibility that to the limited arithmetic value of simple RF titres in the prediction of persistence, we may be able to find qualitative differences in the RF produced in SLS and PS. This may be particularly relevant in very early disease before the simple titres are high enough to be reliable.

1.6.3 The antigen (IgG)

Introduction:

RF are now defined as immunoglobulins with specificity for the Fc fragment of IgG and do not include those antibodies with specificity

for other parts of the IgG molecule[95]. In view of the discussion below (page 64) about altered antigenicity of IgG, the defining of sites on the Fc fragment of IgG, antigenic for RF, is of pivotal importance. Early work concentrated on the genetically defined markers antigenic for RF, the Gm antigens in the heavy chain[96,97]. In addition it was realised[98] that the specificity of IgMRF was often not for Gm. The Ga antigen is an isotypic marker on the Fc fragment at or near the $C\gamma 2/C\gamma 3$ junction of human IgG subclasses 1,2 and 4 but not 3[99,100]. RF has specificity for IgG1, 2 and 4 but usually not for IgG3 and Ga is held to be important in conferring upon IgG its antigenicity for IgMRF[86]. This is reinforced by the finding[101] that seropositive RA sera all had antibody activity directed against Ga. It is interesting to note that IgG3 has at amino acid position 435 (consistent with $C\gamma 2/C\gamma 3$ junction) an arginine with its long side chain, instead of a histidine as in subclasses 1,2 and 4. A particular variant of IgG3 actually has histidine at this position and it does bind some RF[102].

Even allowing for the IgG sub-class variations the binding constants of RF for IgG Fc are considerably lower than those for other defined Ag/Ab systems[54], and so theoretically IgG may not be the primary Ag for RF. Even so, these low avidity interactions may be sufficient for the RF to perform a putative physiological role. In addition, however, there are conditions under which RF/IgG binding is enhanced and these may be highly relevant to synovitis and in particular to its persistence.

<u>IgG in inflammation: increased affinity for RF</u>:

IgG can exist in rheumatoid synovial sera in aggregated form. That aggregates can link to IgMRF and form stable complexes as a result of

stearic stability without change in the individual RF-Fc bonds has been discussed by Carson54. Further to this mechanism there is evidence that as a result of inflammation IgG can undergo structural changes which enhance its binding of RF.

Oxygen derived free radical species have the potential to denature proteins including IgG 103. Exposure to free radicals in-vitro produces changes in IgG which can be visualised in a number of ways104,105 detailed below (page 65). This altered IgG binds at least IgG and IgA RF with two or threefold increased avidity106. Similarly altered IgG can be visualised in vivo, in RA synovial fluids and serum107. Still under investigation108 are the relative contributions (to the increased antigenicity) of damage to the usual Fc sites for binding RF versus damage exposing new sites for binding (eg at the hinge region, rich in aromatic amino-acids). Certainly damage uncovering new sites might partly explain the apparently contradictory finding that in synovial fluid there is RF-IgG3 interaction109 although it has to be admitted that that particular study used a plaque assay and hence the IgG3 being studied may have been undamaged. Before seeking radical-damaged IgG in the present study it is necessary to evaluate the techniques for visualising such damage.

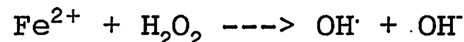
<u>Detecting oxygen-derived-radical damaged IgG</u>:

<u>Fluorescence</u>: One method utilises the development of characteristic auto-fluorescence (excitation 360nm, emission 454nm). It has been suggested that such fluorescence is due at least in part to damage to

and loss of aromatic amino acids in IgG, particularly in the hinge region of the molecule[107].

Aggregation: In the same work it was also shown that in addition to fluorescent monomer, increasing exposure to oxygen derived radical species so alters IgG that it can aggregate into polymers.

Some deductions can be made about which free radicals are responsible for the damage observed, since scavengers are available that preferentially remove particular radicals. It has been shown that catalase, which preferentially scavenges hydrogen peroxide (H_2O_2), greatly reduced resultant IgG damage in the above study[106]. However the concurrent finding that desferrioxamine (a chelator of metal ions) also almost completely protects, suggests that H_2O_2 is not the direct mediator of the damage. Rather, through the Fenton reaction:



$$Fe^{2+} + H_2O_2 \text{ ---> } OH^{\cdot} + OH^{-}$$

it suggests that iron catalyses the conversion of H_2O_2 to the hydroxyl radical ($OH^{\cdot}$) and it is this which is responsible for the fluorescence and aggregation. Superoxide dismutase, which reduces the level of the superoxide anion (by converting it to H_2O_2), had less protective effect.

Relevance of altered IgG to persistence of synovitis:

It has been shown in vitro that artificial stimulation of neutrophils (using phorbol myristate acetate) results in the production of oxygen derived free radical species, resulting in alteration to IgG in the medium, visualised as aggregation, fluorescence and reactivity with RF[106]. It has been shown that similar neutrophil activation results from the presence, at their cell surface membrane, of immune

complexes[110,111]. It has been suggested[106] that a cycle of self-perpetuation could arise in the joint with its high local concentration of neutrophils: inflammation, however initiated, includes the production of oxygen derived free radicals from neutrophils; the free radicals then damage the IgG, resulting in its aggregation and its more avid complexing with RF; the complexes could then further stimulate neutrophils and complete the cycle. Thus with or even without invoking local production of RF in the joint, fluorescent IgG has a theoretical role to play in the persistence of inflammation. That the aggregated IgG can also stimulate RF production[54] is an additional justification for its study.

If the above mechanism has anything to do with persistence of inflammation it would be important to seek differences in the measured products (eg fluorescent IgG) between those patients with persisting versus those with self-limiting synovitis. Specifically it would be important to study each of these groups as early in the course of the disease as possible, in order to try to circumvent the ever present problem that an observed disorder can be the consequence rather than the cause of the larger abnormality.

1.7 Summary of introduction

Patients with Rheumatoid Arthritis (RA) may benefit from disease modifying treatment being introduced as early in the disease as possible. Because of the high incidence of serious side effects with established treatments, it will be necessary to distinguish patients with RA from those with more benign, self-limiting arthritis. For the present study patients were recruited as early in their disease as possible.

A number of variables may help to predict those who will have persistent disease, and for this study the clinical pattern of disease was studied in detail. In addition, patterns of T-cell and B-cell activity which are found in established RA were sought in the study patients. Finally, evidence of specific viral infection was sought.

The results will be discussed primarily for their predictive value but where opportunities to elucidate underlying pathogenetic mechanisms arise, these will be explored.

CHAPTER 2

METHODS

CHAPTER 2

METHODS

This chapter presents an outline of the methods used in each part of the work for this thesis.

2.1 Clinical studies

2.1.1 Recruitment

Patients were recruited to the study by means of a letter circulated to General Practitioners in the South and Central health districts of Birmingham inviting them to refer those with a history of synovitis of up to six months duration. The letter indicated that although the main objective was to see patients with Rheumatoid Arthritis (RA) as early in its evolution as possible, it acknowledged that such diagnosis was difficult and not necessary before referral. The letter offered to see referrals within days, as opposed to the routine waiting time of three months.

2.1.2 Intervention

The data being presented was all taken at the patient's first clinic visit. It is relevant to comment on intervention, however, since the allocation to diagnostic groups was made retrospectively on the basis of eventual outcome. Many patients had been started on non-steroidal anti-inflammatory drugs (NSAIDs) prior to being seen in clinic. To minimise the possibility of altering the natural history of an arthritis, patients were managed only by NSAIDs plus simple analgesia plus physiotherapy. They did not receive "second-line" drugs such as gold or penicillamine unless their disease was persistent, severe and

not responding to "first-line" therapy, by which stage data collection and diagnostic categorisation had been completed.

2.1.3 Clinical and routine laboratory variables

An Early Synovitis Clinic was established and held once weekly to screen and follow patients at six weekly intervals, until either a diagnosis became apparent or their symptoms resolved. The patients were seen exclusively by myself and a standard proforma was completed at each visit.

The proforma was designed by myself to ensure uniform data collection and to facilitate its subsequent transfer to computer for analysis. Although the proforma was to be used primarily for eventual formal statistical analysis, it was also a clinical management tool. To avoid duplication of effort, the proforma was designed to include, within a full clinical history, the variables of particular interest to the thesis. An abbreviated version of the proforma was also completed at subsequent visits to determine progress. A copy of the full proforma is included in appendix 1, and the variables extracted for analysis are listed below. I am grateful to Dr A Silman, Senior Lecturer in Clinical Epidemiology at the London Hospital, for his helpful comments on the proforma design.

The variables to be measured were grouped under three main headings: Clinical, derived from the patient history and examination; Routine Laboratory, performed on blood sent to the hospital service laboratories; and Research, performed on blood taken at each visit and stored for later analysis in the research laboratories.

History:

Name and Number		
Sex		
Age		
Onset date		
Date of first visit		
Duration of symptoms (at first visit)		
Type of onset (rate: hours, days or weeks)		
Pain severity (5 point scale)		
Morning stiffness (duration in minutes)		
Morning stiffness (severity, 5 point ordinal scale)		
Joint stiffness	}	yes/no
Joint pain	}	for
Joint swelling	}	each joint (i)
Prodromal symptoms		
Family history		

Examination:

Rheumatoid nodules		
Rash		
Reduced range of movement	}	yes/no
Tenderness	}	for
Swelling	}	each joint
Ritchie articular index[112]		
Number of inflamed joints (ii)		
Mean grip strength (iii)		

Table 2.1 Clinical variables

Notes:
i) see text for grouping of joints
ii) "inflamed" = tender or swollen
iii) mean, 3 pairs of readings with standard bag and manometer[113]

Grouping of joints:

The joints examined were: temporo-mandibular (TMJ), neck, shoulder, elbow, wrist, metacarpo-phalangeal (MCP), proximal inter-phalangeal (PIP), distal interphalangeal (DIP), back, hip, knee, ankle, metatarso-phalangeal (MTP). Paired joints or groups of joints were recorded separately on the proforma but at analysis were recorded together. Thus although in the analysis (chapter 3) MCP swelling means involvement of any of the left and/or right MCP joints,

patients with asymmetric disease were specifically excluded from the groups under study.

Haemoglobin[114]
White blood cell count
Platelets
Erythrocyte sedimentation rate[115]
RA latex
Rose Waaler
Anti-nuclear antibody[116]
C-reactive protein[117]
X-rays of hands and feet

Table 2.2 Routine laboratory tests

RA latex and Rose Waaler tests:

These two assays were routinely available in the local service laboratories. The RA latex slide test (RA80 Eiken, Tokyo, Japan) is simple to perform and, having human IgG as the antigen, has the advantage of sensitivity and was used to screen all patients. Those with positive results were tested by the Rose-Waaler assay[118] which uses rabbit IgG as the antigen, and has increased specificity for RA (at the expense of sensitivity). The results of these assays will be presented with the clinical and routine laboratory variables in chapter 3.

T-lymphocytes:

Number CD4+
Number CD8+
Number TFR+
Number TFR+ also CD4+
Number TFR+ also CD8+

B-lymphocytes:

Quantitation of IgM RF	(ELISA capture assay)
Quantitation of IgM RF idiotype	(ELISA inhibition assay)
Ratio: fluorescent IgG / whole IgG	

Virology:

Anti- B19 Human Parvovirus antibody (IgM and IgG)
Anti- Rubella virus antibody (IgG and IgM)

Table 2.3 Research variables

Details of the methods for the research variable assays: see text.

2.2 T-Lymphocyte studies

Dr M Salmon, Dr G Kitas and Mr D Pilling taught me the cell separation technique and smear preparation but they also performed these operations for most of the patients and did the monoclonal antibody staining, blind to the diagnostic coding. They also offered constructive criticism of my analysis and interpretation of the data.

2.2.1 Cell separation

At each visit and at the same time of day, 10 to 20 ml of venous blood was collected into a heparinised tube, and transported to the laboratory immediately. The blood was layered onto Ficoll-hypaque and centrifuged for 30 min at 300G[118]. The harvested mononuclear cells were smeared onto glass slides which had been previously been coated with high molecular weight polymers of L-lysine[120]. The slides were dried and foil wrapped before storing at -20°C. Duplicate slides were made for each sample. The slides were later labelled in

batches with monoclonal antibodies to cell surface markers.

2.2.2 Single labelling

Smears were incubated for 60 min with monoclonal antibodies: UCHT-1 pan-T cell, anti-T3 [121]; OKT4-helper/inducer T cells[122]; OKT8-suppressor/cytotoxic T cells[123]; OKT9-anti-TFR[75]. After incubation smears were washed in phosphate buffered saline (PBS) pH 7.4 for 10 min and labelled with fluorescein conjugated goat-anti-mouse IgG (Ortho Diagnostics, Raritan, New Jersey, USA) for 30 min. Smears were then counterstained with ethidium bromide and mounted in phosphate buffered (pH 8.6) glycerol containing p-phenylamine diamine to retard photobleaching during microscopy[124].

2.2.3 Double labelling

The problem with double labelling a smear of cells is that if the same species is used to raise both marker antibodies, then the dye-conjugated detecting antibody will label all positive cells indiscriminately. To circumvent this, smears were double labelled for TFR according to the method developed by Dr Salmon in this laboratory[77]. Smears were labelled with fluorescein-conjugated monoclonal antibodies to T-cell surface antigens as above, but without the ethidium bromide counterstain. They were then incubated with normal human serum (as a source of transferrin) for 15 min and washed for 40 min in three changes of PBS. Smears were then exposed to rabbit anti-transferrin IgG (DAKOPATTS, Weybridge, UK) for 25 min. To visualise the TFR+, labelled cells, the smears were incubated with swine-anti-rabbit IgG conjugated to rhodamine (DAKOPATTS, Weybridge, UK) for 30 min. Since OKT9 does not compete with transferrin for binding sites on the receptor, it was used to control for the sensitivity and specificity of the transferrin - anti-transferrin

binding system. As a negative control transferrin was removed from the smears by incubation with acid desferrioxamine (pH 5.5) for 10 min and then stained with rabbit-anti-human transferrin followed by sheep-anti-rabbit IgG: these controls were all negative.

Smears were examined using a Zeiss fluorescence microscope equipped for incident illumination and using separate filter sets for fluorescein and for rhodamine/ethidium bromide.

2.3 B-Lymphocyte studies

At each visit, during routine venipuncture, ten to twenty ml of blood was taken into a plain tube, allowed to clot and the serum stored in aliquots at -70°C. Most assays were done using serum thawed only once: no aliquot was used for more than three freeze - thaw cycles. Two principle groups of assays were performed: those looking at Rheumatoid Factors and those studying their antigen, IgG.

2.3.1 IgG as an antigen for RF

For the purposes of these experiments, no stored serum had been thawed more than once previously. Serum proteins were separated by size using high performance liquid chromatography (HPLC) on a TSK 3000 SW column at room temperature (pH 7.4). 10microL of serum were diluted 1:2 in phosphate buffer (0.067M KH2PO4 + 0.1M KCl) containing 100microM cytochrome C. The proteins were eluted at 1ml/min with the phosphate buffer as the mobile phase. The protein at the eluate volume (and hence molecular weight) characteristic of IgG was measured in two ways:

1. Total protein was measured by optical densitometry (Uvicord spectrophotometer, Pye Unicam) at wavelength 280nm (OD 280).

2. The fluorimeter (Gilson Instruments Ltd, Villiers le Bel, France) using an excitation wavelength of 360nm measured fluorescent protein by use of filters to read emission at 460nm. Emission at 460nm is characteristic of oxygen derived free-radical species induced damage to IgG[104,105]. Thus the quantity of fluorescent IgG could be compared to the total IgG. This relationship was expressed as a ratio and related to the internal standard, the height (intensity) of the cytochrome C peak in the eluate[107]:

$$\text{Ratio} = \frac{\text{Fluorescent IgG}}{\text{Total IgG}} \times 100\%$$

2.3.2 Rheumatoid factors

These were assayed using a number of different, complimentary techniques. The RA Latex and the Rose-Waaler tests have been described above.

The following methods describe work performed by Dr R Mageed and Dr R Jefferis, Dept of Immunology, University of Birmingham, using serum samples from 40 age and sex matched patients (SLS n = 20, PS n = 20), drawn from the full cohort of 65 patients studied for this thesis. The samples were taken at the patient's first visit to the early synovitis clinic, stored as described above and analysed later, once the diagnostic allocation to SLS or PS had been made. These assays were performed by persons blind to the patient's diagnostic grouping. The following techniques have been described previously[125] and are described here in outline.

Direct binding Enzyme Linked Immunosorbent Assay (ELISA)

i) Total IgMRF: Polystyrene microtitre plates (Flow, UK) were sensitized with Fc-Per (an IgG1 Fc fragment) at a concentration of 20 microg/ml in PBS, pH 7.4 (50microL/well) by incubation at 37°C for 2hrs then washing the plates three times with PBS containing 0.05% Tween 20 (PBS/Tween). Sera from the study patients were titrated 1:100, 1:250, 1:1000 in PBS/Tween across the plates and incubated at 37°C for 2hrs. The plates were washed three times with PBS/Tween. Binding of RF was revealed with peroxidase-conjugated sheep anti-human IgM (The Binding Site, Birmingham Research Institute, UK). Bound peroxidase-conjugated antibody was revealed by peroxidase substrate (O-phenylene diamine) and the results were recorded as OD values at 492nm using a Titertek multiscan reader (Flow Labs, UK). The quantity of RF bound to the plate was obtained by comparing the optical density obtained with the standard curve, derived using a known quantity of a purified IgMRF (IgMRF Fr) as a standard.

Direct binding ELISA

ii) IgG sub-class specificity of IgMRF: Additional information may be gleaned from knowledge of the sub-class specificity of the circulating RF. Accordingly the following sensitizing antigens were also substituted for the Fc-Per in the above procedure: Cri (IgG1), Ware (IgG1), Pe (IgG2), Campbell (IgG2), Renwick (IgG3), Goe (IgG3), Carter (IgG4), Reeder (IgG4). The sera from 18 healthy subjects were also tested in these assays to determine in the normal population the level of IgMRF with specificity for these sub-classes of IgG.

Total IgM and idiotype positive IgM by Capture ELISA

Introduction: Specific monoclonal antibodies (McAb) were used to sensitize ELISA plates (using incubation and washing as above) at an

optimized concentration of 10microg/ml and a McAb of irrelevant specificity was used as a control. The SLS and PS patients serum samples were added in the same dilutions as for the direct ELISA. Captured IgM was again revealed by the peroxidase conjugated sheep anti-human IgM (specifically anti-Fc) developed with the peroxidase substrate as above. The concentration of captured IgM or IgMRF was obtained by using a standard optical density curve derived from known quantities of a standard IgMRF (Kok).

i) Total IgM: Using the capture ELISA the total circulating IgM (ie including RF and non-RF IgM) was measured. The plates were sensitized with a mouse anti-human μ-chain McAb (AF6) used as the intact IgG.

ii) Idiotope-positive IgM: Again using the capture ELISA, the patient's serum was assayed for IgM bearing specific idiotopes (ie including RF and non-RF IgM). In this case the plates were sensitized with the $F(ab')_2$ fragments of the mouse McAbs listed below.

Inhibition of direct binding assay using anti-idiotypic Ab

The above method of quantifying idiotope-positive IgM includes IgM with non-RF activity even though the anti-idiotypic Ab were raised using RF as immunogen (see below). The most interesting portion of the idiotope-positive IgM in the context of the Early Arthritis study, was that having RF activity. This was studied using the direct binding ELISA as described above, with Fc-Per as a bound target for the patient's serum. Inhibition of that binding (ie the IgMRF activity) was achieved by pre-incubating the patient's serum sample, diluted to twice its maximum dilution before reaching a plateau as measured from its binding curves (in the direct ELISA), with a doubling dilution series of each of the listed McAbs or polyclonal antisera, from 0.2mg/ml. The plates having been washed three times with PBS/Tween, 50microL of the mixtures were added to each well. Any

uninhibited RF binding was revealed using peroxidase-conjugated goat $F(ab')_2$ anti-human μ-chain (Kallestad, USA) and the plates developed as above.

McAb	Specificity for:
17-109:	A light chain cross-reactive idiotope associated with RF from the Wa group. Immunogen Sie-IgMRF. Kindly supplied by Dr DA Carson[126]
C7:	A κIII associated epitope independent of H-chain class or sub-class but with preferential association with IgM RF paraproteins. Immunogen Ko-RF, a monoclonal IgM-κRF from a patient with Waldenstrom's macroglobulinaemia. Ko-RF is a member of the Wa idiotype group[127].
H1:	A heavy chain idiotope on a subset of RF from the Wa idiotype group. Immunogen Ko-RF[125].
G6:	A heavy chain cross-reactive idiotope restricted to RF from the Wa group. Immunogen Ko-RF[125].
G8:	A conformation dependent epitope expressed on some IgMRF. Immunogen Ko-RF. (Method:[128])
AF6	Non-idiotopic, binding to human μ-chain. Used to quantify total IgM.

Table 2.4 Antibodies used to detect cross-reactive idiotopes (CRI)

The monoclonal anti-idiotypic antibodies used In this set of experiments are listed above (Table 2.4). Although monoclonal antibodies have value in relative specificity, even allowing for some cross-reactivity they will detect only a limited number of epitopes (idiotopes) on, for example, Ko-RF. In screening populations for patterns of epitope expression it is useful to be able to detect a broader range of epitopes which can be further characterised later. Accordingly, we have used a polyclonal antiserum raised against Ko-RF.

The above hybridoma antibodies have an additional problem in that they were raised using malignant clonal RF as immunogen. Therefore they give information only on those few selected RF idiotopes. This may or may not be relevant to the polyclonal RF in RA. One way towards circumventing this is to use hopefully more relevant immunogens. Accepting that the RF produced by Epstein-Barr virus transformed B-cells may not be entirely similar to that in RA[129], a monoclonal RF produced by EBV transformed B cells from patient with RA (RF-AN)[130] has been used as an immunogen. The resulting polyclonal antiserum was used in the present study. The polyclonal antisera had been rendered specific for Ko-RF and RF-AN by passage on affinity columns with IgMκIII, and IgM and λ light chains respectively (Table 2.5).

Polyclonal antisera	Immunogen
Rabbit anti RF-AN:	RF-AN is a monoclonal IgMλRF from an EBV established B cell line of a patient with RA[130]
Rabbit anti Ko:	Ko-RF as for C7, H1 and G6 above.

Table 2.5

2.4 Viruses

Routine viral serology was performed by the Regional Virology Service laboratory at East Birmingham Hospital. The specific viral antibodies were sought using radio-immunoassay (RIA): the ELISA plate is coated with anti-human IgM (or G) and then incubated with the patient's serum. The viral antigen is then layered on and captured by any specific antibody present. Development of the plate in the case of Rubella is by an I^{125} labelled Mc anti-Rubella antibody[131]. In the

case of B19 Parvovirus no such labelled Mc antibody exists so a mouse Mc anti-B19 is added before finally layering with an I^{125} labelled anti-mouse antibody[132].

2.5 Statistical analysis

The proforma design allowed consistent data collection and systematic entry into a computerised database, DBase III+ [133]. Data held in those files was manipulated and then was able to be imported directly into a commercial statistical analysis program, Statgraphics[133]. This program permits the required range of analyses, both parametric and non-parametric, univariate and multivariate.

2.5.1 Differences

This thesis addresses differences between two groups of patients: those with persistent and those with self-limiting synovitis (PS, SLS). Since all patients arriving in clinic who fulfilled the entry criteria were admitted to the study and grouping was on the basis of later outcome, this was a cohort study rather than a case controlled study.

Categoric variables: These were compared using the Chi-squared test[135], using Yates' correction for continuity[136] where the expected frequency of a cell was less than 5. The Relative Risk of PS for a given variable was obtained from[137]:

$$\frac{\text{\% with PS, of population positive for variable}}{\text{\% with PS, of population negative for variable}}$$

The confidence intervals (CI) for the Relative Risk (RR) were derived from the value of Chi[135]:

$$CI = RR^{(1 + or - z / Chi)}$$

where z is the value of the standard normal distribution associated with the desired level of confidence (taken as 95% confidence level giving z = 1.96).

Ordinal variables: These were compared using the Mann-Whitney U ranking test[138].

Interval variables: Differences between the groups were studied using the unpaired t-test, with CI for the differences in means being given at the 95% level[135]. Since utilising biological variables often produces results whose distribution is not strictly Gaussian, it was felt important to check the results of the t-tests. Accordingly the distribution of values obtained for each variable used was plotted in a frequency histogram and these have been included in the results chapters where appropriate. In addition, the non-parametric ranking test (Mann-Whitney U test[138]) was used to check the t-test results. No significant discrepancies were found but individual results have been presented in more detail, as appropriate.

2.5.2 Correlations

Pearson product moment correlation coefficients[139] are given particularly for the rheumatoid factor analyses. Correlations between variables have also been revealed in the course of conducting the multivariate analysis. Since the objective was the prediction of persistence of synovitis, the correlations have been used to explain how the original data set could be reduced to a few variables. The correlations also reveal that the many variables measured are not completely independent of each other. Thus in the biological setting the apparent risk, of a statistically significant result being found

by chance due to many variables being admitted to univariate analysis, is far less than were the variables to be entirely independent of each other.

2.5.3 Multivariate analysis

The objective of this part of the analysis was to take the widely used clinical and laboratory variables and explore ways of enhancing the prediction of persistence of synovitis. Two contrasting outcomes were possible: either several variables would be combined to generate a scoring system more strongly predictive of persistence together than as individual predictors; or several variables might be shown to correlate so closely with each other that measuring just one of them would yield as much information as measuring all of them.

The steps undertaken are presented in some detail in chapter 3. Briefly, multiple regression was conducted using the computer based program Stepwise Variable Selection[134] to construct a model for the prediction of the numerically coded outcome (SLS or PS). The forward selection mode was used, meaning that the strongest predictive variable was inserted into the model first and the other variables were invited to contribute further to accounting for the variability in outcome. Of the remaining variables, the strongest was again added to the model and the process was repeated until all variables outside the model failed to reach the chosen significance level (95%).

CHAPTER 3

RESULTS

CLINICAL AND LABORATORY VARIABLES

CHAPTER 3

RESULTS - CLINICAL AND ROUTINE LABORATORY VARIABLES

3.1 AIMS

The aim of this section was to identify variables routinely available in the out-patient clinic which, when recorded in the earliest stages of Rheumatoid-like polyathritis, might identify those patients whose disease would become chronic. Even if the null hypothesis were proved and no difference could be seen between those destined for persistent disease and those who were to undergo spontaneous remission, then that would be of value in considering the latter group as a model for RA especially if a triggering agent were to be identified.

3.2 Patients selected

In response to the circular letter (page 70) to General Practitioners and to requests for referrals from other clinics within the Department of Rheumatology, 112 patients were admitted to the study for initial screening. As described above (page 71), each of these patients was questioned and examined by myself and the standard proforma completed at the first visit. The entry criteria to this stage of the study were deliberately lax, simply including patients with any history of joint symptoms of up to and including 6 months duration. Given that the aim was to study symmetrical peripheral polyarthritis, some 47 of the original 112 patients were excluded from further study (Fig 3.1): these included patients with, for example, asymmetrical oligoarthritis (eg associated with psoriasis), osteoarthritis, ankylosing spondylitis and even non-rheumatological conditions including alcoholic liver disease.

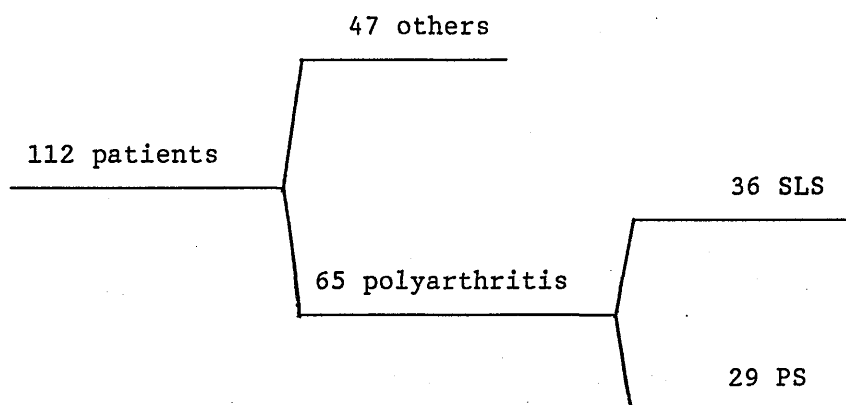


Figure 3.1 Patients studied, subdivisions

The remaining 65 patients were followed in the early synovitis clinic at 6 week intervals for at least 1 year, unless their symptoms resolved entirely before then. In the case of such complete spontaneous remission the patients were discharged from the clinic with instructions to report back if there was any recurrence. The date of remission was agreed between patient and physician and the total disease duration (mean 14.8 weeks) at remission was recorded (See Table 3.3 ii). At each follow-up visit an abbreviated proforma was completed by myself, this being used in an effort to standardise assessment and management. Thus at the end of 1 year each of these 65 patients was allocated to one of 2 groups: Self-limiting Synovitis (SLS) meaning complete remission; and Persistent Synovitis (PS) for those with continuing joint inflammation. Although it does not form part of this thesis, a 5 year recall will be conducted to check on the veracity of the outcome, both symptomatically and objectively.

The results presented in this chapter are derived from the data collected at the patients' first visit and analysed on the basis of the eventual allocation to the groups SLS or PS. The results are presented in four stages. First the simple demographic data will be

given; second the pattern of joint involvement (symptoms and signs) will be analysed; third the routine clinical and laboratory variables (eg duration of morning stiffness and erythrocyte sedimentation rate) will be presented; finally in this chapter, all of the above first visit variables were admitted to a process of multivariate analysis, to see if any combination would select those patients going on to persistent disease.

3.3 Demographic data

	SLS	PS
Men	7	12
Women	29	17

Relative risk of maleness for PS	= 1.71
Confidence interval	= 0.99 - 2.94
Chi Squared	= 3.74
p value	= 0.053

Table 3.1 Sex

Sex: The clear preponderance of women in both PS and SLS is seen in Table 3.1. The proportion of men is slightly higher in the PS group but this was not statistically significant.

	SLS	PS
Mean (yrs)	39.5	49.9
Range	15 - 83	23 - 71
Standard deviation	18.2	13.6
Confidence interval for difference in means		-18 to -2.5
p value (t test)		0.013

Table 3.2 Age

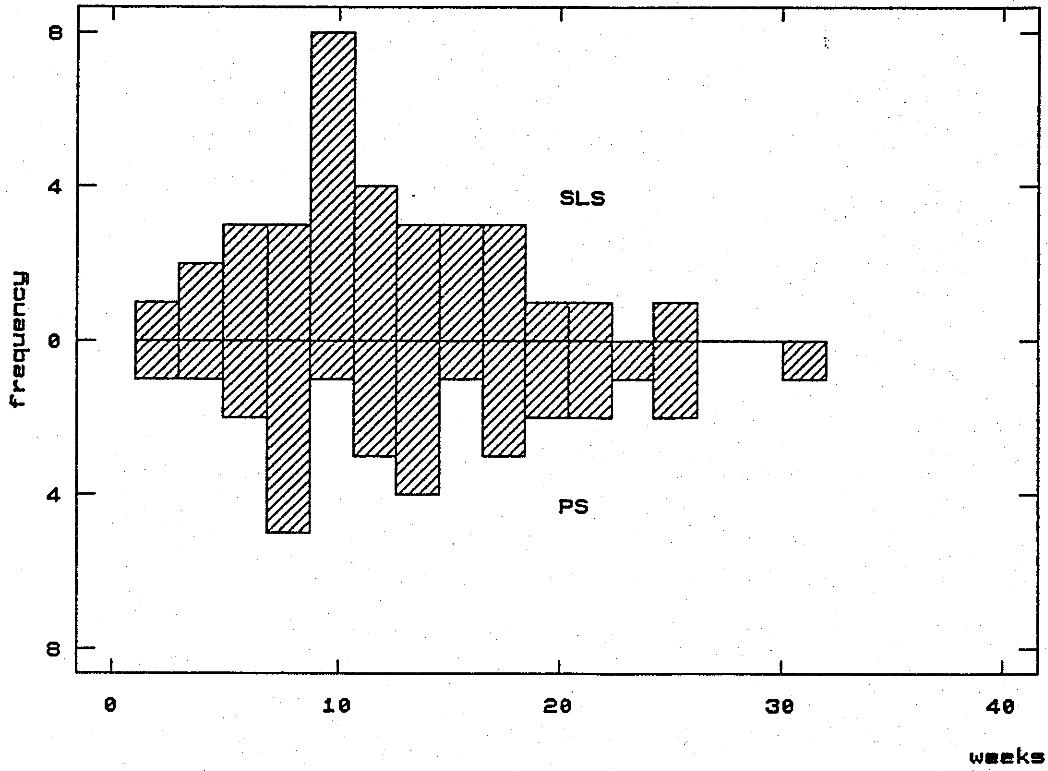


Figure 3.2 Duration of disease at presentation

<u>Age</u>: The mean age of the PS group was 10 years older than that of the SLS group (Table 3.2) and this difference did reach statistical significance, although the similar range of ages reduces the biological usefulness when applied to an individual new patient.

<u>Duration of disease at presentation</u>: Patients were asked at first clinic visit to date the onset of their joint symptoms. The aim of seeing patients with very early disease seems to have been met (Table 3.3). There was a slightly earlier mean presentation for the patients with SLS although the histogram (Figure 3.2 opposite) shows there is more similarity than difference.

(i) Duration of Disease at Presentation

	SLS	PS
Mean (weeks)	10.6	14.2
Range	1 - 26	2 - 32
Standard deviation	5.8	7.4
Confidence interval for difference in means	-6.9 to -0.17	
p value (t test)		0.034

(ii) Duration of Disease at Remission

	SLS
Mean (weeks)	14.8
Range	1 - 44
Standard deviation	9.5

PS was defined as being present for at least 1 year.

<u>Table 3.3</u> Duration of disease

3.4 PATTERN OF JOINT DISEASE

At the patient's first visit 6 variables per joint were recorded on the proforma diagram (appendix 1). Three of these were subjective, namely stiffness, pain and swelling with the patient being asked to report those currently present in any given joint. Three further variables which were "objective" in the sense that the observer decided on their presence or absence in a given joint, included diminished range of movement, tenderness and swelling.

3.4.1 Statistics

Each of the six variables of joint disease was simply recorded as present or absent for a given joint. Although left and right joints were recorded separately, for the purposes of analysis left and right were grouped together: so for example a positive score for wrist stiffness means that for that variable either the left or the right or both wrists were affected. These categoric variables were analysed by the Chi Squared test, the number of patients with, for example, wrist swelling being compared between the two groups, PS and SLS. The relative risk of PS was calculated for the presence of each variable for each joint, together with the confidence intervals for that relative risk (page 82). The sensitivity and specificity for each variable at each joint, in identifying PS was also calculated. For a given variable at a given joint analysis was only carried out if there were sufficient patients positive for that variable to give an expected value of greater than 2 for each cell in the Chi Squared table. As well as not being amenable to statistical analysis, such variables would be of little biological use if they were positive so infrequently. The raw numeric data is presented in Appendix 1 Table A1.1 and in this section are presented in graphic form. The data collection for this section was complete for all patients, with no

missing values.

Caution: It is interesting to note (below) that shoulder pain is significantly different between the groups (p = 0.0132) whereas shoulder stiffness, although in the graph appearing to show a difference, did not reach statistical significance (p = 0.07). Similarly MTP pain is statistically different between the groups (p = 0.0265) but MTP stiffness is not (p = 0.17). Perusal of Appendix 1, Table A1.1 reveals that the lower of the 95% Confidence Limits for the Relative Risk (of a positive variable for PS) for shoulder and MTP stiffness and pain are very close to unity, with the limits for stiffness including unity but those for pain just avoiding doing so. This reflects the relatively small numbers of patients involved and even though the 95% Confidence Intervals are not particularly broad, it emphasises the need for cautious interpretation of a "statistically significant" result. The Confidence Intervals help in that interpretation even though in general, the lower the p - value, the further are the Confidence Intervals for the Relative Risk from including unity.

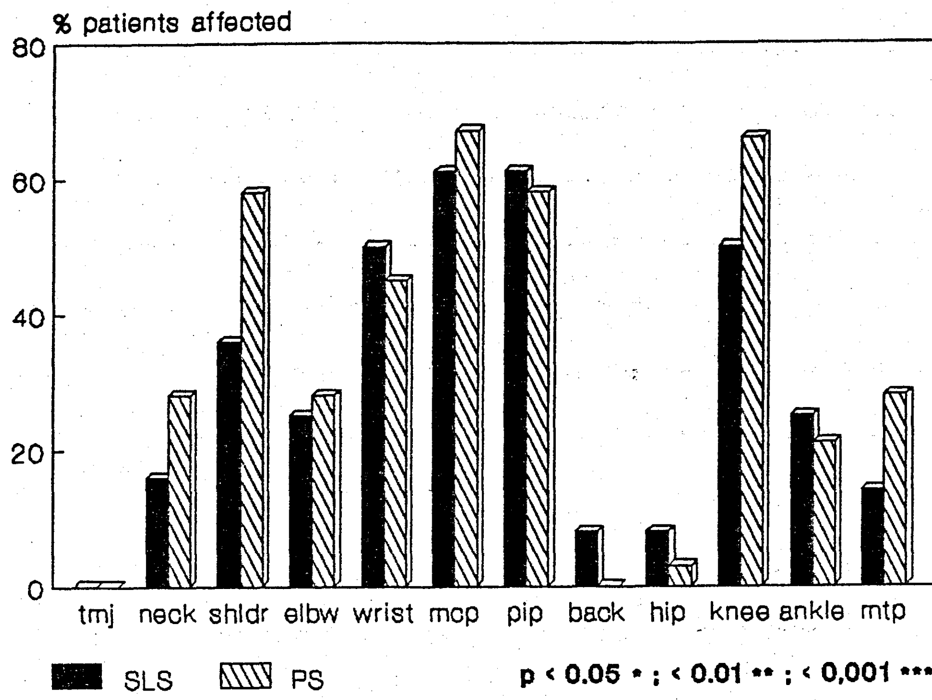


Figure 3.3 Pattern of joint stiffness

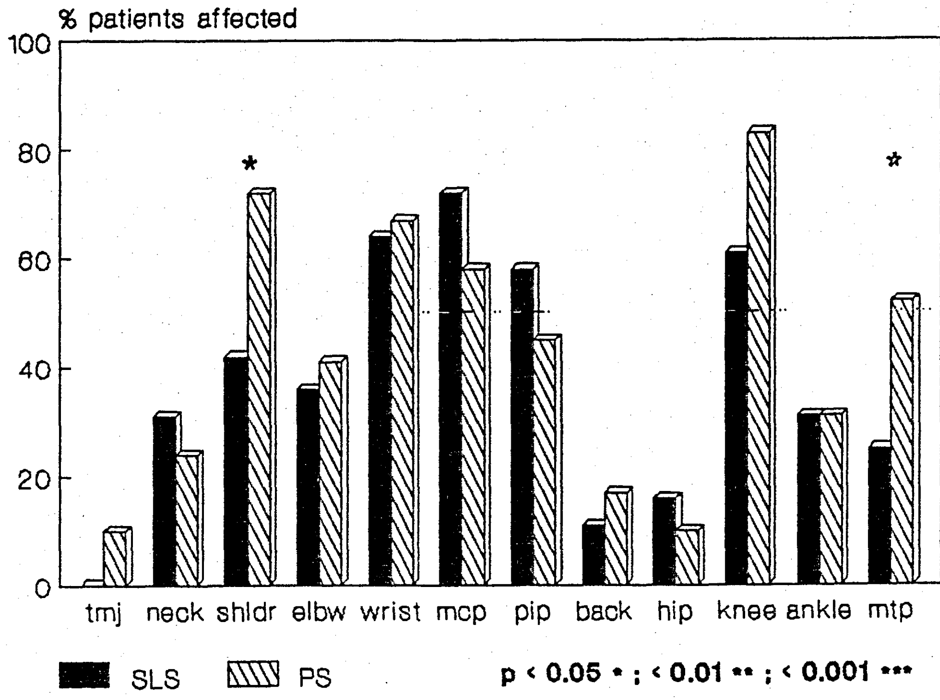


Figure 3.4 Pattern of joint pain

3.4.2 Joint Stiffness

There is remarkable similarity between the 2 groups, PS and SLS, with no statistically significant difference being seen in the distribution of symptomatic joint stiffness (Figure 3.3). The preponderance of peripheral joint involvement reflects the intentional preselection of patients with symptoms suggestive of RA. The question of severity of (morning) stiffness is addressed later (page 97) but was examined using an unvalidated and crude ordinal scale which revealed little useful information.

3.4.3 Joint Pain

Since the overall pattern of joint pain (Figure 3.4) is very similar to that recorded for stiffness, the same comments apply. At the shoulder and MTP there is a significant difference between the groups although as discussed above this is relatively weak.

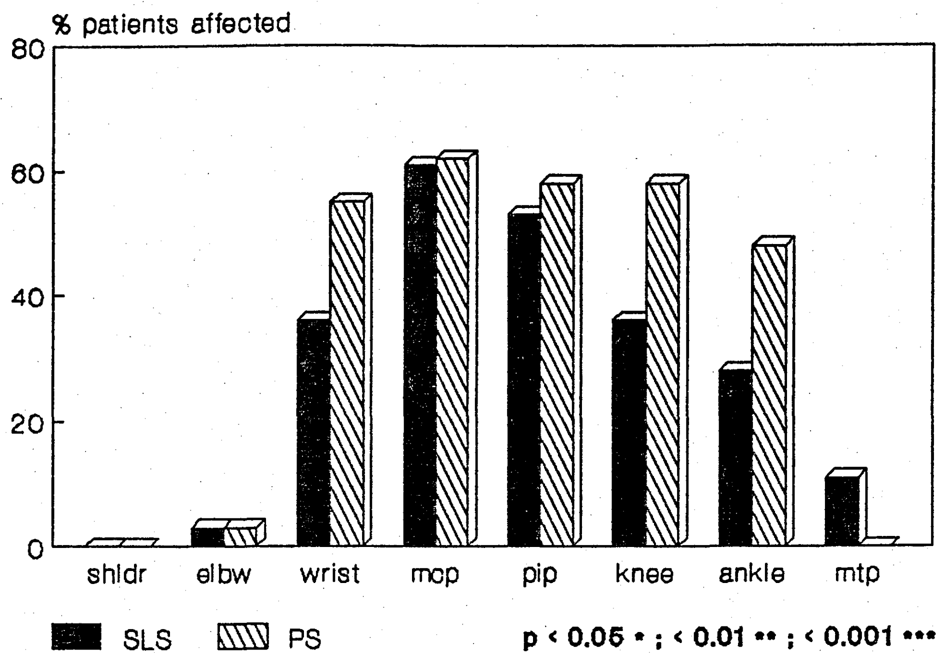


Figure 3.5
Pattern of subjective joint swelling

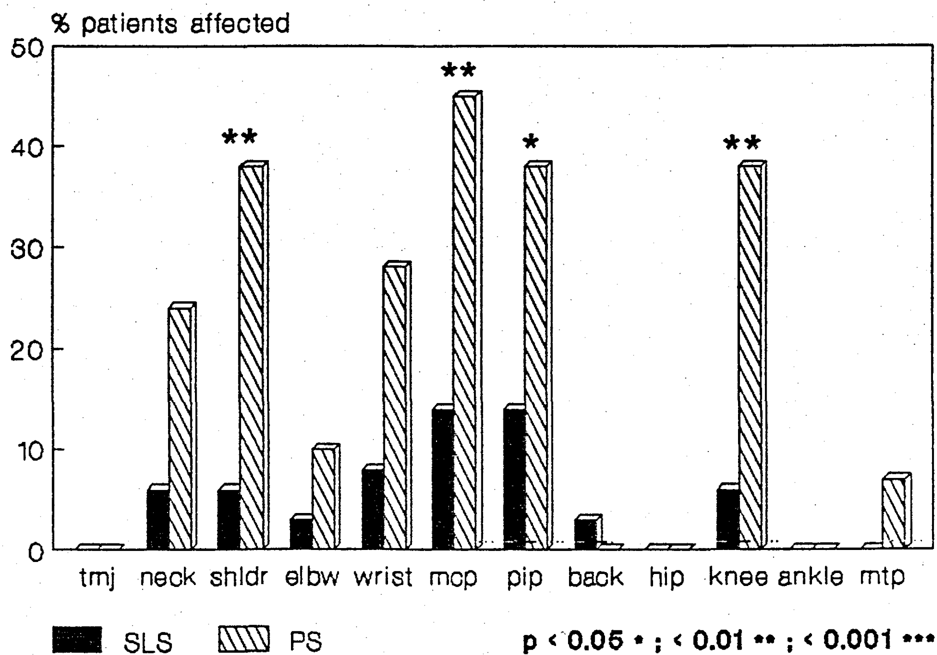


Figure 3.6
Pattern of objectively decreased ROM

3.4.4 Subjective Joint Swelling

No statistically significant difference was found in the numbers of patients reporting joint swelling (Figure 3.5). Overall the less patients reported swelling than stiffness or pain. Naturally patients would not be expected to report swelling of joints deep in tissue and indeed none did for TMJ, neck, back or hip.

3.4.5 Decreased Range of Movement (ROM)

In contrast to the above three variables which were reported by the patients, this variable was recorded (as were the next 2 variables) as present or absent at the first visit by the single observer, ie myself. Although noted on the proforma, for the purposes of analysis no grading of severity of reduced ROM was made. At first clinical visit, diminished ROM at shoulder, MCP and knee were significantly associated with an outcome of PS (Figure 3.6), the Relative Risk, its 95% Confidence Intervals and the p - value all strongly suggesting this (Appendix 1, Table A1.1). Diminished ROM in the PIP joint was also more frequent in the PS group but although this too was statistically significant it was more marginal: $p = 0.025$, Relative Risk 1.86, CI 1.08 - 3.20.

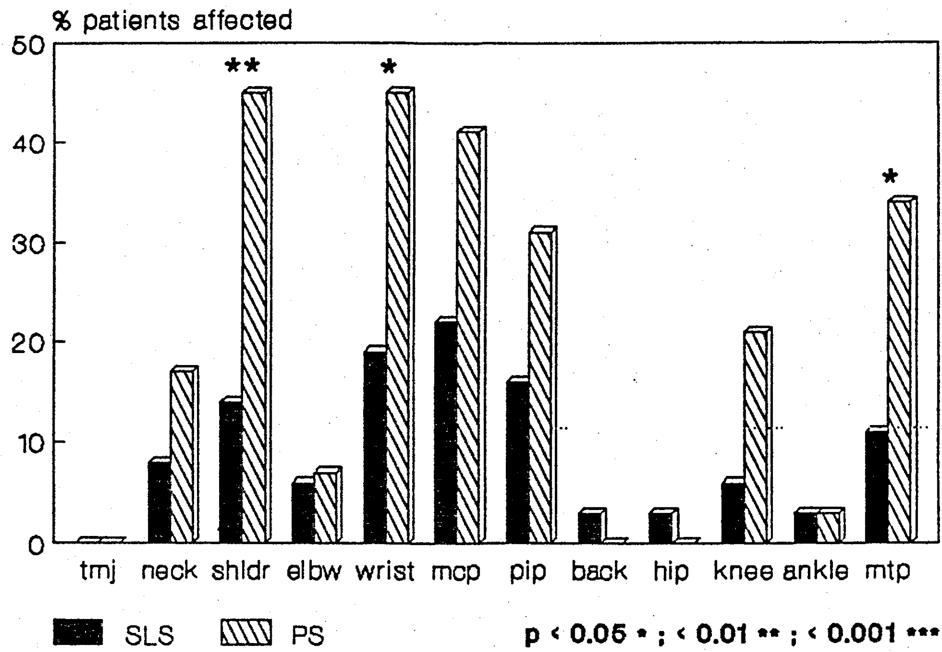


Figure 3.7
Pattern of objective joint tenderness

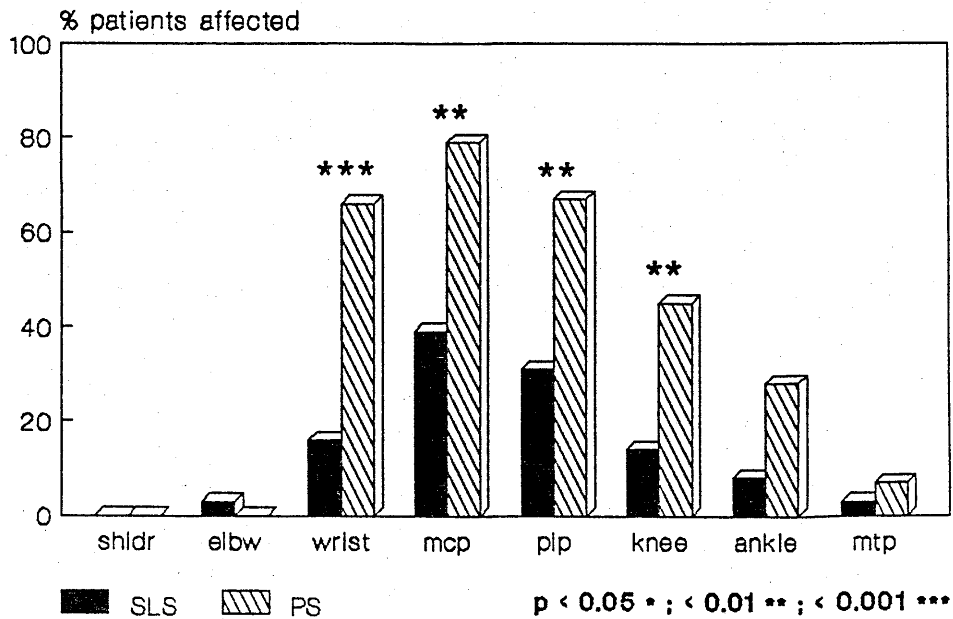


Figure 3.8
Pattern of objective joint swelling
(tmj, neck, back, hip: nil recorded)

3.4.6 Joint Tenderness

As for ROM and Pain (above) tenderness (on pressure and/or joint movement) at the shoulder was significantly increased in the PS group (Figure 3.7). There were also statistically more members of the PS group with tenderness at wrist or at MTP but again the Confidence Intervals for the Relative Risk only just avoid including unity (Wrist CI 1.06 - 3.04, MTP CI 1.09 - 3.36) (Appendix 1, Table A1.1).

3.4.7 Observed Joint Swelling

Joint swelling recorded by the single observer as present at the first clinical visit, was significantly more frequent in the PS group at wrist, MCP, PIP and knee joints (Figure 3.8). The strong statistical values (Appendix 1, Table A1.1) support the suggestion of biological significance, particularly at the wrist ($p < 0.0001$, Relative Risk 3.04, CI 1.77 - 5.22). However, biologically relevant numbers of SLS patients had joint swelling recorded at first visit. This emphasises the clinical difficulty in predicting on the basis of joint swelling at first clinic visit, whether a given patient's synovitis will become persistent.

3.5 Clinical and Routine Laboratory Results

The variables included in this section of the analysis are those which are available from the patient history (excluding pattern of joint involvement), upon clinical examination or from a District General Hospital laboratory service. Those investigations requiring research laboratory facilities are analysed in later chapters. The values presented are those recorded at the patient's first visit to the Early Synovitis Clinic.

3.5.1 Statistical Analysis

As before, the values for each variable were compared between the two eventual outcome groups PS and SLS.

For each categoric variable the Chi Squared test was used, and the Sensitivity, Specificity, Relative Risk (of PS) and its 95% Confidence Interval were calculated.

For ordinal variables the Mann-Whitney test was used to assess the difference between the groups.

In the analysis of the interval (sometimes called continuous) variables, both the unpaired t-test and the Mann-Whitney test were used to study differences between the PS and SLS groups in this cohort study. This was because the distribution of values for the groups approached the normal distribution with varying degrees of closeness (histograms will be presented where illustrative). It was important to use the t-test in this univariate analysis since the variables were later used in a form of multiple linear regression, which relies on distributions similar to the t-test. Fortunately the t-test proved robust since the p - values derived from the parametric and non-parametric tests were very similar indeed. The only minor exception was haemoglobin and this will be discussed.

3.5.2 Categoric Variables

The positive response rate for questions about recent pregnancy (n = 2), and rheumatoid nodules (n = 1) was too low to permit analysis.

	No SLS	Pos PS	Risk	Conf-Int	Sens %	Spec %	Chi^2	p
Family History	3	3	1.14	0.48 - 2.70	10	92	0.02	0.88
Rash	6	2	0.53	0.24 - 1.16	7	83	0.66	0.42
RA Latex	4	23	5.31	2.9 - 9.6	79	89	30.0	$4x10^{-8}$

Data complete for first two variables: SLS n = 36, PS n = 29
RA latex - one missing value: SLS n = 35, PS n = 29

Table 3.4 Clinical and Laboratory: Categoric variables

Family History: Patients were asked if there was any history of inflammatory arthritis in their families. The clinical impression was that the answers were vague and very few patients could be considered as having a positive family history. This question did not discriminate between the groups (Table 3.4).

Rash: A few patients gave a positive history of a new and generalised rash within days of the onset of the arthritis. In all cases this was a mild erythematous macular rash which had resolved by the time of first clinic visit. A history of rash did not discriminate between the groups (Table 3.4).

RA Latex: Contrary to the preconception (page 61) that a simple positive or negative RA Latex result was sensitive but not specific (and so only useful as a screen leading to the Rose-Waaler test), this test emerged as one of the most powerful predictors of PS (Table

3.4). The Relative Risk of developing PS for patients with a positive test was 5.25 (CI 2.9 - 9.5) and as can be seen from the Table the Specificity is actually higher than the Sensitivity when used in the particular setting of recent onset polyarthritis. The value of the test is also seen when it is admitted to the multivariate analysis (page 109).

3.5.3 Ordinal Variables

	n SLS PS	Med SLS PS	Range SLS PS	Z	2-tailed p Mann-Whitney
EMS severity	36 29	2 2	0 - 3 0 - 4	2.22	0.026
Pain	36 29	2 2	1 - 3 1 - 3	0.70	0.48
ANA	29 27	0 0	0 - 1600 0 - 400	0.12	0.90
Rose Waaler	35 29	0 16	0 - 64 0 - 4096	4.1	$4x10^{-5}$

Table 3.5 Clinical and Laboratory: Ordinal variables

EMS = early morning stiffness. ANA = antinuclear antibody.
ANA and Rose-Waaler expressed as reciprocal of dilution titre.

Early Morning Stiffness (EMS) Severity: Although Table 3.5 indicates a statistically significant increase in the PS group, the analysis is based on a crude ordinal scale. It was found to be very difficult to decide on a grade for the patients' description of their EMS and the scale should be seen as very crude indeed. The identical medians and near-identical ranges further question the biological usefulness of this variable in predicting persistence when in the early stages of synovitis. It perhaps has a descriptive value in indicating that even of those patients who subsequently recovered, many described their

stiffness in terms similar to those used by the patients whose symptoms were to persist, emphasising similarity rather than difference.

Pain: The same comments apply as for EMS, since here again the score was based on a crude ordinal scale of pain severity, by patients' description. No difference was seen between the PS and SLS groups (Table 3.5).

ANA: The antinuclear antibody did not predict persistence when using the results as the reciprocal of the dilution titre, in the ranking test (Table 3.5). Since this variable was to be admitted to the multivariate analysis, it was decided to convert it to a categoric variable as well: a titre of less than 100 scoring 0 and titres of at least 100 scoring 1. Again there was no predictive value in the univariate analysis (Table 3.6).

	n SLS	Pos PS	Risk	Conf-Int	Sens	Spec	Chi^2	p
RWaaler	2	14	2.8	1.7 - 4.7	48	94	15.3	$9x10^{-5}$
ANA	5	4	0.9	0.3 - 2.3	15	83	0.06	0.80

Table 3.6 ANA and Rose Waaler as Categoric Variables

Rose Waaler: There is uncertainty about the biological significance of single increments in the dilutional titre of the Rose-Waaler, so this variable was first analysed as the reciprocal of the titre by the ranking method and shown to be raised in the PS group (Table 3.5). It was then analysed as a categoric variable (positive titre > 1:16) in the Chi Squared test. It was again positive statistically more often in the PS group (Table 3.6). It should be noted however,

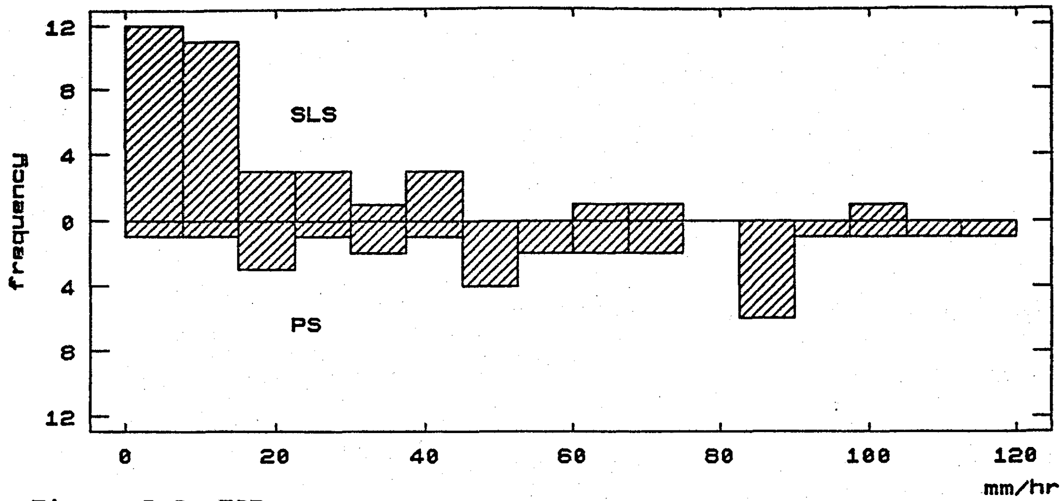


Figure 3.9 ESR

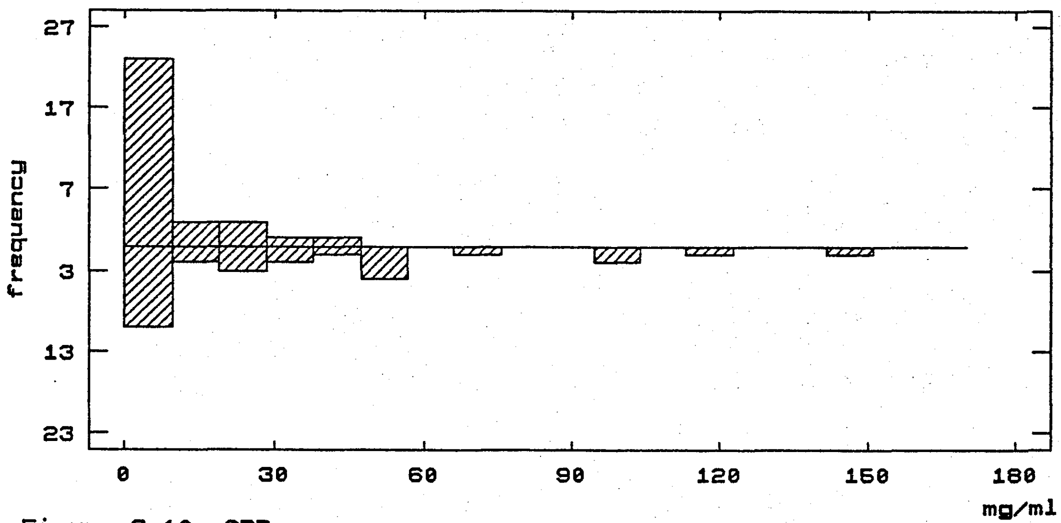


Figure 3.10 CRP

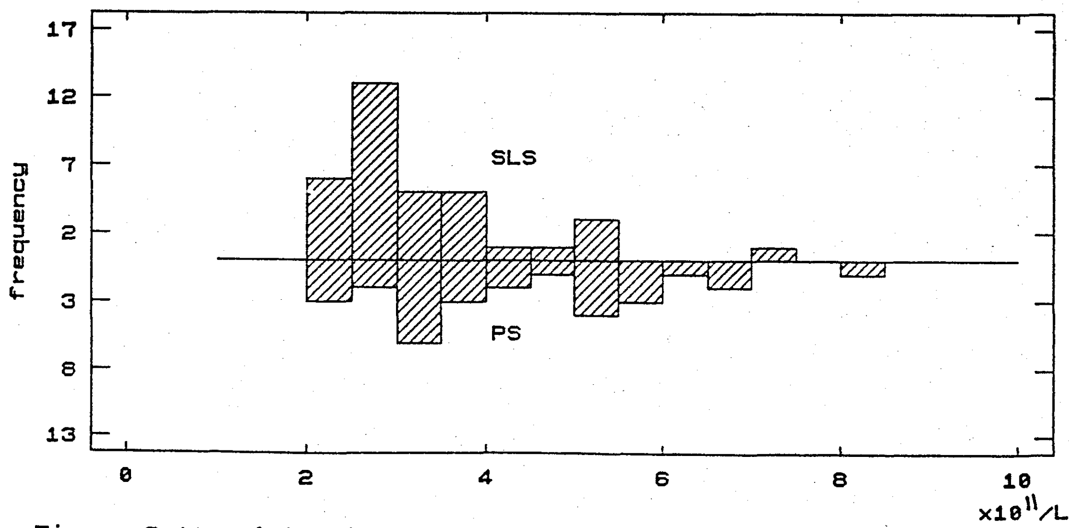


Figure 3.11 Platelets

that a clinician faced with a new patient with recent onset synovitis, will be disappointed by the Sensitivity of only 48% in that stage of disease. This Sensitivity was much lower than that for the RA Latex (79%), while gaining little in Specificity (94% as against 89% for RA Latex).

3.5.4 Interval variables

ESR: Of the interval variables, the erythrocyte sedimentation rate was the most clearly predictive of PS, when assayed at first visit (Tables 3.7 and 3.8). However the similar ranges in both groups (Table 3.8 and Figure 3.9 opposite) is reflected in the ESR only accounting for a rather small proportion of the variability in outcome as will be discussed in the section on multivariate analysis. In clinical terms it would not be possible to predict the group into which a patient would fall if the first visit ESR fell in the 20 to 40 range and indeed patients with ESRs as high as 70 subsequently underwent spontaneous remission.

The C-reactive protein (CRP) and the Platelets behaving as acute phase reactants also showed a strong statistical difference (Table 3.7) with the PS group having a higher mean level of each. This difference shown by the t-test was entirely supported by the non-parametric analysis (Table 3.8). Once again it is important to note from the ranges of these variables (Table 3.8 and Figures 3.10, 3.11) that some of the SLS patients had, at first visit, significantly elevated levels which reflected the clinical disease severity.

The Inflamed Joint Number included any joint (small joints grouped together as one, page 72) which was tender and/or hot and/or swollen.

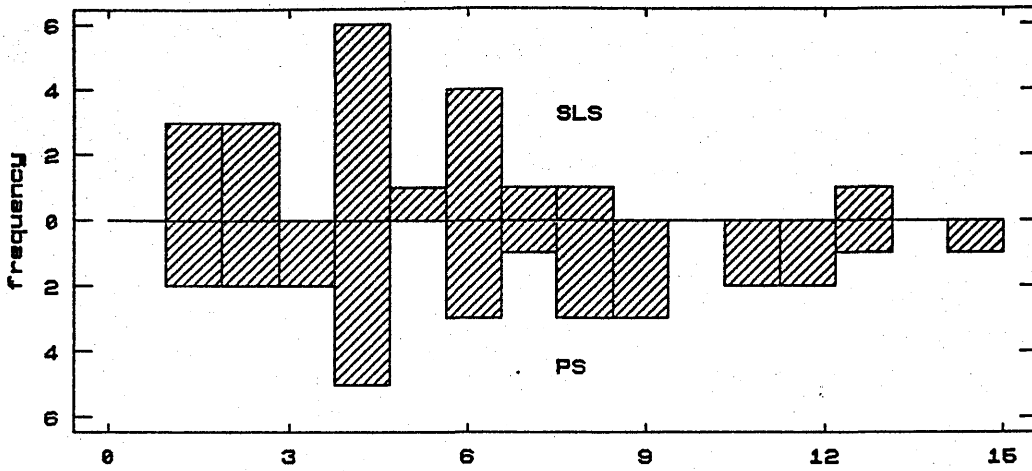


Figure 3.12 Inflammed joint number

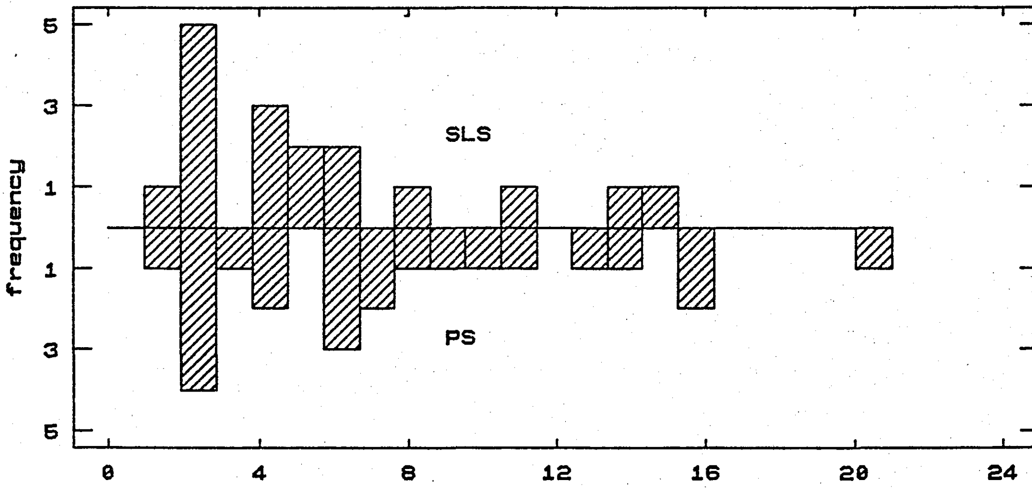


Figure 3.13 Ritchie

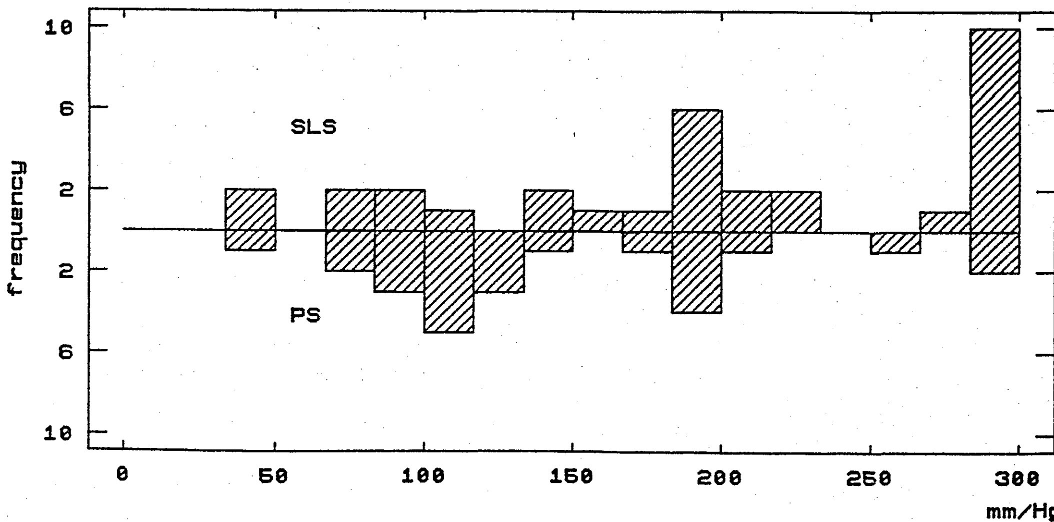


Figure 3.14 Grip strength

Since swelling was included and this has already been shown in the section on pattern of joint involvement (page 94 and Appendix 1) to be predictive of persistence, it is not surprising that looking at the data assembled as here also shows significantly higher scores in the PS group (Tables 3.7, 3.8, Figure 3.12).

The Ritchie Articular Index[112] also shows statistically higher scores in the PS group (Table 3.7) but contrasts slightly with the Inflamed Joint Number in relying on joint tenderness which, in the raw joint data (page 94 and Appendix 1) was rather less useful than joint swelling. Once again however, attention is drawn to the high upper limit of the range of the Ritchie Index for the SLS group (Table 3.8 and Figure 3.13) indicating the clinical disease severity in some of those patients.

The Grip Strength while significantly worse (ie lower) in the PS group (Tables 3.7, 3.8 and Figure 3.14), again indicates that patients from both the PS and SLS groups were affected at the time of first clinic visit. The observed difference between the means of the two groups is of clinically meaningful magnitude and would remain so even if the "infinitely large study" revealed a "true" value approaching the lower end of the 95% Confidence Interval (Table 3.7).

In contrast, for Haemoglobin the observed difference in the means of the two groups (both of which lie within the normal range) would not be clinically relevant even at the upper end of the Confidence Interval and in the t-test the statistics concur with clinical interpretation (Table 3.7). Non-parametric ranking analysis of the haemoglobin (Table 3.8) finds a statistically significantly lower level in the PS group (tending to reflect worse disease severity) but

the clinical relevance of the difference in the medians (0.9 G/dL) is dubious.

The peripheral venous White Blood Cell count (WBC) did not depart from the laboratory's range for the normal population and was not significantly different between the PS and SLS groups.

Early Morning Stiffness (EMS) duration: Both groups of patients reported lengthy periods of morning stiffness with no significant difference being seen between the groups (Table 3.7, 3.8).

	Mean SLS	Mean PS	SD SLS	SD PS	Conf-Int	p
EMS duration (mins)	177	222	305	331	-204 to 114	0.57
Ritchie	2.58	5.86	3.99	5.83	-5.8 to -0.7	0.009
Inflamed joint no.	2.50	6.27	3.10	4.10	-5.6 to -1.9	$8x10^{-5}$
Grip strength mm Hg	201	147	86	70	11.7 to 97.9	0.0136
Haemoglobin G/dl	13.6	13.0	0.97	1.59	-0.07 to 1.28	0.063
WBC 10^9/L	8.25	9.64	2.7	2.9	-2.8 to 0.04	0.056
Platelets 10^9/L	334	437	107	154	-172 to -34	0.0027
ESR mm/1st hr	19	60	21.3	31.3	-54 to -26	$1x10^{-7}$
CRP mg/L	9.6	36	10.2	39.6	-43 to -11	0.0006

Table 3.7 Interval variables: t-test

SD = standard deviation; Conf-Int = 95% confidence interval for the difference in the means; Missing values, see Table 3.8 for number of patients included for each variable.

	n SLS PS	Med SLS PS	Range SLS PS	Z	2-tailed p
EMS duration	36 28	60 60	0 - 998 0 - 998	1.2	0.2
Ritchie	36 29	0 4	0 - 15 0 - 21	2.7	0.007
Inflamed joint no.	36 29	1 6	0 - 13 0 - 15	3.8	0.0001
Grip mm HG	32 24	200 125	38 - 300 50 - 300	-2.5	0.0134
Haemoglobin G/dl	36 29	13.5 12.6	11.5 - 15.8 9.9 - 16.4	-2.1	0.033
WBC 10^9/L	36 28	8.3 9.2	3.4 - 14.8 5.0 - 17.3	1.7	0.09
Platelets 10^9/L	35 28	290 405	207 - 703 220 - 812	2.8	0.0047
ESR mm 1st hr	36 29	11.5 57.0	2 - 98 4 - 114	5.0	$4x10^{-7}$
CRP mg/L	31 27	4 19	4 - 46 4 - 150	3.6	0.00033

Table 3.8 Interval variables: Mann Whitney

Med = Median; WBC peripheral blood white cell count; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein.

3.6 Joint Pattern, Clinical and Routine Laboratory Results: Summary

From the apparent complexity of statistical significance, range overlap and clinical interpretation, a clear picture emerges. Irrespective of whether symptoms were to persist or remit, the patients in this study appeared to have very similar problems at their first clinical visit. The similarities resided particularly in those variables described subjectively by the patients. The similarities existed but were reduced when the patients were examined by the clinician, arguably a more "objective" assessment, and it was

in those variables where the strongest statistical differences were found. This may be because patients unintentionally embellished with recent memory, their interpretation of an abnormality being "currently present".

Such speculation should not detract from the difficulty which the single clinical observer (often in consultation with colleagues) experienced in trying to make management decisions for these patients. Without the benefit of the retrospective analysis of the first visit data from all the patients, it was difficult to decide whether an individual patient's disease would persist or remit. In practice, prognosis and intervention were decided over several weeks of observation. The univariate analysis presented above would not help to accelerate that process greatly because of the overlap between the groups. In an attempt to improve on prediction of outcome at the earliest opportunity for intervention, the next section presents the multivariate analysis which was carried out to see if a combination of measurements might account for a higher proportion of the variability in that outcome.

3.7 Multivariate Analysis Results

This section studies the relationships between individual variables listed in the previous sections (pattern of joint involvement, clinical and routine laboratory variables) and between them and the patient's outcome ie SLS or PS. From the large list of variables recorded it is likely that a much smaller list of variables can be derived to explain as much of the variability in the outcome as does the complete list. Whatever number of variables used, if the amount of variability in outcome explained by them is large then those variables are effective in discriminating between the groups. If the amount of the variability in outcome (PS or SLS) explained is small, then the groups are similar at first visit in terms of the variables used. To put that in biological context, if the variability accounted for by the variables in this study is small, then patients with SLS start out appearing very similar to those with PS.

3.7.1 Statistical Analysis

The selection of variables was carried out by a form of multiple linear regression analysis called stepwise variable selection using the statistical package Statgraphics[134] on a personal computer. In this method the dependent variable, outcome, was called diagnosis and set at 1 for SLS and 3 for PS. These values were chosen for historical reasons and although by convention 0 and 1 might have been chosen, it makes no difference to interpretation. The absolute values of the results do have to be interpreted with caution. The coefficient in the tables refers to the slope of the fitted regression line for that variable: a positive slope for a variable indicates that the patient suffering that symptom or sign is more likely to have PS than SLS.

The programme conducts an analysis of linear regression on the dependent variable (diagnosis) for each independent variable in turn. The variable which explains the most of the variability in diagnosis is selected first and entered into the model. While controlling for this selected variable, the regression analysis is recalculated for the remaining variables. In effect those remaining variables are invited to contribute further to explaining the variability in diagnosis. Many of those remaining variables will be excluded, either because they simply do not discriminate between the groups or because, although they do discriminate, they also correlate very closely with one of the already selected variables. That is, any variability which such a measure could have accounted for, has already been explained in the model.

A degree of caution is required in interpreting the model since in addition to one or two strong variables (others having been excluded by their close correlations) there may be selected some variables for which univariate analysis revealed rather weak differences between the groups. However they happen to measure a slightly different part of the variability in outcome and are accordingly added to the model. The biological significance of such variables needs to be assessed carefully.

The number of candidate variables is very large and to allow the programme to handle them they were broken down into sections, with the selected, strongest variables from each section model being carried forward to the next modelling stage. The three preliminary sections were: Joint pattern, upper limb; joint pattern lower limb; clinical/routine laboratory variables. Particularly among the weaker variables there is a slight risk that the sectioning and sequence of

the variable selection may influence the choice of variables. To check this the sequence of selection was varied. For example the "best of upper limb variables" were added to "all of lower limb variables" and then the model was rebuilt using "best of lower limb variables" and "all of upper limb variables". The effect as seen below was detectable but insignificant in biological terms. However, this does raise the important point that the variable selection presented below need not be the ultimate in biological usefulness. For example, this study might select a variable which is strong in these particular statistical terms and yet reject another variable because it accounted for only slightly less of the variability in outcome. If the rejected variable had a lower coefficient of variation between observers it might actually be more clinically useful. Only a single observer was used in this study.

3.7.2 Stepwise Selection of Upper Limb Variables

All of the upper limb variables as listed in Appendix 1, Table A1.1 were entered for consideration in the model. Data for all of these variables was complete for all 65 patients. Wrist swelling judged by the clinician as present or absent was the strongest of these variables and accounted for 24% of the variability in outcome (Table 3.9). Its presence at the patient's first visit was positively associated with an outcome in the PS group. The presence of any DIP involvement was negatively associated with an outcome in the PS group and accounted for a smaller additional fraction of the variability. This pattern of smaller and smaller increments in the explained variability continued with the addition in turn of diminished shoulder range of movement, TMJ pain, and PIP stiffness. The coefficient of determination (R^2), adjusted for the number of independent variables in the regression, was 0.468 meaning that some

47% of the variability in outcome (SLS or PS) was accounted for by this combination of variables.

Step	Variables in Model	Coefficient	F-Remove	R^2 Adjusted
1	Wrist swelling	1.02	20.90	0.24
2	Wrist swelling	1.07	26.21	
	DIP involvement	-1.12	10.14	0.33
3	Wrist swelling	0.93	20.82	
	DIP involvement	-1.07	10.44	
	Shoulder ROM	0.71	8.27	0.40
4	Wrist swelling	0.86	18.26	
	DIP involvement	-1.01	9.74	
	Shoulder ROM	0.79	10.55	
	TMJ pain	0.97	4.60	0.43
5	Wrist swelling	0.91	21.46	
	DIP involvement	-0.97	9.58	
	Shoulder ROM	0.87	13.29	
	TMJ pain	1.15	6.58	
	PIP stiffness	-0.41	4.55	0.468

Table 3.9 Stepwise Variable Selection: Upper Limb

Abridged version of information available at each stage: fuller details for final modelling stage will be presented.
The working statistic F-Remove (and F-Enter) set at 4, to equate to 95% significance level.

3.7.3 Stepwise Selection of Lower Limb Variables

The above procedure (3.7.2) was repeated for all of the lower limb variables as listed in Appendix 1, Table A1.1, with data complete for all 65 patients. Only diminished range of movement at the knee was selected and this accounted for only 15% of the variability in outcome (Coefficient 1.0, F-remove 12.17).

3.7.4 Combining Upper and Lower Limb Variables

The stepwise variable selection procedure was continued by offering

to the next modelling stage all of the lower limb variables and the best of the upper limb variables, ie those selected above (3.7.2).

Step	Variables in Model	Coefficient	F-Remove	R^2 Adjusted
7	Wrist swelling	0.87	21.28	
	TMJ pain	1.36	10.33	
	DIP involvement	-0.93	10.02	
	Shoulder ROM	0.72	9.6	
	Knee ROM	0.55	5.80	
	Hip pain	-0.57	5.23	
	PIP stiffness	-0.93	4.49	0.540

<u>Table 3.10</u> Stepwise Variable Selection: Upper and Lower Limb

Abridged analysis information showing values for last step of this stage of modelling.

As can be seen from the above (Table 3.10) a rather lengthy list of variables emerges and again during the process of selection, each subsequent variable recruited to the model added smaller and smaller increments to the amount of variability that the model accounted for. In all some 54% (from R^2 adjusted = 0.540) of the variability in outcome (ie PS or SLS) was accounted for by this list of joint involvements. The list comprises not only the best of the upper limb variables (from 3.7.2) and the best of the lower limb variables (from 3.7.3) but hip pain emerged as having a negative association with PS. This indicates the slightly different parts of variability explained by differing combinations of variables. Hip pain inclusion only emerged because slightly more patients in the SLS group (n=6) had hip pain than in the PS group (n=3), and it only accounted for less than 3% of the variability. Indeed in univariate analysis, the difference in numbers of patients with hip pain was not statistically different between the SLS and PS groups (Appendix 1, Table A1.1).

Because of the meticulous nature of the statistical analysis, it is possible that some of the weaker upper limb variables which failed to be selected above (section 3.7.2) might have ousted the weaker lower limb variables, had they been given the opportunity. Even though this was unlikely to be of biological significance, the analysis combining upper and lower limb variables was repeated, this time with the best of the lower limb and all of the upper limb variables. The only difference seen was that PIP stiffness, which had been the last variable selected for Table 3.10, was ousted by PIP swelling as reported by patients. This indicates that once the other variables had been selected for Table 3.10, the symptom of PIP swelling accounted for more of the remaining variability than PIP stiffness. For all that statistical pedantry, the Coefficient and F-value were virtually identical for PIP stiffness and swelling symptoms (and were therefore interchangeable) and the Coefficient of Determination for the model remained at 0.540.

3.7.5 Stepwise Selection of Clinical and Routine Laboratory Variables

All of the variables of section 3.5 were included in the first run of this analysis. From section 3.5 it can be noted that some patients had missing values for some of the variables, and any such patients with even one missing value were excluded from the first run of the analysis. Of the original 65 patients, only 44 patients could be included at this stage. The ANA and Rose Waaler results were entered as categoric variables as above (section 3.5.3).

When the variables are entered into the model programme, the amount of variability in outcome that each individual variable accounts for is calculated independently of all the others (and the strongest on this basis is entered first into the model). For age and sex, the

amount of variability which each could explain independently did not reach statistical significance: F-values 1.24 and 1.08 respectively (F < 4 equates to significance < 95%). The RA Latex test and the EMS severity emerged to account for 52% of the variability in outcome (11) in this set of patients selected for their complete data record.

Step	Variables in Model	Coefficient	F-Remove	R^2 Adjusted
1	RA Latex	1.36	36.36	0.45
2	RA Latex	1.25	33.87	
	EMS Severity	0.28	7.33	0.52

Table 3.11 Stepwise Variable Selection: Clinical and Laboratory (a) 44 patients included (see also Table 3.12)

It was possible to discern during the analysis that some variables were clearly contributing nothing to the variability either because they correlated closely with a selected variable (eg Rose Waaler with RA Latex, Pearson product-moment correlation 0.68, p<0.0001; CRP with ESR, 0.75, p<0.0001) or because they in any case did not discriminate between the groups (eg on univariate analysis WBC, ANA). These variables, shown to be rejected when offered to the statistical program, included some with many missing values so the modelling was retried without them. Thus CRP, ANA, Platelets, WBC and Grip strength were excluded for the next run of the modelling process. In view of its historical importance the Rose Waaler and RA Latex were included despite one patient having a missing value for these variables. An EMS duration result was also missing but only for one patient, so this variable was included. 63 patients (SLS 34, PS 29) were therefore eligible for this stage of analysis.

Variables in Model	Coeff.	F-Remove	Variables not in Model	F-enter
			RA Latex	51.80
			ESR	35.47
			Rose Waaler	17.77
			Inflamed Joint No.	16.11
			Ritchie	6.47
			EMS severity	5.63
			Age	5.23
			Duration at 1st visit	4.2
			Sex	3.97
Step 0:			Haemoglobin	2.76
			Pain score	0.46
R^2 Adj. = 0			EMS duration	0.29
RA Latex	1.37	51.80	ESR	5.99
			EMS duration	5.06
			Inflamed Jt. No.	2.59
			EMS severity	2.32
			Duration at 1st visit	1.1
			Age	0.51
			Ritchie	0.51
			Haemoglobin	0.49
Step 1:			Pain score	0.30
			Sex	0.29
R^2 Adj. = 0.45			Rose Waaler	0.10
RA Latex	0.99	17.16	EMS duration	2.85
ESR	0.009	5.99	EMS severity	2.19
			Inflamed Jt. No.	1.7
			Duration at 1st visit	1.5
			Pain score	0.50
			Age	0.25
			Ritchie	0.16
Step 2:			Rose Waaler	0.06
			Haemoglobin	0.04
R^2 Adj. = 0.49			Sex	0.00

No further variables selected. Number of patients = 63

Table 3.12 Stepwise Variable Selection: Clinical and Laboratory (b)

The data presented in Table 3.12 are amongst the most revealing obtained from the entire study, in terms of the inter-relationships between individual variables. At the top of the Table, at step 0, the independent contribution of each variable is ranked by the F-enter

value. Quite unexpectedly the RA Latex emerged as the strongest variable. When the RA Latex was entered into the model (step 1) accounted for 45% of the variability in outcome. This is better than any other single variable in this chapter and as good as a fairly long lists of joints in combination (see above).

As soon as the RA Latex was entered into the model, the F-enter value for the remaining variables fell sharply. This is because at least some of the variability which they might have accounted for was taken by the RA Latex. This is particularly true of the Rose Waaler and reflects its close correlation with RA Latex (correlation coefficient 0.68, $p<0.0001$). With only one further step, the insertion of ESR to the Model, the variability accounted for rose to 49% and the remaining variables failed to be selected ($F < 4$).

3.7.6 Combining Joint Pattern and Clinical and Laboratory Variables

To the final modelling stage were carried forward those variables selected from the joint involvement data (section 3.7.4) and from the clinical and routine laboratory variables (section 3.7.5). From the latter section the variables inserted were all 12 of those from step 0 of Table 3.12. 63 patients were entered for this stage of the modelling, those 2 with missing values being excluded. As might have been predicted from the above Models, the strongest variable was RA Latex, accounting for its 45% of the variability (Table 3.13). This time however, the next variable selected was the (diminished) knee range of movement, ousting the ESR. Together those two selected variables accounted for 52% of the variability. The subsequent inclusion of DIP involvement and EMS severity only increased the variability accounted for to 57%. Given the already stated dubious reliability of the measurement of EMS severity, it might be best not

to include that variable in clinical practice. Similarly "DIP involvement" included patients' subjective complaints and was only positive in 6 of the 65 patients. Its prominence in the multivariate analysis was because all of those 6 patients were in the SLS group.

Step	Variables in Model	Coefficient	F-Remove	R^2 Adjusted
1	RA Latex	1.37	51.81	0.45
2	RA Latex	1.25	47.22	
	Knee ROM	0.69	9.85	0.52
3	RA Latex	1.89	44.14	
	Knee ROM	0.69	10.47	
	DIP involvement	-0.63	4.60	0.55
4	RA Latex	1.11	39.4	
	Knee ROM	0.71	11.50	
	DIP involvement	-0.60	4.45	
	EMS severity	0.17	4.15	0.57

Table 3.13 Stepwise Selection: Joint and Clinical and Laboratory

17 variables offered to the model (see text): no further variables were selected. Number of patients = 63

Emphasising the marginal biological significance of DIP involvement, the statistical significance was also marginal: the 95% confidence intervals for the coefficient estimates for DIP involvement and EMS severity were very close to including zero (Table 3.14).

Variable	Coefficient	95% Conf. Interval	t-value	sig.level
RA Latex	1.115	0.760 to 1.471	6.28	<0.01
Knee ROM	0.709	0.291 to 1.128	3.39	<0.01
DIP inv.	-0.603	-1.176 to -0.030	-2.11	0.03
EMS sev.	0.169	0.003 to 0.335	2.04	0.04

Table 3.14 Coefficient details for variables in Table 3.13

3.7.7 Summary of Stepwise Variable Selection

For several variables, univariate analysis was able to indicate that there were statistically significant differences in the values returned at first clinic visit by patients whose synovitis was destined to remit (SLS), compared to the values for patients whose synovitis persisted (PS). However, the biological significance of those differences was less because of the considerable overlap in values obtained from the two groups. Furthermore, it can be argued that when conducting many univariate analyses, correction in the level of statistical significance has to be made for the number of independent variables recorded within the study. In analysing the pattern of joint involvement where 47 variables were recorded on each patient, this means that strictly only those variables showing difference between the groups to a p-value of 0.001 or less upon isolated analysis should be considered statistically significant at the 95% level when considered in the context of the number of variables recorded. However, the variables are clearly not strictly independent of each other with, for example an observed (by the clinician) joint swelling likely to be accompanied by a complaint (from the patient) of joint swelling. Furthermore, the presentation of the isolated univariate analysis results is valid if considered as components contributing to the understanding of pathogenesis and if interpreted with caution. When the routine laboratory variables are included and the correction is made for the number of variables, the essential finding in the data is not changed, with the wrist swelling, RA Latex, Rose Waaler, inflamed joint number, ESR and CRP ("major variables") remaining significantly different between the groups. Such strict univariate analysis is only one part of the interpretation, however, since multivariate analysis, in seeking to

account for variability, may recruit variables ("minor variables") which on their own are not significant predictors of outcome. In addition multivariate analysis can remove statistically significant but redundant variables from the selected data set.

It is likely that combining univariate and multivariate analyses would, from several independent groups of patients with the same disease process, select similar "major variables" but with differences in "minor variables". In that context it is interesting to note that the present study, in identifying for example RA Latex, wrist swelling and the acute phase proteins as being important in predicting outcome, is consistent with the ARA criteria for the diagnosis of RA[1,55] and other studies of recent onset synovitis[140] but there is considerable divergence in the minor variables.

Multiple regression analysis, in the form of stepwise variable selection, has permitted a description of how much of the variability in outcome could be explained by the variables included in this study and recorded at the patient's first clinic visit. The final Model selected (section 3.7.6) might be used to explain up to 57% of that variability, although if the two weakest of the four selected variables are considered too unreliable for future widespread clinical use, then 52% would be a more realistic working figure. The possibility of avoiding a variable which might be prone to inter-observer error (eg diminished knee range of movement) should be considered. A combination of RA Latex and ESR (Table 3.12) would leave little inter-observer disagreement and still result in 45% of the variability in outcome being explained.

One possible use for these combinations of variables would be in the field of therapeutic intervention studies. It has been argued that treatment initiated in the very early stages of RA might be more effective than treatment which is delayed for some months (page 50). One of the major ethical considerations in early disease is that some patients may remit spontaneously and if entered into a trial, would be needlessly exposed to potentially toxic drugs. From the present study the RA Latex alone offers 89% specificity but if a positive result is combined with an ESR of at least 30mm/hr or with positive knee ROM involvement, the specificity rises to 94% and 97% respectively (Table 3.15). This gain is of course at the expense of sensitivity but until a treatment was proven to be of great benefit, this would be an acceptable loss at least using ESR, although the loss using knee ROM is probably impracticably large.

	n SLS	Pos PS	Risk	Conf-Int	Sens %	Spec %	Chi^2	p
RA Latex + ESR>30mm/hr	2	20	4.33	2.52 - 7.44	69	94	28.1	$1x10^{-7}$
RA Latex + Knee ROM	1	7	2.23	1.21 - 4.11	24	97	6.6	0.01

Table 3.15 Combining RA Latex with ESR or Diminished Knee ROM

A further loss shown in Table 3.15 is the reduction in relative risk (over RA Latex alone), whichever variable is added to "enhance" the usefulness of the RA Latex. This again arises because of the reduction in sensitivity.

Whichever of these combinations is used, still only around half of the variability in outcome is explained. This suggests that whether destined for SLS or for PS, patients presenting with recent onset polyarthritis are more remarkable for their similarities than for their differences. The single outstanding variable arising from this chapter of the study is the RA Latex. More detailed study of rheumatoid factors in early synovitis will be presented in chapter 5.

CHAPTER 4

RESULTS

T-LYMPHOCYTE STUDIES

CHAPTER 4

RESULTS - T-LYMPHOCYTE STUDIES

4.1 Aims

Since activated lymphocytes are a feature of established disease, the question was posed: would closer study of T-lymphocyte populations in peripheral blood help in predicting disease persistence in patients with recent onset arthritis, if measured on their first visit to clinic?

4.2 Patients selected

The patients admitted to this part of the study were drawn from those 65 with polyarthritis of recent onset as detailed above (Section 3.2). These assays are complex and data on only 28 patients (18 SLS, 10 PS) was available for analysis. Clearly, with small numbers such as these, interpretation of the results should be guarded.

The lymphocyte subsets described below were studied in two phases. All 28 patients' subsets were analysed in the peripheral blood, which includes both active and inactive cells (page 55). Of these 28 patients, only 15 had sufficiently high numbers of activated, (transferrin receptor positive, TFR+) cells for the subsets to be measured within the activated population.

4.3 Statistical analysis

In each group values for each variable were scrutinised for equal variances and a statistically normal distribution. The t-test was then used but because of slight deviations from the normal distribution (histograms will be given where appropriate), the

results were checked using the non-parametric Mann-Whitney ranking test. No discrepancy was found between the results obtained with the parametric and the non-parametric analyses. Only univariate analysis was carried out for this section, particularly in view of the small number of subjects studied. Patients with incomplete data were excluded from the appropriate parts of the analysis.

4.4 Subsets of lymphocytes in whole peripheral blood PBM

4.4.1 CD4:CD8 Ratio

The ratio of CD4 positive PBM to CD8 positive PBM, when measured at the patients' first visit to the clinic, was not different between the two outcome groups (SLS and PS) and was therefore not predictive of persistence (Tables 4.1, 4.2).

4.4.2 CD4 PBM

The number of CD4 positive cells in the PBM fraction of peripheral blood (expressed per millilitre of whole peripheral blood) also failed to show any difference between the SLS and PS groups (Tables 4.1, 4.2).

4.4.3 CD8 PBM

Similarly the CD8 positive cells were not statistically different between the two outcome groups Tables 4.1, 4.2).

	Mean SLS	Mean PS	SD SLS	SD PS	Conf-Int	p
CD4:CD8 PBM ratio	2.54	2.79	1.77	1.17	-1.5 to 1.0	0.70
CD4 PBM 10^6/ml	0.81	0.91	0.36	0.44	-0.4 to 0.2	0.53
CD8 PBM 10^6/ml	0.40	0.36	0.18	0.16	-0.1 to 0.2	0.52
TFR+ 10^3/ml	76	132	45.9	47.9	-95 to -19	0.005
TFR+ % of PBM	4.66	6.99	3.05	2.83	-4.7 to 0.1	0.058

Table 4.1 Lymphocyte Subsets in Peripheral Blood: t-test

SD = standard deviation; Conf-Int = 95% confidence interval for the difference in the means; n = 18 (SLS), 10 (PS). PBM = peripheral blood mononuclear cells.

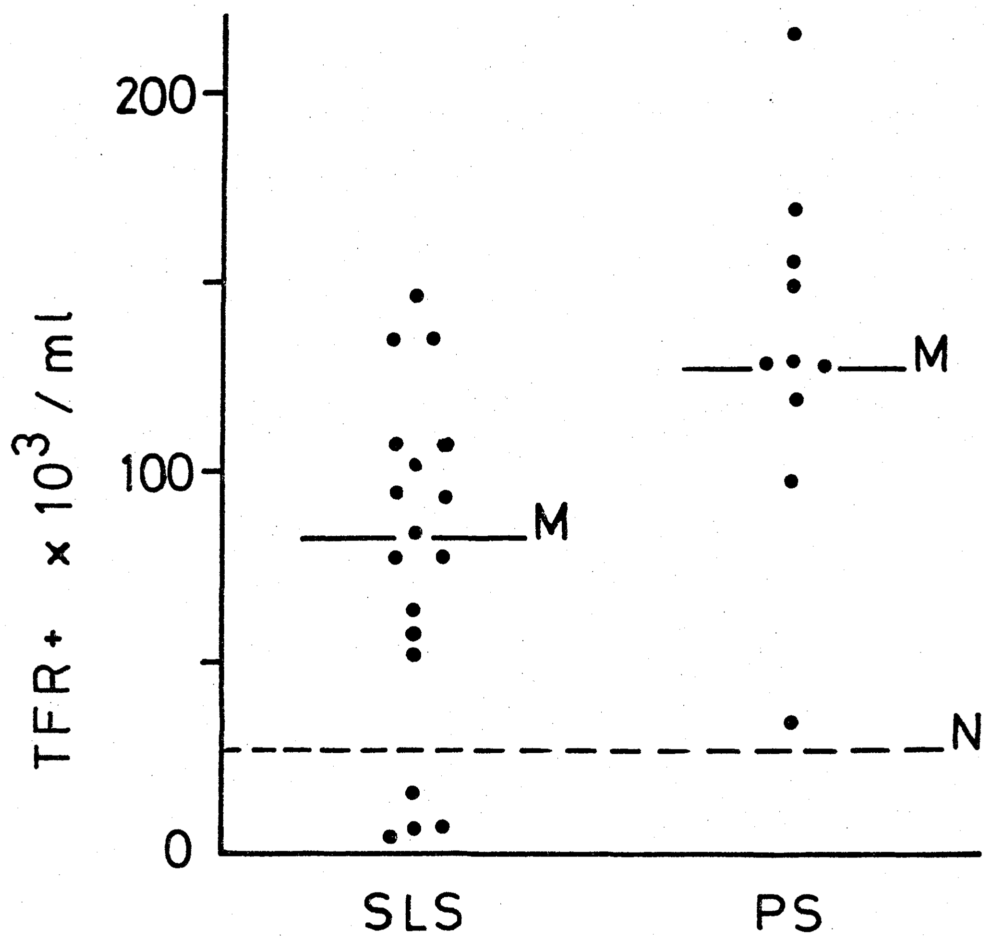


Figure 4.1 TFR+cell numbers
N= normal 95th centile
M= median

	n SLS PS	Med SLS PS	Range SLS PS	Z	2-tailed p
CD4:CD8 PBM ratio	18 10	2.25 2.70	0.45 - 8.66 0.93 - 4.67	0.96	0.34
CD4 PBM 10^6/ml	18 10	0.77 0.80	0.33 - 1.59 0.43 - 1.96	0.55	0.58
CD8 PBM 10^6/ml	18 10	0.42 0.32	0.06 - 0.73 0.12 - 0.72	-0.79	0.43
TFR+ 10^3/ml	18 10	81.5 128.0	2 - 147 34 - 218	2.76	0.0058
TFR+ % of PBM	18 10	5.25 6.95	0.1 - 9.2 2 - 12	1.85	0.06

Table 4.2 Lymphocyte subsets in peripheral blood: Mann-Whitney

Med = Median; PBM = peripheral blood mononuclear cells.

4.4.4 TFR+ cells

The mean number of TFR positive cells was elevated above the upper limit of the normal range[77] (95th centile of control population 27×10^3/ml, in both the SLS and the PS groups (Tables 4.1, 4.2). The range obtained in the SLS group included both normal and supra-normal values, whereas the range for PS was entirely above normal (Table 4.2, Figure 4.1). Furthermore, the TFR+ numbers were significantly higher in the PS group compared with SLS ($p = 0.005$). It was therefore of interest to further characterise this population of activated, TFR+ cells.

4.5 T-cell subsets within the activated TFR+ subpopulation of PBM

It is not possible to know the normal values for the CD4:CD8 ratio and the numbers of CD4+ and CD8+ cells within the activated TFR+

fraction of the PBM because healthy subjects simply do not have sufficiently high numbers of circulating TFR+ cells to allow the necessary double labelling technique. Accordingly the values obtained have to be compared between disease groups (here SLS and PS) and between the values in the activated and in the heterogeneous (active and inactive) whole PBM population.

4.5.1 CD4:CD8 ratio within the TFR+ subpopulation

The ratio of CD4+ to CD8+ cells within the activated, TFR+ population was significantly higher in the PS group than in the SLS group (Tables 4.3, 4.4). The mean ratio in the activated population was, in the SLS patients, not significantly different from the mean ratio in the whole PBM for the SLS group. However, it is interesting to note that for PS patients, the CD4:CD8 ratio was significantly higher in the activated TFR+ population compared with the ratio in the whole PBM preparation ($p = 0.024$, by unpaired t-test).

As discussed above (page 55), it might be expected that in RA any increased ratio would be due to a rise in the number of CD4 positive cells. Similarly, it was discussed that recent viral infections, with a fall in the ratio, were thought possibly due to a rise in the CD8 positive population. With the working hypothesis that the PS patients being reported here probably had RA, and that at least some of the SLS patients had their disease triggered by a viral infection, the numbers of CD4+ and CD8+ cells within the TFR+ population, were therefore analysed.

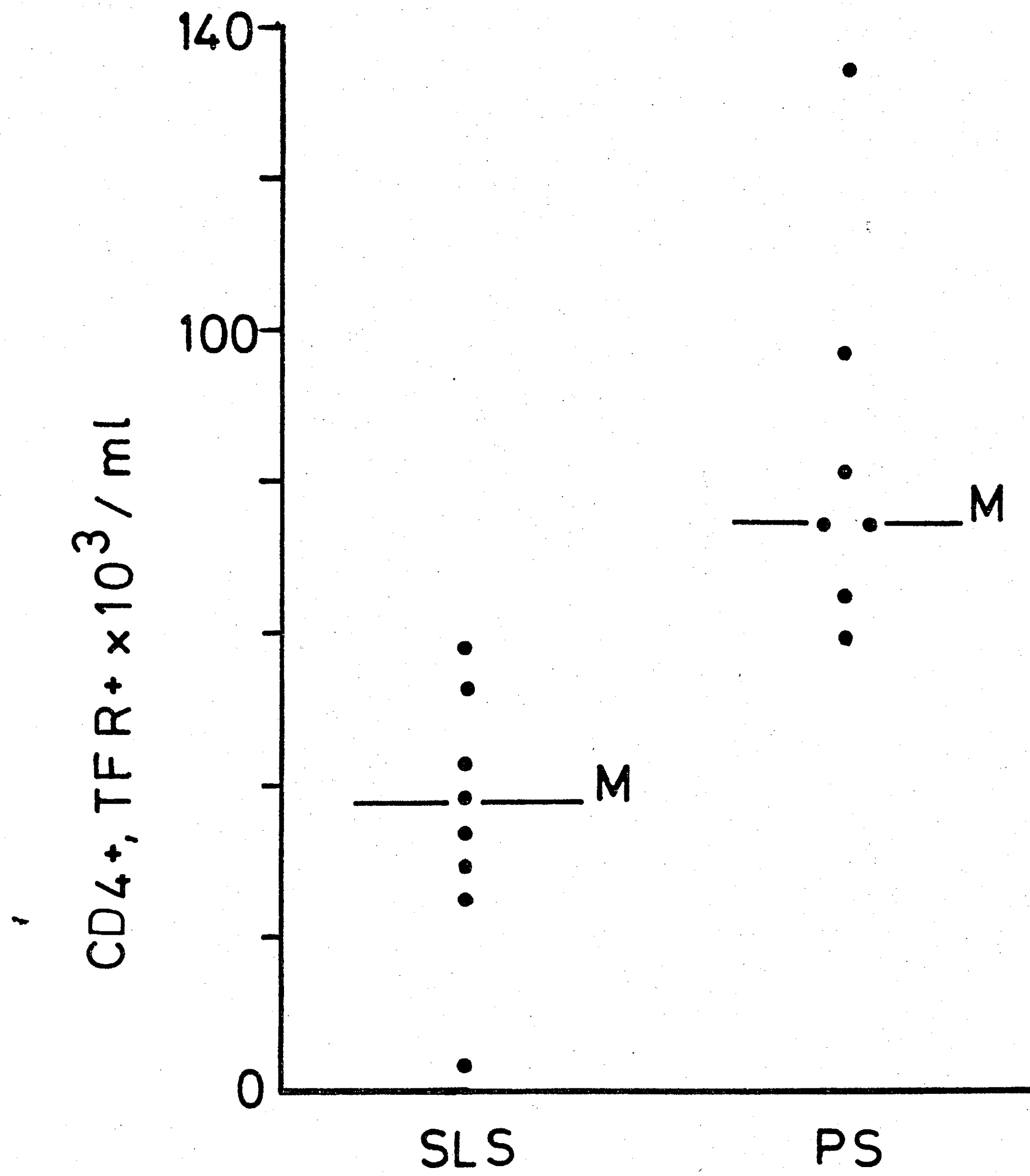


Figure 4.2 CD4TFR double positive cells
M = median

	Mean SLS	Mean PS	SD SLS	SD PS	Conf-Int	p
CD4:CD8 TFR+ ratio	2.09	4.95	1.49	2.37	-5.2 to -0.58	0.014
CD4 TFR+ 10^3/ml	35.7	84.4	17.2	25.6	-73 to -23	0.0007
CD8 TFR+ 10^3/ml	27.5	19.6	18.5	8.4	-8.3 to 24.1	0.32

Table 4.3 Lymphocyte Subsets in TFR+ population in PBM: t-test

SD = standard deviation; Conf-Int = 95% confidence interval for the difference in the means; n = 8 (SLS), 7 (PS); PBM = peripheral blood mononuclear cells.

4.5.2 CD4+ TFR+

The number of CD4+ TFR+ cells was significantly higher in the PS group compared with SLS (Tables 4.3, 4.4). Indeed, the ranges of values obtained for this variable are almost unique in this study in that there is no overlap at all between the SLS and PS groups (Table 4.4, Figure 4.2).

	n SLS PS	Med SLS PS	Range SLS PS	Z	2-tailed p
CD4:CD8 TFR+ ratio	8 7	1.49 4.00	0.61 - 4.33 3.5 - 10.2	2.37	0.018
CD4 TFR+ 10^3/ml	8 7	37.0 75.5	3.12 - 58.3 59.8 - 135	3.18	0.0015
CD8 TFR+ 10^3/ml	8 7	23.3 18.7	0.72 - 49.7 5.88 - 34.9	-0.52	0.6

Table 4.4 Lymphocyte subsets in TFR+ population in PBM: Mann-Whitney

Med = Median; PBM = peripheral blood mononuclear cells.

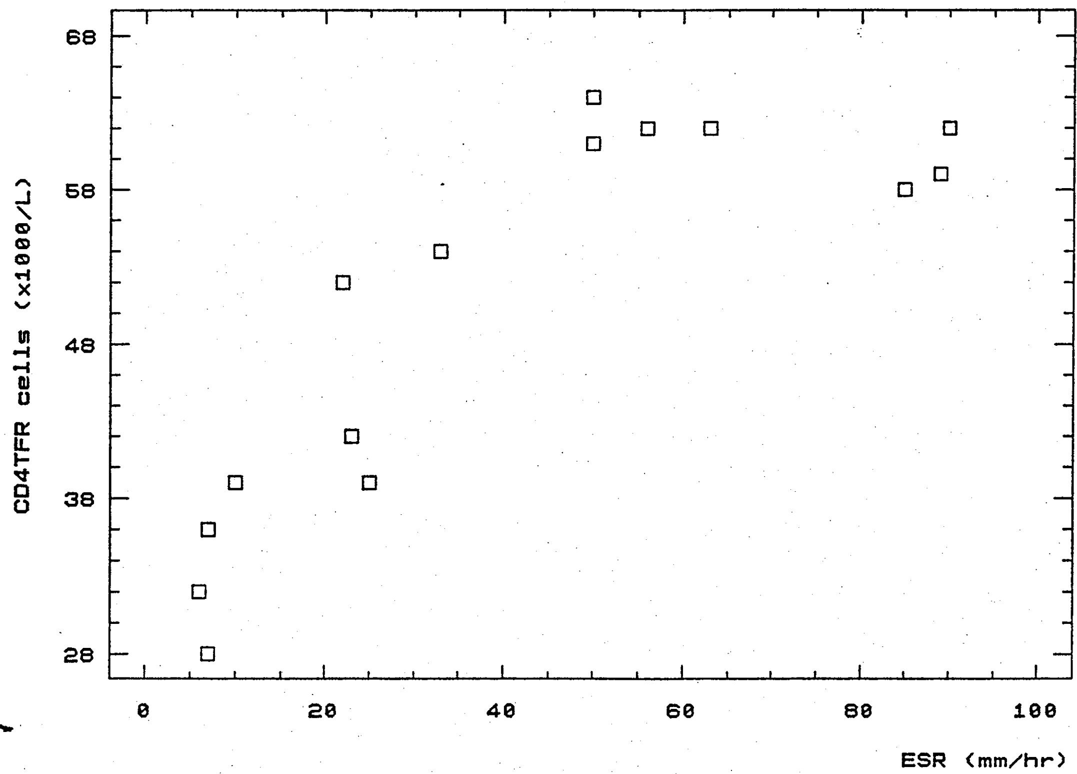


Figure 4.3 Plot of ESR v CD4TFR cell numbers

4.5.3 CD8+ TFR+

In contrast to the CD4+ TFR+ results, the numbers of CD8+ TFR+ cells was not significantly different between the SLS and PS groups (Tables 4.3, 4.4).

4.6 Summary of T-cell population analyses

From the studies in the whole PBM population, which includes both activated and resting cells, it is clear that in patients presenting with recent onset synovitis, the possibilities for predicting persistence of disease lie within the activated TFR+ subpopulation. Both the PS and the SLS patients had elevated numbers of these TFR+ cells.

Practically all of that higher number of TFR+ cells in the PS patients (Table 4.1) was accounted for by the higher numbers of their CD4+ TFR+ cells (Table 4.3). There was no correlation of the activated T-cell numbers with age (Table 4.5). Previous studies in established RA have found no correlation of the activated T-cell numbers with the acute phase response[77] which suggests that the two phenomena are not directly connected. In the present study of patients with early (evolving) disease there is a weak correlation between the ESR and the number of TFR+ cells (Table 4.5) and a much stronger correlation between the ESR and the double positive CD4+,TFR+ cell numbers (Table 4.5, Figure 4.3). These results suggest that the two phenomena (acute phase response and number of activated CD4 cells) can occur concurrently in patients with recent onset synovitis.

		TFR+	CD4+,TFR+
Age			
	Correlation	0.29	0.41
	Significance	0.13	0.13
	n subjects (SLS+PS)	28	15
ESR			
	Correlation	0.45	0.83
	Significance	0.02	0.005
	n subjects (SLS+PS)	28	15

Table 4.5 Activated T-cell numbers: correlation with age and ESR

The most outstanding result was the finding that the ranges of CD4+TFR+ double positive cell numbers did not overlap at all between SLS and PS, unlike all the other variables in this thesis. Unfortunately the assay is very complex and in this form is not a practical proposition for routine screening. Nevertheless, the implications of these results for the understanding of the pathogenesis of persistence will be discussed in chapter 7, when they can be put together with the implications of the B-lymphocyte studies which are presented next.

CHAPTER 5

RESULTS

B-LYMPHOCYTE STUDIES

CHAPTER 5

RESULTS - B-LYMPHOCYTE STUDIES

5.1 Aims

The primary intention of this section of the study was once again to seek variables which, when measured in the earliest stages of synovitis, would be predictive of persistence. The variables used in this section are, however, all derived from assays under development, so the secondary intention was to see if any of them would cast light on mechanisms of disease persistence. Three questions were addressed:

1) Were there higher circulating levels of damaged IgG in PS, resulting in increased drive for the production of rheumatoid factor (RF)?

2) Were there quantitative differences in the levels of RF between the SLS and PS groups?

3) Could qualitative differences in RF production be determined by examining the specificity and idiotypy of the RF in the two groups?

5.2 Patients selected

For section 5.4, IgG as antigen for RF, all 65 patients were of interest. First visit serum samples from 52 of those patients (29 SLS, 23 PS) were available for study in the assay which was performed (blind to the diagnostic coding) by Dr J Lunec.

For section 5.5, serum samples from the first clinic visits of 40 patients (20 SLS, 20 PS) were selected from storage at -20^{0}C, by the clinician (EJT) once the diagnostic outcome had been decided. The samples were then assayed by Dr R Mageed, who was blind to the diagnostic coding. If for any patient there was insufficient serum

for a given assay, that patient was excluded from the relevant part of the data analysis.

5.3 Statistical analysis

The distributions for most of these variables were skewed so the non-parametric ranking test (Mann-Whitney) was used. The exceptions were the IgG Ratio and the Total IgM (see below) where the distributions were more normal, permitting use of the t-test.

As a consequence of the skewed distributions (for both SLS and PS) with most patients having low results and a few (generally PS) having high results, even where there was a statistically significant difference between the groups, interpretation of the biological significance needs to be guarded.

5.4 Immunoglobulin as antigen for RF: free radical altered IgG

In the fluorescent IgG assay the established normal range (the mean of 100 normal subjects +/- 2SD) for the ratio is 4.8 to 9.0%. For both SLS and PS the majority of the patients were found to have values within the normal range (Table 5.1). Accordingly the finding of a statistically significant difference between the means of values for SLS and PS by t-test is of less biological than statistical significance. Using the Chi^2 test to look at the number of patients in each group with ratio results above the upper limit of normal, the assay failed to discriminate between the two outcome groups (Table 5.1).

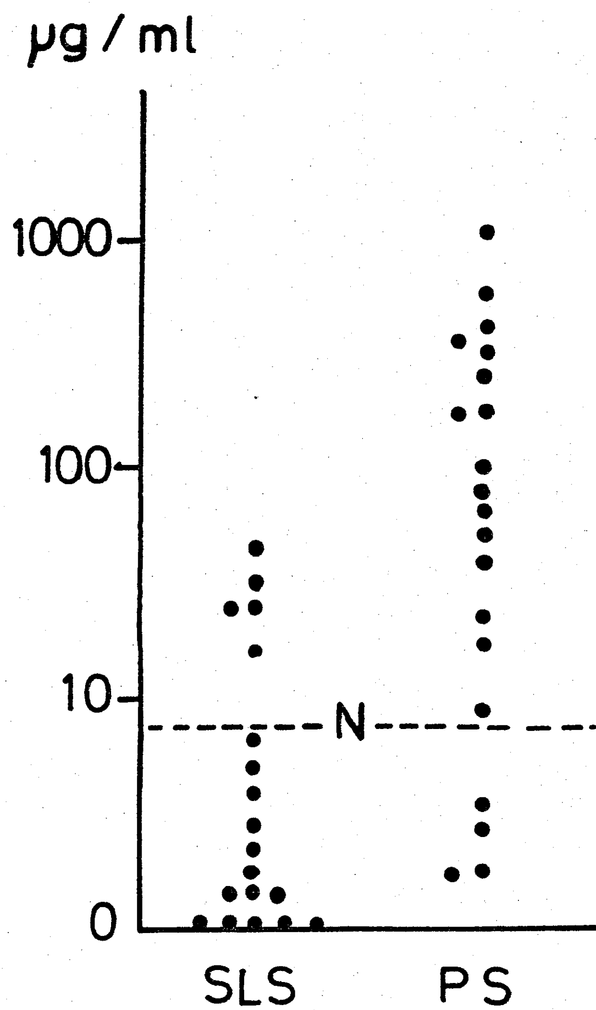


Figure 5.1

IgMRF (Per)

N = normal 90th centile

(i) t-test	Mean SLS	Mean PS	SD SLS	SD PS	Conf-Int	p
IgG Ratio (%) (Normal range: 4.8 to 9.0%)	7.67	5.71	3.29	2.58	0.25 - 3.67	0.025

SD = standard deviation; Conf-Int = 95% confidence interval for the difference in the means; n = 29 (SLS), 23 (PS).

(ii) Above-normal values for the ratio: predictive of persistence?

	Above normal SLS	Above normal PS	Risk	Conf-Int	Sens	Spec	Chi^2	p
Ratio	8	3	0.55	0.2 - 1.4	13	72	1.6	0.20

Table 5.1 Fluorescent IgG : total IgG ratio (%)

5.5 Immunoglobulin as rheumatoid factor (IgMRF)

The RA Latex emerged in chapter 3 as the best of the rather poor predictors of persistence of synovitis. In an attempt to understand the pathogenesis and perhaps enhance the predictive value, both quantitative and qualitative studies of the IgMRF were performed.

5.5.1 Direct binding ELISA: quantitation of total IgMRF

Using Fc-Per as the standard antigen for the IgMRF in the direct binding ELISA, levels of IgMRF were found to be significantly higher in the PS patients compared with either SLS or normal subjects (Table 5.2, Figure 5.1). The upper limit of normal was defined as 8.4µg/ml, the 90th centile of the distribution of values found in normal healthy volunteers (n=15). Using the Chi^2 test to estimate the predictive usefulness in early synovitis, the finding of a supra-normal value carried a relative risk for developing PS of 3.43 (Table 5.3), with sensitivity and specificity comparable to the RA Latex,

but the RA Latex remains the stronger predictor (page 96).
Clearly IgMRF was present at first clinic visit in greater than normal quantity for PS as a group but not in SLS as a group. However some individual SLS patients did have supra-normal levels of IgMRF and some individual PS patients did have normal IgMRF levels. As a result of this overlap, quantitation offers a statistical but not categoric ("all or nothing") distinction between the two outcomes of recent onset synovitis. The next question addressed was whether any qualitative differences in the circulating IgMRF could be found between the normal subjects, SLS patients and PS patients. The IgMRF was examined first by its specificity for IgG of differing subclasses and then by its expression of cross-reactive idiotopes.

5.5.2 Direct binding ELISA: IgG subclass specificity of IgMRF

Instead of Fc-Per, the ELISA plates were sensitized with IgG from subclasses 1, 2, 3 and 4, two different examples of each subclass being used, making a total of eight potentially different target antigens for the polyclonal IgMRF in each patient's serum.
Normal v. SLS: Comparison was made between the normal subjects and the SLS group (Table 5.2, Figures 5.2 to 5.9): both the median values and the Mann-Whitney analysis indicate a trend for the SLS group to have lower levels than normal, of IgMRF of the defined specificities. This trend is consistent using each IgG subclass as antigen, with the exceptions of the IgG1 example Cri and the IgG3 Ren, where the two groups are essentially equal. This trend to lower than normal levels of IgMRF in SLS does not reach statistical significance for IgG1 but does for both examples of IgG2 and for one example each of IgG3 and IgG4. This is perhaps because the normal control population were slightly older (Mean 49.9yrs, Std Dev 10.0, n = 14) than the SLS patients, (but had the same mean age as the PS group)

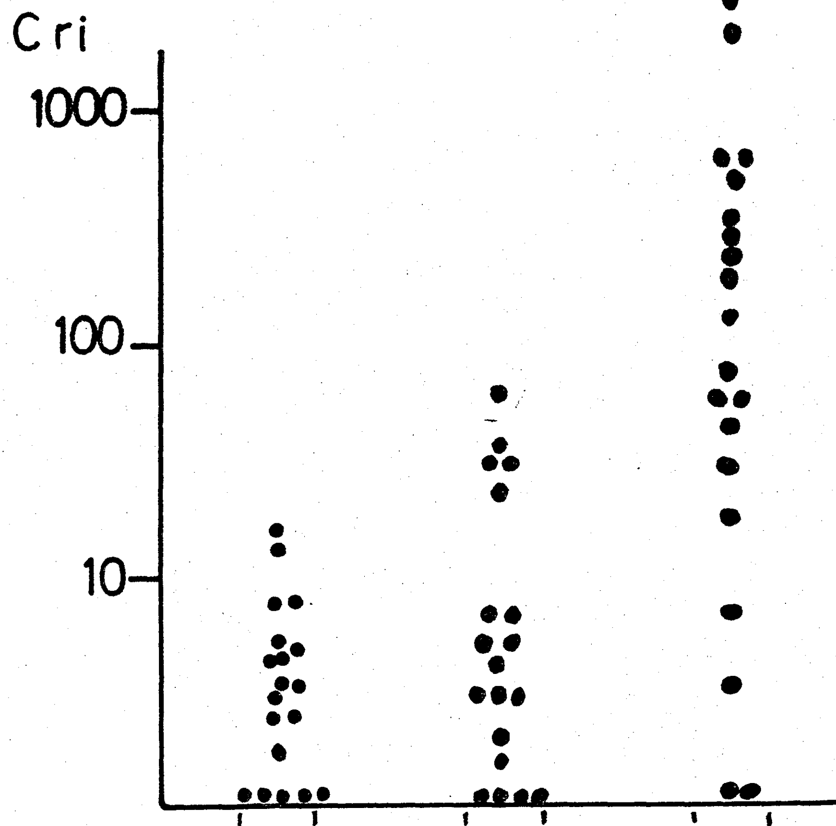


Figure 5.2

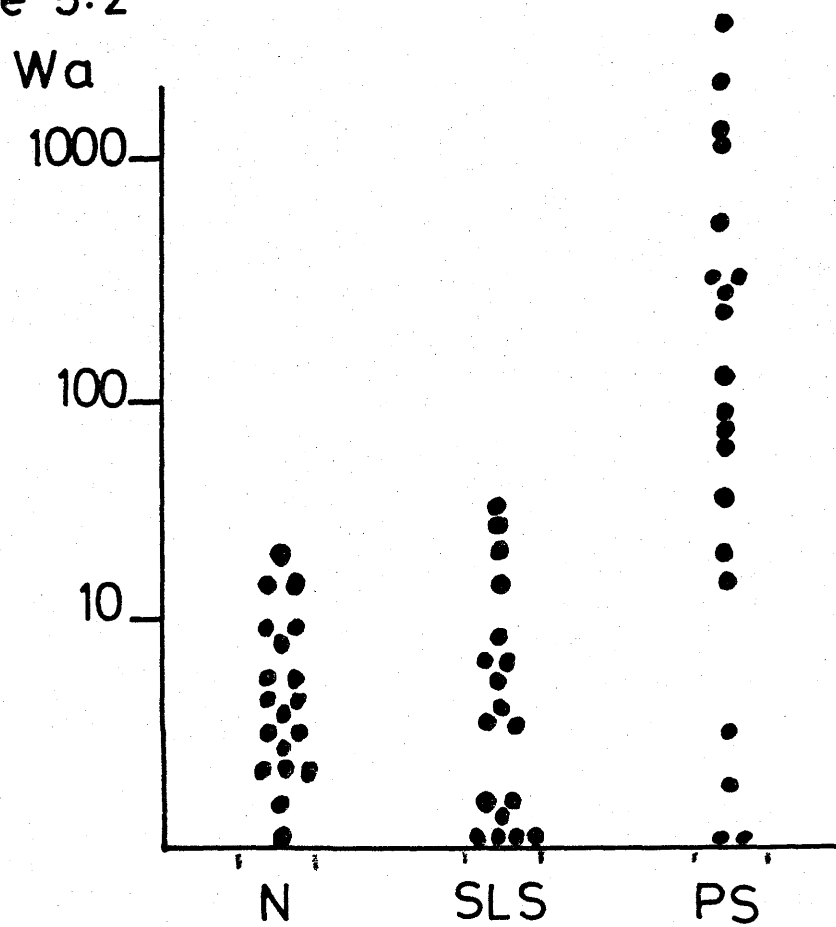


Figure 5.3 IgMRF v IgG1 (μg/ml)

Pet

1000

100

10

Figure 5.4

Cam

1000

100

10

Figure 5.5

N SLS PS

IgMRF v IgG2 (μg/ml)

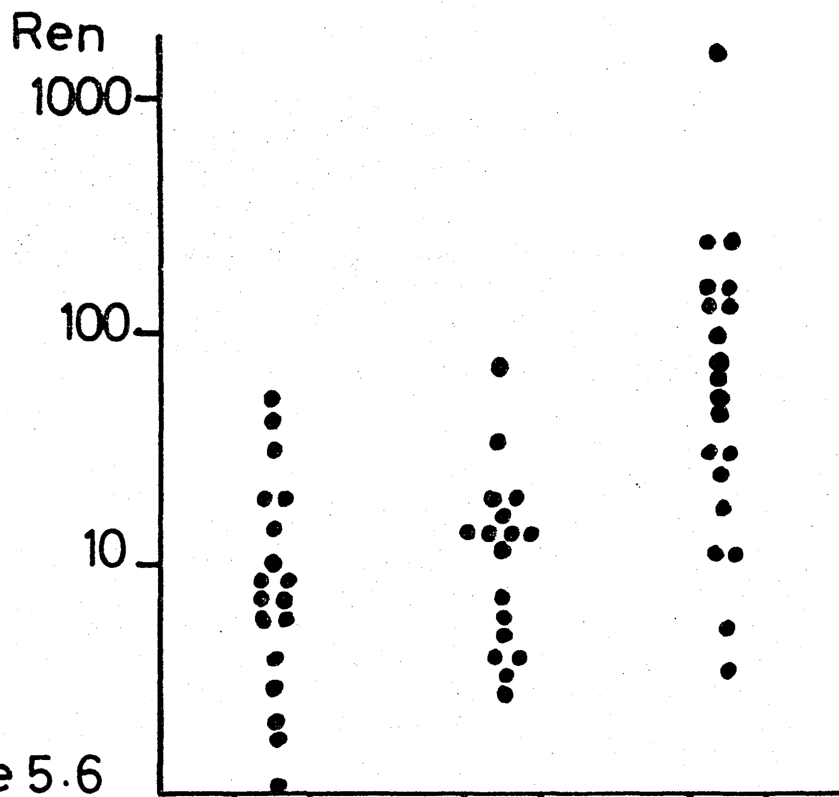


Figure 5.6

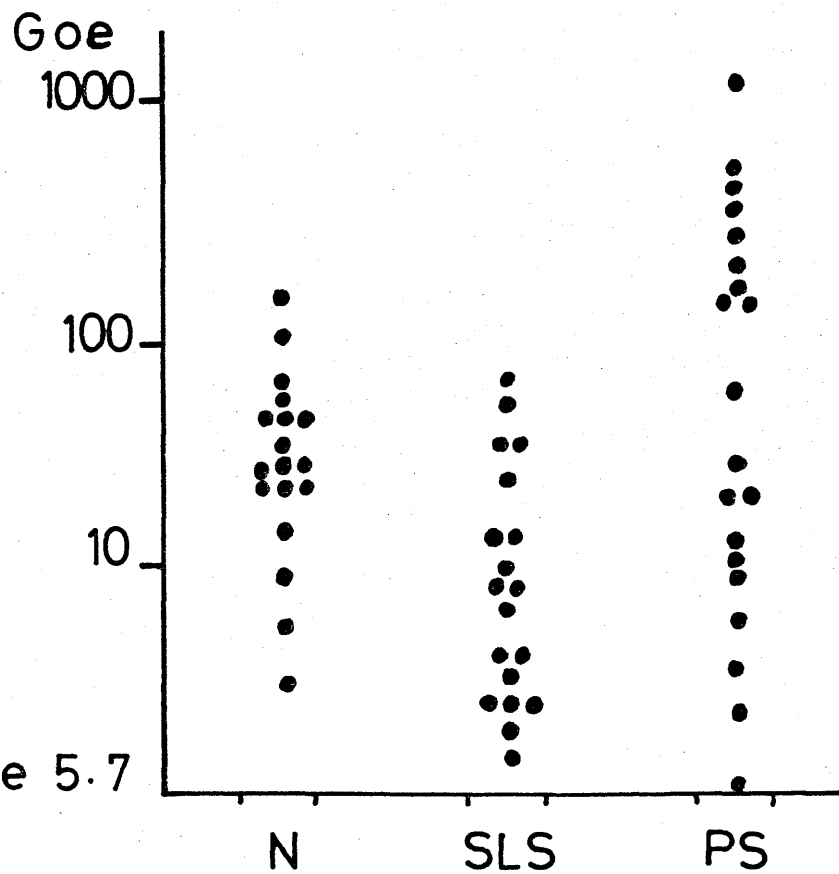


Figure 5.7

IgMRF v IgG 3 (ug/ml)

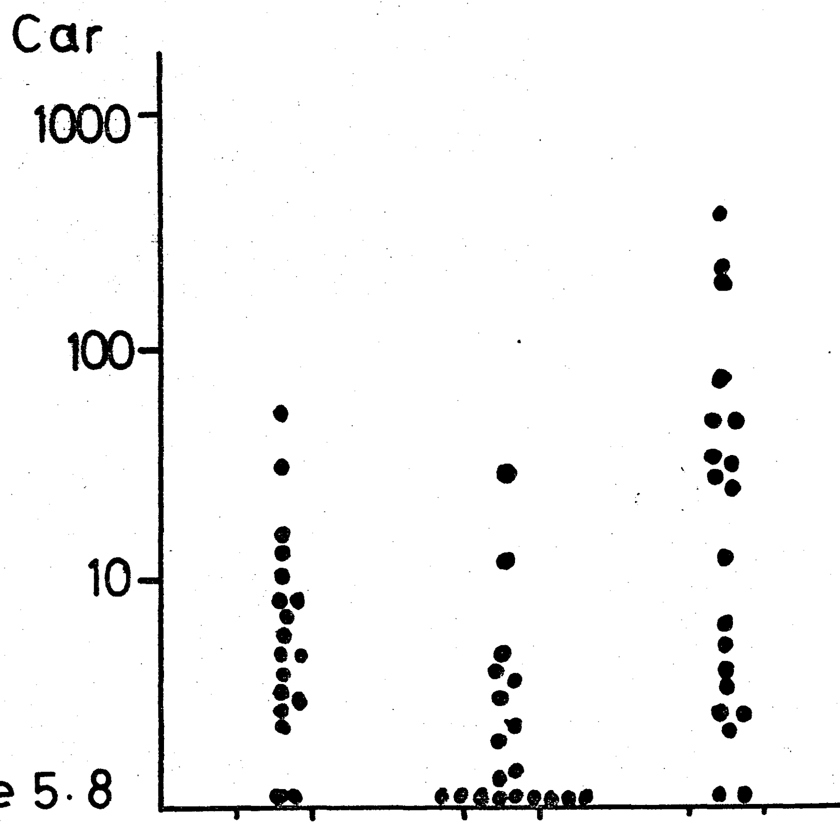


Figure 5·8

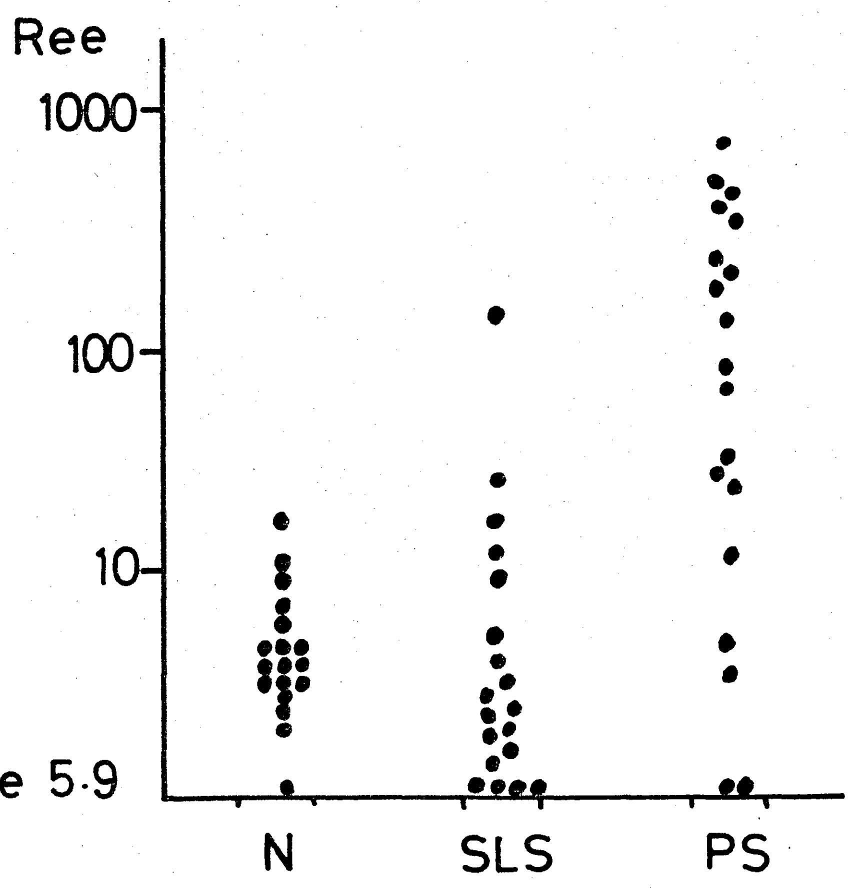


Figure 5·9

IgMRF v IgG4 (ug/ml)

Antigen	n	Median	Range	Z-value	p-value	
	SLS PS N	SLS PS N	SLS PS N	N v SLS N v PS SLS v PS		
Fc-Per	19	3.4	0 - 46	-0.36	0.71	
(IgG1)	20	90	2 - 1114	3.97	7.0x10^{-5}	***
	18	5.1	0 - 17.7	4.06	4.8x10^{-5}	***
Cri	18	2.9	0 - 70	0.05	0.96	
(IgG1)	19	149	1 - 2598	4.29	1.7x10^{-5}	***
	18	3.25	0 - 15.2	3.96	7.3x10^{-5}	***
Ware	18	3.6	0 - 33	-0.38	0.7	
(IgG1)	19	112	0 - 2022	3.66	2.5x10^{-4}	***
	18	4.35	0.7 - 18.5	3.57	3.5x10^{-4}	***
Pe	18	5	1 - 23	-2.07	0.038	*
(IgG2)	19	37	0 - 1549	2.60	0.0094	**
	18	11.45	1.1 - 33	3.19	0.0014	**
Camp	18	2.65	0 - 23	-2.06	0.04	*
(IgG2)	19	156	0 - 2304	3.36	7.8x10^{-4}	***
	18	4.9	1.3 - 13.8	3.48	4.9x10^{-4}	***
Ren	18	9.5	3 - 73	0.05	0.96	
(IgG3)	19	74	5 - 1101	3.90	8.3x10^{-5}	***
	18	8.75	0.8 - 53	3.96	7.3x10^{-5}	***
Goe	18	9	1 - 67	-2.90	0.0032	**
(IgG3)	19	51	0 - 1005	0.68	0.5	
	18	27.65	5.3 - 140	2.45	0.014	*
Carter	18	1.4	0 - 28	-2.80	0.0048	**
(IgG4)	19	21	0 - 499	1.50	0.12	
	18	5.2	0.2 - 57.5	3.35	7.9x10^{-4}	***
Reeder	18	2.4	0 - 134	-1.30	0.19	
(IgG4)	19	130	0 - 2624	3.90	9.4x10^{-5}	***
	18	3.55	0.8 - 16.8	3.66	2.5x10^{-4}	***

Table 5.2 IgMRF levels (μg/ml): Mann-Whitney: N v SLS v PS

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Normal v. PS: In contrast to SLS, the PS patients had significantly higher levels of IgMRF as tested against most of the target antigens, the exceptions being the IgG3 example Goe and IgG4 Carter, where the

trend was nevertheless consistent (Table 5.2, Figures 5.2 to 5.9).
SLS v. PS: Given the above contrast, it is not surprising to find that the PS patients had significantly higher levels than in SLS, of IgMRF when tested against each of the eight target antigens (Table 5.2, Figures 5.2 to 5.9). As for IgMRF assayed by Fc-Per, despite the statistically significant differences between SLS and PS it should be noted that there was considerable overlap between the groups, when IgMRF was assayed by these alternative antigens.

To test whether a supra-normal circulating IgMRF level (assayed by each of the above antigens) in early synovitis would predict persistence of disease, the upper limit of normal was defined as the 90th centile of the values for the normal subjects. Using the Chi^2 test, the finding of a supra-normal IgMRF level carried a significantly increased relative risk for PS of between 2.38 and 5.0 depending on the test antigen (Table 5.3). These values for relative risk and the corresponding sensitivities and specificities are very similar to those for the RA Latex (page 96) which, however, remains marginally the stronger predictor.

Antigen (90cle)	Above normal SLS	PS	Risk	Conf-Int	Sens %	Spec %	Chi^2	p
Fc-Per (8.4)	5	16	3.43	1.7 - 7.0	80	74	11.3	0.0008
Cri (12.8)	4	16	4.53	2.1 - 9.9	84	78	14.3	0.0004
Ware (16.6)	4	15	3.6	1.6 - 8.1	79	78	9.7	0.0018
Pe (32.0)	0	11	3.25	1.7 - 6.3	58	100	12.2	0.0005
Cam (13.0)	1	15	4.9	2.3 - 10.3	79	94	17.4	$3x10^{-5}$
Ren (42.5)	1	11	2.86	1.4 - 5.6	58	94	9.3	0.0023
Goe (104)	0	9	2.8	1.4 - 5.5	47	100	8.8	0.0029
Carter (32.3)	0	6	2.38	1.1 - 5.3	31	100	4.6	0.031
Reeder (10.2)	3	16	5.0	2.2 - 11.7	84	83	14.3	0.0002

Table 5.3 IgMRF: Specificity for IgG subclass as predictor of PS

For Fc-Per n = 19 SLS, 20 PS; for others n = 18 SLS, 19 PS
90cle = 90th centile of normal population (μg/ml)

Correlations between IgMRF of differing IgG subclass specificity: The above Chi^2 analysis measures the quantitative usefulness of the different assays but to appreciate the qualitative pattern of IgMRF levels it is important to know if a given patient was producing similar levels of each specificity of IgMRF. Table 5.4 shows the high correlation coefficients between the levels of IgMRF of each specificity tested, taking the SLS and PS patients all together.

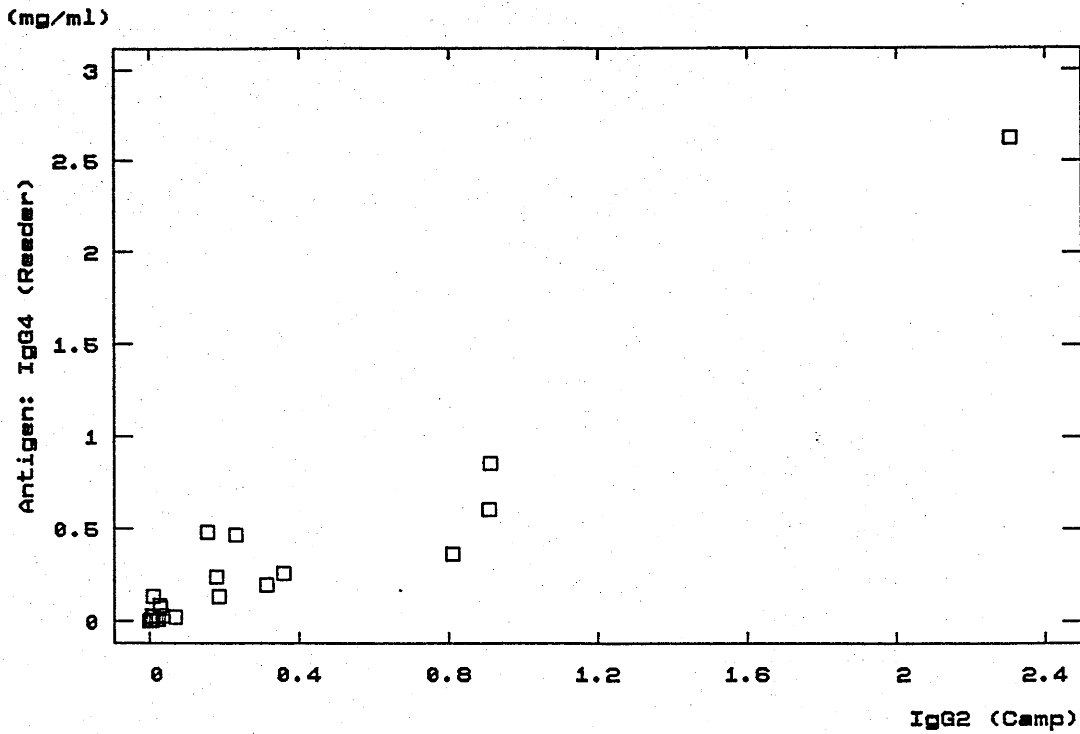


Figure 5.10 Correlation of IgMRF specificity for IgG2 v IgG4

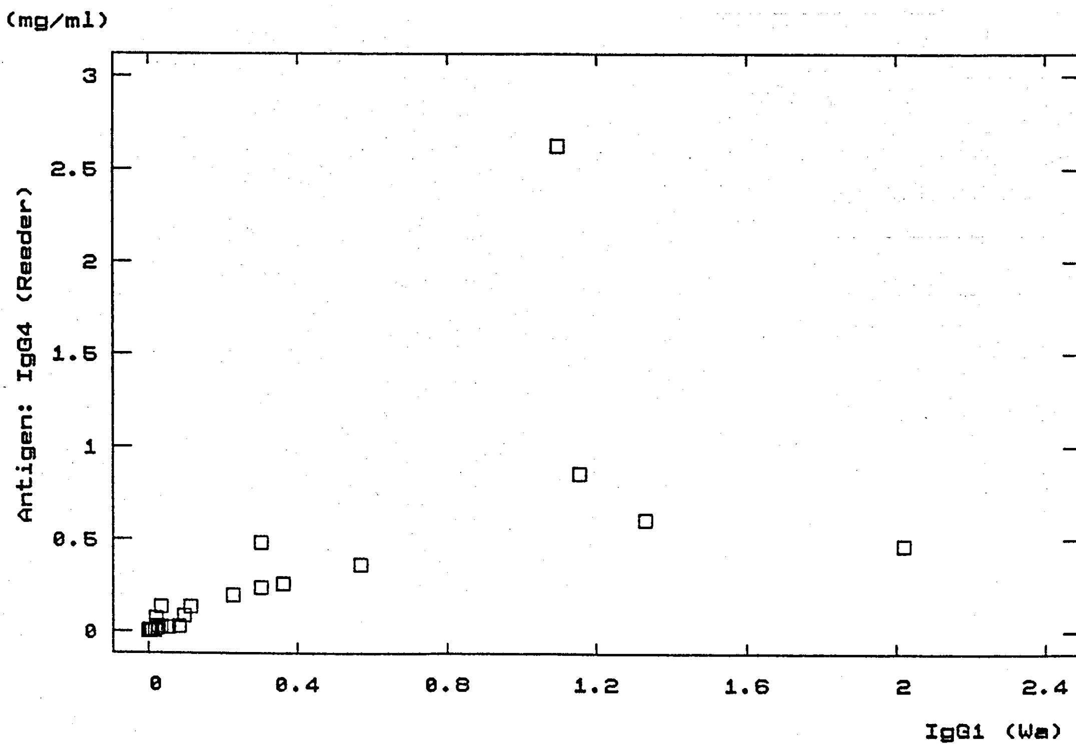


Figure 5.11 Correlation of IgMRF specificity IgG1 v IgG4

	Fc-Per	Cri	Wa	Pe	Cam	Ren	Goe	Carter	Reed
Cri	0.68								
Wa	0.81	0.92							
Pe	0.77	0.66	0.66						
Cam	0.74	0.66	0.65	0.98					
Ren	0.63	0.80	0.69	0.92	0.92				
Goe	0.72	0.83	0.82	0.88	0.92	0.93			
Carter	0.75	0.58	0.60	0.98	0.97	0.89	0.87		
Reeder	0.72	0.70	0.64	0.96	0.96	0.95	0.88	0.95	
Age	0.09 .59	0.01 .97	0.04 .83	0.02 .91	0.01 .96	0.01 .98	0.01 .99	0.01 .97	0.01 .95
ESR	0.24 .14	0.09 .58	0.16 .34	0.13 .43	0.21 .21	0.10 .54	0.19 .25.	0.13 44.	0.13 .42
RA Latex	0.49 .002	0.38 .018	0.43 .008	0.34 .040	0.38 .021	0.35 .030	0.41 .010	0.32 .050	0.34 .036

Table 5.4 IgG subclass specificity of IgMRF:
Correlation coefficients; SLS and PS taken together

Significance level < 0.0001 unless stated (given as the lower figure for: age, ESR, RA Latex)

If the specificities for IgG subclasses 2, 3 and 4 are considered first, the correlations between them are very high indeed, with coefficients at least as high as 0.88 and an example plot is shown in Figure 5.10. Slightly lower but still significant correlations are seen between levels of IgMRF with specificity for IgG1, and the other subclasses. This is in part due to a small number of outliers, having particularly high levels of IgG1 specific IgMRF, without a parallel increase in levels of IgMRF of other specificities (Figure 5.11, as an example). In general, however, if there was an increase in IgMRF in early synovitis, this was contributed to by IgMRF of each tested specificity in parallel.

It is worthy of note that the levels of these IgMRF did not correlate with either age or ESR, and only weak correlations were seen with the RA Latex.

	Fc-Per	Cri	Wa	Pe	Cam	Ren	Goe	Carter
Cri	0.59							
Wa	0.62	0.96						
Pe	0.16	0.52	0.55					
Cam	0.19	0.54	0.68	0.66				
Ren	0.20	0.39	0.51	0.69	0.84			
Goe	-0.10	0.36	0.39	0.86	0.70	0.69		
Carter	0.12	0.72	0.57	0.59	0.20	0.09	0.52	
Reeder	0.32	0.87	0.72	0.40	0.24	0.06	0.30	0.92

Table 5.5 IgG subclass specificity of IgMRF: Correlation coefficients SLS patients only

____ (double underline) = significance level greater than 0.05

____ = coefficient appreciably less than for SLS and PS together

A study of the correlations between the IgMRF of differing subclass specificities within the groups SLS and then PS allows comparison of the pattern of IgMRF production. Table 5.5 reveals the much lower correlation values and often loss of significance when the SLS group alone is examined. The change is partly due to the low absolute values for each variable with, in any one patient, some specificities being undetected and other specificities having moderate levels. In contrast, for PS (Table 5.6), levels of IgMRF of the varying specificities tended to parallel each other in a given patient.

	Fc-Per	Cri	Wa	Pe	Cam	Ren	Goe	Carter
Cri	0.60							
Wa	0.76	0.91						
Pe	0.73	0.60	0.59					
Cam	0.69	0.60	0.58	0.97				
Ren	0.55	0.76	0.63	0.91	0.91			
Goe	0.65	0.79	0.78	0.87	0.90	0.92		
Carter	0.71	0.52	0.53	0.97	0.97	0.88	0.85	
Reeder	0.67	0.65	0.57	0.96	0.95	0.95	0.86	0.95

Table 5.6 IgG subclass specificity of IgMRF: Correlation coefficients PS patients only

____ = significance level greater than 0.05

____ = coefficient appreciably less than for SLS and PS together

Summary of IgG subclass specificity of IgMRF: Quantitative differences were seen between the SLS and PS groups but with considerable overlap. Qualitatively, IgMRF of each tested specificity was detected in both SLS and PS, with strong correlation in the levels of IgMRF of the different specificities in PS but a rather less consistent pattern in SLS. In an attempt to examine further any possible qualitative differences between the SLS and PS groups, the expression of cross-reactive idiotopes (CRI) on the IgM and IgMRF was studied.

5.5.3 Capture ELISA: Cross-reactive idiotope (CRI)-bearing IgM

In this set of experiments the subjects' sera were added to ELISA plates pre-coated with monoclonal antibody (McAb) with specificity for CRI as detailed in the Methods section, the focussing on IgM

being achieved by the use of an anti-human IgM antibody at the visualisation stage. It is re-emphasised that this quantitation includes IgM both with and without RF activity. Previous work, in the laboratory where the assays were performed for this chapter of the thesis, showed that in a panel of 163 IgM paraproteins 35% of IgMRF (9 of 26) expressed the G6 CRI but only 3% (5 of 137) non-RF paraproteins expressed this CRI[141]. This tended to suggest that McAb raised against monoclonal IgMRF, would detect expression of CRI mainly on IgM with RF activity rather than without. In addition, for two patients from the PS group (pt 97 and 104) who had high levels of IgMRF (by direct binding ELISA to Fc-Per, above), RF was isolated from the sera by affinity chromatography on immobilized IgG. The expression of G6 and G8 CRI were quantified in the IgMRF and IgM non-RF fractions. That the CRI were expressed mainly within the IgMRF fraction (Table 5.7) suggested that the levels of CRI expression in the whole sera (IgM and IgMRF) would be due mainly to the RF fraction.

Patient	CRI	Total μg/ml	% in RF	% in non-RF	%CRI+IgMRF
97	G6	60.16	92.3	7.7	2
	G8	188.20	87.0	13.0	5
104	G6	8.16	72.0	28.0	2
	G8	35.84	83.5	16.5	4

Table 5.7 G6 and G8 CRI-bearing IgM in RF and non-RF serum fractions

CRI	n	Median	Range	Z-value	p-value	
	SLS PS N	SLS PS N	SLS PS N	N v SLS N v PS SLS v PS		
C7	19	132	34 - 458	2.60	0.0093	**
	20	147	7.5 - 627	3.20	0.0012	**
	32	46.2	2.0 - 516	0.80	0.40	
17_109	9	2.3	0.5 - 10	1.80	0.068	
	11	18.5	0 - 495	3.10	0.002	**
	11	1.0	0.25 - 4.3	2.58	0.0098	**
H1	19	0.0	0 - 15.6	-0.46	0.64	
	20	1.8	0 - 25.6	1.57	0.12	
	32	0.11	0 - 1.0	1.57	0.11	
G6	19	3.4	0 - 30.8	2.70	0.0068	**
	20	5.2	0.8 - 33.3	4.17	2.9×10^{-5}	***
	32	1.07	0 - 14.1	1.18	0.24	
G8	19	6.3	0 - 114	2.46	0.014	*
	20	20.1	0.8 - 116	4.50	6.6×10^{-6}	***
	30	1.85	0.1 - 25.5	2.60	0.0093	**

Table 5.8 Cross-reactive idiotope (CRI) bearing IgM levels (μg/ml): Mann-Whitney: Normal subjects (N) v SLS v PS

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Normal v. SLS: In some contrast to the IgG subclass specificity of IgMRF, CRI-bearing IgM in SLS show a trend to higher than normal levels (Table 5.8), reaching statistical significance for C7, G6 and G8 but failing to do so for 17_109 (for which small numbers of patients were tested). For H1 the trend is reversed but the apparently lower levels of this CRI-bearing IgM do not reach statistical significance.

Normal v. PS: For each CRI tested the levels of IgM in the PS group were significantly higher than in the normal subjects.

SLS v. PS: For three of the CRI-bearing IgM tested (C7, H1, G6), no significant difference was seen between the two groups of early

synovitis patients. The levels were significantly higher in PS for 17_109 and G8.

To ascertain whether a supra-normal level of CRI-bearing IgM was predictive of persistence of early synovitis (ie PS rather than SLS), the upper limit of normal for a given CRI-bearing IgM was set at the 90th centile of the distribution of values obtained from normal subjects: the number of normal subjects studied for each CRI are given in Table 5.8. Only for G8 did the Chi^2 test show a significantly increased relative risk of PS (Table 5.9). The apparently higher risk for 17_109 was negated by the small numbers studied and hence the wide confidence interval and poor p-value.

CRI (90cle)	Above SLS	normal PS	Risk	Conf-Int	Sens %	Spec %	Chi^2	p
C7 (292)	5	9	1.46	0.8 - 2.7	45	74	1.48	0.22
17_109 (3.17)	4	9	2.42	0.6 - 9.5	82	55	1.60	0.2
H1 (0.92)	6	11	1.58	0.9 - 2.9	55	68	2.17	0.14
G6 (5.6)	5	9	1.46	0.8 - 2.7	45	74	1.48	0.22
G8 (14.4)	4	12	2.16	1.2 - 4.0	60	79	6.1	0.013

Table 5.9 CRI-bearing IgM: do supra-normal levels predict persistence?

90cle = 90th centile of the normal population (μg/ml); for n see table 5.3

CRI-bearing IgM as a proportion of total IgM: Some understanding of the pathogenesis of persistent synovitis might be obtained from an

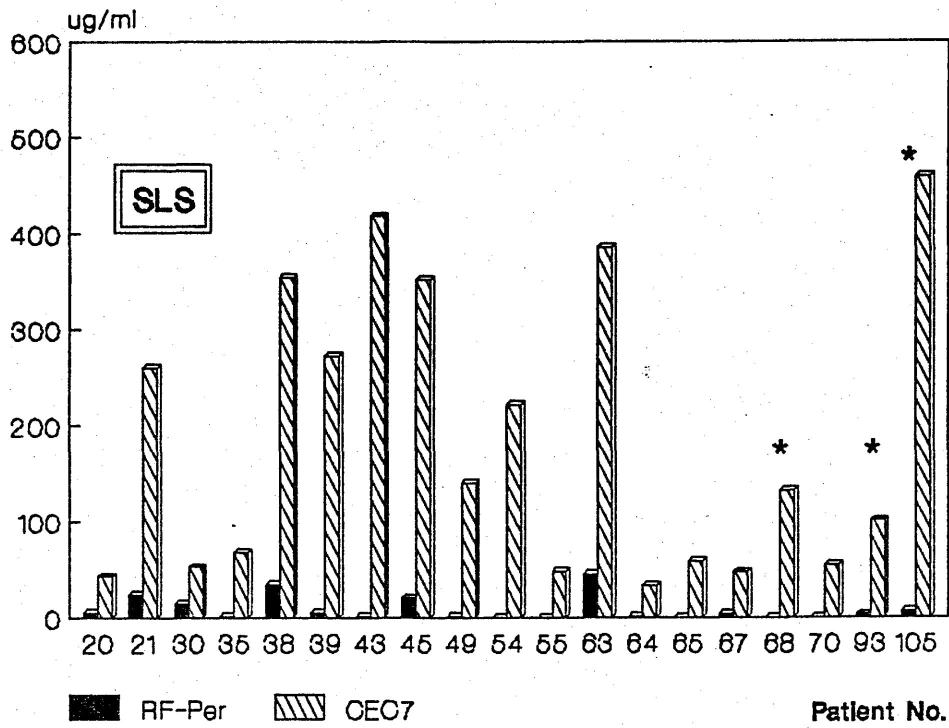
appreciation of what proportion of the total circulating IgM is CRI-bearing, comparing SLS with PS. For C7 the median value for CRI-bearing IgM is just over 5% of the total IgM for both groups (Table 5.10), in a small number of cases ranging as high as 20 to 45%, but with no significant difference between the groups. For the remaining CRI tested the proportions of IgM that bore CRI were much smaller, with median values of around 1% or less. For the CRI 17_109 and G8 the PS group had significantly higher proportions of IgM expressing CRI, but for H1 and G6 the groups were not significantly different.

CRI	n	Median	Range	Z-value	p-value	
	SLS PS	SLS PS	SLS PS			
C7	19	6.4	2.1 - 45.3			
	20	5.5	1 - 22.4	-0.69	0.49	
17_109	9	0.21	0.03 - 0.59			
	11	1.1	0 - 7.9	2.28	0.023	*
H1	19	0	0 - 0.24			
	20	0.04	0 - 1.27	1.39	0.16	
G6	19	0.18	0 - 0.78			
	20	0.22	0.03 - 1.72	0.72	0.47	
G8	19	0.39	0 - 1.76			
	20	0.87	0.03 - 4.17	2.65	0.0079	**

<u>Table 5.10</u> CRI-bearing IgM as percentage of total IgM: Mann-Whitney
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

The significance of the above data is difficult to evaluate but is presented as it will be referred to in broader discussion below.

<u>CRI-bearing IgM as a proportion of IgMRF</u>: Since it was thought that the majority of the CRI expression would be within the RF rather than



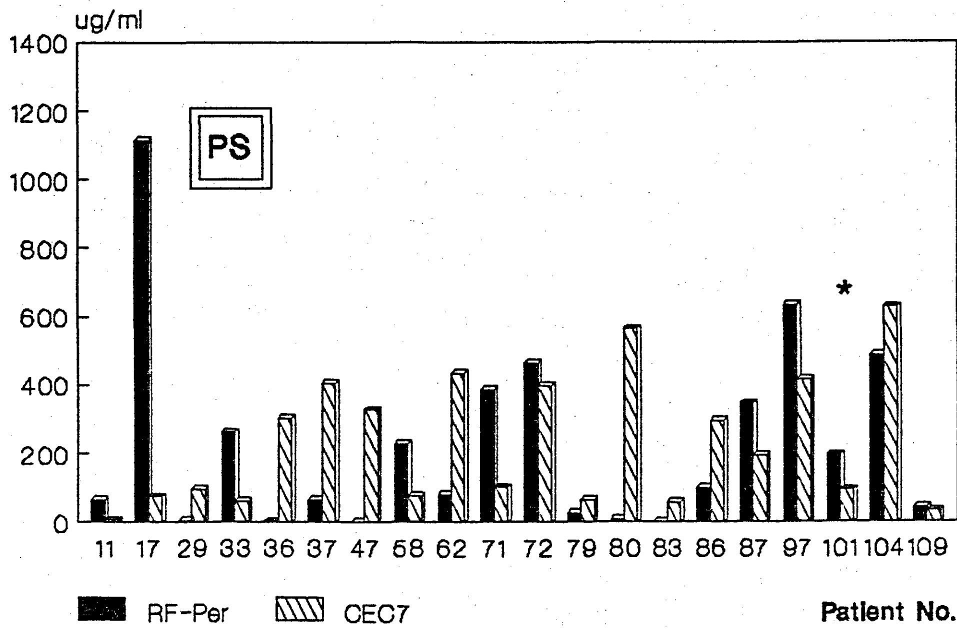
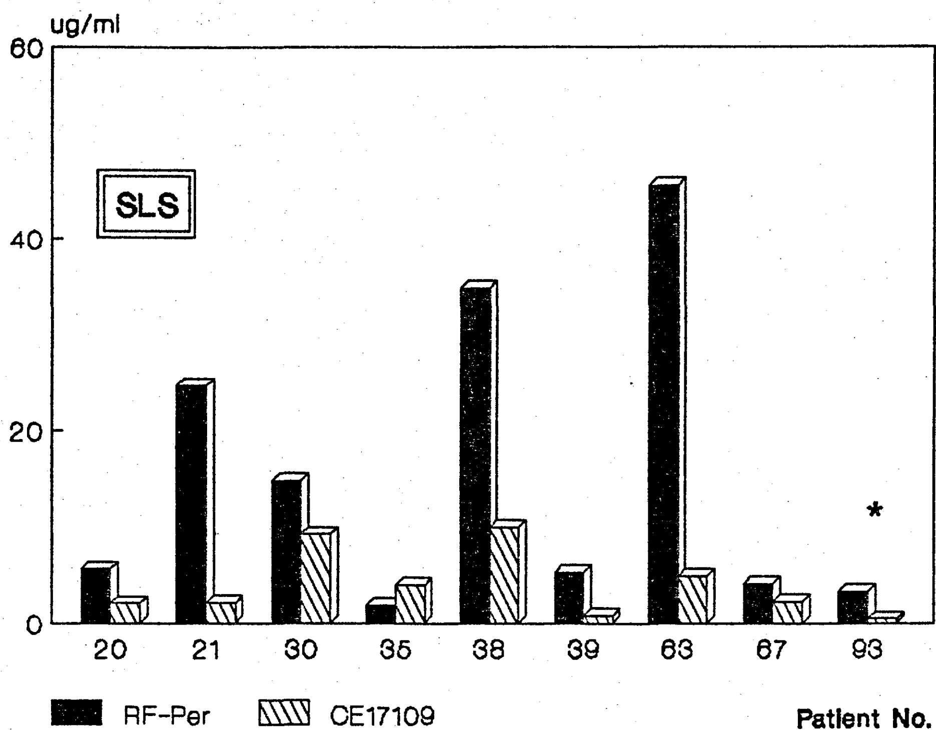


Figure 5.12 CRI+ IgMRF v total IgMRF
* = patient B19 IgM positive
Note different scales

non-RF fraction of the IgM (and hence possibly be of relevance to the pathogenesis of persistent synovitis) the levels of CRI-bearing IgM were compared to the levels of IgMRF (defined by binding to Fc-Per as above). In both the SLS and PS groups there were individuals who had unrecordable values for given CRI. In addition in the SLS group some individuals had undetectable levels of IgMRF (5 of 19) but detectable levels of CRI-bearing IgM. Accordingly the results are presented graphically for each individual and each CRI tested.

CRI C7: As noted above (Table 5.8) there was no difference in the values (μg/ml) for the SLS and PS groups but the PS group had higher levels of IgMRF (Table 5.2). In SLS patients the level of C7-bearing IgM was always higher than the IgMRF level (Figure 5.12 top) so clearly the McAb was detecting IgM other than in the measured RF compartment. In PS this is reflected in the C7-bearing IgM having no relationship to the IgMRF, being higher in exactly half of the 20 PS patients and lower in the other half (Figure 5.12 bottom). This is in keeping with the epitope recognised by McAb C7 being preferentially associated with but not restricted to IgM with RF activity[127].



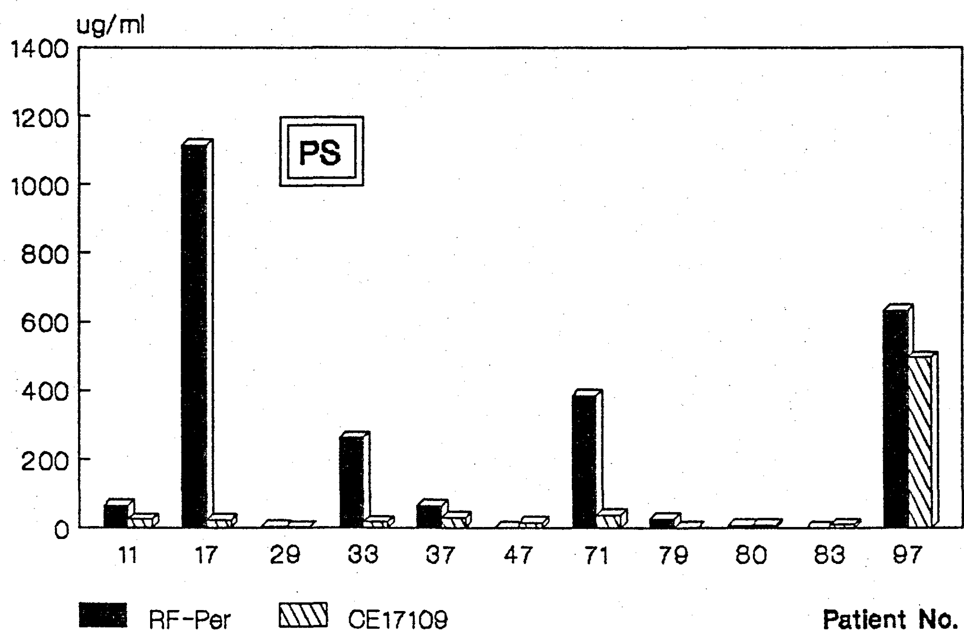
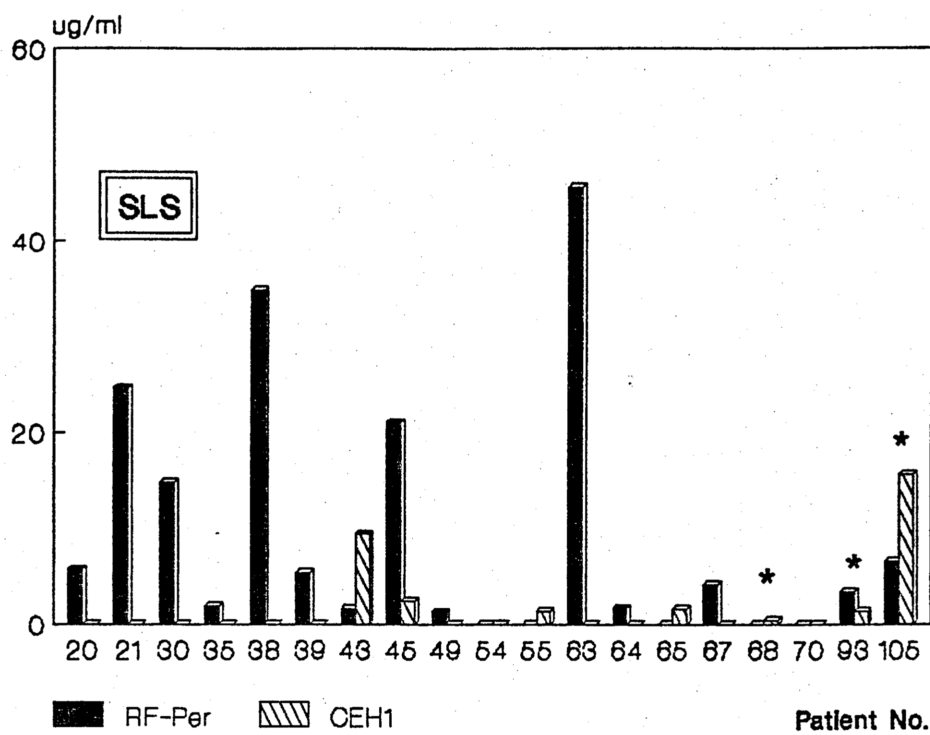


Figure 5.13 CRI+ IgMRF v total IgMRF
* = patient B19 IgM positive
Note different scales

CRI 17-109: While there was a measurably higher level of IgM bearing this CRI in the PS group (Table 5.8), this accounted for very little of the one or two orders of magnitude difference between SLS and PS in the total IgMRF levels (Table 5.2) and in general the proportion of the IgMRF bearing this CRI was smaller in PS than in SLS (Figure 5.13). Only in three patients (2 SLS, 1 PS) was the CRI-bearing value higher than that for IgMRF and those were when both variables had very low values. This in keeping with the 17-109 epitope being more restricted to IgM with RF activity, although it is known that such restriction is not complete[126].



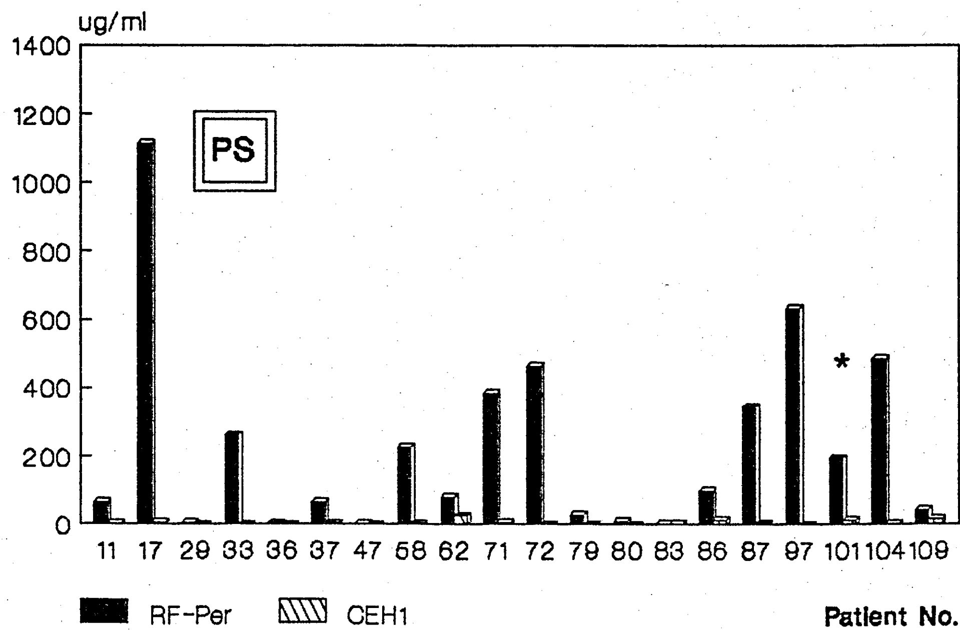
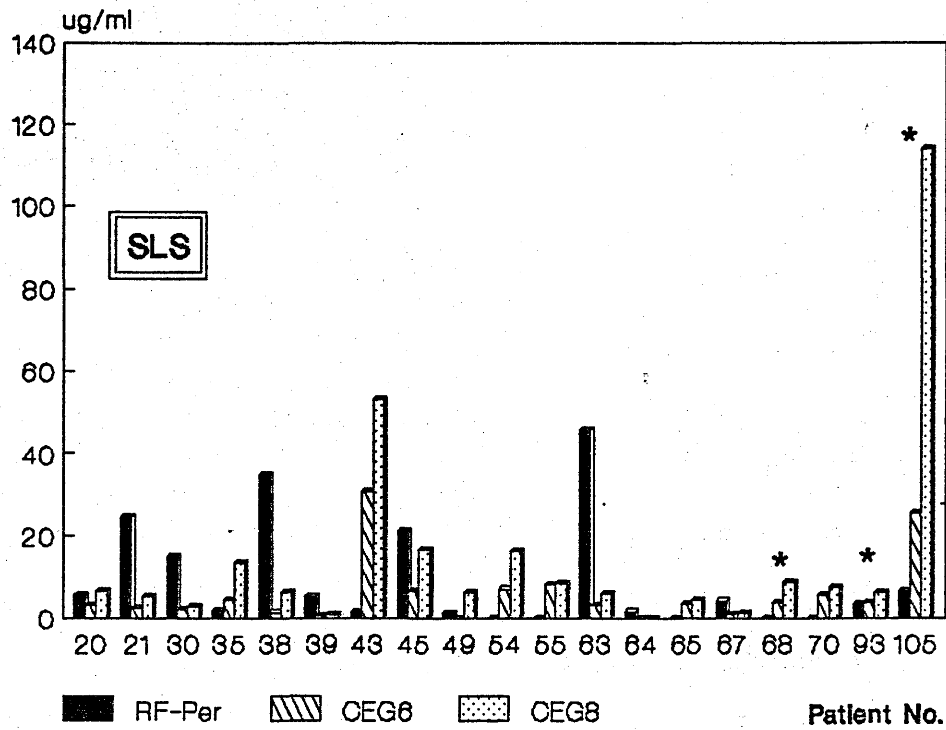


Figure 5.14 CRI+ IgMRF v total IgMRF
* = patient B19 IgM positive
Note different scales

CRI H1: Where detected (Figure 5.14), this CRI was present in only very low levels, with it being undetectable in half of the patients in each group (11/19 SLS, 9/20 PS). With no significant difference between the groups in the CRI level, it made up proportionately less of the IgMRF. The finding of only 4 patients with this CRI level higher than their IgMRF level, and those at very low values for both the IgMRF and the CRI-bearing IgM, is in keeping with the H1 epitope being detected by the H1 McAb being mainly in the IgM compartment with RF activity[125].



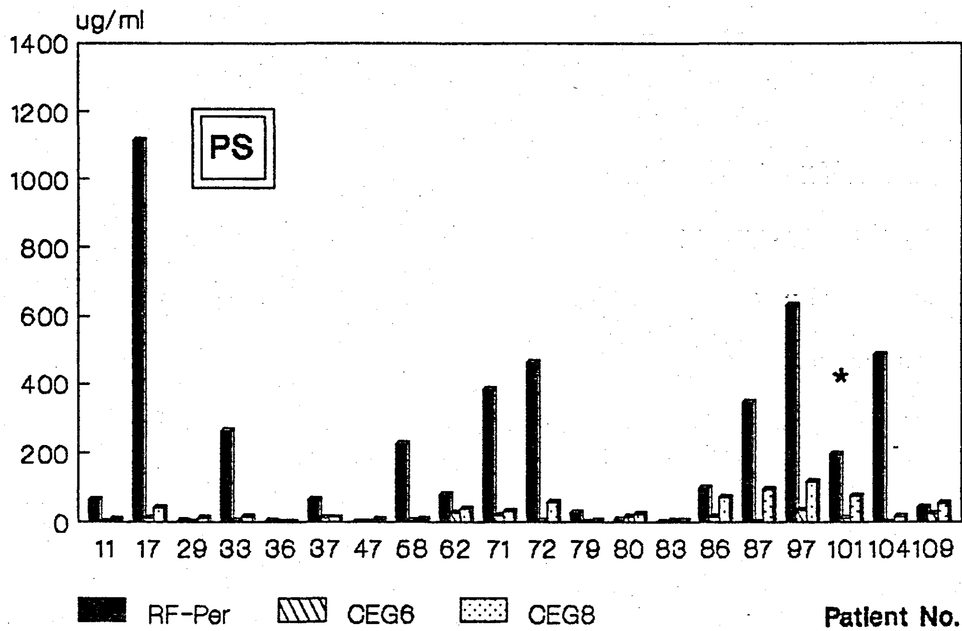


Figure 5.15 CRI+ IgMRF v total IgMRF
* = patient B19 IgM positive
Note different scales

<u>CRI G6 and CRI G8</u>: Again the CRI-bearing IgM accounted for a very small percentage of the higher value of IgMRF in the PS patients (Figure 5.15). As indicated above the epitopes recognised by McAbs G6 and G8 are mainly within the RF compartment of IgM. In keeping with this the CRI value was higher than that for IgMRF in only those patients with very low values for IgMRF. The finding of low or absent IgMRF being accompanied by low but measurable levels of the G6 and G8 CRI is of interest when it is noted that three such patients (Pt 68, 93, 105 but not 43) had evidence of recent parvovirus infection, both clinically and by specific IgM in the same first visit serum sample. The only other patient with recent parvovirus infection (Pt 101) also had detectable levels of CRI G8.

<u>Summary: CRI-bearing IgM as proportion of IgMRF</u>: Within the constraint that the expression of CRI is mainly but not entirely within the IgM with RF activity, the expression of CRI in PS is similar to or slightly higher than in SLS but only a very small proportion of the IgMRF in PS bears the CRI tested.

5.5.4 Direct binding ELISA: estimation of CRI-bearing IgMRF

An alternative approach to the study of CRI expression on IgMRF was to use the McAb to inhibit its binding to Fc-Per. For these complex assays a limited number of patients were selected (n: SLS = 8; PS = 10) and values for the normal population are not available. Each patient's serum was incubated with one Mc anti-CRI antibody before being added to the Fc-Per sensitized ELISA plates. Reduction of the subject's control level of binding of IgMRF to Fc-Per was ascribed to inhibition, by the Mc anti-CRI antibody, of CRI-bearing IgMRF. The CRI-bearing IgMRF could be expressed in μg/ml. Three questions were

then asked: (i) did the assay discriminate between SLS and PS? (ii) what proportion of the IgMRF bore the CRI tested? (iii) how much of the total CRI-bearing IgM had RF activity?

CRI	n SLS PS	Median SLS PS	Range SLS PS	Z-value	p-value
C7	8 10	0.65 0	0.27 - 4.2 0 - 2.8	-1.8	0.08
17_109	8 10	0 0	0 - 3.6 0 - 1.43	-0.4	0.67
H1	8 10	0 0	0 - 5.1 0 - 14.3	-0.7	0.46
G6	8 10	0 0	0 - 1.4 0 - 5.9	0.2	0.87
RABKO	8 10	1.31 0.20	0 - 3.4 0 - 7.37	-0.7	0.44
RABAN	8 10	1.34 0	0 - 4.5 0 - 5.7	-1.4	0.17

Table 5.11 CRI-bearing IgMRF (μg/ml): SLS v. PS: Mann-Whitney (inhibition assay)

RABKO and RABAN are rabbit polyclonal antibody preparations.

SLS v. PS: Although there was a trend for the PS patients to have lower levels of CRI-bearing IgMRF (Table 5.11), this did not reach statistical significance for any CRI tested.

Percentage of the total IgMRF bearing CRI: Using the inhibition assay there was, as for the capture assay, a consistent trend for the PS patients to have low proportions of their IgMRF expressing CRI. However. the only statistically significant difference between the groups was seen for IgMRF bearing the C7 CRI, with the SLS group

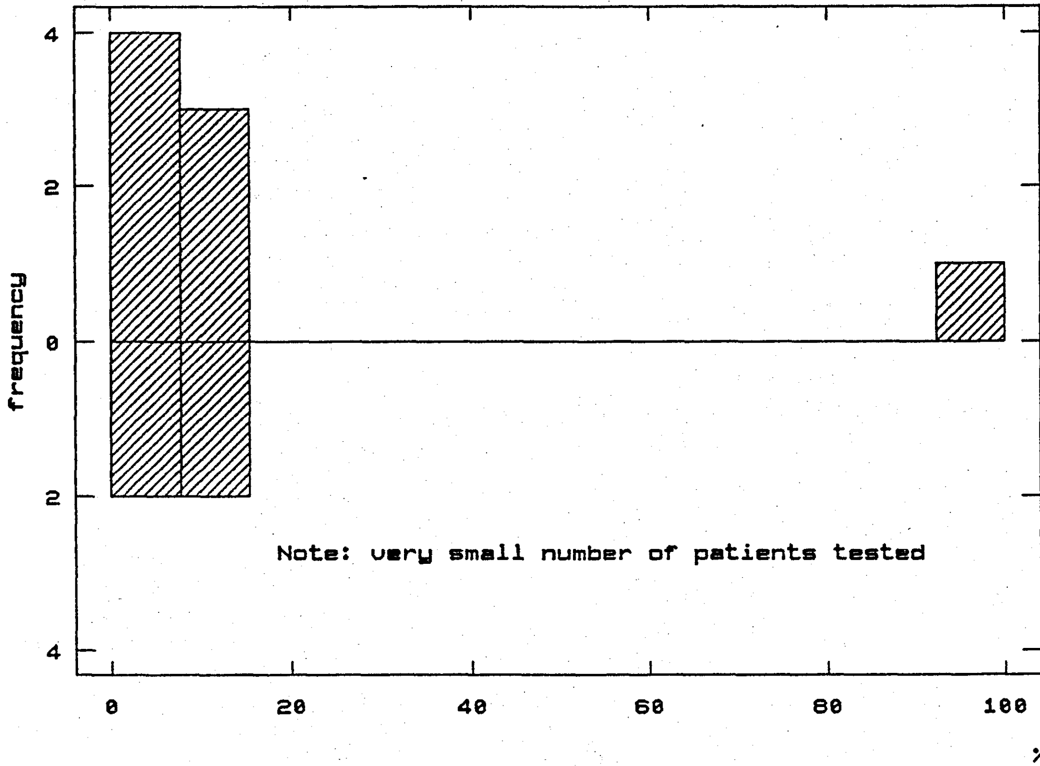


Figure 5.16 C7 as % IgMRF: highly skewed distribution

having higher levels (Table 5.12). The caution needed in interpreting the statistical analysis of such small numbers of subjects is emphasised by the plot of C7 CRI-bearing IgMRF (Figure 5.16) which shows a highly skewed distribution.

CRI	n	Median	Range	Z-value	p-value	
	SLS PS	SLS PS	SLS PS			
C7	8 10	6.8 0	0.7 - 100 0 - 14.4	-2.1	0.033	*
17_109	8 10	0 0	0 - 36.5 0 - 6	-0.4	0.669	
H1	8 10	0 0	0 - 92.1 0 - 21.8	-0.8	0.399	
G6	8 10	0 0	0 - 33.3 0 - 25.9	0.1	0.95	
RABKO	8 10	8.9 4.9	0 - 81.0 0 - 51.9	-0.6	0.55	
RABAN	8 10	9.6 0	0 - 100 0 - 61.7	-1.7	0.096	

Table 5.12 CRI-bearing IgMRF (% total IgM): SLS v. PS: Mann-Whitney

RABKO and RABAN are rabbit polyclonal antibody preparations.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

5.5.5 Comparing IgMRF values by the inhibition and capture assays: It would help to confirm the accuracy of the assumption that the capture ELISA assay (section 5.5.3) was measuring CRI expression on IgMRF as opposed to non-RF, if the values from the inhibition studies could be compared. However the values were not comparable, with for a given CRI the value obtained for some patients in the one assay being measurable but absent by the other assay: in other patients the reverse applied. The discrepancies applied to both SLS and PS groups.

Clearly the bonding forces in the inhibition assay are more complex than in the capture assay with, in different patients, differing relative affinities between the McAb, the IgMRF and the Fc-Per.

5.6 Discussion of B-lymphocyte studies: The value of the results in this chapter of the thesis can be summarised by returning to the three questions laid out in the aims at the beginning of the chapter.

5.6.1 The fluorescent IgG : total IgG ratio

This ratio was not significantly different between the two groups of early synovitis patients and the levels were not different from the normal population. Furthermore the value of the ratio did not correlate (Coefficient -0.23, sig = 0.16) with the level of RF (by ELISA, using Fc-Per as antigen). It seems unlikely that damaged IgG circulating in the peripheral blood is a principle drive for RF production. Study of the same phenomenon in synovial fluid would be interesting but impractical in early synovitis patients since even in the PS patients at first visit there were very few joint effusions to aspirate.

5.6.2 Quantitation of IgMRF

There were indeed quantitative differences in IgMRF levels between SLS and PS. If the ESR can be considered a reasonable reflection of the acute phase response and hence of disease severity, then the poor correlation between ESR and IgMRF levels is worthy of note (Table 5.4). This suggests that IgMRF levels represent a dimension of the disease process other than severity. There were, however considerable overlaps between the groups in the quantitative levels of IgMRF, suggesting that the production of IgMRF was part of the inflammatory process in synovitis, rather than the cause of its persistence.

5.6.3 Qualitative study of IgMRF

Using the specificity of the IgMRF for differing IgG subclasses to seek qualitative differences between SLS and PS, no particular specificity was exclusive to either group. Rather than categoric the differences between the groups were arithmetic, the main difference being that the quantities of IgMRF of the different specificities were more closely correlated in PS. It is open to speculation that this more uniform pattern in PS may suggest that they are a group of patients with a more homogeneous disease, perhaps in terms of the trigger factor, or in the genetic determinants of the host response. In contrast the slightly less uniform pattern in SLS may reflect differing triggering infections, or alternatively phenotypic differences in the host response.

Extending the qualitative evaluation to the IgMRF idiotypy, the differences between SLS and PS were again arithmetically continuous rather than categoric, with each specificity being expressed by at least some patients in both groups. The most important observation was that when high levels of IgMRF are produced (and this is mainly in PS) the majority of it is not accounted for by CRI-bearing IgM. There is evidence to suggest that the finding of CRI-bearing IgMRF arises from the expression of germ line genes, with minimal somatic mutation[142-5]. Accordingly the finding of a predominance of non-CRI-bearing IgMRF, particularly in PS, suggests a departure from use of those germ line genes. This lowered proportion of CRI-bearing IgMRF in PS may represent polyclonal activation of IgM secreting B-lymphocytes in a non-specific manner. Alternatively or in addition it may represent maturation of the immune response, with somatic mutation of the germ line genes, perhaps resulting from specific but unidentified antigenic drive.

CHAPTER 6

RESULTS

VIRAL STUDIES

CHAPTER 6

RESULTS - VIRAL STUDIES

6.1 Aims

The aim of this section of the thesis was to determine whether any of the patients suffered specific viral infections coincident with the onset of their synovitis. Since the potential infecting viruses are numerous a decision was made to limit the investigation to only those viruses of specific interest in polyarthritis, as reviewed in the introductory chapter. In particular, evidence of recent infection with either Rubella virus or Human Parvovirus B19 was sought.

6.2 Patients selected

Serum was collected from all patients at first visit and stored in aliquots at -70°C. The samples were analysed in one batch by the routine service laboratory (blind to the diagnostic coding) at the end of the clinical period of the study. The allocation of patients to diagnostic groups (SLS or PS) was made independently of the viral serology results, although the results were known (the test having been performed by the referring clinician) for two of the B19 positive patients (1 SLS, 1 PS) at the time of allocation. Serum was available for analysis from 30 SLS and 24 PS patients.

6.3 Viral serology

Using the single, first visit sample, there was considered to be evidence of recent infection if specific anti-viral IgM antibody was detected.

6.3.1 Rubella

No patient, in either the SLS or PS groups, had evidence of recent Rubella infection. Seventeen patients (9 SLS, 8 PS) had no evidence of distant past infection, the other two-thirds of each group having circulating anti-Rubella IgG antibody.

6.3.2 Human Parvovirus B19 (B19)

Six patients (5 SLS, 1 PS) had specific anti-B19 IgM evidence of recent B19 infection and their case histories are outlined below. Nineteen patients had no evidence of distant past infection, as determined by the absence of circulating anti-B19 IgG antibody: this is in keeping with the finding of anti-B19 IgG in around two-thirds of the normal adult population as discussed previously.

6.4 Patients with recent B19 infection - case histories

6.4.1 Patient number 68: This 27yr old housewife was first seen in the Early Arthritis Clinic on 2nd September 1985. From the age of 7yrs to the age of 14yrs she had attended a District General Hospital complaining of neck, shoulder and hand pain, with pins and needles in the hands. No specific diagnosis had been reached and she was managed conservatively with reassurance and a soft cervical collar.
During the subsequent 13 years she had been entirely well, until the middle of May 1985 when she developed a sore throat and hoarse voice and was treated with penicillin. Within one week of the onset of sore throat she developed pain, swelling and 3hrs morning stiffness in an apparently symmetrical distribution affecting hands, wrists, elbows, shoulders, knees and ankles but not feet. At worst she could barely get out of bed but after a further two weeks, using only aspirin 600mg tid, her symptoms had almost resolved. There was no history of

rash or contact with rash and neither of her children (aged 5yrs and 8yrs) nor her husband had suffered a similar illness.

By the time of her first clinic visit (symptom duration 15 weeks) there was only slight residual pain in the knees and ankles and clinical examination revealed no abnormality. Confirming the clinical impression of remission, the ESR was 3mm/hr and CRP normal. The haemoglobin was 14.3G/dL, RA Latex negative. Viral serology showed Rubella HAI negative, B19 IgM 16U, IgG >100U indicating recent B19 infection, with the values obtained being consistent with the clinical suspicion of an infection just before the onset of joint symptoms.

She was reviewed on three occasions over the subsequent seven months and the remaining symptoms had resolved within the first month. There were some pins and needles in the fingers, reminiscent of her teenage symptoms but clinical examination and nerve conduction studies were normal. It was considered that this symptom was unrelated to her acute polyarthritis and she was reassured and discharged from clinic with instructions to report any recurrence of the arthritis. She was assigned to the SLS group.

6.4.2 Patient number 92: This 27yr old housewife was first seen in the Clinic on 10th March 1986. Six weeks previously, having been completely well, she developed pain, swelling and 90mins morning stiffness in many joints. This began as an ache in one knee but "suddenly" spread to involve hands, wrists, shoulders, hips, knees and ankles (but not feet) in an apparently symmetrical distribution. At worst, during the first week, she could not fully open or close her hands and was given diclofenac by her General Practitioner. Within two weeks of the onset, the worst had eased and by the time of first clinic visit (duration 6 wks) there was only mild joint pain

and morning stiffness persisting in the above listed joints. She had no history of rash but both of her children (aged 6yrs and 7yrs) were said to have had chickenpox in early January 1986, about the time of onset of her symptoms.

Examination at first clinic visit revealed pain at the extremes of neck movement, tender muscles in the lumbar region where there was slight global restriction in range of movement, and there was slight ankle swelling although those joints were cool and had full range of movement. The ESR was 2mm/hr, CRP normal, RA Latex negative and haemoglobin 14.4G/dL. Viral serology revealed Rubella HAI 1:64, Rubella IgM negative, B19 IgM 27U and B19 IgG 38U indicating recent B19 infection, the values being consistent with the six week history. At the first follow-up visit six weeks later the symptoms had almost completely resolved apart from mild pain in her back and knees and no abnormality was found on examination. She was allocated to the SLS group. Over two further visits, covering a total of six months from disease onset, all remaining symptoms resolved.

6.4.3 Patient number 93: This 35yr old quality assurance manager was first seen in clinic on 3rd February 1986. As a child he "had a problem" with one knee and a doctor had "put in a needle and drawn some fluid" but this was a transient problem and he considered himself entirely well so that he regularly played badminton until the presenting events. About the beginning of January 1986 he noticed a macular non-pruritic rash on only one arm, lasting only for a day or two. About the same time his daughter (aged 5yrs) had a runny nose and similar rash on her arms and chest. Just over two weeks after his rash he developed pain, swelling and 60mins morning stiffness in PIP, MCP, knees, ankles and MTP joints in an apparently symmetrical distribution, plus in one wrist. At worst he was unable to manipulate

buttons, walking was painful and his General Practitioner had commenced ibuprofen.

At the first clinic visit, two weeks from the onset of the arthritis, the symptoms had abated only slightly. The only positive findings on clinical examination were small, cool but definite effusions in both knees, although those joints were not tender and had full range of movement. In addition this previously fit man had grip strength of only 78mmHg (normal >300). The ESR was 11mm/hr, CRP normal and haemoglobin 14.5G/dL. The RA Latex was not measured. From one knee 4ml of transparent, straw coloured synovial fluid was aspirated and found to have a leucocyte count of 1.6×10^9/L, mostly lymphocytes. Viral serology revealed Rubella HAI 1:32, Rubella IgM negative, B19 IgM 50U, B19 IgG 18U, indicating distant past Rubella infection and recent B19 infection.

The symptoms resolved within two weeks of the first clinic visit, confirmed upon clinical examination at the six week follow-up visit which revealed no abnormality and the grip strength had returned to normal (>300mmHg). He was discharged from the clinic with a request to report any recurrence and assigned to the SLS group.

6.4.4 Patient number 105: This 31yr old housewife, having been completely well with no significant past medical history, was admitted as an emergency to Selly Oak hospital under the care of the general medical team on 22nd March 1986 and reviewed for this study two days later. Two days prior to admission she had developed a non-pruritic rash "like measles" on her forearms, thighs and abdomen, associated with a feeling of tiredness. The rash faded within two days. None of her three children nor her husband had a similar illness, although her youngest had been vaccinated against measles one month earlier. On the same day as the rash started she developed

pain, swelling and 30mins morning stiffness affecting hands ("sausage-like swelling"), wrists, shoulders, knees, ankles and MTP joints. At worst she was unable to fully open or close her fingers and was commenced on aspirin.
When first reviewed for this study (duration 4 days) the symptoms were past their peak. There was slight swelling of wrists, MCP, PIP, and ankle joints in a symmetrical pattern but all joints had full range of movement. The grip strength on the left was normal (>300mmHg) but reduced in her (dominant) right hand (151mmHg). The ESR was only 8mm/hr, haemoglobin 12.6 and RA Latex negative.
Interestingly the complement components were low: C3 0.68G/L (normal 0.75 - 1.75), C4 0.08G/L (0.14 - 0.54), suggesting possible complement consumption by an immune complex disease. Viral serology revealed Rubella HAI 1:16, Rubella IgM negative, B19 IgM >100U, B19 IgG 38U, suggesting distant past infection with Rubella and recent infection with B19.
Within one week of onset her symptoms resolved sufficiently for her to be discharged to home. After a total of two weeks from onset, the symptoms had resolved to only slight pre-menstrual aching in the joints and <u>she was assigned to the SLS group</u>. She was followed in clinic for a total of six months by which time even the slight residuum had resolved and she was discharged from clinic with a request to report any recurrence.

<u>6.4.5 Patient number 113</u>: This 44yr old nun was first seen in the Early Arthritis Clinic on 19th May 1986. She was completely well until mid-March 1986 when she had diarrhoea and aching limbs for one day. Four days later, while bathing, she noticed a non-pruritic rash on arms, chest and thighs "like measles". During that same day she developed pain, swelling and (on subsequent days) 60mins morning

stiffness. The peak of symptoms was reached over three days and at worst she had difficulty dressing and walking. After one week from onset the symptoms began to resolve. She did not live in an enclosed order and one week prior to the onset she had visited her young nieces and nephews but there was no history of similar illness in any of her contacts.

At first clinic visit (symptom duration eight weeks) she volunteered the continuing improvement in symptoms although examination revealed tenderness in wrists, MCP and PIP joints with slight swelling of the MCP joints. The ESR was 18mm/hr, CRP normal, haemoglobin 13.7G/dL and RA Latex negative. The viral serology revealed Rubella HAI negative, B19 IgM 8U, B19 IgG 100U suggesting no previous infection with Rubella but recent infection with B19.

At the first follow-up visit six weeks later all symptoms had resolved except mild morning stiffness in the hands and shoulders, and clinical examination was normal. She was assigned to the SLS group and discharged from clinic with an invitation to report any recurrence.

6.4.6 Patient number 101: This 33yr old dental receptionist was first seen in the early arthritis clinic on 18th March 1986. She had no significant past medical history and had been completely well, with in particular no previous joint symptoms until three months prior to being seen. On 26th December 1985 she developed a mild non-specific illness with headache in the evenings for two weeks. On 7th January 1986 she developed a slightly pruritic, macular, erythematous rash "all over" but mainly on legs feet and arms "like Rubella" which lasted three days. The day after the rash cleared she developed pain in her wrists, evolving within days to joint pain, swelling and 5hrs morning stiffness affecting: PIP, MCP, wrists, elbows, shoulders,

hips, knees, ankles and MTP joints in an apparently symmetrical pattern. At worst she had great difficulty with bathing, dressing, walking and stairs and experienced only partial relief on starting ibuprofen in mid-February. During the two weeks after Christmas 1985 her husband and two daughters (aged 10yrs and 16yrs) but not her third daughter (aged 14yrs) had the same rash and headache for one day each, without joint symptoms.

At the first clinic visit (duration of symptoms three months) there was: tenderness in the shoulders with slight restriction of movement; swelling of wrists; swelling of most of the MCP and PIP joints (symmetrically) with inability to completely open or close the hands; tenderness, swelling and slightly reduced flexion of knees; and tenderness on forefoot (MTP) squeeze. The grip strength was bilaterally reduced to 50mmHg. The ESR was 67mm/hr at this visit and interestingly her General Practitioner had noted it to be rising: 7th January 1986 - 19mm/hr; 20th January - 28mm/hr; 6th February - 40mm/hr. The CRP in clinic was 17 and the RA Latex was positive but the Rose-Waaler test was negative. Viral serology arranged by the referring physician had shown B19 IgM positive on 24th January and at her first visit to the Early Arthritis Clinic in March 1986 the showed Rubella HAI only 1:8, B19 IgM 9.2U, B19 IgG 60U. The serology thus confirmed the clinical suspicion of a B19 infection around the time of onset of her symptoms.

She was admitted to hospital in early May 1986 (duration of symptoms four months) when her symptoms were so poorly controlled by piroxicam that she was incapacitated, with severely restricted walking, diminished grip, and painful restriction of movement in hands and shoulders. The persistent swelling of PIP, MCP and knees joints was confirmed on examination. The ESR remained high at 105mm/hr and the haemoglobin had gradually fallen to 8.3G/dL, from 11.6G/dL in the

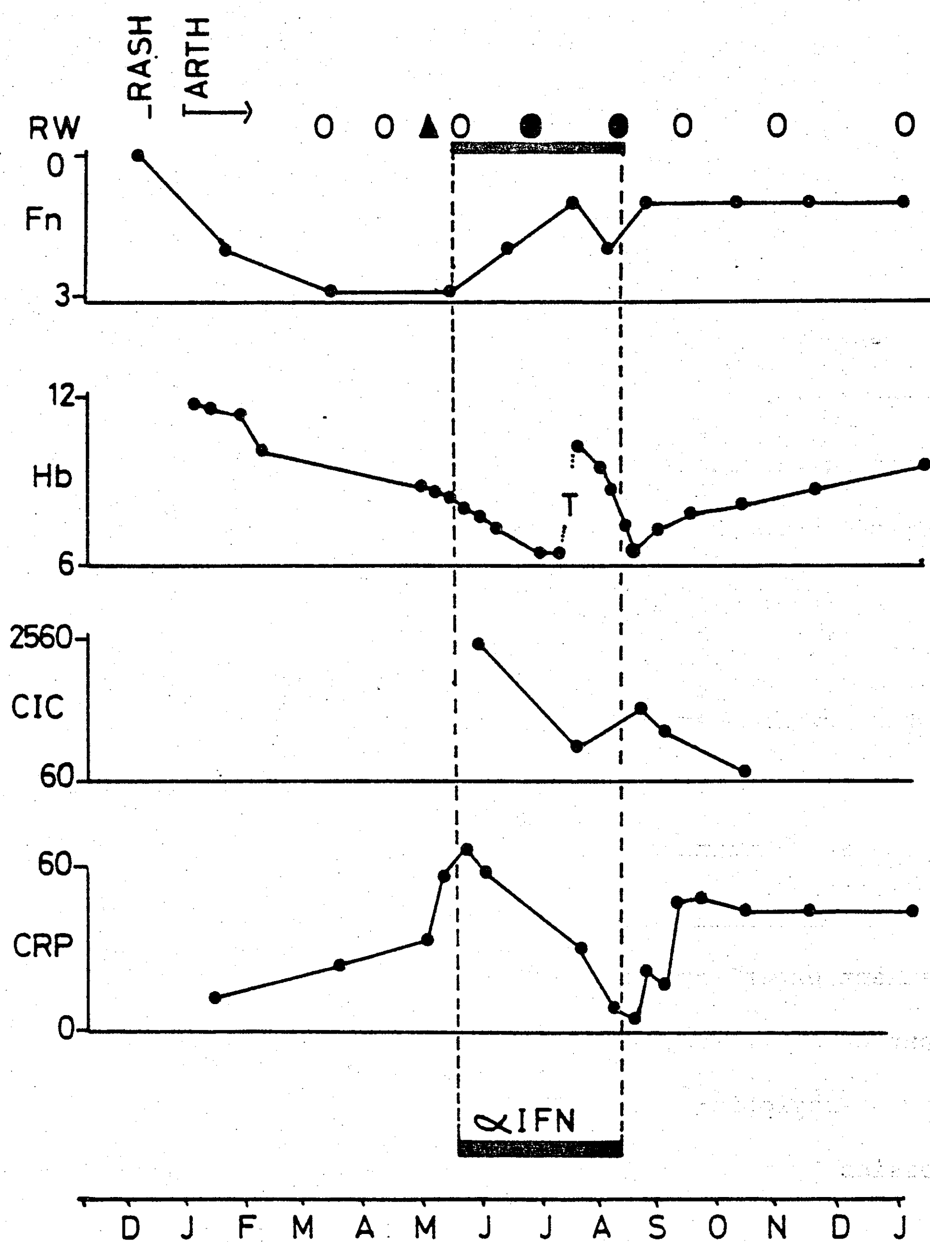


Figure 6.1 Response to alpha interferon therapy .

RW = Rose Waaler positive (filled) or negative (open) in serum (circles) or synovial fluid (triangles); Fn = functional class; Hb = haemoglobin; T = transfusion; CIC = circulating immune complexes; shown over a year, from December to the following January.

January. She was assigned to the PS group and invited to take part in trials of experimental therapy.

Alpha Interferon - rationale and protocol (Patient 101 continued):
Persistence in the host of virus after primary infection is well described for hepatitis B virus and because this is associated with chronic inflammation, a number of anti-viral treatments have been used with some success including alpha interferon[146]. The question of Parvovirus persistence is currently being studied for the animal and human hosts (page 46). Because of the possibility of viral persistence we elected to treat our patient (Number 101) with alpha interferon (Wellferon, kindly supplied by the Wellcome Foundation Ltd, Crewe) with the same regimen as used for hepatitis B. During the first week of treatment with alpha interferon the daily dose was gradually increased to the target dose of 10 Megaunits per square metre body surface area, given as a single subcutaneous injection. For the subsequent twelve weeks she received 10MU/sq.m on three days per week. She continued taking piroxicam throughout this period.

Alpha Interferon - problems during treatment (Patient 101 continued):
Our patient experienced mild influenza-like symptoms, well controlled with paracetamol. The main problem during treatment was a continued fall in haemoglobin: whether this was due to disease activity, marrow suppression by the alpha-interferon or by the B19 virus itself is not known, but she had been having heavy menstrual flow which resolved when an intra-uterine contraceptive device was removed shortly after the interferon trial was completed. The pattern of the changes in the haemoglobin are summarised in Figure 6.1. The Mean Corpuscular Volume showed a steady fall before and during interferon therapy until intervention with blood transfusion and iron, in keeping with the iron deficiency seen on subsequent bone marrow examination (see below). A fall in the platelet count (from 388 before, to a nadir on

interferon of 121 ($x10^9/L$)) closely followed that of the CRP and rose with the CRP after the end of interferon treatment, suggesting the recognised phenomenon of platelet count reflecting the acute phase response rather than necessarily a direct suppression of megakaryocytes by interferon. The lymphocyte count was lower than normal before ($0.82x10^9/L$), during (nadir $0.45x10^9/L$) and after (regularly $< 1.3x10^9/L$) interferon. The neutrophil count fell with interferon (from 5.98 to a nadir of 1.37 ($x10^9/L$)) and recovered after cessation of treatment. When the haemoglobin reached 6.7G/dL the dose of interferon was halved for two weeks (treatment weeks 6 and 7) and she was transfused three units of blood, after which the interferon was returned to full dose.

Alpha Interferon - response to treatment (Patient 101 continued): There was an apparent improvement during treatment in symptoms, clinical signs and in the acute phase response, summarised in Figure 6.1. The functional capacity improved from great difficulty arising from a chair or walking across the consulting room pre-treatment, to keeping up with her family on a country walk in the last week of treatment and then relapse two weeks later, sufficiently severe to require admission to hospital for bed rest. The CRP closely supported the subjective assessments. The fall in circulating immune complexes may be of pathogenetic significance. The RA Latex has been consistently positive in this lady but interestingly the Rose-Waaler has only been positive in blood twice (titre 1:64) and both occasions were during the interferon treatment. One month prior to interferon the Rose-Waaler had been positive (1:64) in the synovial fluid from her knee.

D-Penicillamine/Placebo (Patient 101 continued): Our patient was managed conservatively (one week of bed rest then out-patient physiotherapy, co-proxamol and piroxicam) for five months after the

interferon trial had finished. She experienced fluctuating mood and disease activity but was able to resume her occupation, shopping and housework although all with some difficulty. However, in January 1987, one year from onset of her symptoms, the symmetrical, peripheral poly arthritis was still active clinically and supported by: haemoglobin 9.6G/dL, ESR 50mm/hr, CRP 40 (normal <10). Her existing treatment was continued and she gave informed consent to be admitted to a double-blind, placebo controlled trial of D-penicillamine (designed to study the effect of the drug over one year, in recent onset arthritis of the rheumatoid type). She remained on the "study tablets" for four months without clinical or serological improvement. When she developed mildly painful 5mm diameter mouth ulcers (the only time she had ever these) the trial tablets were stopped (May 1987), the code broken and she was found to have been taking placebo.

Gold (Patient 101 continued): She experienced relief after intra-articular injection of triamcinolone to both knees but this was short lived. In August 1987, twenty months from onset of symptoms, she still had clinically apparent symmetrical, peripheral polyarthritis of the rheumatoid type, with ESR 95mm/hr and CRP 75. She was commenced on treatment with gold as Myocrisin 50mg intramuscularly each week and the coproxamol and piroxicam were continued. She began to experience improvement after two months and in January 1988, five months after starting gold and two years from onset of the first symptoms she was in symptomatic, clinical and serological remission. She was able to run, ride an exercise bicycle, work part-time and conduct activities of daily living with nil difficulty. She complained of slight pain in the first MTP joint of each foot, but of no other joint symptoms. The only abnormality on clinical examination was slight ulnar deviation of the fingers but there was neither

active synovitis nor accumulated damage in any joint. The ESR was 13 and CRP only 21, confirming the clinical impression of disease remission. The gold was continued in the reduced dose of 50mg per fortnight. When reviewed in January 1989, three years from onset of symptoms, the remission was sustained apart from cool but definite synovial swelling of one thumb inter-phalangeal joint. She has never had rheumatoid nodules and no definite joint erosions have yet been seen on X-ray of hands and feet.

Probing for persistence of Human Parvovirus B19: In patient 101 there was the possibility of persistent infection with the virus as an explanation for the continued immune response. Dot-blot hybridisation[33] was kindly carried out by Dr BJ Cohen, Virus Reference Laboratory, London but no B19 DNA was found in a synovial biopsy taken from the patient's knee in May 1986, four months from onset of symptoms and just before starting the interferon treatment. A sternal marrow aspirate performed three weeks after completing the interferon treatment revealed a normocellular pattern but reduced iron stores. It was hoped that with removal of any suppressant effect of the alpha-interferon therapy on erythropoiesis there might be a resurgence of erythropoietic activity. Since the main host cell infected by B19 is the erythroid precursor, it was hoped that if there was any B19 present in this patient, then it might replicate with the increased erythropoiesis. A faint positive signal was obtained but its significance is uncertain. The polymerase chain reaction method of DNA detection[147] is being developed for this virus and will be applied to this marrow sample.

6.5 Review of measured variables in B19 arthritis patients

With only 5 SLS and 1 PS patient having had recent B19 infection, it would be inappropriate to compare them with each other or with their

respective outcome groups (SLS or PS) in a formal statistical analysis. Review of the median and range values for the clinical variables (chapter 3) would suggest, however, that the B19 patients are representative of their groups for example in terms of age, sex, duration of disease at presentation and grip strength. It is particularly interesting that the strongest selected variable, the RA Latex, was able to discriminate between the B19 IgM positive SLS patients (all RA Latex negative) and the B19 IgM positive PS patient (RA Latex positive). Unfortunately none of the B19 IgM positive patients were among the 18 SLS and 10 PS patients (chapter 4) who had T-lymphocyte subsets assayed at first clinic visit.

Antigen	Fc-Per	Cri	Ware	Pe	Camp	Ren	Goe	Cart	Reed
median SLS	3.4	2.9	3.6	5.0	2.65	9.5	9.0	1.4	2.4
range SLS	0 - 46	0 - 70	0 - 33	1 - 23	0 - 23	3 - 73	1 - 67	0 - 28	0 - 134
Pt 68	0	3.0	6.3	7.4	6.7	3.6	9.8	1.1	2.3
Pt 93	3.4	2.9	1.5	4.3	1.3	11.7	1.7	0	1.6
Pt 105	6.6	28.8	22.3	23.1	23.4	73.5	66.8	3.7	11.0
median PS	90	149	112	37	156	74	51	21	130
range PS	2 - 1114	1 - 2598	0 - 2022	0 - 1549	0 - 2304	5 - 1101	0 - 1005	0 - 499	0 - 2624
Pt 101	195	336	304	48	179	91	237	31	236

Table 6.1 IgMRF levels (μg/ml) in B19 positive patients

Four B19 IgM positive patients (3 SLS, 1 PS) were assayed for IgMRF: their values were representative of their outcome groups (Table 6.1) but interestingly (as was the case for the clinical variables) patient 101 (in the PS group) had markedly different values from the other B19 IgM positive patients (who were in the SLS group). The

results of the capture ELISA assay quantifying CRI-bearing IgM levels were presented graphically and discussed for the B19 IgM positive patients (chapter 5). The values obtained (Table 6.2) seem to be representative of the respective outcome groups in the case of C7, H1, G6, G8 and the one value for 17_109. The possible exception was patient 105 (SLS) who had the highest values in the SLS group for H1, G8 and almost for G6. However one B19 IgM negative patient (Patient 43) had similar levels, so the phenomenon is not confined to B19 positive SLS.

CRI	C7	17_109	H1	G6	G8
median SLS	132	2.3	0.0	3.4	6.3
range SLS	34 - 458	0.5 - 10	0 - 15.6	0 - 30.8	0 - 114
Pt 68	132	-	0.4	3.7	8.8
Pt 93	101	0.5	1.4	3.8	6.3
Pt 105	458	-	15.6	25.6	114
median PS	147	18.5	1.8	5.2	20.1
range PS	7.5 - 627	0 - 495	0 - 25.6	0.8 - 33.3	0.8 - 116
Pt 101	93.8	-	11.8	11.5	76.4

Table 6.2 Cross-reactive idiotope (CRI) bearing IgM levels (μg/ml) in B19 positive patients

Only one B19 IgM positive patient (Patient 93) was included in the inhibition of direct binding ELISA assay of CRI-bearing IgMRF (chapter 5) and his results were very close to the median for his outcome group.

6.6 Discussion of viral studies

6.6.1 Discussion - viral serology results:

Rubella: The finding of no patients with post-Rubella arthritis presumably reflects the increasing level of community immunity as a result of screening women near puberty and vaccinating those found to be non-immune, with further monitoring of the immunity in the obstetric service. The role of Rubella in arthritis will possibly reduce further with the introduction of the combined vaccination against mumps, measles and rubella for all children prior to entering school.

Human Parvovirus B19: In view of the wide range of disease duration at presentation, we chose to use the single serum sample obtained at first visit, to determine recent infection on the basis of specific anti-B19 IgM. Previous studies have also included patients with rising titres of anti-B19 IgG[42]: however since we were able to detect IgM as late as three months after the clinically apparent infection, when the IgG titres would have reached a plateau, it is unlikely that paired IgG titres would have included any more patients. The recruitment period for this study was from May 1984 to May 1986. All six of our positive patients experienced infection in the winter of 1985/86. The availability of serological testing for B19 was not widely known at that time so there are no accurate figures for the incidence of B19 infection in the community over the same period. However one community based study of a B19 epidemic in a school[11] revealed that of the 54 anti-B19 IgM positive adult contacts, fully 26 had acute symmetrical polyarthropathy, but lasting a median of only 10 days. Our figures confirm the benign nature of B19 arthritis with the majority of cases being sub-clinical and of those who do come to the attention of their General Practitioner, spontaneous remission usually ensues before any hospital referral, even when the

hospital is actively recruiting such cases.

Perhaps more intriguing than the anti-B19 IgM positive patients are the 24 SLS patients with unexplained polyarthritis, where the nature of the trigger remains elusive, as it does in all but one of the PS patients.

6.6.2 Discussion - case histories:

The analysis of the clinical data (chapter 3) suggested that the SLS patients had a very similar disease pattern to PS in all but persistence. The concept of SLS as a model for PS (and hence for RA) is further refined in this chapter with the identification of a specific trigger for SLS in five patients who would appear to be representative of the SLS group, in terms of the variables measured for this study.

Even though B19 arthritis is usually benign the cases described here indicate that for some patients the arthritis is sufficiently severe to seek medical help and even to require hospital admission. Their subjective complaints are supported by clinical observation with, for example, very real reduction in grip strength in the earliest weeks with subsequent recovery. The ability of a virally induced arthritis to mimic rheumatoid arthritis in all but persistence (and arguably in severity) suggests that many components of the inflammatory process we recognise as polyarthritis, are common to both SLS and PS and hence independent of the characteristic of persistence.

The case of patient 101 has been described in detail precisely because she would appear to be the exception to the rule of a benign prognosis for post-B19 arthritis. There is little doubt that she and her family suffered infection with B19 and that within days she experienced her first ever joint symptoms. We were unable to demonstrate conclusively any persistence of the B19 virus but

although the methods used and the tissues chosen were the only ones available at the time, it is clear that the search could not be exhaustive. Whether, in susceptible hosts, persistence of B19 virus drives a chronic immune reaction, or alternatively transient infection triggers an appropriate immune reaction which escapes the normal controlling mechanisms, remains open to further investigation. Although not germane to the prediction of persistence in recent onset synovitis, the details of treatments for patient 101 are given lest they influenced the outcome. There was little effect on symptoms or signs from the placebo D-penicillamine phase of treatment. The timing of onset of remission once gold was started is in keeping with this having been due to the gold. It remains possible that the remission was coincidental and spontaneous but she continues to have very limited but definite synovitis in one joint, which is not the experience to date of spontaneously remitting B19 arthritis where there may be continuing non-specific symptoms but not definite synovitis. The temporary benefit from alpha interferon may have been due to incomplete elimination of putative viral persistence or to temporary immunomodulation and the further investigation of these possibilities, using serial data from our patient may help to elucidate the mechanism of her persistent synovitis.

It is clear that in patient 101 the B19 arthritis was severe and remained so for longer than any previously reported B19 arthritis. On many occasions her arthritis fulfilled the ARA criteria for the diagnosis of RA. The B-lymphocyte data obtained from this patient would support the clinical decision to assign her to the PS group. If she were to develop characteristic joint erosions or rheumatoid nodules it would be hard to refute the diagnosis of RA. In the meantime it is a matter of philosophy whether her diagnostic label should be "a persistent post-viral polyarthritis of the rheumatoid

type" or simply RA with an identified trigger. In the context of possible mechanisms of persistence it was particularly interesting to find she is homozygous for HLA DR4, but that will not resolve the question of the diagnostic label until more information is available from epidemiological studies which include the relationship of HLA phenotype to speed of recovery from B19 arthritis. Any study of B19 arthritis as a model for RA will have to consider the two identified outcomes, the commoner being SLS but now with PS as a possibility.

CHAPTER 7

DISCUSSION

AND

CONCLUDING COMMENTS

CHAPTER 7

DISCUSSION

7.1 The patients

A total of 112 patients with musculo-skeletal symptoms of recent onset has been screened, with 65 of these having the symptoms of a symmetrical, peripheral polyarthritis similar to that of Rheumatoid Arthritis (RA). As a result of specifically soliciting from general practitioners their patients with recent onset of disease, these 65 patients were seen earlier than would have been possible through more routine methods of referral. Since the study excluded anyone with more than six months of symptoms, the 65 patients reported here were seen earlier than in most published studies of early RA. Accordingly they are a particularly rare and valuable group in the study of RA. Conceived on similar lines to the present work, a previous study[140] also included patients with recent onset arthritis but had one entry requirement that patients should have at least probable RA (by ARA criteria) during the study and hence patient selection was possibly more specific and less sensitive than for the present study.

7.2 Constraints on data interpretation

The following considerations are important because they underlie the formal data analysis for each results chapter.

7.2.1 Selection

The response from general practitioners was varied, with some referring several patients and others (the majority) referring none. This study therefore is not (and was not designed to be) epidemiologically sound in the sense of representing the experience of the entire at-risk population. However the study was designed to

address two main areas of ignorance: (i) if a treatment is to be applied to early RA, how easy will it be to select appropriate patients? and (ii) what can be learned of the pathology of chronic RA from study of its earliest stages?. If either of these questions are applied in routine clinical practice, the recruitment method used here will be common, and is therefore relevant.

7.2.2 Definition of outcome

I allocated a patient to one of the disease groups (SLS or PS) based on the patient and myself agreeing to disease being present or absent. Unlike the broader clinical experience where patients may complain of vague symptoms even after the clinician judges there to be no disease present, the patients in this study had, in general, dramatic symptoms at the outset and were keen to welcome remission if it came. I would suggest that although this study was not designed to measure it, the definition of outcome was so categoric that the inter-observer variation would be low.

The time at which an outcome was defined for a patient was generally within six to twelve weeks of their first visit to clinic. This allocation was much earlier than in a previous study[140] where the analysis was made on the basis of patients being followed for a minimum of three years. That study included three outcome groups: category 1 - no swelling or joint erosions; category 2 - soft tissue swelling but no erosions; category 3 - erosions. The SLS group in the present study corresponds to the category 1 of the previous study. The PS group is likely to include categories 2 and 3, assuming that in the present study the development in time of joint erosions will be confined to the PS group. No patient in the present study is known to have crossed over after allocation to an outcome group but long

term follow-up studies are in progress and will include comparison of the radiographic examination of the hands and feet performed on all patients at entry to the study and at follow-up: clearly a disease duration of three to six months is too early to comment on development of erosions[52].

7.2.3 Single observations at point time

All of the analysis is based on each variable being measured only once at a single point in time, namely the patient's first visit to the early arthritis clinic. By design this thesis examined only those first visit data. It is likely that in clinical practice the selection of a patient for entry to a therapeutic study would depend on the gradient of disease activity based on measuring selected variables at a minimum of two time points. Serial data available on most of the patients presented here will be the subject of further study to examine whether they confirm or change the current conclusions.

7.2.4 Statistical techniques and validity

In the univariate analysis of many variables, the need for the correction in the level of statistical significance for the number of variables recorded has been discussed in chapter 3. In chapters 4 and 5 the T-lymphocyte and B-lymphocyte studies can be considered as independent studies with small numbers of variables. The significance values in those (most of which would remain significant and would not alter interpretation if corrected for the number of variables recorded) have been used as a guide to interpreting the pattern of changes, in the hope of understanding underlying pathogenesis. Notwithstanding significant differences, in univariate analysis the striking finding was of overlap between the groups (SLS and PS). This

overlap was further confirmed by the multivariate analysis (stepwise variable selection) which, although identifying the most predictive of the recorded variables, was still only able to account for about half of the variability in outcome.

7.3 Clinical variables

7.3.1 Natural history

The critical observation first noted by others and studied in detail here, is that people experiencing acute, severe, disabling peripheral polyarthritis can expect one of two outcomes: spontaneous remission within about six months, or chronic disease that is similar or identical to RA. It is worth emphasising the point which does not emerge clearly from previous studies: even those patients whose recent onset synovitis is destined to remit, experience a severe arthritis. A typical story of a patient with SLS, which cannot be reflected in bare statistical analysis would be as follows:
"I awoke early one morning - I was well the night before - and I could not move. I was in terrible pain, all stiff. My husband had to help me out of bed and to the toilet. I cried because I was terrified it would never leave me".
For the fortunate patient with SLS the emotional impact of the disease will soon resolve with the clinical remission, unlike in the PS patient. For the statistician the possibility of predicting outcome on the basis of presenting features has been explored in detail in the clinical data analysis (chapter 3) and by previous authors with, for example the rapid rate of onset of symptoms being considered a benign prognostic feature by some[59] but not by others[58]. However, if the long term review of the present study's patients confirms the allocation to outcome groups, then prediction of outcome on very early data will be unnecessary since spontaneous remission

was found to declare itself within one or two follow-up visits (interval six weeks) and generally by disease duration six months. That duration would be suitable for all but the most aggressive early treatment protocols.

7.3.2 Clinical variables - SLS as a model for PS

The outstanding clinical observation is that in the earliest stages the patients with SLS have disease that is apparently very similar to PS and although symmetry was not formally analysed, the pattern of joint involvement was pre-selected for being symmetrical. This similarity between SLS and PS is supported by the detailed statistical analyses of the clinical variables which are more remarkable for the overlap between the groups than for the discrimination between them. The main outcome of the clinical analysis then is not the prediction of persistence but rather the realisation that in the brief but severe arthritis of SLS lies a human model for the study of PS and hence for the study of rheumatoid arthritis. The absence of chronicity of inflammation and accumulated joint damage enhance the value of the model by allowing a reductionist approach to the understanding of their aetiology: it should help to differentiate between those phenomena of inflammation common to any polyarthritis from those mechanisms intrinsic only to chronic disease.

In considering the pattern of joint involvement, the similarities between the two groups (SLS and PS) were emphasised particularly by the poor combined sensitivity and specificity of most of the joint variables (Appendix 1, Table A1.1). Considering the combined sensitivity and specificity (referred to as "accuracy" in the description of the 1987 ARA criteria[55]) it is interesting to note

that the most predictive (for PS) of the joint variables in the present study are those defined as being present by the clinician, namely swelling at wrist, MCP and PIP joints (sensitivity/specificity (%) 65/83, 79/61, 69/69 respectively). The values for sensitivities and specificities in the present study of recent onset disease are only slightly lower than in the description of the ARA criteria for the diagnosis of RA[55], which were developed in patients with longer established disease. It emerges that by the time of presentation two-thirds or more of the SLS patients did not have (objective) swelling at those joints: this is partly because many were already undergoing remission (compare with the much lower specificities of the (subjective) swelling reported by patients in those same joints) and hence as a group the SLS patients had less severe disease at first clinic visit, than the PS patients. This difference in severity between the groups is seen also in the routine laboratory variables, with for example the ESR being higher in the PS group. Clearly, however, it is possible for a patient to have a high ESR and still undergo remission: the acute phase response is therefore likely to be part of any polyarthritis rather than be a determinant of chronicity. This is consistent with the production of acute phase proteins by the liver being induced by interleukin-1 (IL-1), produced in turn by the non-antigen specific monocyte/macrophage lineage[148,149] (although now thought to be in conjunction with IL-6 [150]) and so likely to occur if there is inflammation of sufficient synovial tissue, irrespective of cause or chronicity.

7.4 B-lymphocyte studies - a dichotomous discrimination?

7.4.2 RA Latex and IgMRF quantitation

The simple slide latex test for RF, returning merely a positive or negative result, has been regarded as a useful screening test which

lacks specificity and, for diagnostic purposes, has to be supported by a more specific test (eg Rose-Waaler test or quantitation of the latex result). It was therefore surprising to find a near-dichotomy in the results for the RA Latex in the present study with very little overlap between SLS and PS (sensitivity/specificity for PS being 79/89 %). It was even more remarkable to find the RA Latex positive in only one of the six post-Human Parvovirus B19 patients, and that was in the patient with Persistent Synovitis. This strongly suggests that the production of RF (so defined) is not common to all inflammatory polyarthritis but is almost restricted to persistent synovitis. That its presence in the peripheral blood is not, however, necessary for persistence is evidenced by the six PS patients in whom the test was negative: in turn this is in keeping with the finding of a positive RF in only some 70% of patients with otherwise typical RA[86]. It could be argued that a positive RA Latex reflected disease severity, since the correlation between RA Latex and ESR was moderate (coefficient 0.65, $p > 0.0001$) but the weakness of the apparent correlation (between a categoric variable and a continuous variable) was revealed on regression analysis, with the ESR accounting for only 42% of the variability in RA Latex. That the RA Latex is not simply a marker of disease severity is further supported by the finding that the quantity of IgMRF did not correlate with the ESR either (chapter 5, Table 5.4). The increased levels of IgM (with and without RF activity) in PS compared with SLS would be consistent with the provision of increased T-cell help for B-cells, suggested by the finding in PS of higher numbers of activated TFR+CD4 lymphocytes in the peripheral blood.

This then suggests a specific role for RF in PS. That such a role might be physiological, aimed at binding circulating immune complexes

and enhancing their removal from the circulation[151], would be supported by the finding of a close correlation between IgMRF levels irrespective of the IgG subclass used as target in the assay (chapter 5, Table 5.4). Thus the main antigen for IgMRF would be self IgG-Fc of a range of subclasses. Superficially there is a suggestion that the RA Latex (using rabbit IgG as antigen) is more discriminating between SLS and PS (relative risk for PS 5.31, Chi^2 30.0, table 3.4) than is a supra-normal IgMRF level using human IgG as antigen (relative risk for PS 3.43, Chi^2 11.3 (RF-Per) table 5.3). However, the respective sensitivities and specificities are remarkably similar, especially considering that the RA Latex is a naturally categoric variable and the supra-normal IgMRF level depends on the validity of the normal population sample, and considering that the IgMRF levels were performed on a smaller number of subjects than for RA Latex.

7.4.3 Qualitative differences in IgM(RF) between SLS and PS

If the above model of IgMRF as having a physiological, clearance role with wide specificity for IgG is true, it begs the question of why the idiotypy of the IgMRF is different in SLS versus PS. It appears that in both SLS and in PS there is IgMRF with specificity for IgG but the levels are higher in the PS patients (albeit with considerable overlap with SLS and indeed with normal subjects). In both SLS and PS expression of cross-reactive idiotopes (CRI) on IgMRF can be detected. (The argument that the assays measure CRI expression mainly IgM with RF activity has been addressed in detail in Chapter 5). The levels of some but not all of the measured CRI are slightly higher in PS than in SLS. However the striking feature in PS is that the much higher total levels of IgMRF in the PS patients are largely accounted for not by CRI-bearing IgMRF but by greatly increased

levels of non-CRI-bearing IgMRF. Indeed there is a disproportionate elevation in the non-CRI-bearing IgMRF in the PS group of patients. This additional production in PS of IgMRF which does not express the tested CRI suggests alteration in fine structure of the variable region of such IgMRF, arising from a departure from the use of germ-line sequences in PS, through somatic mutation. This would mean that in addition to so-called "natural antibodies" (being generally low affinity, cross-reactive and of IgM class) the PS patients were also producing more specific, higher affinity, "pathogenic antibodies".

The CD5 antigen is expressed on the surface of a subset of B-cells in normal mice[152] (previously referred to as Ly-1 positive cells) and in humans[153, 154] (previously known as Leu-1 positive cells). This subset produces IgM antibodies with activities including anti-RBC, anti-IgG and anti-ssDNA. CD5+ B cells are present in patients with RA and indeed have been found in increased numbers compared with normal subjects[153, 155] but their numbers do not correlate with the levels of total IgMRF in the patients. The autoantibodies they produce are thought to be of low affinity and to have been translated and transcribed from germ-line genes without somatic mutation. Since the CD5+ cells occur in health as well as disease (albeit in different amounts) the antibodies they produce are thought to have a physiological role[156]. It seems likely that in both SLS and PS patients these CD5+ B-cells will be found to be present, to be secreting CRI+ IgMRF, and perhaps with increased numbers in the PS patients. However the increase in non-CRI-bearing IgMRF in the PS patients would suggest that in addition they have CD5- (negative) B-cells present, producing IgMRF of a "pathogenic" nature, with altered specificity as a result of somatic mutation.

Support for the concept of somatic mutation in IgMRF production in PS comes from the murine models of lupus where the pathogenic (IgG) antibodies appear to result from somatic mutation. Variability in the specificity of auto-antibodies from genetically identical (within (NZBxNZW)F1 and also within MRL-lpr/lpr) groups of mice cannot arise from the germ-line[157,158]. Furthermore DNA sequence analysis supports the use of somatic mutation in auto-antibody production in MRL mice[159]. The somatic mutations are believed to occur randomly and those B-cell clones producing higher affinity antibodies are selected by their preferential binding to antigen[160-2].

It appears then that somatic mutation may be occurring in PS and the question can then be asked about the nature of the driving antigen. One possibility is that the change in fine structure results in increased affinity for the putative main target antigen, the Fc portion of IgG. Arguably this might enhance clearance of IgG complexed to foreign antigen or, through cross-linking of the Fc component of IgG molecules specifically bound to a microbial cell surface, enhance opsonisation[163]. For the future that hypothesis could be tested by comparing the affinity, for IgG bound to antigen, of CRI-bearing and non-bearing IgMRF. Work to date suggests that IgMRF in RA are generally of low affinity for autologous circulating IgG[54].

An alternative hypothesis would suggest that normal IgG is not the main target antigen for IgMRF and that its affinity for IgG arises from cross reactivity with an alternative (exogenous or self) primary antigen. Recruitment of clones of B-lymphocytes producing non-CRI-bearing IgMRF could be antigen driven, and might persist through either persistence of the primary antigen, or through cross-

reactivity with self IgG.

As discussed in chapter 1, one such antigen could be IgG that had been damaged at a site of inflammation. However, the levels of free-radical damaged IgG were not abnormal in the peripheral blood of the SLS and PS groups of patients. This could be because the hypothesis is wrong and free-radical damaged IgG is not a persistent stimulus for RF production. The hypothesis could alternatively be correct if the avid binding of RF to damaged IgG resulted in the clearance of the latter from the circulation and the findings in the PS patients reflect antibody (RF) excess. Work is in progress to attempt to quantify free-radical altered IgG extracted from circulating immune complexes.

Any departure from considering IgG as the main antigen for IgMRF would have to account for the continued ability to bind to IgG despite affinity maturation in favour of binding to a putative alternative antigen. Candidates for consideration as target antigens for IgMRF would include those of infectious agents. Such a model would have, at its simplest, the IgMRF binding the microbial antigen directly, without the need for an antigen-specific IgG molecule as intermediary. A formal study of any affinity of non-CRI-bearing IgMRF for the antigens of arthritogenic organisms would be worthwhile, including for example the structural proteins of the Human Parvovirus B19 in patients who have recently been infected. However, although some RF have been identified as having specificity for antigens other than IgG, these have been the exception, with most RF not having an identified alternative specificity[54].

The complexity of the exogenous antigen model can be increased by

considering the IgMRF as an anti-idiotypic antibody. The expression of receptors for Fc on the surface of some micro-organisms (allowing them to sequester host anti-microbial antibodies) might lead to a host response with antibody (Ab1) specific for any unoccupied microbial Fc receptor and would have Fc as its internal image. An anti-idiotypic (controlling) antibody (Ab2) would then have anti-Ab1 specificity and as a consequence also anti-Fc specificity, ie RF-activity[160]. The relative affinities of CRI-bearing (germ-line) versus non-CRI-bearing (possibly non-germ line) IgMRF for IgG-Fc might test the alternatives. If simple cross-reactivity between an exogenous antigen and IgG were true, then with affinity maturation towards the exogenous antigen, the (cross-reactive) affinity for IgG-Fc might be expected to decrease. If the anti-idiotype model were more accurate, the same affinity maturation might be parallelled by increased affinity (of Ab2) for IgG-Fc.

7.4.4 B-lymphocyte studies: summary

The studies of both the quantity and quality of IgMRF production suggest differences in the nature of the inflammation in PS compared with SLS. These differences do not simply reflect disease severity and their near-dichotomous nature may indicate a role for specific forms of IgMRF in the chronicity of disease in PS. Whether the role is causative of chronicity or in response to it, remains an open question which might be addressed by the study of antigen-specific arthritis models.

7.5 T-lymphocyte studies

Although only phenotypic studies were performed it is interesting to combine the results with those from the B-lymphocyte studies to speculate on corresponding functional mechanisms.

7.5.1 Activated T-lymphocytes

It was clear from the results presented in chapter 4 that in terms of circulating T-lymphocytes, the main differences between the SLS and PS groups lay within the activated population of T-lymphocytes, and in particular within the CD4+ subset of the activated cells. The greatly increased levels of TFR+,CD4+ cells suggests the presence of increased availability of T-cell help (by cytokine production) for B-lymphocytes. The IgM levels in PS being higher than in SLS would superficially support that assertion.

7.5.2 Failure of Ig class switching
- possible differential cytokine production?

The probability that affinity maturation is taking place in PS was discussed above, and in a normal immune response one would expect this to be accompanied (within about 10 days from first antigen challenge) by a rising specific IgG and then (after some weeks) by a fall in the specific IgM level. The existence in a primary immune response of that IgM antibody with specificity for an antigen, indicates that at least a degree of affinity maturation may take place in advance of isotype switching. This suggestion is supported by evidence from experimental systems that somatic mutation takes place before, during and after isotype switching[162, 164-5].

In order to develop this discussion further a degree of extrapolation is now required, since the observations for this thesis were made at a single point in time for each patient and sequential data has not been presented. In addition no studies of IgGRF were performed on these patients. Equating the PS patients to RA would suggest that the elevated IgMRF levels observed in this study can be expected to

persist: supra-normal levels of IgMRF are found in RA patients decades after the onset of their disease and indeed this phenomenon forms one of the ARA criteria for the diagnosis of RA. In contrast, although IgGRF is identified in RA, it is not usually the predominant Ig class and indeed when it becomes so, it is associated with a change in the disease manifestations towards systemic vasculitis[54]. I would suggest that in PS although affinity maturation of the immune response (compared to that of SLS) appears to have taken place (mean disease duration at first visit PS = 14.2 weeks), the immunoglobulin class-switching is at best incomplete. It might be possible on future PS patients follow this possible affinity maturation serially from the earliest days of symptoms and to include examination of the cytokines produced by their CD4+ lymphocytes. It may be that in PS the CD4+ cells supply cytokines required for the proliferation of B-lymphocytes and their secretion of immunoglobulin, but fail to supply the cytokines required to complete class-switching. A simple scheme has been suggested for the cytokine control of Ig production, with IL-4 involved in B-cell activation (in conjunction with antigenic stimulation), IL-5 being involved in B-cell proliferation and IL-6 involved in B-cell differentiation to plasma cells[166]. In that simple scheme affinity maturation and isotype switching occur in presence of IL-6 but the authors pointed out that IL-6 increases the mRNA for both gamma and mu chains, ie that the effect is not isotype specific. Since that review was produced (about one year ago) it has become clear that the picture is far more complex: individual (recombinant) cytokines, tested in purified B-cell preparations, can have contradictory effects (enhancement or suppression) depending on the state of pre-activation of the B-cell, and the relative amounts of other cytokines present[167]. Furthermore, a degree of isotype specificity has been suggested, with IL-4 being associated with IgG1

and IgE production (depending on how the B-cells are stimulated), IL-5 with production of IgA, and gamma interferon with IgG2a[167]. In addition recent work has suggested that not all the cytokines have been fully characterised, with a possible novel B-cell differentiation factor being involved in the very late stages of B-cell differentiation[168].

All this leaves room for speculation that the persistence of RF of the IgM isotype, despite somatic mutation, arises as a result of an aberration (fault or virtue) in the control of isotype switching, due to a differential cytokine effect (at any stage of the signalling: transcription, secretion, binding or transduction).

Whether the incomplete isotype-switching were advantageous (in avoiding systemic vasculitis, for example) or an embarrassment (in promoting persistence), it would of course have to be restricted to a very limited part of the host's immune repertoire: patients with RA can mount normal class switching in response to common infecting organisms.

7.6 Viral studies

The search for a viral aetiology for the arthritis in these patients was deliberately confined to those viruses which may be amenable to further study in the foreseeable future. Human Parvovirus B19 (B19) is known to infect the susceptible population in epidemics. The resulting "fifth disease" in children is mild but the rash alerts the school and medical authorities to the epidemic. That awareness, added to the high incidence of arthritis in affected adult contacts (especially the mothers), offers the opportunity to study useful numbers of patients with an arthritis arising in response to an

identified and well characterised antigen. Rubella was studied in addition partly because of the similarity in the clinical picture but also because the assay performed on the serum to detect a specific immune response uses a near identical method to that for B19: the Rubella results are therefore a control for any non-specific immune response.

7.6.1 Clinical picture of B19 arthritis

Of the six patients shown to have specific anti-B19 IgM near the onset of their arthritis, five were typical SLS patients. Even though there was variation in the severity of B19 arthritis, the patients here demonstrate a consistency in the pattern of joint involvement which is further supported by observations by previous authors[42, 43]. The general conclusion arising from the clinical studies (chapter 3), that SLS offered a model for the study of PS (and hence for the study of RA), can therefore be refined to using B19 arthritis as a model for RA. Hence with a known antigen and with the usual outcome known, the host responses should be amenable to specific study and comparison with the wealth of observations on the immune system in RA.

The finding of one patient with B19 arthritis which evolved to a persistent synovitis was unexpected but potentially increases the value of the model. In response to a given antigenic challenge (serological and DNA sequencing studies suggest little or no variation in the structure of the B19 Parvovirus recovered in different epidemics[169]), the finding of diversity of outcome suggests that the differences lie at the level of the host response to the antigen. At the simplest level, a limited number of serial serum samples are available from the B19 patients and it might be possible

to further study affinity maturation through any changes over time in the idiotope expression on IgMRF, and to more formally examine the possibility of incomplete Ig class-switching. If future patients could be seen sufficiently early, serial functional studies of the T-lymphocytes, in particular cytokine production, might help to explain the persistence of IgMRF in RA.

7.6.2 Persistent infection or persistent host response?

The question of the possible persistence of Human Parvovirus B19 has been discussed above (page 46) and so far no definite conclusion can be drawn. Our results so far have failed to demonstrate any persistence of the virus (see chapter 6, case 101) but development of the polymerase chain reaction method[147, 170-1] with its increased sensitivity might define how long virus persists, although the studies are limited by the availability of host tissue.

Animal models of persistent immune response have suggested that the triggering antigen may only be necessary early in the disease, with host T-lymphocytes responsible for persistence, and with disease transfer mediated by antigen specific T-lymphocytes[172]. The association of RA with particular class II major histocompatibility complex (MHC) antigens[173], together with the recognition of the role played by the MHC molecules in presentation of antigen to the T-lymphocyte receptor[63], suggests that T-lymphocyte recognition of antigen may be crucial in the pathogenesis of RA. In RA the difficulty lies in choosing which antigen to study from a large number of candidates. Previous viral studies in RA have been

inconclusive[26] indicating the need to use more precisely defined viral antigens[174].

7.7 B19 arthritis as a model for RA - work in progress

From the observations in this thesis I have developed the hypothesis that B19 provides a model for the study of RA, with differing outcome of infection with a given antigen being determined by differing host response. I will present in this section further justification for choosing this virus and the specifics of the work which has now begun to test the hypothesis. Having previously discussed the possibility of persistence in the host of the B19 virus and the intention to continue to probe for its presence, I will now concentrate on the study of the host response. Precedents for this kind of work are established in other virus systems (for example Foot and Mouth Disease virus[175] and influenza virus[176] but have not been applied to the rheumatic diseases.

7.7.1 Human Parvovirus B19 antigens available for study

B19 arthritis provides the opportunity to study T-lymphocyte responses to defined viral antigens. B19 is a structurally simple, well characterised, single strain, single stranded DNA (5.5Kb) virus. Culture of the virus requires human bone marrow[39] and so the only practical but rare source of the whole virus remains viraemic plasma. Small quantities of viraemic plasma are available but the planned studies will require larger amounts of antigen, and so the supply of whole virus will be reserved for very selected studies. Recent cloning[33-4], and sequencing[35] of the viral genome has improved the understanding of its structure. Furthermore, expression of some partial B19 cDNA clones to yield antigenic polypeptides has permitted the description of epitopes recognised by anti-viral antibodies

(B-cell epitopes)[177]. Equivalent studies on T-cell defined epitopes have not yet been performed. To date, there are no published studies of T-cell responses to whole virus. The search for T-cell epitopes can be narrowed further: firstly by considering the published transcription map[178] to determine the likely proteins encoded by the virus and secondly, by examining the sequence of a given protein, for peptides containing amino-acid sequences that correspond to algorithms designed to predict sequences which can be recognised by T-cells when bound to MHC antigens[179]. Once selected, candidate peptides can be synthesised directly (the algorithms generally require peptides of some 8 to 10 amino-acids in length) or alternatively, viral constructs which contain these relevant sequences may be used.

I have now obtained, from several of the cited workers in the field, peptides synthesised from the B19 sequence on the basis of the algorithms, protein expressed by partial cDNA B19 clones in E. Coli, and expressed protein from a partial cDNA clone in a mammalian cell line (unpublished data). These materials are therefore ready to be applied to the study of the study of the immune response to specific antigens, in different hosts.

<u>7.7.2 B19 arthritis - possible mechanism for differing host response</u>

The association of RA with particular MHC antigens[173], for example DR4, suggests that it would be worth exploring the processing and presentation of antigen to T-cells by antigen processing/presenting cells of differing MHC phenotypes. Preliminary evidence in B19 arthritis suggests that no particular MHC phenotype is associated with the development of arthritis, but its persistence may be associated with DR4 while DR1 appears to be protective[12]. Given the

structural similarity between DR1 and some of the sub-types of DR4[180], that dichotomy is surprising and would have to be confirmed by further work. Nevertheless it is known that for a given antigen, the different epitopes which are recognised by T-cells are determined by MHC antigens[181]. It is possible therefore, that different B19 antigenic epitopes are recognised according to the MHC phenotype in the host. These differences may influence the persistence of B19 arthritis either by deficient elimination of the virus or by cross-reactivity between particular B19 epitopes and self antigens.

The work now in progress is therefore examining T-lymphocyte in vitro proliferative responses to defined B19 antigens in a number of clinical groups of subjects: these are defined according to recent, distant or nil evidence of B19 infection (by presence or absence of specific anti-B19 IgG and IgM), the presence or absence of an acute arthritis (self-limiting or persistent), and the presence or absence of rheumatoid arthritis (classically defined). The results will then be viewed in the context of the subjects' MHC phenotypes.

7.8 Concluding comments

In this study of patients with recent onset arthritis two main conclusions emerge, one pragmatic and related to clinical practice, and the other an hypothesis, that there is a new model for the study of pathogenetic mechanisms in RA.

The clinical studies reveal that at their very first visit to clinic, patients with recent onset polyarthritis can be assigned to either SLS or PS with only limited certainty. However within a further six weeks the certainty is greatly enhanced. This still means that at less than six months of disease duration we can identify those

patients with serious, persistent disease who may benefit from early treatment. It may be that this opens an early therapeutic window, after disease onset but before "chronicity" is established, when it would be possible to alter the course of the disease.

The second important conclusion lies in the realisation that the phenomenon of SLS might serve as a human disease model for the study of rheumatoid arthritis. It may be hypothesised that in PS and SLS the triggers are the same but the difference in outcome is determined by the variation in host response. This hypothesis can be tested by further refining the model to suggest that infection with Human Parvovirus B19 offers the specific opportunity to investigate the molecular mechanisms of the MHC restriction of host immunity and possible autoimmunity. This may sharpen the advancing edge of our knowledge of man's interaction with his environment through an immune system so sadly perturbed in people with rheumatoid arthritis.

APPENDIX

APPENDIX 1

EARLY ARTHRITIS CLINIC

PRELIMINARY SCREENING FORM
(First Visit)

Name______________________ Survey No_____________
Address___________________ Date__________________
__________________________ Unit No_______________
__________________________ NHS No________________

Telephone_________________
Sex
DOB
Occupation Now:

Past:

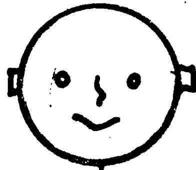
Presenting complaint

History

Onset	Date		
	Duration of disease at presentation		
	Age		
	Type	Hours =1 Days =2 Weeks =3	[ ]
Pain	Severity	0= nil 1= slight 2= moderate 3= severe 4= very severe	[ ]
Stiffness		0= No 1= Yes	[ ]
EMS	duration in minutes		[ ]
	Severity	0= nil 1= slight 2= moderate 3= severe 4= very severe	[ ]

NAME______

DATE______

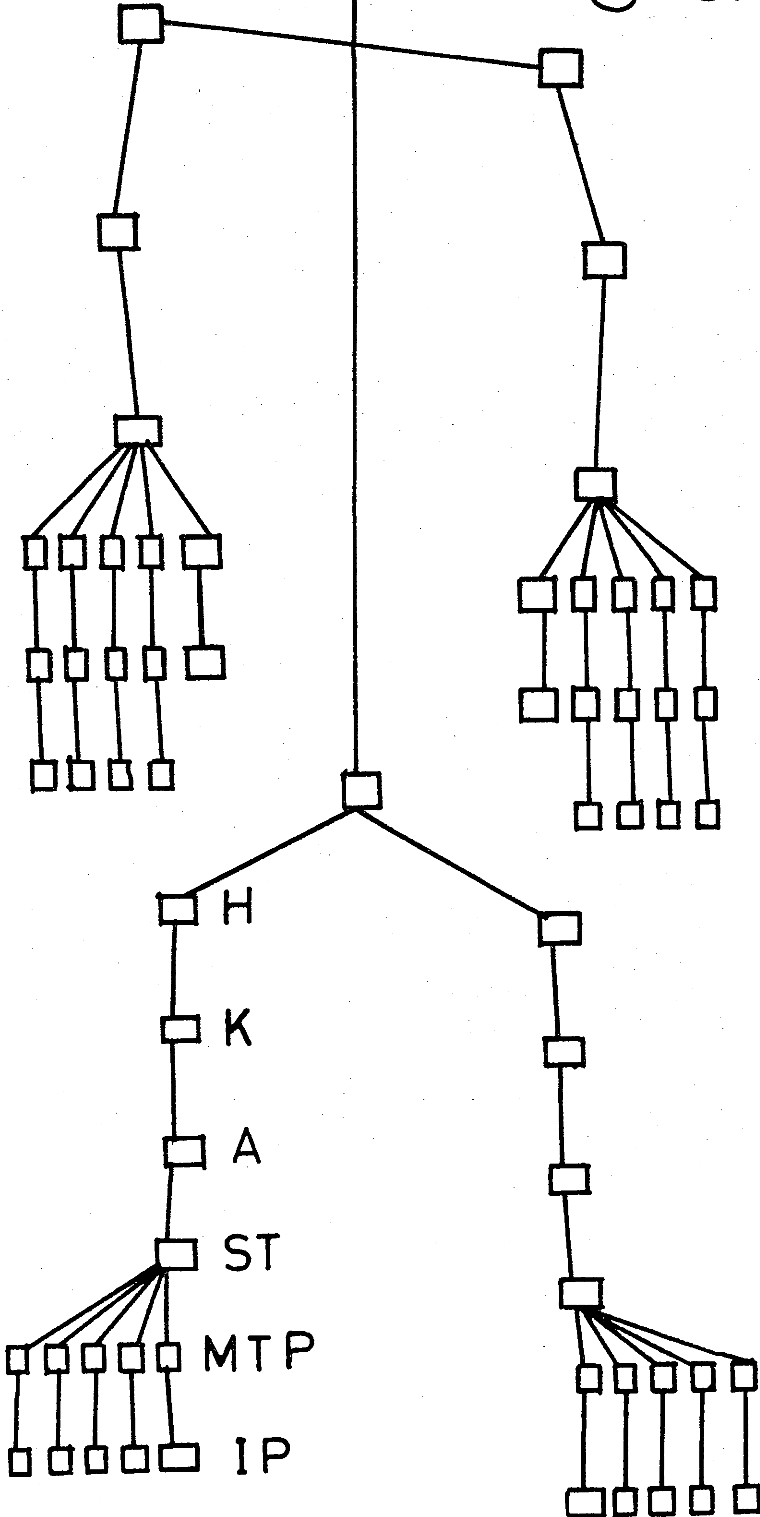


Hx.

▨ = stiff

☒ = pain

◎ = swelling



For the following 0= No
1= Yes
3= Don't know

Swelling		[ ]
Prodromos		[ ]
Recent systemic illness		[ ]
Recent immunisation		[ ]
Recent life event		[ ]
Associated symptoms:		
	Red eyes	[ ]
	Dry eyes	[ ]
	Dry mouth	[ ]
	Rash	[ ]
	Backache	[ ]
	GI upset	[ ]
	Weight loss	[ ]
	Other	[ ]
Non-RA symptoms:		
	Mouth ulcers	[ ]
	Genital ulcers	[ ]
	Dysuria	[ ]
	Photosensitivity	[ ]
	Psoriasis	[ ]
	Muscle pain	[ ]
	Muscle weakness	[ ]
	Dysphagia	[ ]
	Raynauds	[ ]
	Other arthritis	[ ]

Family history

Sexual history

Past medical history

Drug history

GENERAL EXAMINATION

General:	Nodes	[ ]
	Nodules	[ ]
	Rash	[ ]

Cardiovascular

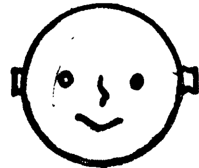
Respiratory

Abdominal

Central Nervous

NAME_____

DATE_____

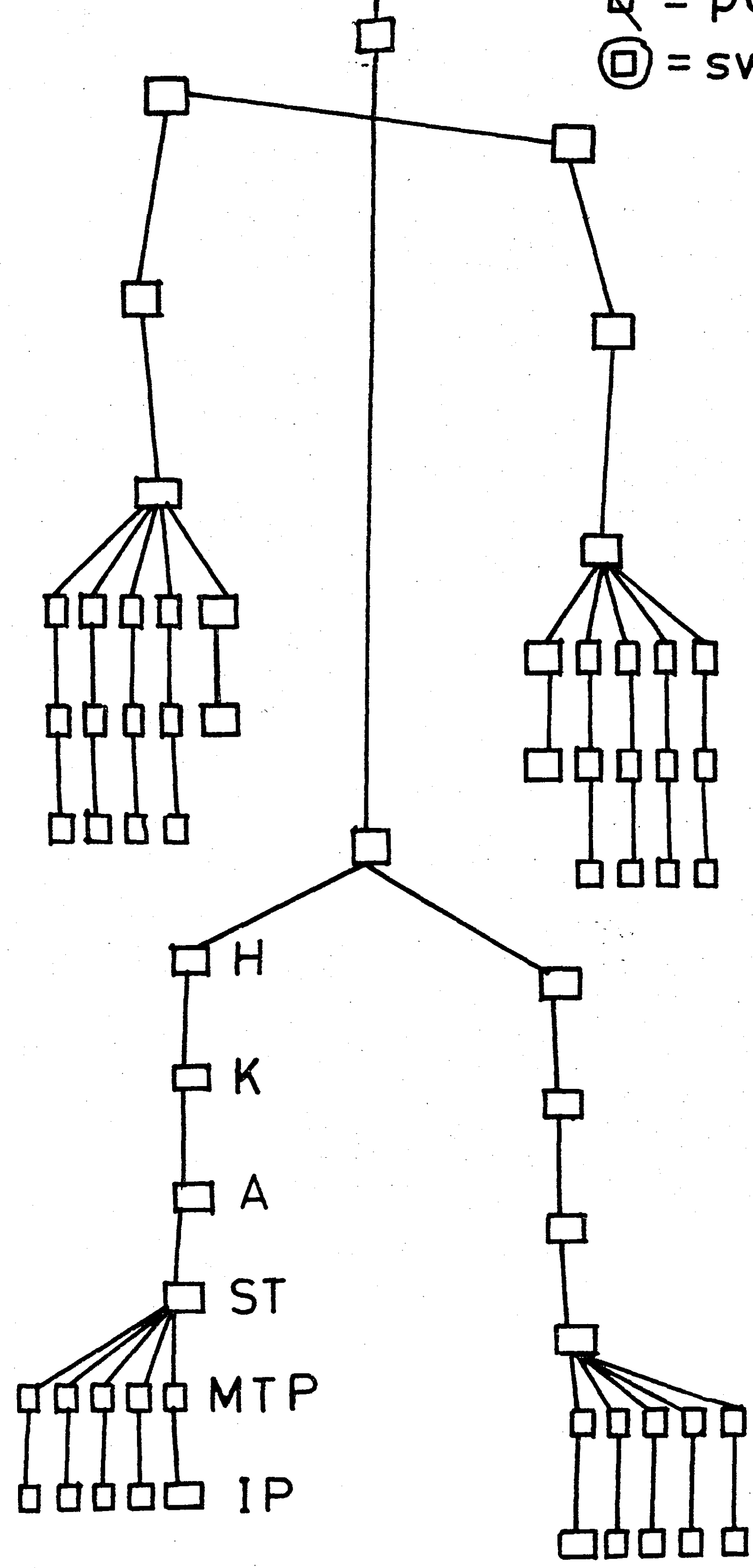


O/E

■ = ↓ rom

☒ = pain

◎ = swelling



METROLOGY

Ritchie: 0= No tenderness
1= Pain
2= Pain and winces
3= Pain, winces and withdraws

	RIGHT		LEFT
TMJ	____		____
Neck		____	
Thoraco/lumbar		____	
Acromio-clavicular	____		____
Sterno-clavicular	____		____
Shoulder	____		____
Elbow	____		____
Wrist	____		____
MCP	____		____
PIP	____		____
DIP	____		____
Hip	____		____
Knee	____		____
Ankle	____		____
Subtalar	____		____
MTP	____		____
Total		____	
No of inflammed joints		____	

Grip strength: Right i)____ ii)____ iii)____

Left i)____ ii)____ iii)____

IMPRESSION

Main diagnosis:

Concurrent conditions

PLAN

1) Investigation screen (see below)
2) Advice and simple analgesia
3) Review in 6 (?) weeks
4) Other

First visit screening investigations:

Haemoglobin	[ ]
WBC	[ ]
Platelets	[ ]
ESR	[ ]
RA Latex	[ ]
Rose Waaler	[ ]
ANA	[ ]
CRP	[ ]

Store 10ml serum at -70°C

X-Ray if indicated: Hands
Wrists
CXR
Feet

Urinalysis:

Table A1.1 Pattern of joint involvement

Jnt	Varble	No SLS	Pos PS	R Risk	Conf-int	Sens	Spec	Chi^2	p
TMJ	Pain	0	3	2.30	0.70-7.50	10	100	1.9	0.176
NECK	Stiff	6	8	1.39	0.76-2.55	28	83	1.13	0.29
	Pain	11	7	0.84	1.52-0.46	24	69	0.33	0.56
	ROM	2	7	2.00	0.93-4.26	24	94	3.22	0.073
	TND	3	5	1.48	0.50-4.38	17	92	0.50	0.48
SHDR	Stiff	13	17	1.68	0.96-2.94	53	63	3.27	0.07
	Pain	15	21	2.07	1.16-3.67	72	58	6.14	<u>0.0132</u>
	ROM	2	11	2.43	1.42-4.15	38	94	10.52	<u>0.0011</u>
	TND	5	13	2.12	1.24-3.61	45	86	7.67	<u>0.0056</u>
ELB	Stiff	9	8	1.09	0.87-1.36	28	75	0.56	0.81
	Pain	13	12	1.14	0.88-3.38	41	64	0.19	0.66
	ROM	1	3	1.74	0.40-7.50	10	97	0.55	0.46
	TND	2	2	1.14	0.88-4.90	7	94	0.09	0.76
WRST	Stiff	18	13	0.89	1.55-0.51	45	50	0.17	0.67
	Pain	23	20	1.12	0.66-1.89	69	36	0.18	0.67
	Big	13	16	1.53	0.89-2.63	55	64	2.36	0.12
	ROM	3	8	1.87	0.92-3.81	28	92	2.98	0.08
	TND	7	13	1.80	1.06-3.04	45	81	4.86	<u>0.0275</u>
	SWL	6	19	3.04	1.77-5.22	65	83	16.19	<u>0.0001</u>
MCP	Stiff	22	20	1.23	0.66-2.27	69	39	0.43	0.51
	Pain	26	17	0.72	1.26-0.41	59	28	1.33	0.25
	Big	22	18	1.02	0.62-1.68	62	39	0.01	0.93
	ROM	5	13	2.12	1.25-3.61	45	86	7.68	<u>0.0056</u>
	TND	8	12	1.58	0.92-2.71	41	78	2.77	0.096
	SWL	14	23	2.95	1.54-5.64	79	61	10.70	<u>0.0011</u>
PIP	Stiff	22	17	0.96	1.42-0.64	59	39	0.04	0.84
	Pain	21	13	0.73	1.29-0.41	45	42	1.17	0.28
	Big	19	17	1.15	0.64-2.06	59	47	0.22	0.64
	ROM	5	11	1.86	1.08-3.20	38	86	5.00	<u>0.025</u>
	TND	6	9	1.50	0.84-2.68	31	83	1.87	0.17
	SWL	11	20	2.46	1.39-4.36	69	69	9.50	<u>0.002</u>
DIP	ANY	6	0	0.00	0.00-0.00	0	83	3.52	0.06
Back	Pain	4	5	1.30	0.30-5.63	17	89	0.12	0.73
HIP	Pain	6	3	0.67	0.15-2.95	9	83	0.28	0.60
Knee	Stiff	18	19	1.42	0.82-2.45	65	50	1.58	0.21
	Pain	22	24	2.00	0.51-4.08	83	39	3.64	0.056
	Big	13	17	1.68	0.96-2.95	59	64	3.27	0.07
	ROM	2	11	2.43	1.42-4.15	38	93	10.52	<u>0.0012</u>
	TND	2	6	1.87	0.55-4.35	21	94	2.15	0.14
	SWL	5	13	2.12	1.25-3.61	45	86	7.68	<u>0.0056</u>
Ankl	Stiff	9	7	0.94	0.53-1.66	24	73	0.04	0.83
	Pain	11	9	1.02	0.43-2.43	31	69	0.01	0.97
	Big	10	14	1.57	0.93-2.64	48	72	2.90	0.09
	SWL	3	8	1.87	0.92-3.81	28	92	2.98	0.084
MTP	Stiff	5	8	1.52	0.83-2.76	28	86	1.88	0.17
	Pain	9	15	1.82	1.07-3.09	52	75	4.92	<u>0.0265</u>
	TND	4	10	1.92	1.09-3.36	34	55	5.19	<u>0.0227</u>

Univariate comparison of joint involvement patterns in SLS and PS with those variables with $p<0.05$ being underlined. The abbreviations indicate the joint, variable, number of patients positive in each group, the relative risk (and its 95% confidence limits) for PS of a positive variable and the sensitivity and specificity for PS.

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