IMMUNOLOGICAL ASPECTS OF POLYMYALGIA RHEUMATICA AND GIANT CELL ARTERITIS

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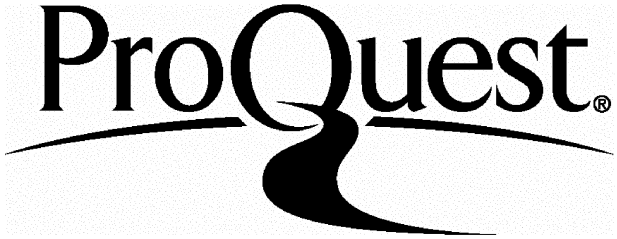

ABSTRACT

Forty four patients with PMR/GCA have been followed from presentation for a period of 2-4 years. Immunological investigations have been carried out in a search for useful tests to assist in the diagnosis of PMR/GCA and in assessing disease activity.

This study has confirmed that ESR and CRP are useful investigations at presentation of PMR/GCA, although even at this stage these tests may not be elevated. During relapses of PMR/GCA both ESR and CRP remain in the normal range in the majority of patients so no reliance should be placed on these investigations to confirm a clinical diagnosis of relapse.

Alpha-1-antichymotrypsin (ACT) has shown an interesting pattern of response, in that the raised levels at presentation (1.0g/l) did not fall rapidly on prednisolone treatment but fell gradually over 2-4 years reaching normal levels (0.6g/l) in those patients satisfactorily off prednisolone treatment. An ACT concentration of ≤0.8g/l at 12 months and ≤0.7g/l at 18 months indicated a reduced risk of subsequent relapse. Hence this investigation may be a useful tool in tailoring prednisolone reduction for the individual patient with PMR/GCA.

Measurement of the cytokines IL1ß, IL6 and soluble IL2 receptor, using ELISA methods, did not add any useful information to the assessment of the individual patient. However the fact that IL1ß levels were raised at presentation and relapse (albeit to only 4pg/ml and 5pg/ml respectively) does illustrate that this mediator of inflammation is involved in PMR/GCA. The elevation of soluble IL2 receptor at presentation (476 U/ml) compared with controls (366 U/ml) also illustrates that there is immune system activation in PMR/GCA. IL6 levels were not significantly elevated in this study.

This study did not find low CD8+ cells in PMR/GCA prior to treatment. %CD8+ cells were significantly reduced after prednisolone treatment commenced, and a study in volunteers confirmed that this was an effect of the prednisolone itself, particularly in the older volunteers.

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ABBREVIATIONS

ACT	-	α1-antichymotrypsin
AGP	-	α1-acid glycoprotein (=orosomucoid)
AIDS	-	acquired immunodeficiency syndrome
AIF	-	antibodies to intermediate filaments
Con A	-	concanavalin A
CRP	-	C-reactive protein
DTT	-	dithiothreitol (Cleland's reagent)
EDTA	-	ethylene diaminetetraacetic acid
ELISA	-	enzyme-linked immunosorbent assay
ESR	-	erythrocyte sedimentation rate
FACS	-	fluorescent activated cell sorter
GCA	-	giant cell arteritis
HIV	-	human immunodeficiency virus
Ig	-	immunoglobulin
IL1	-	interleukin 1
IL1α	-	interleukin 1 alpha
IL1ß	-	interleukin 1 beta
IL1ra	-	interleukin 1 receptor antagonist
IL2	-	interleukin 2
IL2R	-	interleukin 2 receptor
IL6	-	interleukin 6
ITP	-	idiopathic thrombocytopenic purpura
JCA	-	juvenile chronic arthritis

kD	-	kiloDalton
LPS	-	lipopolysaccharide
NK	-	natural killer (cells)
NS	-	not statistically significant at 5% level
OA	-	osteoarthritis
o.d.	-	once daily dosage
PMR	-	polymyalgia rheumatica
PMR/GCA	-	polymyalgia rheumatica and/or giant cell arteritis
RA	-	rheumatoid arthritis
RNA	-	ribonucleic acid
r_s	-	Spearman's rank correlation coefficient
SAA	-	serum amyloid A protein
SD	-	standard deviation
SLE	-	systemic lupus erythematosus
sIL2R	-	soluble IL2 receptor
TNFα	-	tumour necrosis factor alpha

RELEVANT PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Pountain GD, Keogan MT, Brown DL, Hazleman BL. Circulating T cell subtypes in polymyalgia rheumatica and giant cell arteritis: variation in the percentage of CD8+ cells with prednisolone treatment. Ann Rheum Dis 1993; 52: 730-3.

Pountain GD, Keogan MT, Hazleman BL, Brown DL. Effects of single dose compared with 3 days prednisolone treatment of healthy volunteers: contrasting effects on circulating lymphocyte subsets. J Clin Pathol 1993; 46: 1089-92.

Pountain G, Keogan M, Brown D, Hazleman B. Circulating T cell subtypes in polymyalgia rheumatica and giant cell arteritis: variation in the percentage of CD8+ cells with prednisolone treatment. (Letter - Matters arising) Ann Rheum Dis 1994; 53: 283.

Pountain GD, Keogan MT, Brown DL, Hazleman BL. The Cambridge Dilemma. (Letter - Matters arising) Ann Rheum Dis 1994; 53: in press.

Pountain G, Keogan M, Hazleman B, Brown D. Lymphocyte subpopulations analysis in peripheral blood in polymyalgia rheumatica / giant cell arteritis. (Letter) Br J Rheumatol 1994; 33: 194-5.

Pountain GD, Calvin J, Hazleman BL. α1-antichymotrypsin, C-reactive protein and erythrocyte sedimentation rate in polymyalgia rheumatica and giant cell arteritis. Br J Rheumatol 1994; 33: 550-4.

Calvin J, Pountain GD, Hazleman BL. Effects of short-term corticosteroid administration on the concentration of circulating proteins. Ann Clin Biochem 1994; 31: In press.

POSTER PRESENTATIONS

Pountain GD, Hazleman BL, Cawston TE. ESR, CRP, IL1ß and IL6 in polymyalgia rheumatica and giant cell arteritis. British Society for Rheumatology, Oxford, April 1991; 30: Abstracts Supplement 1; 24.

Pountain G, Hazleman BL. Soluble IL2 receptors in polymyalgia rheumatica and giant cell arteritis, and during prednisolone treatment per se. British Society for Rheumatology, Southampton, March 1992. Abstract in Br J Rheumatol 1992; 31: Abstracts Supplement 1; 7.

Pountain G, Calvin J, Hazleman BL. Alpha-1-antichymotrypsin levels in polymyalgia rheumatica and giant cell arteritis. British Society for Rheumatology, London, July 1992. Abstract in Br J Rheumatol 1992; 31: Abstracts Supplement 2; 38.

Pountain GD, Keogan MT, Brown DL, Hazleman BL. Circulating lymphocyte subsets A) in polymyalgia rheumatica/ giant cell arteritis, and B) in healthy volunteers taking prednisolone. British Society for Rheumatology, Cambridge, September 1993. Abstract in Br J Rheumatol 1993; 32: Abstracts Supplement 2; 36.

Calvin J, Pountain GD, Hazleman BL. Effects of short-term corticosteroid administration on the concentration of circulating proteins. Association of Clinical Biochemists National Meeting, Brighton, May 1994. Abstract in Proceedings of ACB, May 1994: 110.

CHAPTER 1

GENERAL INTRODUCTION

1.1 OBJECTIVES

Polymyalgia rheumatica (PMR) and giant cell arteritis (GCA) are related conditions whose aetiology is as yet unknown. In GCA the predominance of activated CD4+ T cells in the infiltrate around the internal elastic lamina (Banks et al 1983, Cid et al 1989) suggests that the immune response drives the vascular inflammation in GCA. This hypothesis is supported by the fact that the particular amino-acid sequence of the HLA-DRß1 chain which is associated with GCA lies in the floor of the peptide-binding cleft. This suggests that binding of an antigen at this site may be the trigger for this disease (Hunder et al, 1993). No specific antigen has been demonstrated as causative in GCA, although elastin has long been under suspicion (Andersson 1991). In PMR, although no site of inflammation is observed, an acute phase response is seen in most cases as in GCA, and pronounced morning stiffness usually occurs, suggesting an inflammatory basis for PMR even in the absence of overt GCA.

Diagnosis of PMR/GCA may be difficult (Kyle and Hazleman 1983), particularly in PMR where there is no diagnostic test, and other similar diseases must be excluded. GCA may be more straightforward to diagnose, particularly if a

positive arterial biopsy is obtained, but the patchy nature of the arteritis means that even in a tender, accessible artery such as the temporal artery, biopsy may miss the lesions.

As well as the difficulty in diagnosing PMR/GCA there are also problems in monitoring the disease during treatment. The duration of corticosteroid treatment required varies from patient to patient (Jones and Hazleman 1981, Bengtsson and Malmvall 1981, Kyle and Hazleman 1990) and ascertaining the corticosteroid requirements in a particular patient is largely a matter of trial and error. The usual practice is to reduce the corticosteroid dose according to a schedule which suits the average patient (Anonymous 1993). If our particular patient relapses then we know we have reduced too quickly, whereas if they do not relapse at all then we may have subjected them to more corticosteroid treatment than was necessary. Even the diagnosis of a relapse may be open to doubt, in the absence of a reliable indicator of disease activity.

The purpose of the studies described here was to investigate immunological aspects of PMR/GCA, to obtain a clearer understanding of the mechanisms of this disease. Also, in studying possible markers of disease activity, the aim was to identify useful tests in the management of the individual patient with PMR/GCA. I have pursued 3 main threads of investigation. The role of cell-mediated immunity in GCA (Hunder et al 1993), as well as the reported changes in

circulating T cell subsets in PMR/GCA (Dasgupta et al 1989, Chelazzi and Broggini 1984) suggest that ascertaining the numbers and activation of T cells could be useful in diagnosing PMR/GCA. Secondly, the known elevation of ESR in most cases of PMR and GCA suggests that further characterisation of the acute phase response in PMR/GCA might be helpful in assessing disease activity. Finally, the role of the cytokine network in mediating inflammation and the acute phase response suggests that circulating cytokine levels might also be useful in assessing disease activity in PMR/GCA.

1.2 PATIENTS

1.2.1 Patient details

All patients were seen at Addenbrooke's Hospital, Cambridge, between 1989 and 1993. Although many of the 44 PMR/GCA patients were common to all the studies in this thesis, there were some different patients in each study so the demographic details are given separately in the section "Subjects and Methods" for each chapter.

1.2.2 Diagnosis of PMR/GCA

The diagnosis of PMR and/or GCA was made according to the criteria of Jones and Hazleman (1981) excluding the requirement for a raised ESR or C-reactive protein (CRP). These two laboratory tests themselves were being studied and might also have associations with other laboratory variables, hence to use them as inclusion criteria might

have biased the results obtained. In practice, the great majority of patients in each study did have raised ESR or CRP in addition to the clinical criteria for PMR/GCA.

Where GCA was diagnosed clinically, temporal artery biopsy was arranged where possible, but negative histology did not change the diagnosis.

Where PMR was diagnosed, other diagnoses were first ruled out, such as rheumatoid arthritis, polymyositis, underlying carcinoma or myeloma, using clinical examination and any necessary laboratory tests. All PMR patients had normal creatine kinase levels and normal protein electrophoresis pattern.

In both PMR and GCA, a rapid and pronounced response to prednisolone treatment was required to confirm the diagnosis.

1.2.3 Treatment

Prednisolone was commenced at a dose of 40mg daily for GCA and 10-20mg daily for PMR. The initial dose was maintained for one month and then the dose was reduced as described in standard guidelines such as those in the Drugs and Therapeutics Bulletin (Anonymous 1993), modified according to clinical progress. Relapse of PMR or GCA was diagnosed on clinical grounds without reference to the results of laboratory blood tests. In the event of clinical relapse, the dose of prednisolone was increased as necessary to control symptoms.

1.2.4 Follow-up

Patients were seen at the same time of day on each visit, between 10am and 2pm, in a research clinic. They were assessed by the author before treatment and during treatment at 10 days, 3 weeks, 6 weeks, 3 months and then every 3 months up to 3 years, or longer if active disease continued. In addition to the routine follow-up, patients were asked to report any relapse of symptoms, and were seen at the time of the relapse and then in remission on increased prednisolone. Relapses were diagnosed on clinical grounds including a good response to increased dose of prednisolone. Laboratory results were not available at the time of diagnosing relapse, and only the ESR result was known at the time of the subsequent visits.

1.3 CONTROLS

1.3.1 Matched controls - on no corticosteroids

Age- and sex-matched controls were obtained for all the PMR/GCA patients involved in any of the studies described in this thesis. Age was matched to within ± 2 years. Ethical committee approval was obtained to approach control subjects for blood tests. These subjects were:-

spouses of PMR/GCA study patients;

attenders at the geriatric day hospital;

volunteer staff at the WRVS canteen;

patients with osteoarthritis from rheumatology clinics.

All were seen at Addenbrooke's Hospital, Cambridge. None had any inflammatory or neoplastic disease and none was receiving any corticosteroid treatment.

1.3.2 Volunteers taking prednisolone

12 younger volunteers participated in an investigation of the effects of prednisolone on lymphocyte subsets and these volunteers are described in that particular study (i.e. Chapter 7, section 7.B.2.1).

1.4 STATISTICS

The data were analysed using the computer software of Number Cruncher Statistical System, Version 5.0. All the variables examined had a non-Gaussian distribution and therefore non-parametric statistical tests have been used throughout. The individual statistical tests used are referred to in the "Methods" sections of the relevant chapters. Where statistical probabilities were not included in the Number Cruncher analysis (e.g. for Spearman's rank correlation), statistical tables were referred to (Siegel and Castellan 1988).

In graphs, the medians and interquartile ranges have been plotted for all group data.

CHAPTER 2

ERYTHROCYTE SEDIMENTATION RATE AND C-REACTIVE PROTEIN IN PMR/GCA

2.1 INTRODUCTION

2.1.1 Erythrocyte sedimentation rate (ESR) at presentation of PMR/GCA

A raised ESR is widely considered to be important in diagnosing PMR/GCA. Healey et al (1971) documented a consensus statement on the definition of PMR in which an ESR of at least 50mm/h was considered necessary for the diagnosis. However Mowat and Hazleman (1974) disputed this, having described an ESR less than 50mm/h in 39% of patients with clinical PMR. Bird HA et al (1979) found an initial ESR ≥40mm/h to be a valuable diagnostic criterion for PMR. Jones and Hazleman (1981) included ESR >30mm/h or CRP >6mg/l as a necessary criterion for the diagnosis of PMR or GCA. The American College of Rheumatology 1990 criteria for GCA (Hunder et al 1990) include ESR ≥50mm/h as one of three criteria (out of a possible five criteria) required to make the diagnosis of GCA. However Ellis and Ralston (1983) questioned the emphasis placed on a raised ESR in diagnosing PMR/GCA, describing an ESR ≤30mm/h in 22.5% of cases at presentation. In a letter replying to this paper Jones and Hazleman (1983) cited 10/108 patients where repeating the ESR gave one normal and one elevated result (5 with the

normal result first and 5 with the normal result second). They suggested that, because of this fluctuation, the test should always be repeated if initially normal in suspected PMR/GCA. Mallya et al (1982) showed that even the time of day relative to feeding affected the ESR in 7 patients with rheumatoid arthritis.

One of the difficulties in assessing the importance of a raised ESR in PMR is that there is no absolute diagnostic test for PMR. Any series of PMR patients that requires a raised ESR for patient inclusion will obviously record raised ESR's, whereas series which include subjects without a raised ESR may contain some subjects without PMR. Even a series professing not to require a raised ESR may suffer from investigators' being more impressed by the described symptoms when accompanied by a raised ESR, and in any case general practitioner referral to investigators is likely to favour a raised ESR. In biopsy-proven GCA there is no such problem of doubt about the diagnosis, and several such cases of GCA have been reported in the presence of a normal ESR. Bruk (1967) described 3 patients with histologically-proven GCA with ESR's of less than 20mm/h, as did Dare and Byrne (1980) in a series of 25 patients with biopsy-proven GCA. Kansu et al (1977) reported 2 cases of normal ESR in biopsy-confirmed GCA, one with an ESR of 13mm/h and the other with an ESR of 22 then 30mm/h. Graham et al (1981) in a study of 90 cases of biopsy-proven GCA found 3 cases with ESR <30mm/h and 6 cases with ESR <40mm/h. Several other

cases have been reported of ESR <30 in the presence of a positive biopsy (Heptinstall et al 1954, Harrison and Bevan 1967, Eshaghian and Goeken 1980, Espinoza and Espinoza 1980, Biller et al 1982, Wong RL and Korn 1986, Jonasson et al 1989). Although some of these cases with normal ESRs may have been seen so acutely that the ESR had not yet risen, it is likely that most would not be seen so quickly. In two of these cases of biopsy-proven GCA the interval from the onset of disease to the measurement of an ESR in single figures was specified as two weeks (Bruk 1967 and Biller et al 1982).

Another difficulty in using the ESR as an aid to diagnosis in PMR/GCA is that the "normal" value increases with age and there is no general agreement on what would be considered "normal" in the elderly. Most cases of PMR/GCA occur in the elderly age group and, as has been illustrated above, different PMR/GCA study groups have chosen different levels of ESR in defining PMR/GCA. In a study of 2,458 normal subjects aged from 20 to 70 years, Bottiger and Svedberg (1967) described the normal ranges for ESR, using the mean ± 2SD. In females aged from 50 to 69 years the normal range for ESR was 0-28mm/h, while in males of this age the normal range was 0-19mm/h. Miller et al (1983) analysed ESR results from 26,836 men and 1,076 women aged from 20 to 65 years undergoing routine health screening. The 98th percentile for ESR in men approximated to (age in years)÷2, and in women to (age in years + 10) ÷2, and the authors

suggested this be considered the maximum normal ESR for any age. However Griffiths et al (1984) commented that, although a value above this level would indicate pathology, a value within this "normal" range would not necessarily exclude pathology, there being an overlap of the ESR's in diseased and normal populations. In a group of 200 subjects aged 60 to 89 years able to attend a general practice, the authors found that the 23 men with disease had a mean ESR of 19mm/h and the 22 women with disease had a mean ESR of 22mm/h. They suggested that values higher than this should be investigated. Hence quite a disparity in opinion can be seen in what constitutes an acceptable ESR.

2.1.2 C-reactive protein (CRP) at presentation of PMR/GCA

CRP has theoretical advantages over ESR in confirming a clinical diagnosis of PMR/GCA. CRP responds more quickly than ESR following the inflammatory stimulus, beginning to rise at about 6 hours and peaking at 48 hours (Colley et al 1983, Thompson D et al 1992). Unlike ESR, CRP does not depend on the age of the patient and is therefore easier to interpret. Pepys (1993) has shown that circulating levels of CRP depend solely on its rate of synthesis, and hence he recommends its measurement for monitoring disease activity. Whicher and Dieppe (1985b) consider CRP adequately sensitive to diagnose significant inflammatory disease, without being as sensitive as serum amyloid A protein (SAA) to mild viral infections (Whicher et al 1985a). However Pepys (1993) points out that, with a lower limit of detection of

5-10mg/l, many commercial CRP assays could fail to detect an increase of 10 to 100-fold (40% of healthy subjects having a CRP in the range 0.07-0.5mg/l).

In PMR/GCA at presentation Kyle et al (1989c) described CRP >6mg/l in 49 out of 55 cases, although the usefulness of this finding is limited by the requirement for either ESR >30mm/h or CRP >6mg/l for inclusion in the study. Mallya et al (1985) similarly restricted recruitment into their study, and hence described elevated CRP in all 13 of their cases. They showed a rapid fall in CRP at the start of prednisolone treatment, coincident with clinical improvement, whereas the fall in ESR was delayed.

Eshaghian and Goeken (1980) reported raised CRP in 10 out of 11 patients with biopsy-proven GCA prior to treatment. They used a variety of semi-quantitative methods which have been superseded, but it is interesting that one case of biopsy-proven GCA had no detectable CRP.

2.1.3 ESR in relapses of PMR/GCA during prednisolone treatment

A raised ESR is often used to determine relapses in PMR/GCA (Jones and Hazleman 1981, Mason and Walport 1992). However the wisdom of this is questioned by Mason and Walport (1992), and several studies have described normal ESRs during clinical relapse. Ellis and Ralston (1983) found that ESR and symptoms of relapse parallelled each other in only 41 out of 191 occasions (i.e. 23%). Bengtsson and Malmvall (1981) described an ESR ≥20mm/h in 64 out of 86

clinical flare-ups during treatment (=74%), i.e. the ESR was less than 20mm/h in one quarter of flare-ups. Even in relapses off treatment in this study 6 out of 33 occurred with ESR <20mm/h. Kyle et al (1989c), using the higher threshold of >30mm/h for considering the ESR raised, described a correspondingly lower percentage (48%) of ESRs raised at relapse during treatment. However they also found that 9/44 patients had an ESR >30 at the previous visit when they were clinically well.

Rynes et al (1977) have reported a case of biopsy-proven GCA during prednisolone treatment of PMR where the ESR was normal on 3 occasions (20, 19 and 20mm/h) between the occurrence of symptoms and the positive biopsy. Sheehan et al (1993) have reported a fatal relapse of GCA proven at post-mortem, where ESRs were 5-20mm/h prior to death.

A study by Paulsen and Iversen (1971) apparently shows the ESR to be a reliable parameter of disease activity in PMR/GCA. However, closer scrutiny reveals that a rise in sedimentation rate was one of the criteria needed to diagnose relapse, so it is not surprising that this prophecy was fulfilled.

2.1.4 CRP in relapses of PMR/GCA during prednisolone treatment

Kyle et al (1989c) documented a normal CRP (<6mg/l) in 41 out of 73 relapses of PMR/GCA during treatment (=56%). These authors noted that, in those patients whose CRP was raised, 10 out of 38 had a raised CRP at the clinic

attendance **prior** to relapse, when they were still clinically well. In the 73 relapses there was discordance between the ESR and CRP on 22 occasions: 13 relapses with raised ESR and normal CRP, and 9 relapses with normal ESR and raised CRP. Hence although the ESR was more likely than the CRP to be abnormal in a clinical relapse of PMR/GCA, the addition of the CRP did confirm a few extra relapses.

In the study by Eshaghian and Goeken (1980) CRP was detectable in all nine patients with clinical relapse of PMR/GCA on treatment and was not detectable in any patient who was asymptomatic. However the semi-quantitative techniques used to estimate CRP levels in this study are outdated, and no healthy controls were studied. Nevertheless the striking concordance of clinical relapse and raised CRP does suggest a real association.

2.2 SUBJECTS AND METHODS

2.2.1 Patients and controls

The patients consisted of 44 patients (31 female) with PMR and/or GCA seen before treatment. Their ages ranged from 51 to 87 years (median 71 years). 26 patients had PMR alone, 7 had GCA alone, and 11 had both at some stage. Of the 7 patients diagnosed as GCA alone, temporal artery biopsy was carried out in 6 cases of which 3 had positive histology. Of the 11 patients with both PMR and GCA on clinical evidence, a temporal artery was biopsied in 7 cases of which 4 had a positive histology.

Blood samples for ESR and sera for storage were taken before treatment and during routine follow-up. In addition, 49 clinical relapses were documented in 23 of the patients, requiring an increase in prednisolone dosage. Blood samples were taken during and after these relapses, however neither ESR nor CRP results were known at the time of diagnosing relapses.

Control samples were obtained as described in Chapter 1, section 1.3.1.

2.2.2 Technical methods

ESR was measured by the routine hospital haematology service, using the Westergren method. The laboratory does not determine a coefficient of variation for ESRs but acknowledges a wide variation due to environmental factors.

CRP was measured in the Rheumatology Research Laboratory on sera stored at -70^{o}C. A Beckman rate nephelometer and Beckman reagents were used (Beckman, California). This system was designed to measure serum concentrations of CRP chiefly in the range 4-120mg/l but does quantify levels of CRP down to 1mg/l. The coefficient of variation between runs was <8% for control sera at 3 concentrations.

2.2.3 Statistical methods

Statistical analysis for the comparison of patients and controls was carried out using the Mann-Whitney test, while the Wilcoxon rank sum test was used for the "within-patient"

paired comparison of results at different stages of follow-up. The Chi-square test (with Yates' correction) was used to examine the distribution of ESR and CRP either side of threshold values. To test for correlations between ESR and CRP, the Spearman rank correlation coefficient was used.

2.3 RESULTS

2.3.1 ESR and CRP from Time 0 to 3 years

In PMR/GCA patients prior to treatment ESR and CRP were both significantly higher than in controls (Figure 2.1). Of the 44 patients, 6 individuals had a normal ESR (<30mm/h), and 9 had a CRP <6mg/l, 4 of these having both results normal.

By 10 days of treatment the ESR and CRP values in patients were not significantly different from controls and remained low during regular follow-up for 2 years (Figure 2.1).

Beyond 2 years of follow-up, the comparison of patients still requiring prednisolone with those successfully off treatment revealed little difference in ESRs (Table 2.1) or CRPs (Table 2.2).

2.3.2 ESR and CRP in relapses

When relapses during treatment were analysed, ESR was not significantly higher than controls (Figure 2.2) but, in the paired comparison with pre-relapse ESR, it had risen from 14 to 16 which was statistically significant (p=0.007). CRP at relapse was significantly raised both in comparison with controls (Figure 2.2) and in the paired comparison with

pre-relapse CRP (p=0.008). However the median value of CRP at relapse was only 5.6mg/l (compared with 3mg/l in controls and 3.4mg/l in patients before relapse).

2.3.3 ESR and CRP in biopsy-proven relapses

Two relapses (in two patients) satisfied "the gold standard" for diagnosis in that temporal artery biopsy showed active arteritis at the time of clinical relapse. Both occurred in female patients who had had only PMR previously and then developed clinical GCA whilst on prednisolone treatment. One of these patients developed amaurosis fugax whilst on prednisolone 6.25mg daily, yet ESR 24 hours later was only 18mm/h and CRP only 7.8mg/l (having been 10mm/h and <6mg/l 2 months earlier). The positive temporal artery biopsy was obtained only a few hours after the relatively normal blood tests. In the other patient, her ESR and CRP were abnormal on prednisolone 1.25mg daily at relapse with headache and scalp tenderness. Three months prior to this relapse, ESR had been 17mm/h and CRP 4.2mg/l, but 3 weeks prior to the relapse, although there were no symptoms of PMR or GCA, ESR had risen to 28mm/h and CRP to 19.2mg/l. By the time of the relapse with GCA symptoms ESR was 58mm/h and CRP 13.1mg/l, and biopsy a few hours later confirmed active giant cell arteritis.

2.3.4 The effect of prednisolone dosage on ESR and CRP at relapse

The above two anecdotes of biopsy-proven relapses raise the possibility that a prednisolone dose of 6.25mg daily was

suppressing the acute phase response whereas 1.25mg daily was not. To investigate this possibility I examined the relationship between the prednisolone dosage and the ESR or CRP in all documented relapses. In fact there was no correlation in the 49 relapses seen (in which corticosteroid dosage ranged from 0-30mg daily). For ESR and prednisolone dose, Spearman rank correlation coefficient r_s=-0.087 ($p>0.25$), while for CRP and prednisolone dose r_s=-0.063 ($p>0.25$).

2.3.5 Threshold levels of ESR and CRP

Table 2.3 shows the numbers and percentage of controls and patients (at presentation and at relapse) with ESR at or above the thresholds of 30mm/h and 40 mm/h respectively, and the numbers with CRP at or above the threshold of 6mg/l. At presentation, ESR and CRP had similar sensitivity (around 80%), but a CRP ≥6mg/l was much less specific than a raised ESR, with 23% of controls having a raised CRP by these standards. At relapse, only the CRP ≥6mg/l differentiated patients and controls, but again with considerable overlap between patients and controls.

2.3.6 Relationship of ESR and CRP

As expected there was a strong correlation between ESR and CRP levels before treatment (Spearman rank correlation coefficient r_s=0.617, $p<0.0005$) and at relapse (r_s=0.526, $p<0.0005$). During remission the correlation between ESR and CRP was weaker (r_s=0.361, $p<0.01$).

Table 2.4 shows the agreement of raised ESR and CRP in the majority of patients before treatment of PMR/GCA. This applied whether the threshold for "raised" ESR was 30 or 40mm/h. A small number had both normal ESR and normal CRP before treatment.

Table 2.5 shows the lack of agreement of raised ESR and CRP in relapses during treatment. In a third of cases the CRP was raised with a normal ESR (whichever threshold was used, q.v.). In not a single case was the ESR raised to even the 30mm/h level if the CRP was normal. In other words the ESR at relapse gave no additional information compared with the CRP in any patients at all.

2.4 DISCUSSION

2.4.1 ESR and CRP at presentation of PMR/GCA

This work has confirmed the presence of a raised ESR in the great majority of cases of PMR/GCA at presentation although, as in some previous studies (Bruk 1967, Kansu et al 1977, Dare and Byre 1980, Graham et al 1981, Ellis and Ralston 1983), several patients were exceptions to this rule (whichever threshold was used for considering the ESR raised).

Previously a raised CRP has been assumed to occur in PMR/GCA at presentation, but this has not been tested with modern quantitative methods. In this study a raised CRP or ESR was not an inclusion criterion so the finding that the great

majority of cases had a CRP ≥6mg/l is meaningful. However, as with the ESR, there were several patients with a normal CRP before treatment (using CRP <6mg/l as a definition of "normality"), and conversely several controls with a raised CRP.

ESR or CRP alone detected very similar percentages of cases of PMR/GCA at presentation. The combination of the two investigations did detect more abnormalities at presentation than either investigation alone. It is therefore advisable to carry out both of these tests in PMR/GCA at presentation. However, as a small number of cases had both a normal ESR and a normal CRP before treatment, it may not be safe to exclude PMR/GCA on the basis of both tests being normal. The gold standard of biopsy-proven GCA has not yet been reported with a normal CRP as well as a normal ESR.

Although this study did not use a raised ESR or CRP as a diagnostic criterion, there is a possibility that referral of patients from general practitioners and from other rheumatologists was biased in favour of patients with these tests raised. In particular the ESR would usually be checked by a GP considering the diagnosis of PMR/GCA and might influence the decision to refer the patient, so there may be some overestimation of the proportion of patients with raised ESR. On the other hand, those cases diagnosed as PMR/GCA in this study despite a normal ESR and CRP may not be true PMR/GCA, so the importance of a raised ESR or CRP may be underestimated. Of the patients with both the

ESR and the CRP normal before treatment, none had histologically-proven GCA, so there is no absolute proof that they had PMR/GCA.

2.4.2 ESR and CRP in relapses of PMR/GCA during prednisolone treatment

In this study the CRP was more useful than the ESR in confirming relapses during prednisolone treatment. However in just over half of relapses the CRP was still less than 6mg/l, i.e. a rise would not have been detected in those laboratories reporting only values of 6mg/l or more. This obviously limits the usefulness of the test in managing the individual patient.

These data for raised CRP in relapses are similar to those reported by Kyle et al (1989c) but, unlike that study, I have found a raised ESR in very few cases in relapses. Those authors found an ESR greater than 30mm/h in just over half of relapses. They also reported ESRs >30mm/h in a fifth of patients **prior** to clinical relapse and CRP >6mg/l in a quarter of patients before clinical relapse (no control data is given). My data for CRP are similar to this, with one third of CRPs ≥6mg/l before relapse, but this is not statistically different from the quarter of elderly controls with CRP ≥6mg/l. ESR was raised on only 4% of patients before clinical relapse, which is similar to the control data. In other words, there is no evidence that either of these tests **predict** relapse in PMR/GCA.

Park et al (1981) reported that ESR had a better correlation

with disease activity than CRP in PMR/GCA, but they did not distinguish between presentation and relapses on treatment. As almost half of their data were in pre-treatment samples, the results are still compatible with my findings that in relapses CRP was more likely to be raised.

Eshaghian and Goeken (1980) also found that CRP was more useful than ESR in confirming clinical relapse of PMR/GCA.

In this study the addition of the ESR result to the CRP result gave no further sensitivity in detecting relapses on treatment. This is therefore unlike the situation at presentation where the combination of the two investigations did give greater sensitivity, and this has been shown in Tables 2.4 and 2.5.

In practice neither the ESR nor the CRP is a reliable indicator of relapse during treatment of PMR/GCA. Kyle and Hazleman (1990) and Mason and Walport (1992) have stressed the importance of using clinical judgment when assessing prednisolone requirements, rather than using ESR and CRP results. However, in practice, laboratory results are still used by many doctors to diagnose relapse during treatment. Chakravarty et al (1994), in an audit of GPs and hospital physicians in Norwich, found two-thirds of each group of doctors would increase prednisolone in response to a raised ESR in the absence of symptoms. Very few doctors considered increasing the steroid dosage based on symptoms alone.

2.4.3 Possible mechanisms for the lack of response of ESR or CRP in relapses during treatment

Studies requiring a raised ESR or CRP to diagnose relapse are likely to overestimate the occurrence of one or both of these investigations being raised. On the other hand, excluding ESR and CRP as a criterion for diagnosing relapse (as in this study) may result in the inclusion of patients without a true relapse and hence the importance of ESR and CRP may be underestimated. Certainly, symptoms of relapse of PMR/GCA may be vague, but the risk of overdiagnosing relapses was minimised here by requiring a good response to increased prednisolone. Also, there was histological proof of active GCA in two relapses, as described, and in one of these cases the ESR was normal and the CRP barely raised. This would tend to confirm that there are genuine relapses of PMR/GCA where ESR and CRP are not raised.

There are three possible explanations for the failure of ESR and CRP to rise in relapse, all of which may play a part:

(i) It may be that mild symptoms are recognised as relapses of PMR/GCA by patient and doctor, compared with presentation where more florid disease may be required to alert the doctor to the diagnosis.

(ii) It is possible that prednisolone treatment suppresses the acute phase response directly e.g. at the hepatocyte, in addition to suppressing inflammation at the disease site. In this case the ESR and CRP might remain low on prednisolone despite recurrence of disease activity. In

fact, the evidence available tends to suggest that corticosteroids do not suppress biosynthesis of acute phase proteins. On the contrary, serum orosomucoid and haptoglobin are said to increase on corticosteroids, while CRP, serum amyloid A protein and α-1-antitrypsin remain unchanged (Whicher and Dieppe 1985b). Laurell (1985) states that corticosteroids have a very limited effect, if any, on the biosynthesis of most protective plasma proteins. My own data showed no evidence that higher doses of prednisolone were associated with lower ESRs or CRPs at relapse. If prednisolone were directly suppressing the acute phase response, then one might expect to see such a relationship with the prednisolone dose.

(iii) It has been suggested that in chronic inflammation there may be downregulation of the acute phase response (Whicher and Dieppe 1985b). For example, in uveitis a much smaller acute phase response is seen in patients who have had several previous attacks despite clinical evidence of similar inflammation (Yorston et al 1985). This mechanism might also operate in relapses of PMR/GCA.

TABLE 2.1

Comparison of ESR in patients successfully off prednisolone and in those still requiring prednislone, between 2 and 3 years follow-up. Results as medians (mm in the 1st hour).

	(Controls)	PMR/GCA patients (follow-up in months)				
		24m	27m	30m	33m	36m
ESR in patients successfully off prednisolone	(14.5) (n=44)	19 (n=16)	18 (n=15)	15 (n=13)	14.5 (n=12)	16.5 (n=14)
p for comparison with controls	-	p=0.042	NS	NS	NS	NS
ESR in patients still requiring prednisolone treatment	(14.5) (n=44)	12 (n=21)	14 (n=20)	13 (n=17)	14 (n=14)	18 (n=11)
p for comparison with controls	-	NS	NS	NS	NS	NS
p for comparison between patient groups	-	NS	NS	NS	NS	NS

TABLE 2.2

Comparison of CRP in patients successfully off prednisolone and in those still requiring prednisolone, between 2 and 3 yrs follow-up. Results as medians (mg/l).

	(Controls)	PMR/GCA patients (follow-up in months)				
		24m	27m	30m	33m	36m
CRP in patients successfully off prednisolone	(3.0) (n=44)	3.8 (n=16)	3.6 (n=15)	3.6 (n=13)	3.0 (n=11)	3.0 (n=14)
p for comparison with controls	-	NS	NS	NS	NS	NS
CRP in patients still requiring prednisolone treatment	(3.0) (n=44)	3.0 (n=21)	3.6 (n=20)	4.2 (n=17)	6.6 (n=14)	6.0 (n=11)
p for comparison with controls	-	NS	NS	NS	p=0.007	p=0.061
p for comparison between patient groups	-	NS	NS	NS	NS	NS

TABLE 2.3

Numbers (and percentage) of subjects with ESR ≥30mm/h, ESR ≥40mm/h and CRP ≥6mg/l

	Controls	PMR/GCA			
	n=44	Before treatment n=44	Before relapse n=49	At relapse n=49	Relapse suppressed n=48
ESR ≥ 30mm/h number (%)	1(2)	38(86)	2(4)	6(12)	3(6)
p for comparison with controls (Chi-square)	-	p<0.0001	NS	NS	NS
ESR ≥40mm/h number (%)	1(2)	32(73)	2(4)	4(8)	2(4)
p for comparison with controls (Chi-square)	-	p<0.0001	NS	NS	NS
CRP ≥6mg/l number (%)	10(23)	35(80)	16(33)	24(49)	18(38)
p for comparison with controls (Chi-square)	-	p<0.0001	NS	p=0.009	NS

TABLE 2.4

Concordance of raised ESR and raised CRP in 44 patients before treatment. Numbers of patients in each group are given (and in brackets the percentage of the total).

	ESR <30mm/h	ESR ≥30mm/h	ESR <40mm/h	ESR ≥40mm/h
CRP <6mg/l	4(9)	5(11)	7(16)	2(5)
CRP ≥6mg/l	2(5)	33(75)	5(11)	30(68)

TABLE 2.5

Discordance of raised ESR and raised CRP in 48 relapses on treatment. Numbers of patients in each group are given (and in brackets the percentage of the total).

	ESR <30mm/h	ESR ≥30mm/h	ESR <40mm/h	ESR ≥40mm/h
CRP <6mg/l	25(52)	0	25(52)	0
CRP ≥6mg/l	17(35)	6(13)	19(40)	4(8)

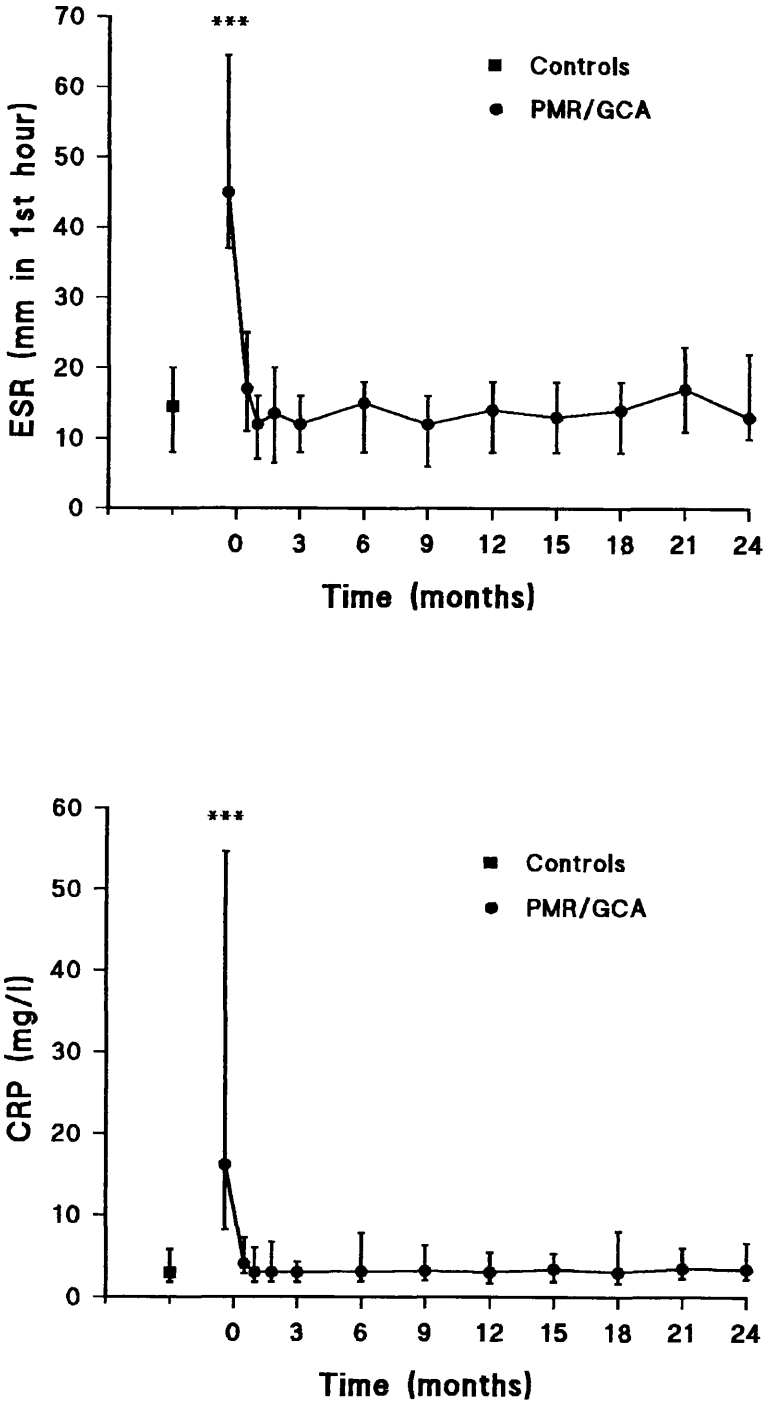

Figure 2.1 ESR (top) and CRP (bottom) in 44 PMR/GCA patients before treatment and up to 2 years treatment. Medians + interquartile range. (*** = $p<0.001$ for the comparison with controls.)

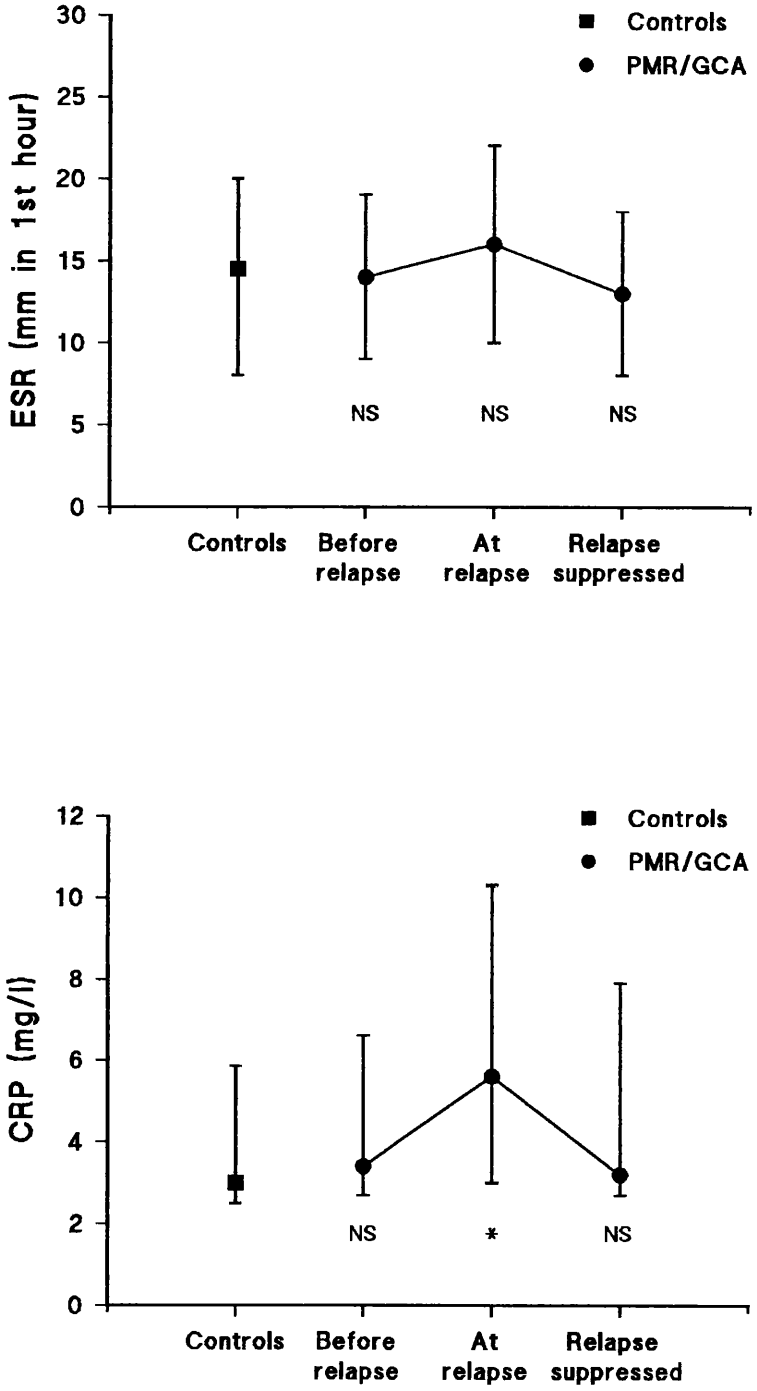

Figure 2.2 ESR (top) and CRP (bottom) in PMR/GCA patients before, during and after relapse (n=49). Medians + interquartile range. (NS = not significantly different from controls, * = $p<0.05$ for the comparison with controls.)

CHAPTER 3

α1-ANTICHYMOTRYPSIN LEVELS IN PMR/GCA

3.1 INTRODUCTION

3.1.1 α1-antichymotrypsin (ACT) in inflammatory disease

ACT is a protease inhibitor which begins to rise by 6-8 hours after an inflammatory stimulus, with serum levels reaching a peak at 2-3 days (Laurell 1985). This is similar to the rapid response of CRP, although ACT has a longer half-life than CRP and is therefore somewhat slower to return to normal (Whicher and Dieppe 1985b, Laurell 1985). ACT increases to 2-6 times normal in acute inflammation (Whicher and Dieppe 1985b, Laurell 1985). Unlike orosomucoid (= α1-acid glycoprotein [AGP]), it is said not to be elevated by corticosteroid therapy (Laurell 1985) and, unlike fibrinogen, haptoglobin and α1-antitrypsin, it is reported not to be reduced by consumption (Whicher and Dieppe 1985b, Thompson D et al 1992). ACT has therefore been recommended by Laurell (1985) as the preferred indicator of the inflammatory response. SAA, as a very sensitive marker of inflammation, has been suggested as a valuable acute phase protein (Pepys 1993), but its very sensitivity may be a disadvantage in that even the common cold produces a rise in SAA (Whicher et al 1985a).

Calvin et al (1988) found ACT to be the most sensitive acute phase protein in gastrointestinal diseases when compared with CRP, orosomucoid, α1-antitrypsin, haptoglobin and ESR, without being any less specific. These authors advocated using a combination of a "short" half-life and "long" half-life protein when screening for inflammation in a wide range of patients. In particular they suggested CRP and ACT as a suitable combination.

Chard et al (1988) studied sequential ACT levels in patients with rheumatoid arthritis (RA) treated with gold or penicillamine. ACT correlated with clinical indices of disease activity and with CRP, orosomucoid (AGP) and ESR. However, in some patients, ACT was elevated corresponding to clinical activity when CRP was inappropriately low, suggesting that measurement of both acute phase proteins was more useful than reliance on one. These authors also found that in some patients ACT levels remained elevated for some months after CRP had fallen to normal on treatment. They suggested that, in addition to the longer half-life of ACT, it may take longer for production to be switched off and hence ACT levels may reflect disease activity over several weeks while CRP reflects only the events of the previous few days. In monitoring chronic diseases such as RA or PMR/GCA both types of indicator would be useful.

Thompson D et al (1992), reporting for the Clinical Laboratory Investigation Subcommittee of the Association of

Clinical Biochemists, recommended that serum ACT analysis should be available in all teaching hospitals, as well as CRP and ESR being available in every District General Hospital.

3.1.2. ACT in PMR/GCA

ACT levels have been studied very little in PMR/GCA. Hachulla et al (1991) did describe levels of SAA, CRP, ACT, AGP, haptoglobin and fibrinogen in PMR/GCA at various stages of the disease. Each of these proteins was elevated in samples taken before treatment but, when clinical activity at different stages of the disease was considered, ACT was exceptional in having no correlation with disease activity. The authors' conclusions focussed on the usefulness of SAA in determining disease activity, but because of the lack of specificity they emphasised the importance of combining this with clinical data. These authors, like Park et al (1981), described AGP (=orosomucoid) and haptoglobin remaining elevated after the suppression of disease, but Hachulla et al also described ACT remaining elevated in this way. AGP is known to be elevated by corticosteroids but ACT is said not to be (Laurell 1985), so the persistence of raised levels in PMR/GCA during treatment is interesting.

Hachulla et al (1990) have looked at microheterogeneity of ACT in PMR/GCA. Using crossed immunoaffinity electrophoresis, they showed a relative increase in those fractions non-reactive and weakly reactive to Con A in clinically active PMR/GCA compared with healthy controls or

inactive PMR/GCA. This pattern was not seen in a group of controls with acute sepsis. Pawlowski et al (1990) similarly showed a relative decrease in Con A reactive fractions of ACT in PMR/GCA compared with healthy controls and compared with polymyositis and dermatomyositis. This investigation may be useful in the future in the diagnosis of PMR/GCA and possibly in confirming relapse, but at present it is not a routine test.

In view of the paucity of information on total ACT levels in PMR/GCA, and the fact that this is an easily performed assay, I have studied ACT at different stages of the disease.

3.2 SUBJECTS AND METHODS

3.2.1 Patients and controls

ACT was measured in 42 patients (30 female) at presentation of PMR/GCA and during treatment. Ages ranged from 51 to 87 years (median 71 years). 25 patients had PMR alone, 7 GCA alone and 10 had both. (Temporal artery biopsy results were as described in Chapter 2, section 2.2.1).

Age- and sex-matched controls were as described in Chapter 1, section 1.3.1.

3.2.2 Healthy volunteers taking prednisolone

ACT levels were available from a group of 12 healthy volunteers taking prednisolone 20mg daily for 3 days. More details of this group are given in section 7.B.2.1. In

addition, a pilot study in one volunteer involved administration of prednisolone for 10 days (20mg x 3 days, 10mg x 3 days, 5mg x 4 days) with blood tests before (x3), during (x7) and after (x3) the course of prednisolone.

3.2.3 Technical methods

ACT measurements were made on sera which had been stored at -70^{o}C. The analysis was carried out in the Department of Clinical Biochemistry at Addenbrooke's Hospital using routine methods. This involved addition of anti-ACT antiserum (Dako, High Wycombe, UK) and measurement of turbidity using a Monarch analyser (Instrumentation Laboratory, Warrington, UK) as described by Calvin and Price (1986). The assay is accurate down to a level of 0.13g/l, i.e. well below the normal range of 0.33-0.64g/l. Quality control, during the period of testing these sera, revealed coefficients of variation between assays of 3-5%, for standard samples of 0.4g/l and 1.0g/l.

Results were reported only to the nearest 0.1g/l in the PMR/GCA patients and their matched controls. In the healthy volunteers taking prednisolone, ACT results were available to the nearest 0.01g/l.

3.2.4 Statistical methods

The Mann-Whitney test was used to compare non-paired data and Wilcoxon's rank sum test for within-patient comparisons. Spearman's rank correlation test was used to examine possible correlations of ACT with other variables. The Chi-

square test was applied (with Yates' correction) when considering the distribution of ACT either side of threshold levels. The serial data in volunteers taking prednisolone was analysed by Friedman's test.

3.3 RESULTS

3.3.1 ACT at presentation and during 3 year follow-up

As shown in Figure 3.1, ACT was significantly raised at presentation: 1.0g/l compared with 0.6g/l in controls ($p<0.0001$). 39/42 patients had ACT >0.6g/l at presentation but 17/42 controls also had ACT above this level. Each of the 3 patients with normal ACT also had CRP <0.6mg/l, although ESR was slightly elevated in 2 of them at 34 and 35mm/h, i.e. in only one patient were all 3 results normal at presentation. Overall at presentation there was a strong correlation of ACT levels with ESR and CRP ($r_s=0.671$ and $r_s=0.659$ respectively, $p<0.0005$).

Unlike the ESR and CRP, ACT did not fall rapidly in response to treatment. In the PMR/GCA group overall, ACT remained significantly raised compared with controls for up to 18 months of treatment. For the comparison of serial ACT results within patients (i.e. the paired data), ACT levels were significantly lower by 6 months of treatment than at presentation.

For the period between 24 and 36 months follow-up, the data have been analysed separately for those patients still

requiring prednisolone treatment and for those patients remaining in remission off prednisolone (Figure 3.1). The group still requiring prednisolone continued to have significantly raised ACT levels (0.8g/l) compared with controls (0.6g/l) (p=0.005 to 0.011), whereas the group who were successfully off prednisolone had normal ACT levels. When these two groups were compared with each other there was a significant difference between them at 24 months (p=0.002) and at 36 months (p=0.014). Because of the smaller number of patients tested at 27, 30 and 33 months the difference between the 2 groups was not significant at these points.

3.3.2 ACT in volunteers taking prednisolone

The persistent elevation of ACT in patients taking prednisolone could be due to the drug rather than the disease. This is unlikely to be the whole story, as the highest ACT levels in patients were seen before prednisolone was started. Nevertheless, to investigate whether prednisolone treatment itself was contributing to the persistently raised ACT, data has been analysed from 12 healthy volunteers taking prednisolone for 3 days. The median ACT level prior to prednisolone was 0.53g/l and after 2 and 3 days of prednisolone was 0.56g/l. This difference just reached statistical significance (p=0.04, Friedman's test) but is too small to account for the high levels of ACT persisting in patients continuing on prednisolone treatment, i.e. 0.2g/l higher than controls. The pilot study in one

volunteer taking prednisolone for 10 days showed a rise of ACT from 0.48g/l to 0.53g/l, with a plateau reached by 3 to 4 days.

3.3.3 ACT during relapses of PMR/GCA on treatment

ACT levels were already significantly raised prior to relapse (0.8g/l) and there was no further rise in ACT during relapse (Figure 3.2). After relapse was suppressed the ACT concentration was slightly higher (0.9g/l), but this rise was not significant (p=0.758).

3.3.4 Does ACT predict the clinical course of PMR/GCA?

The ACT levels **at presentation** did not correlate with the subsequent duration of prednisolone treatment required (r_s=0.233, p=0.10), nor with the number of relapses experienced before treatment was successfully stopped (r_s=0.02, p>0.1). Looking at the 14 patients who were successfully off prednisolone at 3 years, they had presented with slightly lower ACT levels (median 0.9g/l) than the 12 patients still requiring prednisolone at 3 years (median ACT at presentation 1.05g/l), but this difference was not significant (p=0.304).

During follow-up, ACT levels from 12 months onwards correlated with the eventual duration of treatment, as well as with the number of relapses before treatment was successfully stopped (range of r_s =0.30-0.50, p<0.05). Looking for a threshold level of ACT with prognostic significance, an ACT level of ≤0.7g/l at 18 months did

predict a reduced likelihood of subsequent relapse (Table 3.1). Where ACT had fallen to ≤0.7g/l on 2 successive occasions by 21 months this was no more specific in predicting freedom from relapse (and was less sensitive) than using the single ACT level at 18 months (Table 3.1).

Using a value of ACT of >0.8g/l was less reliable as an indicator of future relapse than the value of >0.7g/l. However at 9 months there was a trend for ACT >0.8g/l to be associated with later relapse (p=0.059, Chi-square), and at 1 year this association just reached statistical significance, with 8/9 relapsing subsequently compared with 14/27 if ACT was ≤0.8g/l (p=0.048).

Figure 3.3 shows the median ACT levels at different stages in patients who would subsequently relapse compared with those who would not. From 18 months the ACT levels were significantly higher in the group who would subsequently relapse.

If ACT levels reflect disease activity over the preceding few months, as suggested by Chard et al (1988) in RA, then the association of a raised ACT at 18 months with **subsequent** relapse might merely reflect an association of both of these variables with **recent** relapse. In fact, this does not appear to be the case. Those patients with relapses between 12 and 18 months (n=8) had the same median ACT level at 18 months (0.75g/l) as those patients who had not relapsed between 12 and 18 months (n=26). Nor did relapse between 12

and 18 months predict relapse after 18 months (p=0.153, Chi-square). However, relapse at **any** stage before 18 months was associated with an increased likelihood of relapse after 18 months (p=0.039, Chi-square).

ACT levels **at the time of discontinuing prednisolone** have been analysed to see if they predict subsequent relapse. Those patients who subsequently remained well off prednisolone (n=22) had a lower ACT (median 0.6g/l) than those who later relapsed (n=9) (median ACT 0.7g/l), but this difference was not statistically significant (p=0.617).

In none of the results was the **fall** in ACT level more useful than the **absolute** level, so only the absolute levels have been described.

3.4 DISCUSSION

At presentation of PMR/GCA, ACT was raised in a similar proportion of patients to that in which ESR or CRP was raised. However, once clinical disease activity had been suppressed, ACT then behaved in quite a different way from the other 2 laboratory parameters, falling gradually over 21 months. The persisting raised levels of ACT do appear to be due to the underlying disease rather than the corticosteroid treatment, as the data in volunteers suggests that any corticosteroid effect is much smaller than the observed elevation in PMR/GCA patients. More prolonged administration of prednisolone in the volunteers might have

produced a greater rise in ACT, but the levels did appear to plateau between 2 and 3 days, and in the longer pilot study in one volunteer maximum levels were reached by 3-4 days. We cannot rule out the possibility that the biosynthesis of ACT is increased by corticosteroid in PMR/GCA patients to a greater extent than in healthy volunteers. However, the median daily prednisolone dosage in the PMR/GCA patients on treatment was only 5mg and 2.5mg at 18 months and 24 months respectively. It is unlikely that these low doses would account for the elevation of ACT levels from 0.6g/l to 0.8g/l, when much higher doses in healthy volunteers produced such a small effect.

In view of the persisting raised ACT levels in suppressed disease, it is not surprising that ACT does not rise further in clinical relapses on treatment. However it appears that ACT may be useful in a different way, in that by 12 months the level of ACT was a prognostic indicator of subsequent relapse. By that stage it correlated with the subsequent number of relapses or the duration of treatment required, but it was not until 18 months that a clear division appeared between the ACT levels in those likely to relapse and those not. At this stage an ACT level of $\leq$0.7g/l indicated a significantly reduced risk of subsequent relapse. Hence ACT may be helpful in accelerating the steroid reduction in this group, while indicating the need for caution in those with ACT still at 0.8g/l or more. However, at no stage is the ACT level a substitute for

clinical assessment. Some patients in this study had persistently raised ACT levels yet successfully stopped prednisolone treatment while others with low ACT levels subsequently relapsed.

The risk of side-effects from corticosteroids in PMR/GCA is related to the cumulative dose (Kyle and Hazleman 1989b), so the earlier the prednisolone dose can be tailored to the individual (i.e. minimised) the better. By 9 months in this study there was a trend for ACT $\leq$0.8g/l to indicate reduced risk of relapse and this threshold was a stronger predictor by 12 months. It may be possible at that stage to divide patients according to their ACT levels, into a "fast stream" and a "slow stream" for prednisolone reduction, as long as clinical assessment is still used as the final arbiter of dosage. This study is planned, using a new series of patients. This will examine whether knowledge of the ACT levels in one group of PMR/GCA patients can reduce the overall corticosteroid dose in that group, without increasing the relapse rate, compared with another group where ACT levels are not known.

TABLE 3.1

Subsequent relapse rates in patients grouped according to whether ACT was ≤0.7g/l at particular stages of follow-up.

	Follow-up in months				
	12	15	18	21	24
Subsequent relapse rate if ACT ≥0.8g/l	13/18	14/19	13/17	9/12	8/12
Subsequent relapse rate if ACT ≤0.7g/l	9/18	7/16	5/17	8/22	7/22
p for the difference between the 2 groups (Chi-square)	0.171	0.071	0.006 **	0.031 *	0.050
Subsequent relapse rate if ACT not ≤0.7g/l at 2 consecutive visits	-	-	12/19	11/14	-
Subsequent relapse rate if ACT ≤0.7g/l at 2 consecutive visits	-	-	7/16	7/20	-
p for the difference between the 2 groups (Chi-square)	-	-	p=0.251	p=0.012 *	-

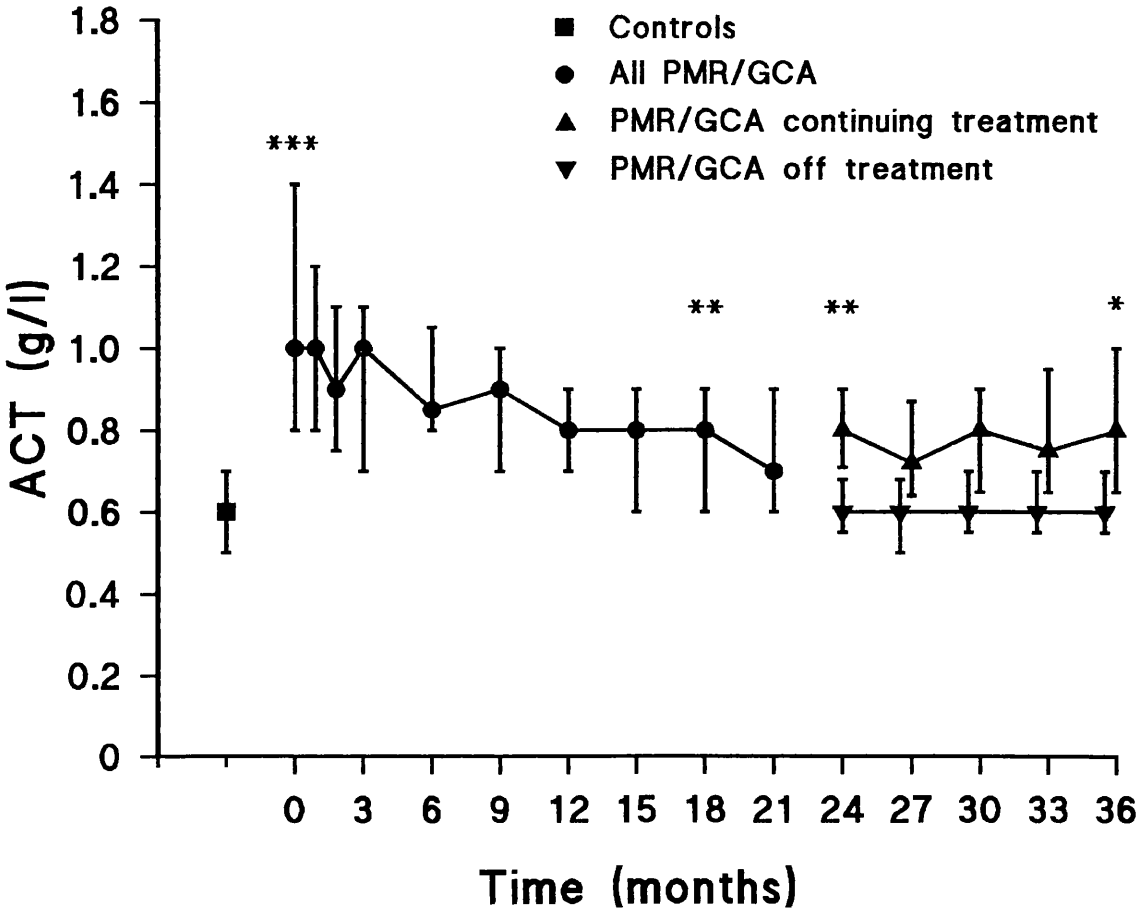

Figure 3.1 3 year follow-up of ACT levels in PMR/GCA. Medians + interquartile range. n=42 at time 0. (3 week point omitted for clarity.) From 24 months onwards the PMR/GCA patients are divided into those continuing prednisolone treatment and those in remission off prednisolone. At 24 months n=21 for the group continuing treatment and n=14 for those off treatment. At 36 months n=11 continuing treatment and n=14 off treatment.
(*** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$ for the comparison at 0, 18, 24 and 36 months with controls.)

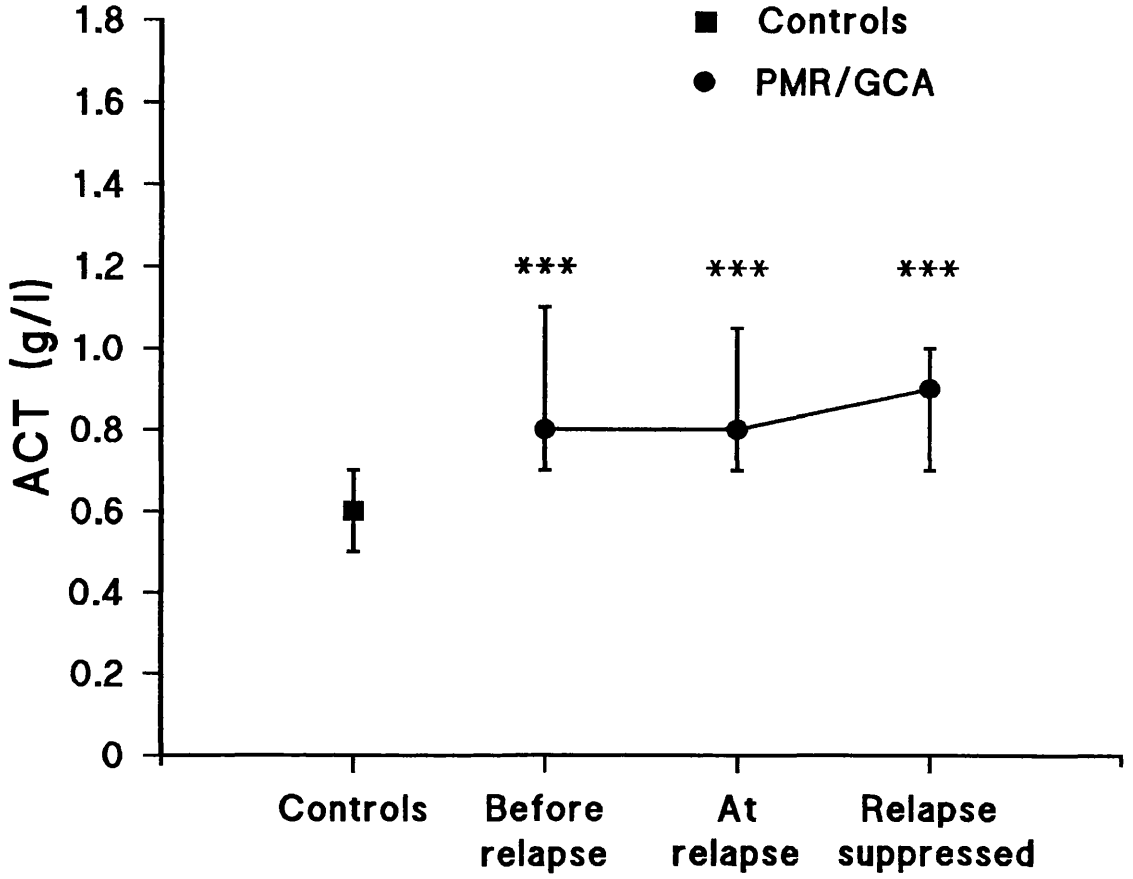

Figure 3.2 ACT levels in controls and in PMR/GCA patients before, during and after relapse. Medians + interquartile range. n=43.
(*** = $p<0.001$ for the comparison with controls.)

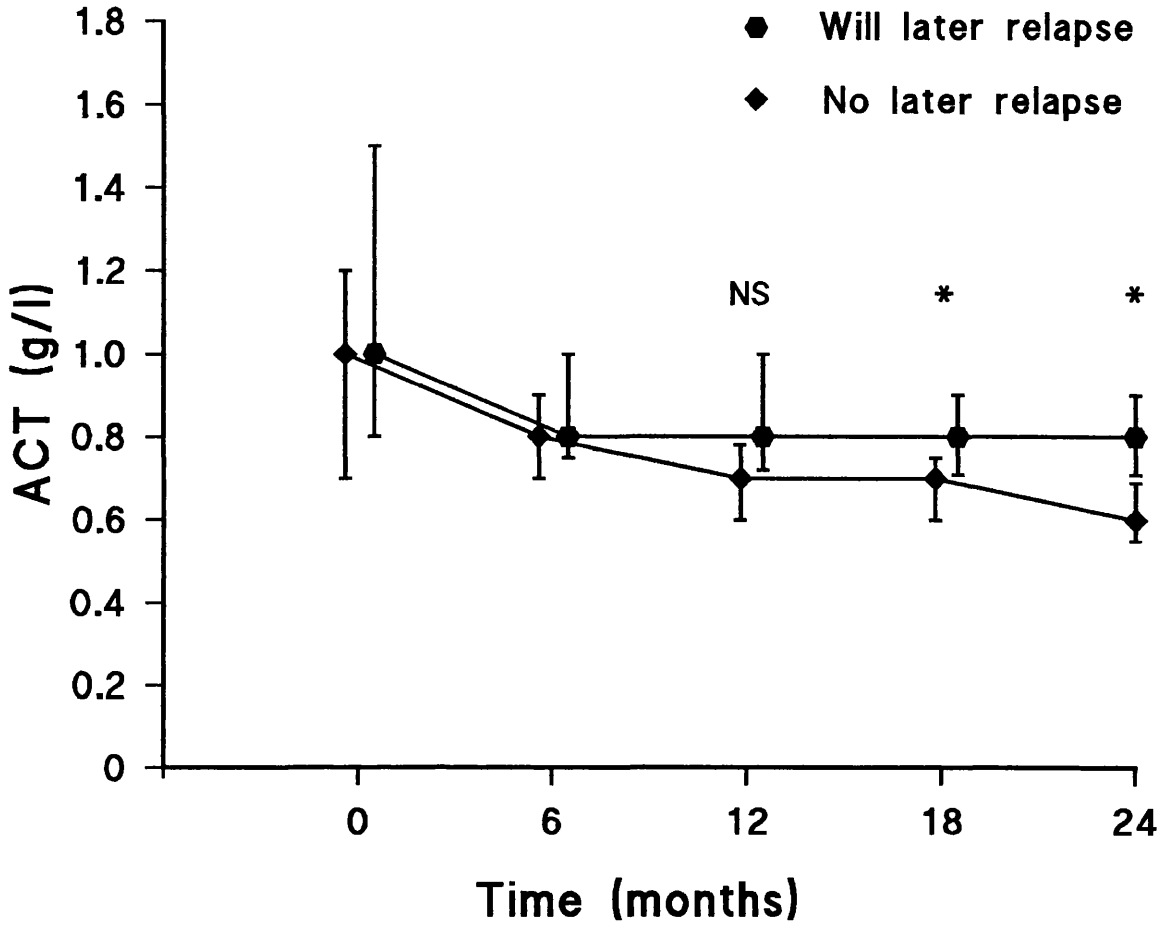

Figure 3.3 ACT levels in patients who will subsequently relapse compared with those who do not subsequently relapse. Medians + interquartile range. (Coincident data points for the two groups are offset for clarity.) n=25 and n=11 at time 0 ranging to n=15 and n=19 at 2 years.
(NS = not significant, * = $p<0.05$, for the comparison between the two groups.)

CHAPTER 4

PLASMA INTERLEUKIN 1ß IN PMR/GCA

4.1 INTRODUCTION

4.1.1 Interleukin 1ß (IL1ß) as a mediator of inflammation

Interleukin 1 (IL1) is produced by the monocyte/macrophage group of cells. It has a wide range of effects directly, and also indirectly by stimulating the release of other cytokines such as IL2 and IL6. These effects include fever, neutrophilia, activation of T lymphocytes, maturation of B lymphocytes, stimulation of bone and cartilage resorption and stimulation of the synthesis of prostaglandins, collagenase and acute phase proteins (Dinarello 1984, Oppeheim et al 1986, Pettipher et 1986, Thompson BM et al 1986).

These actions of IL1 make it a likely candidate in the pathogenesis of inflammatory diseases and hence a possible target for therapeutic intervention in such diseases (Dinarello 1984, Arend and Dayer 1990, Thompson RC et al 1992, Dunn 1993).

March et al (1985) showed that IL1 actually consisted of 2 distantly related polypeptides which they called IL1α and IL1ß. These both bind to the same receptor and have similar activity (Oppenheim et al 1986). Macrophages stimulated

with lipopolysaccharide (LPS), produce messenger RNA (mRNA) for IL1ß in much greater amounts than that for IL1α (March et al 1985). The resulting proteins are cleaved to produce the 17 kD active forms. Most IL1ß is released, whereas IL1α remains mainly cell-associated (di Giovine and Duff 1990). Hence most of the IL1 found in blood, synovial fluid or cell supernatants is IL1ß (Kirkham 1991).

IL1ß injected into animals will produce fever (Dinarello et al 1986) and conversely in febrile illness circulating IL1ß has been shown to be raised in some studies. Girardin et al (1988) found that serum IL1ß correlated with the severity of meningococcal septicaemia in children, and Waage et al (1989) detected bioactive IL1 present only in the most severe meningococcal disease, i.e. rapidly fatal. In contrast to these studies, Cannon et al (1990) found that although plasma IL1ß was raised in septic shock compared with healthy controls, higher levels were present in the patients who survived. Luger et al (1986) also found higher serum IL1 bioactivity in patients surviving sepsis compared with those dying. Hence it is not clear from the available data whether levels of IL1ß are prognostic.

The role of IL1 as a mediator in inflammatory disease is in keeping with the effects of corticosteroids in these diseases. IL1 production in vitro is inhibited by corticosteroids (Palacios 1982, Knudsen et al 1987, Kern et al 1988) and hence these drugs would be expected to inhibit inflammation in vivo. However corticosteroids also have a

separate effect in reducing IL2 synthesis by T cells in vitro, which cannot be overcome by adding excess IL1 (Palacios 1982).

Inhibiting the effects of IL1 with IL1 receptor antagonist (IL1ra) has been shown to reduce inflammation in animal models of ulcerative colitis and rheumatoid arthritis (Thompson RC et al 1992). This effect appears to be a result of suppressing the inflammatory pathway rather than suppressing the immune response, in that humoral and cell-mediated immunity remain intact. Clinical tests are now under way in humans to see if IL1ra could be a useful treatment in rheumatoid arthritis, septic shock and inflammatory bowel disease (Thompson RC et al 1992, Dunn 1993).

4.1.2 IL1 in synovial fluid of rheumatoid arthritis (RA)

The role of IL1 in the rheumatic diseases has been studied particularly in RA. Studies using bioassays to measure IL1 in RA synovial fluid have given differing results. Wood DD et al (1983) and Hopkins et al (1988) found bioactive IL1 in RA synovial fluids as well as in osteoarthritis (OA), whereas Bhardwaj et al (1988) reported a lack of bioactive IL1 in RA synovial fluid, and Holt et al (1992) found minimal IL1 in RA and OA synovial fluids. Studies using immunoassays of IL1ß in synovial fluid have tended to show raised IL1ß in active RA compared with OA (Yamagata et al 1988, Westacott et al 1990, Holt et al (1992), although Hopkins et al (1988) found similar high levels in RA and OA

synovial fluids, while Bhardwaj et al (1988) could detect no IL1ß in either. Even in those studies showing raised immunoreactive IL1ß in RA synovial fluid, the actual levels found have varied considerably. Westacott et al (1990) reported mean levels of 130pg/ml, Rooney et al (1988) levels up to >2,000pg/ml, while Holt et al (1992) found concentrations ≥30pg/ml in only 10/41 patients. These discrepancies might be expected when comparing bioassay with immunoassay, but differences are seen between results when the same type of assay has been used (Smith MD 1989). Bioassays may be variably affected by the presence of inhibitors, and anyway the assay cell lines often respond to more than one cytokine. On the other hand, immunoassays may be influenced by the viscosity of synovial fluids and by the presence of rheumatoid factors (Arend and Dayer 1990). Indeed there is great variation between studies in the pretreatment of synovial fluid for assay, in terms of dilution, centrifugation and use of heparin and/or hyaluronidase (Hopkins et al 1988, Yamagata 1988, Westacott et al 1990, Holt et al 1992). Feldmann et al (1990) have described synovial fluid as a "soup" of enzymes, proteins and hyaluronic acid at very high concentration and they felt that analysis of cytokines in this "soup" would not be productive.

4.1.3 *In vitro* production of IL1 by RA monocytes and macrophages

Monocytes and macrophages from healthy subjects are usually found to produce no IL1 spontaneously (March et al 1985,

Kirkham 1991) but unstimulated monocytes and synovial macrophages from RA patients have been reported to secrete IL1 in vitro, chiefly IL1ß, (Shore et al 1986, Buchan et al 1988b, Firestein et al 1990, Goto et al 1990). However Bhardwaj et al (1988) found no IL1 production by mononuclear cells from RA synovial effusions, unless these cells were stimulated with LPS. Di Giovine and Duff (1990) and Kirkham (1991) have pointed out that LPS, a microbial product, is a ubiquitous laboratory contaminant, so apparently spontaneous secretion of IL1 may be due to inadvertent priming of cells. Two of the above studies of peripheral blood monocytes compared control cells with RA cells; Goto et al (1990) found no spontaneous IL1 secretion from their healthy control or OA cells whereas RA cells did secrete IL1ß; Shore et al (1986) did have some "spontaneous" IL1 production by normal control monocytes with a similar secretion of IL1 by RA monocytes. Despite the background "spontaneous" IL1 production, the latter study shows an interesting increase in the ILß produced by monocytes from RA with recent onset or exacerbation, when compared with controls or "stable" RA.

4.1.4 IL1ß in blood samples in RA

Gordon Duff's group (Eastgate et al 1988) reported raised plasma levels of IL1ß in patients with RA (mean 98.2pg/ml) compared with controls (mean 44.7pg/ml). Furthermore, ILß levels correlated with clinical disease activity and ESR. In this study, plasma samples were collected with EDTA and aprotinin, platelets were removed and samples were extracted

using chloroform and assayed using the Cistron ELISA (Cistron Biotechnology Inc., New Jersey, USA). In none of these samples, including controls, did the IL1ß concentration fall below the limit of detection of the assay i.e. 20pg/ml. However in another study (Holt et al 1992), no detectable IL1ß was found in plasma from RA patients. This study used heparinised and diluted plasma with the Cistron high sensitivity ELISA, giving a sensitivity down to 12pg/ml. Although there was no extraction of plasma samples in this study, the failure to detect IL1ß conflicts with the results of Eastgate et al, where even unextracted samples yielded 85.6pg/ml IL1ß in RA. Unpublished work presented at the British Society for Immunology in 1989 (Sheeran TP, Gray L, Blann A, Lunec J, Emery P) found plasma concentrations of IL1ß <10pg/ml in the majority of RA patients, although a few had much higher levels. With decreasing disease activity on second-line drugs there was no significant change in IL1ß levels. This study used very similar methods to Eastgate et al, including plasma collection with EDTA and aprotinin, and chloroform extraction, but the results do not confirm the high levels of IL1ß found by Eastgate et al.

Duff (1989) has drawn attention to the problems of handling blood samples so as to avoid releasing IL1 from leucocytes in the sample. This may occur during clotting when serum is prepared, or by contamination from the buffy coat when plasma is prepared. Activation of serine proteases during clotting may also damage IL1. Hence Duff recommends the use

of plasma rather than serum to reflect in vivo blood concentrations of IL1. Earlier work by his group (Symons et al 1988a) found very high levels of IL1ß in serum of RA patients (mean 518pg/ml) compared with controls (mean 68pg/ml). This work was superseded by this group's measurement of IL1ß in plasma of RA patients (Eastgate et al 1988), referred to previously, which showed much lower levels than in serum, but nevertheless still significantly raised at 98pg/ml (compared with controls 45pg/ml).

4.1.5 IL1ß in blood samples in PMR/GCA

There is no detailed published work in the English language literature on plasma IL1 in PMR/GCA. One paper in abstract form (Dasgupta and Panayi 1988) refers to serum IL1ß levels in untreated PMR/GCA. Using a radioimmunoassay sensitive to 250pg/ml, no IL1ß was detected in the PMR/GCA sera. As immunoassays more sensitive than this have become available, such as the Cistron ELISA sensitive down to 20pg/ml, and later the Cistron "High Sensitivity ELISA" sensitive down to 2pg/ml, I have investigated circulating IL1ß concentrations in PMR/GCA using these more sensitive methods. To reflect *in vivo* levels as closely as possible (Duff 1989), I have used plasma samples rather than sera.

4.2 SUBJECTS AND METHODS

4.2.1 Patients and controls

The patients consisted of 35 individuals with PMR/GCA (27 female). All were seen before corticosteroid treatment and

during subsequent remission, and 21 relapses were also studied (in 14 patients). Ages ranged from 51 to 87 years (median 70 years). 20 patients had PMR alone, 5 GCA alone and 10 had both at some stage. Temporal artery biopsy was positive in 7 cases.

Age- and sex-matched controls were recruited as described in Chapter 1. In addition, blood samples were obtained from 12 patients with active rheumatoid arthritis and from 12 patients with pyrexia, for comparison with the PMR/GCA samples. The RA patients were outpatients with Ritchie articular index of at least 6 as well as swollen joints. The pyrexial patients were inpatients who had been admitted for treatment of infectious diseases or for the investigation of pyrexia of unknown origin. Some of the latter subjects were found to have infectious diseases, while 3 had malignancy.

4.2.2 Technical methods - Plasma samples

IL1ß was measured in plasma samples prepared by collecting blood in tubes containing EDTA (1.5mg / ml of blood) and aprotinin (Sigma, Poole, Dorset, UK), a serine protease inhibitor (0.67 trypsin inhibitor units / ml of blood). Samples were refrigerated at $4^{o}C$ and cells removed within 2 hours of venesection. Erythrocytes and leucocytes were separated by centrifugation at 400g for 15 minutes and the plasma removed without disturbing the buffy coat. Platelets were then removed by centrifuging at 10,000g for 2 minutes at $4^{o}C$, and the plasma transferred in aliquots of 500µl to

fresh microfuge tubes for storage at -20°C.

4.2.3 Technical methods - Extraction procedures

Immediately prior to IL1ß measurement, plasma aliquots were thawed at room temperature and subjected to an extraction procedure to dissociate IL1ß from carrier proteins. Two techniques for extraction were investigated:-

Chloroform extraction was carried out as described by Cannon et al (1988). 900µl chloroform was added to each 500µl aliquot of plasma and agitated for 5 minutes. After centrifuging at 10,000g at 4°C for 5 minutes, the aqueous phase was removed and subjected to a repeat of the procedure. The aqueous phase was again removed and used for the assay.

Dithiothreitol (DTT) extraction was carried out to dissociate protein binding by reduction of disulphide bonds. 50µl of 100mM DTT (Sigma, UK) was added to each 500µl aliquot of plasma and incubated for 1 hour at room temperature. (Incubation at 37°C caused the sample to clot and, despite resuspension and washing of the clot, recovery of IL1ß was poorer than after room temperature incubation with DTT.) 50µl of 300mM iodoacetate (Sigma, UK) was then added to inhibit rebinding of the dissociated IL1ß. The x1.2 dilution resulting from this procedure was taken into account when calculating IL1ß concentrations in the samples.

To compare the recovery of IL1ß achieved by these 2 extraction procedures, a range of concentrations of IL1ß was added to normal plasma and incubated for 3 hours at room

temperature. The recovery was somewhat more effective using DTT extraction (Figure 4.1) so this method was subsequently used in the preparation of samples for IL1ß assays. Lest the recovery from PMR/GCA plasma should be substantially different from the recovery from normal plasma, 6 patient samples were spiked with IL1ß 200pg/ml. The recovery by DTT extraction ranged from 134-214pg/ml (median 194pg/ml) which is comparable with that obtained from normal plasma (Figure 4.1).

4.2.4 Technical methods - IL1ß ELISA

Measurement of plasma IL1ß was carried out using the Cistron ELISA kit (Cistron Biotechnology Inc., New Jersey, USA). 100μl plasma samples in duplicate were incubated for 3 hours at 37^{o}C in microtitration wells pretreated with monoclonal antibody to IL1ß. After washing the wells, bound IL1ß was detected using polyclonal rabbit antibody to IL1ß followed by anti-rabbit IgG conjugated to horseradish peroxidase. On addition of the substrate for this enzyme (o-phenylene-diamine) to the washed wells, the intensity of the colour developed indicated the amount of IL1ß present in the plasma sample. After stopping the reaction with sulphuric acid, the optical density to light of 490nm wavelength was read and compared with standard concentrations of IL1ß in buffer. The plates were read using the Titertek Multiskan Plus Mark II, and the results calculated using the Titersoft computer programme (ICN Flow, Thame, UK).

All samples from the same patient were assayed in the same

ELISA together with the matched control sample, to eliminate interassay variability as a source of error. The coefficient of variation within assays was 5-7%, and between assays 7-10%, for 3 standard concentrations.

For most of the ELISA kits used, the manufacturer's specifications indicate that the lowest concentration of IL1ß reproducibly detectable from zero (with 95% confidence) was 20pg/ml. However 5 later ELISA's were carried out using the new "High Sensitivity IL1ß ELISA" kit, in which a concentration of 2pg/ml could be distinguished from zero with 95% confidence. Although the majority of results fell below 20pg/ml, and were therefore not accurate as individual results, these values have been used together to illustrate the abnormalities in PMR/GCA.

4.2.5 Statistical methods

For the comparison of patient groups with controls the Mann-Whitney test was used. When paired comparisons were made within the PMR/GCA group then Wilcoxon's rank sum test was used. Spearman's rank correlation coefficient was used to test for any correlation between IL1ß and ESR or CRP.

4.3 RESULTS

4.3.1 Plasma IL1ß concentration in PMR/GCA at presentation

Plasma IL1ß levels were slightly, but significantly, raised in PMR/GCA before treatment (median 4pg/ml) compared with controls (median 0pg/ml), p=0.0001. By 10 days of

prednisolone treatment IL1ß concentration had fallen to normal in the PMR/GCA patients (median 0pg/ml). Figure 4.2 illustrates plasma IL1ß levels before treatment until suppression of disease on prednisolone treatment.

IL1ß concentration in the majority of samples, even those in PMR/GCA before treatment, fell below the reliable limits of detection of the assay (see 4.2.4 Technical methods). The highest level of IL1ß seen in a PMR/GCA patient before treatment was 38pg/ml, while the highest level in a control subject was 12pg/ml. Although most individual results are below the limit of accurate detection, these results have all been used in the pooled group data to enable comparisons to be made.

4.3.2 Plasma IL1ß in relapses of PMR/GCA

Figure 4.3 illustrates plasma IL1ß levels in clinical relapses of PMR/GCA. Even before relapse was evident clinically, plasma IL1ß levels were significantly higher than controls although still extremely low (median 2pg/ml). During relapse, plasma IL1ß rose to 5pg/ml which, although still very low, was significantly raised compared with controls ($p=0.0016$). Once relapse had been suppressed with increased prednisolone, plasma IL1ß concentration fell to control levels (median 0pg/ml). The highest individual level of IL1ß during relapse of PMR/GCA was 72pg/ml, although several patients had no detectable IL1ß whatsoever at relapse.

4.3.3 Relationship of IL1ß, ESR and CRP in PMR/GCA

There was no correlation between IL1ß and ESR or CRP at presentation (r_s<0.1 for both), nor at relapse (r_s<0.2, p>0.1 for both).

4.3.4 Plasma IL1ß in RA and in febrile illness

In 12 patients with active rheumatoid arthritis, the median plasma IL1ß concentration was 0pg/ml (interquartile range 0-4pg/ml) which was no different from healthy controls.

In 12 patients with pyrexia due to infection or malignancy, the median plasma IL1ß was 5pg/ml (interquartile range 2-25pg/ml). This was significantly higher than age-matched controls (p=0.005), and was similar to the PMR/GCA patients (p=0.44).

4.4 DISCUSSION

This work has shown minimal elevation of plasma IL1ß levels in untreated PMR/GCA and in clinical relapses of PMR/GCA. Current immunoassays for IL1ß are not sensitive enough to be useful in confirming diagnosis or relapse of PMR/GCA in individual patients, but the elevation in this group overall suggests that IL1ß is a mediator of this disease. It is interesting that, unlike the ESR (see Chapter 2), IL1ß did rise in relapses of PMR/GCA when corticosteroid treatment was inadequate. Corticosteroids are known to suppress IL1ß production from cells in vitro (Knudsen et al 1987, Kern et al 1988). My work shows that suppression of circulating

IL1ß by corticosteroids corresponds to suppression of clinical disease, whereas suppression of the ESR by corticosteroids does not correspond to control of clinical disease.

It may be that more sensitive immunoassays will eventually make measurement of plasma IL1ß levels a useful adjunct in diagnosis of PMR/GCA and in confirmation of relapses. However absolute levels of particular cytokines may be less important than the relative concentrations of the different cytokines and inhibitors interacting in a complex network (Feldmann et al 1990, Giovine and Duff 1990, Brennan and Feldmann 1992).

Even in RA, where the synovium is a source of circulating IL1, local concentrations of IL1 have not always been shown to be raised. In PMR no similar site of production of IL1 is known, although in GCA a site of active arteritis may be identified. Circulating IL1 would be diluted compared with the concentration at the site of production, so lower levels would be expected in blood samples. The sparse data on plasma IL1ß levels in RA, and the even sparser data in PMR/GCA, suggests that these levels are very low or undetectable. The study of Eastgate et al (1988) conflicts with the other data available in describing plasma levels of 98pg/ml in RA and 45pg/ml even in healthy controls. Using the same ELISA as this group I have not found detectable IL1ß in RA or controls, and barely detectable IL1ß in the PMR/GCA group overall. In the case of RA, rheumatoid

factors may cause false positive results by bridging the 2 antibodies used in an ELISA. Grassi et al (1991) found that all those samples of RA plasma or synovial fluid that had apparently high levels of IL1α or ß (at ng/ml levels), measured by ELISA, had in fact IgM or IgG anti-IgG antibodies (i.e. rheumatoid factors). They found that the interference due to IgM anti-Ig was completely neutralised by DTT treatment, which depolymerised the IgM without significantly altering the recognition of IL1 in the assay. As my samples contained DTT it is likely that they were not subject to such false-positive results due to rheumatoid factors. This may explain the apparent difference between RA plasmas in my assays and those in the study of Eastgate et al (1988). However it is unlikely to explain the differing results in control subjects, who would only rarely possess rheumatoid factors.

Another source of variation might be the extraction procedures themselves. Figure 4.1 suggests that the chloroform and DTT extractions produce recovery curves which cross, although this data is drawn from only 2 assays. If real, this would suggest that either the DTT extraction fails to release a fixed amount of IL1ß, or the chloroform extraction increases non-specific binding. In the absence of a "gold-standard" for IL1ß measurement, it is not possible to be sure whether control plasma does contain significant IL1ß or not.

Immunoassays have the specificity and reproducibility lacked

by bioassays, but many cytokine immunoassays are not sensitive enough to use on blood samples (Whicher and Evans 1990). This seems to be the case with ELISAs for IL1ß at present, where the balance of evidence suggests that RA plasma levels cannot be distinguished from controls. In PMR/GCA too, IL1ß concentrations do not seem to be high enough to reliably indicate active disease. However, the overall higher plasma levels of IL1ß in PMR/GCA compared with controls, does suggest that IL1ß is indeed a mediator of the pathology of this disease. This model is also consistent with the great benefit of corticosteroids in PMR/GCA (Andersson 1991).

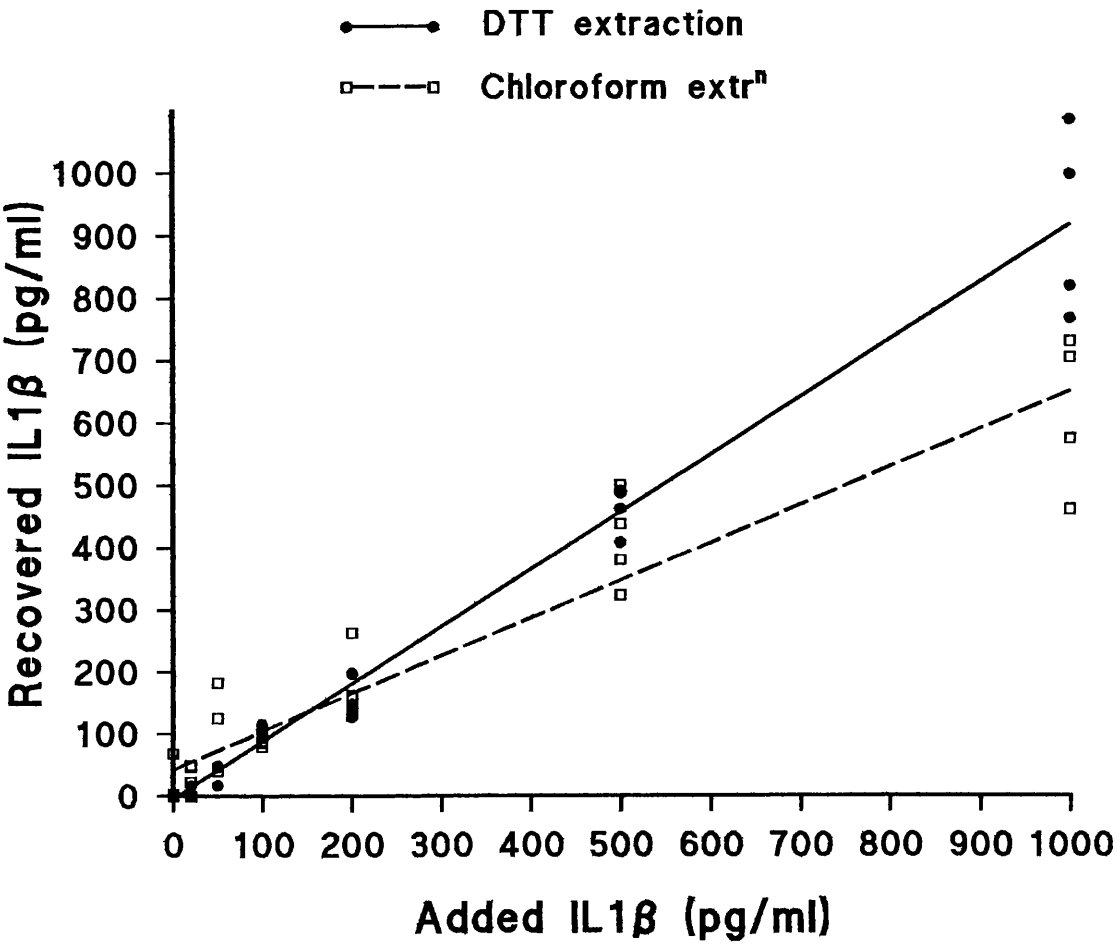

Figure 4.1 Recovery of IL1ß added to normal plasma: a comparison of DTT extraction and chloroform extraction. Recovered IL1ß was measured relative to standards in buffer. At each concentration of added IL1ß the 4 datapoints represent duplicate results in 2 separate ELISAs.

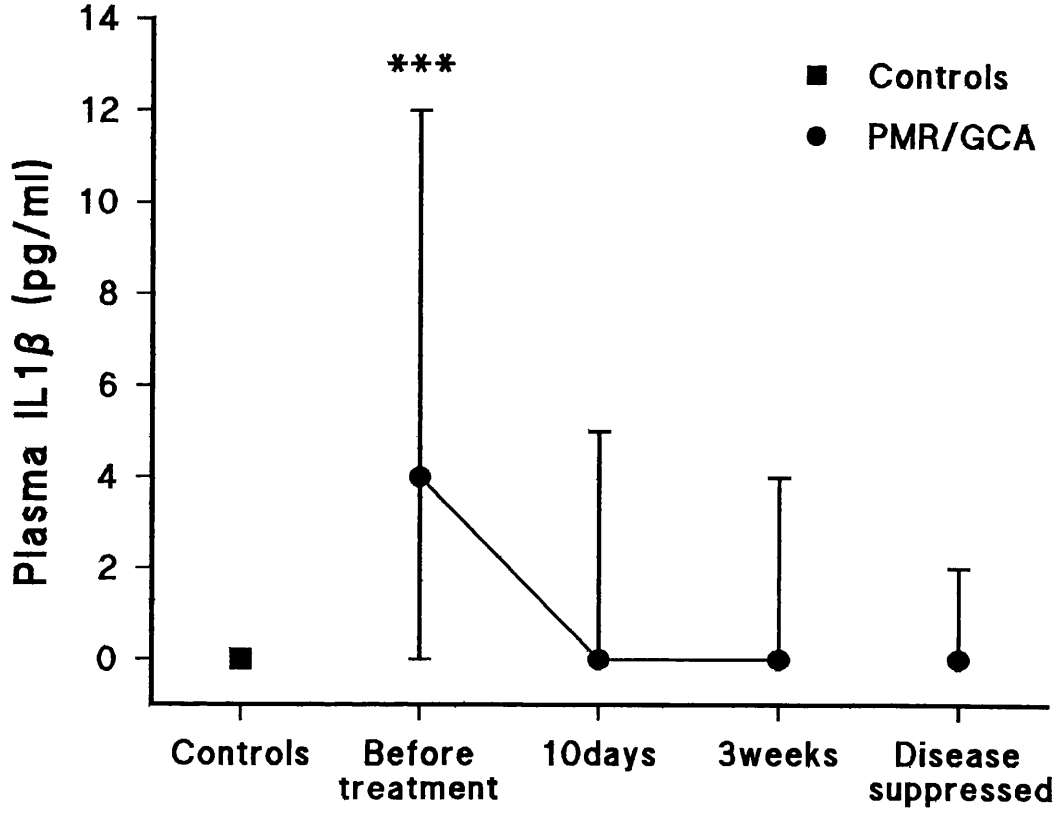

Figure 4.2 Plasma IL1ß concentrations in 35 PMR/GCA patients before treatment and in the early stages of prednisolone treatment. ("Disease suppressed" represents the earliest sample where symptoms were controlled on treatment, which was usually at 3 weeks or earlier.) Results expressed as medians + interquartile ranges. *** = $p<0.001$ for the comparison with controls.

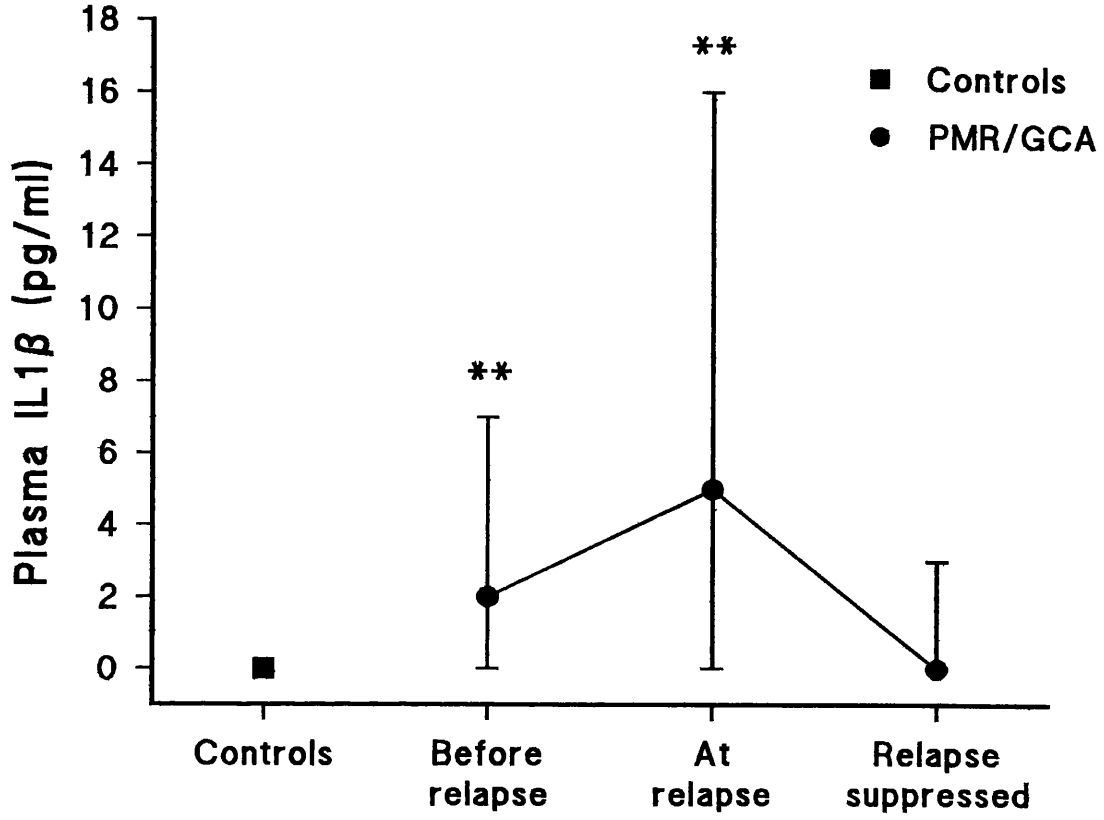

Figure 4.3 Plasma IL1ß concentrations in 21 clinical relapses of PMR/GCA occurring in 14 patients. Results expressed as medians + interquartile ranges. ** = $p<0.01$ for the comparison with controls.

CHAPTER 5

CIRCULATING INTERLEUKIN 6 IN PMR/GCA

5.1 INTRODUCTION

5.1.1 Interleukin 6 (IL6) in the acute phase response

The acute phase response is a protective response by the hepatocytes to distant tissue damage and inflammation. There is increased synthesis and release of the so-called "acute phase proteins", which inhibit tissue damage by their actions as protease inhibitors, opsonisers of cellular debris and promoters of tissue repair (Laurell 1985, Gauldie et al 1992, Thompson D et al 1992). Gauldie et al (1987) showed that the full hepatic acute phase response was induced by IL6. Other cytokines such as IL1 and tumour necrosis factor α (TNFα) have only a partial effect which may be indirect, as IL6 release is itself stimulated by IL1 and TNFα (van Snick 1990, Whicher and Evans 1990). Each of these 3 cytokines is released chiefly from the monocyte/ macrophage population of cells, but IL6 release has been demonstrated from a wide variety of cells (Wolvekamp and Marquet 1990, van Snick 1990, Whicher and Evans 1990). In addition to its effect on the acute phase response, IL6 produces B cell differentiation, stimulates erythropoiesis, induces fever and the release of corticosteroids (Wolvekamp and Marquet 1990, Gauldie et al 1992). In turn,

corticosteroids suppress IL6 production (Whicher and Evans 1990, Heinrich et al 1990), but potentiate the induction of the acute phase response (Baumann et al 1983, Andus et al 1988, Marinkovic et al 1989), probably by their effect in increasing IL6 receptor expression (van Snick 1990).

Unlike other cytokines, IL6 does not seem to activate inflammatory mediators such as the prostaglandins (Hopkins 1990). This is in keeping with its role as the main mediator of the acute phase response which is a protective, "anti-inflammatory" response. It has therefore been suggested that IL6 might be **administered** or **induced** as an approach to treatment of inflammatory diseases (Gauldie et al 1992). This is therefore quite unlike other experimental treatments to modulate inflammation where the aim is **inhibition** of cytokines such as IL1 and TNF (Thompson RC et al 1992, Dunn 1993, Elliott et al 1993).

As IL6 is the main mediator of the acute phase response, its measurement may be useful in anticipating this response, i.e. providing a more rapid indication of inflammation (Whicher and Evans 1990). IL6 has therefore been measured by many researchers in a variety of conditions.

5.1.2. IL6 in trauma, sepsis and malignancy

Aarden's group (Nijsten et al 1987) measured IL6 bioactivity in serum and plasma of patients with severe burns, using the B9 cell line and confirmed by neutralization with anti-IL6 antibody. They found a rise in IL6 to 2-100 times the

normal level within hours of the injury, and a later rise in acute phase proteins. Similarly, after the fall in IL6 levels there was a time-lag before acute phase proteins decreased. Guo et al (1990), also using the B9 assay, found that serum IL6 levels in burn patients were highest in those who did not survive.

In surgical trauma, Nishimoto et al (1989) used a different hybridoma cell line (MH60-BSF2) to demonstrate a rise in serum IL6 levels within 3 hours of surgery, peaking around 24 hours. CRP was detectable from 6 hours, peaking around 48 hours. Another group (Roumen et al 1992), using an ELISA to quantify serum IL6, found that half of their patients undergoing cholecystectomy had no detectable IL6 (i.e. ≤20pg/ml), despite a rise in CRP. Such surgical trauma is, however, relatively mild compared to the tissue damage occurring in burns.

In severe sepsis, several groups have reported very high circulating levels of IL6 and an association with disease severity. Using the B9 bioassay, Hack et al (1989) and Waage et al (1989), in plasma and serum respectively, showed than non-survivors had significantly higher IL6 levels than survivors, and this was confirmed by Munoz et al (1991), using the 7TD1 cell line. Circulating IL6 was also found to be higher in patients with septic shock than in normotensive patients with sepsis (Hack et al 1989, Waage et al 1989, Saladino et al 1992). The latter group used a less sensitive immunoassay (Predicta ELISA, Genzyme,

Massachussets), to quantify serum IL6. These levels were greatly elevated in septicaemic children (mean 407pg/ml) compared with children with minor febrile illnesses, all of whom had undectable serum IL6 (i.e. <18pg/ml).

In malignant disease the relationship between serum IL6 and disease severity does not seem to be as clear-cut as it is in sepsis and burns. Although Gause et al (1991) and Kurzrock et al (1993) both found elevated levels of IL6 in Hodgkin's disease using the Quantikine ELISA (R & D Systems, Minneapolis, USA), only Kurzrock et al found an association between elevated IL6 and systemic symptoms as well as reduced survival time. Gause et al showed IL6 falling to undetectable levels (<10pg/ml) after treatment but this occurred even where treatment was not successful. Kurzrock et al also found raised IL6 concentrations (<22pg/ml) in non-Hodgkin's lymphomas and the same association with systemic symptoms that they had shown in Hodgkin's disease, whereas Gause et al could demonstrate no IL6 in non-Hodgkin's lymphomas despite a detection limit of the assay down to 10pg/ml. In renal carcinoma Tsukamoto et al (1992) detected circulating IL6 in 25% of cases, using an ELISA sensitive down to 10pg/ml. Here again, higher IL6 levels were associated with fever and with reduced survival.

5.1.3. IL6 in inflammatory arthritis - synovial cells and synovial fluid

Synovial tissue cells from RA patients have been shown to express IL6 mRNA. Wood NC et al (1990) localized the IL6

mRNA to the T cells, with macrophages frequently found in association with the IL6-positive cells. Firestein et al (1990) however found 98% of the IL6-positive cells were not T cells, but were mainly type B synoviocytes (i.e. fibroblast type). The latter study also found that the percentage of synovial tissue cells expressing the IL6 gene was significantly lower in OA than in RA, and that cells from synovial fluid had a much lower level of gene expression than did synovial tissue cells. However, in another study (Hirano et al 1988) synovial fluid mononuclear cells constitutively expressed high levels of IL6 mRNA (5 times higher than maximally stimulated peripheral blood monocytes).

Production of actual IL6 protein from RA synovial tissue cells in vitro has been demonstrated by Hirano et al (1988) and Nawata et al (1989). The latter group found that the constitutive production of IL6 was from the mononuclear cells, although the type B fibroblast-like synoviocytes could be stimulated by IL1 to secrete IL6. Hirano's group found that the constitutive production of IL6 was from T and B lymphocytes in synovial tissue. Looking at the free mononuclear cells in RA synovial fluid, Bhardwaj et al (1989) were not able to demonstrate any spontaneous IL6 production, but in response to LPS the monocytes produced IL6, while the lymphocytes did not.

Although these studies give conflicting results as to which type of synovial cell is responsible for the majority of IL6

production in RA, they do all seem to suggest that synovial tissue is an important source of IL6. This is borne out by many studies showing high levels of IL6 in inflammatory synovial fluids. Houssiau et al (1988), Waage et al (1989), Holt et al (1991), de Benedetti et al (1992) and Wood NC et al (1992) have used bioassays, and Holt et al (1992) have confirmed with an ELISA that synovial fluid IL6 concentrations are higher than circulating levels in a variety of inflammatory arthritides, which suggests that the synovium is a major source of circulating IL6. Several studies have shown that synovial fluid levels of IL6 are related to local disease activity (Miltenburg et al 1991 and Holt et al 1991 with bioassays, and Brozik et al 1991 and Holt et al 1992 using ELISAs). Brozik et al also found that synovial fluid IL6 levels correlated with ESR and serum CRP in inflammatory arthritides and OA. Waage et al (1989) found a similar relationship between synovial fluid IL6 and ESR in inflammatory arthritis. Many studies have compared synovial fluid concentrations of IL6 in inflammatory arthritis and OA. Using bioassays Houssiau et al (1988), Holt et al (1991), Wood NC et al (1992) showed much higher levels of IL6 in synovial fluids of inflammatory arthritides than of OA, as did Hirano et al (1988) using a radioimmunoassay. With ELISAs capable of demonstrating pg/ml concentrations of IL6, Brozik et al (1991) and Holt et (1992) found ng/ml levels of IL6 in inflammatory synovial effusions but only pg/ml levels in OA effusions.

5.1.4 IL6 in inflammatory arthritis - circulating levels

Although circulating levels of IL6 are much lower than synovial fluid levels in inflammatory arthritides, sensitive bioassays have shown significantly higher circulating IL6 levels in RA than in OA or in normal controls (Houssiau et al 1988, Waage et al 1989, Wood NC et al 1991 and Madhok et al 1993a using serum, and Holt et al 1991 using heparinised plasma). However, Houssiau et al and Waage et al recorded detectable IL6 in only 30% of these RA sera, while Barrera et al (1993) showed no difference between serum IL6 in RA and controls, with the sensitive B9 bioassay nor with an ELISA. In systemic lupus erythematosus (SLE) serum IL6 levels appear to be higher than in RA (Gabay et al 1993, al-Janadi et 1993).

Some studies have demonstrated a correlation between serum IL6 levels and ESR and CRP in RA (Houssiau et al 1988, Dasgupta et al 1992, Vreugdenhil et al 1992, Madhok et al 1993a). Holt et al (1991), however, found plasma IL6 correlated with ESR and CRP only in non-RA inflammatory arthritides such as psoriatic arthritis and the B27 spondarthropathies. De Benedetti et al (1991 and 1992) showed serum IL6 levels correlated with ESR and CRP in articular juvenile chronic arthritis (JCA) but not in systemic JCA. In SLE, despite normal CRP levels, serum IL6 is higher than in RA (Gabay et al 1993). Clinical disease activity has been shown to correlate with circulating IL6 levels in various inflammatory arthritides (Holt et al 1992,

de Benedetti et al 1991 and 1992, Madhok et al 1993a). RA responding to treatment has been associated with a fall in serum IL6 in some studies (Madhok et al 1993b, Barrera et al 1993). However other studies found no reduction in serum IL6 in RA patients responding to treatment as judged clinically and by the acute phase response (Danis et al 1992, Racadot et al 1992, Wood NC et al 1992). The latter group unexpectedly found rising serum IL6 levels in the 3 individuals whom they tested serially while their RA went into remission.

5.1.5 Circulating IL6 in PMR/GCA

Dasgupta and Panayi (1990) used the IL6-dependent 7TD1 cell line to demonstrate elevated serum IL6 levels in 15 PMR/GCA patients before treatment. There was no overlap at all of the results of these untreated patients and controls matched for age and sex. Although the specificity of their IL6 measurements does not appear to have been confirmed by neutralisation with anti-IL6 antibodies, the cell line used has been shown by others to be very specific for IL6 (Houssiau et al 1988, Bhardwaj et al 1989, van Snick 1990). When Dasgupta and Panayi followed-up their PMR/GCA patients during corticosteroid treatment, they found that, at 6 months, only half of the IL6 levels had fallen into the normal range, despite all patients being in clinical remission with a normal ESR. The 7 patients who were followed up to one year had normal IL6 concentrations at this stage. In active disease there was a significant

correlation between serum IL6 and ESR.

Using a much less sensitive ELISA, al-Janadi et al (1993) found serum levels of IL6 in PMR were not significantly higher than in controls. However, this Inter Test-6 ELISA kit (Genzyme, Massachusetts) had a lower limit of detection of 150 pg/ml, so it is perhaps not surprising that PMR sera could not be distinguished from controls. The PMR data in this study might also be criticised on the grounds that the age range of the patients was 31-47 years old, which casts doubt on the validity of the diagnosis.

Now that more sensitive ELISAs are available for the measurement of IL6, these represent a more practical alternative to the labour-intensive bioassays if IL6 levels are to be measured in large numbers of samples (Whicher and Evans 1990). I therefore investigated whether ELISA measurement of circulating IL6 would be clinically useful in PMR/GCA, using the Quantikine ELISA (R & D Systems, Minneapolis) because of its ability to reliably detect levels of IL6 as low as 3.5 pg/ml.

Since my work, Lecron et al (1993) have reported serum IL6 levels in 20 patients with biopsy-proven GCA using an ELISA sensitive down to 3pg/ml (Medgenix, Brussels). They found elevated serum IL6 before treatment (mean 54pg/ml) compared with controls (mean 3.3pg/ml). On prednisolone treatment there was a significant fall in IL6 levels within one day, followed by a fall in CRP between day 1 and day 7. Despite

the rapid fall in IL6 levels in this study of GCA, the follow-up data at 30 days appear to show that most levels were still above control values. This is similar to the 6-month data in the study of PMR/GCA by Dasgupta and Panayi (1990). Neither of these studies investigated IL6 levels at the time of clinical relapse, which I have included in my study.

Recently Roche et al (1993) found elevated plasma IL6 in untreated PMR (mean approximately 20pg/ml) and GCA (mean approximately 30pg/ml) compared with controls (mean <1pg/ml). In this study the assay used was an ELISA produced by Biosource International (Camarillo, California). Although plasma IL6 began to fall rapidly on corticosteroid treatment, in more than half the patients the levels had not yet returned to nroaml after 4 weeks treatment. These authors also state that "short-term withdrawal of corticosteroids even after several months of treatment, was followed by an immediate increase in plasma IL6 concentrations", but this statement appears to be based on data in one patient only. In this patient, with PMR treated for some 6 months, corticosteroid therapy was deliberately witheld for 48 hours and plasma IL6 levels rose from 0 to 15pg/ml. However relapses of PMR/GCA during normal gradual prednisolone reduction do not appear to have been studied.

5.2 SUBJECTS AND METHODS

5.2.1 Patients and controls

30 patients (24 female) with PMR/GCA were studied. Blood samples were obtained prior to corticosteroid treatment and during subsequent remission, and 18 relapses were also studied (in 14 patients). Ages ranged from 51 to 84 years (median 70 years). 16 patients had PMR alone, 4 GCA alone and 10 had both at some stage. Temporal artery biopsy was positive in 6 patients.

Age- and sex-matched controls were recruited as described in Chapter 1 (section 1.3.1). In addition, disease controls were studied : 12 patients with active RA and 14 patients with pyrexia, as defined in section 4.2.1, as well as 8 patients in the intensive care unit with multiple injuries.

5.2.2 Technical methods

Plasma samples in EDTA and aprotinin were treated as described in section 4.2.2 and were then stored at -20^{o}C. Sera were also stored at -70^{o}C. A comparison of IL6 concentrations in 14 pairs of plasma and serum revealed very similar results. Because of the theoretical possibility of IL6 release during clotting, plasma samples were subsequently used to reflect *in vivo* concentrations.

IL6 was measured using the Quantikine ELISA kit (R & D Systems, Minneapolis). This assay has a lower limit of

detection of 3.5 pg/ml. Prior treatment of plasma or serum samples using the chloroform and DTT extraction procedures described in chapter 4 (section 4.2.3) did not improve the recovery of IL6 from the samples, so these procedures were not used.

200 μl samples of plasma were assayed in duplicate in microtitration wells pretreated with monoclonal antibody to IL6. After 2 hours incubation at room temperature, the bound IL6 was measured by washing the wells and adding polyclonal anti-IL6 antibody conjugated to horseradish peroxidase. After further washing, the substrate for this enzyme was added to the wells (tetramethylbenzidine plus hydrogen peroxide) and the reaction stopped after 20 minutes. The optical density of each well was determined using a spectrophotometer set to 450nm, and compared to a standard curve of human recombinant IL6 in buffer solution. The plate reader and computer software were as described in section 4.2.4. The intra-assay coefficient of variation for this ELISA was 7-9%, while the inter-assay coefficient of variation ranged up to 11%.

5.2.3 Statistical methods

The Mann-Whitney test and Wilcoxon's rank sum test were used for comparison of patients and controls and for paired within-patient comparisons respectively. When testing for correlations between plasma IL6 concentrations and other variables, Spearman's rank correlation coefficient was calculated.

5.3 RESULTS

5.3.1 Plasma IL6 concentration in PMR/GCA at presentation

Before treatment, plasma IL6 levels in PMR/GCA were slightly, but not significantly, raised (median 8.5pg/ml) compared with controls (median 0pg/ml) p=0.078 (Figure 5.1). The full range of PMR/GCA results was from 0 to 65pg/ml. Once the disease had been controlled the median IL6 was 0.5pg/ml but this fall was not significant (p=0.165).

5.3.2 Plasma IL6 in relapses of PMR/GCA

Plasma IL6 levels were not raised at relapse (Figure 5.2.). However, in the first sample where relapse was suppressed, the median IL6 concentration was 9.5pg/ml. This level was not significantly higher than controls, but the paired comparison with the "before relapse" samples did show a significant rise (p=0.022).

5.3.3 Relationship of IL6 to CRP, ESR and IL1ß in PMR/GCA

At presentation, plasma IL6 showed a significant correlation with CRP (r_s=0.545, p=0.001) and with ESR (r_s=0.362, p=0.025). Once the disease was suppressed, this correlation disappeared. During relapses, CRP showed no significant correlation with plasma IL6 levels (r_s=0.326, p=0.10), but a correlation became apparent immediately after relapses when IL6 tended to be raised (r_s=0.580, p=0.01). ESR, on the other hand, was correlated with IL6 levels at the time of relapse (r_s=0.474, p=0.025), but not after relapse (r_s=0.389, p>0.05).

At presentation, there was also a correlation between plasma IL1ß and plasma IL6 concentrations (r_s=0.453, p<0.01) and again this correlation disappeared as the disease was suppressed. At relapse there was no correlation at all between IL1ß and IL6 (r_s=-0.201, NS), but when relapse was suppressed a correlation was evident (r_s=0.668, p<0.005).

5.3.4 Plasma IL6 in RA, in trauma and in febrile illness

12 patients with active RA had a median plasma IL6 level of 9pg/ml (interquartile range 0-16.5pg/ml), which was similar to pre-treatment PMR/GCA and not significantly higher than matched controls (median 0pg/ml, p=0.093).

The 8 trauma cases had higher levels of IL6 (median 29pg/ml, interquartile range 16-98pg/ml) which were significantly greater than controls (p=0.001) and significantly greater than PMR/GCA (p=0.004).

The 14 patients with febrile illness had intermediate IL6 levels (median 18.5pg/ml, interquartile range 0-33pg/ml) which was significantly higher than matched controls (p=0.012) but not significantly higher than PMR/GCA (p=0.057).

5.4 DISCUSSION

In this study, plasma levels of IL6 in PMR/GCA were not significantly higher than in controls. However, in untreated PMR/GCA, there was a non-significant trend towards

elevation of plasma IL6. The fact that plasma IL6 levels at presentation correlated with the raised CRP concentrations, also suggests that IL6 levels are indeed elevated but cannot be accurately enough determined by this method. Using a bioassay, Dasgupta and Panayi (1990) did demonstrate raised serum IL6 in PMR/GCA before treatment, while Lecron et al (1993) found pre-treatment serum levels of 54pg/ml in biopsy-proven GCA, using the Medgenix ELISA, and Roche et al (1993) found pre-treatment plasma levels of 20pg/ml in PMR and 30pg/ml in GCA, using the Biosource ELISA. Patient selection may play a part in the differences between results but in my study the patients with GCA had no higher levels of IL6 than did the patients with PMR.

My results in the patient groups with trauma or fever show that IL6 can be reliably detected by this method when higher concentrations are present, but it appears that the levels in PMR/GCA and in RA are too low to be reliably distinguished from normal. In other studies of RA, circulating IL6 levels have varied greatly, with results ranging from undetectable to significantly higher than controls (Barrera et al 1993, Houssian et al 1988, Waage et al 1989, Holt et al 1991, Wood NC et al 1991, Madhok et al 1993a).

Holt et al (1992) compared bioassay and immunoassay and found that at low levels the bioassay overestimated IL6 concentrations relative to the immunoassay. However, as there is no "gold standard", this finding does not indicate

which of the assay methods gives the "correct IL6 concentration.

Even control values of circulating IL6 have varied considerably between studies. Teppo et al (1991) reported a mean serum IL6 concentration of 83ng/l (i.e. 83pg/ml) in 45 healthy subjects (range <20-290pg/ml), using a radioimmunoassay. Although **plasma** IL6 was not measured in these healthy subjects, extrapolation from recovery experiments suggested that plasma levels would have been some 25% higher. Wood NC et al (1991) and Holt et al (1992), using bioassays, reported serum IL6 levels of 22pg/ml and 27pg/ml respectively in healthy volunteers, while the latter study also found similar levels in osteoarthritic controls. Lecron et al (1993), using an ELISA, found a mean serum IL6 level of only 3.3pg/ml in healthy volunteers, and Guo et al (1990) detected no IL6 at all in normal sera using the sensitive B9 bioassay.

The hope that IL6 might be useful in PMR/GCA by responding before the acute phase proteins, does not seem to have been borne out by this study nor that of Dasgupta and Panayi (1990) who found a prolonged elevation of IL6 in half of PMR/GCA patients where ESR had fallen to normal. However Lecron et al (1993) found that IL6 fell significantly on treatment before CRP did. Neither of these studies looked at IL6 in relapses, but in my study any IL6 response seemed to lag behind the CRP response. This is similar to the finding of Wood NC et al (1992) in RA patients followed into

remission, where serum IL6 levels continued to rise after CRP and ESR had fallen.

This study found circulating IL6 levels less useful than CRP concentrations in the assessment of PMR/GCA, both at presentation and at relapse. Refinements in assay sensitivity and reproducibility could alter this situation in the future, but at present even those studies which have found higher IL6 levels in untreated PMR/GCA than in controls have not shown any additional benefit in terms of monitoring disease activity.

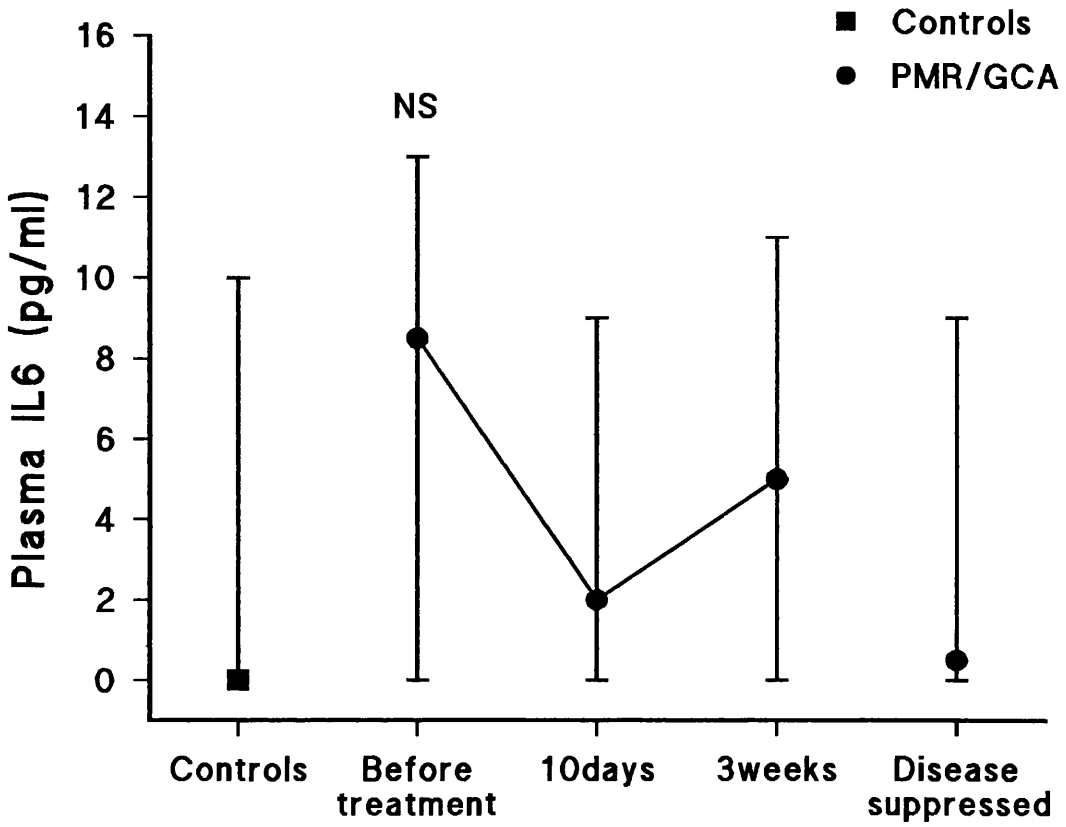

Figure 5.1 Plasma IL6 concentrations in 30 patients with PMR/GCA before treatment and during early prednisolone treatment. ("Disease suppressed" represents the earliest sample where symptoms were controlled on treatment, which was usually at 3 weeks or earlier.) Results expressed as medians + interquartile ranges. NS = not significantly different from controls at the 95% level.

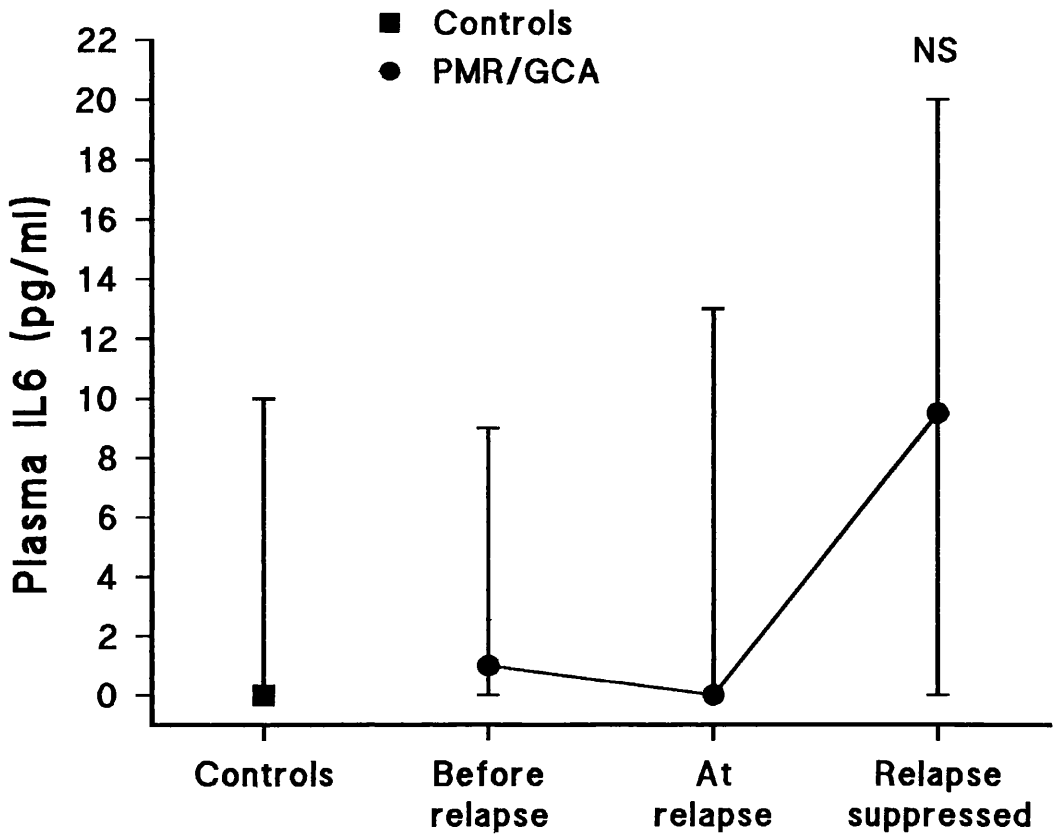

Figure 5.2 Plasma IL6 concentrations in 18 clinical relapses of PMR/GCA in 14 patients. Results expressed as medians + interquartile ranges. NS = not significantly different from controls at the 95% level.

CHAPTER 6

SERUM CONCENTRATION OF SOLUBLE INTERLEUKIN 2 RECEPTORS IN PMR/GCA

6.1 INTRODUCTION

6.1.1 Soluble interleukin 2 receptors (sIL2R) as a marker of immune system activation

Activation of T lymphocytes results in production of interleukin 2 (IL2) and expression of IL2 receptor (IL2R) on the T cell surface. This receptor in turn mediates the effects of IL2 i.e. T cell activation and proliferation (Robb and Greene 1983, Buchan et al 1988a, Boumpas et al 1991). Corticosteroids inhibit *in vitro* production of IL2 and IL2R, but addition of exogenous IL2 restores IL2R expression. This suggests that, in T cell activation, IL2R expression is **secondary** to IL2 production (Boumpas et al 1991).

The IL2R consists of 2 subunits; alpha (α or Tac) with low affinity for IL2, and beta (ß) with intermediate affinity. When linked, these form the high affinity IL2R (Rubin and Nelson 1990a). Even resting lymphocytes express the ß subunit, but only activated mononuclear cells express the α chain, so the appearance of this Tac antigen is an early marker of activation of the immune system.

Rubin et al (1985), using an ELISA for Tac antigen

demonstrated that activated T cells *in vitro* release a soluble form of IL2R (sIL2R). This seems to be produced by enzymatic cleavage and release of cell surface Tac (Rubin et al 1990b). This identity is supported by the fact that the affinity of IL2 for sIL2R is low, similar to that for cell surface Tac. However, despite this low affinity for IL2, high concentrations of sIL2R do appear to be associated with impaired cellular responsiveness to IL2 both *in vitro* and *in vivo* (Rubin and Nelson 1990a). This suggests that sIL2R may act in a negative feedback loop, as an antagonist of IL2 mediated cell responses. This binding of free IL2 by sIL2R may also explain why free IL2 is not detectable in many situations where abundant IL2 message is found (Buchan et al 1988a).

Under normal physiological conditions, healthy adults have detectable levels of serum sIL2R, reflecting a baseline level of immune activation (Rubin and Nelson 1990a). Higher levels of sIL2R are seen in healthy children (Pui 1988), and also in healthy elderly individuals, although in the latter group raised sIL2R is associated with the presence of autoantibodies i.e. increased immune activation (Manoussakis et al 1990).

In addition to its release from normal activated mononuclear cells, sIL2R is also released by neoplastic lymphocytes in many lymphoproliferative disorders (Pui 1988). Other non-haematological tumours are not associated with elevated sIL2R levels unless there is extensive metastatic disease,

perhaps indicating host immune response rather than release from the tumour (Rubin and Nelson 1990a). In many of the lymphoproliferative diseases serum sIL2R levels reflect tumour burden and can be used to monitor response to treatment.

In assessing immune system activation, the measurement of sIL2R in serum which can be stored has obvious advantages over a cell-based system which requires analysis of fresh cells. In addition the low capital cost of ELISAs compared with a fluorescent activated cell sorter (FACS) make measurement of sIL2R an attractive method of assessing immune system activation in various diseases (Rubin and Nelson 1990a).

6.1.2 SIL2R in infections, trauma and AIDS

Raised serum sIL2R has been demonstrated in some infections such as tuberculosis (Brown et al 1989), malaria (Doloron et al 1989), viral hepatitis (Chu and Liaw 1989) and measles but not other viral exanthems (Griffin et al 1989). "Abnormal" immune responses may be associated with particularly high levels of sIL2R; for instance if viral hepatitis progresses to chronic active hepatitis (Chu and Liaw 1989). Similarly, in the delayed hypersensitivity "reversal reaction" in lepromatous leprosy serum sIL2R is markedly raised compared with the reduced levels seen in tuberculoid leprosy (Tung et al 1987). Wong KL and Wong RPO (1991) found that infections were associated with raised sIL2R levels in SLE (in active or inactive SLE), and that in

chronic infections such as tuberculosis sIL2R levels were higher than in pyogenic infections.

Surgical trauma causes a rise in serum sIL2R (Lissoni et al 1990, Fassbender et al 1993). Burns also result in an acute elevation of serum sIL2R, and a further rise is seen in those patients who become immunosuppressed. The sIL2R in the serum of these patients inhibits the effects of exogenous IL2 *in vitro*, which suggests that it may contribute to the patients' immunosuppression (Rubin and Nelson 1990a). Therefore sIL2R, a product of immune activation, may downregulate that immune activation and in some situations even result in pathological immunosuppression.

Serum sIL2R is elevated in HIV infection and AIDS. This may be as a result of activation of CD8+ cytotoxic T cells so that, despite falling numbers of CD4+ cells and total lymphocytes, the release of sIL2R increases as the disease progresses (Rubin and Nelson 1990a).

6.1.3 SIL2R in autoimmune and rheumatic diseases

As would be expected of a test indicating increased immune activation, serum sIL2R levels are increased in graft rejection (Rubin and Nelson 1990a). In the same way, it is not surprising that in many allergic and autoimmune diseases elevated sIL2R levels have been demonstrated. Asthma and atopic eczema are both associated with raised serum sIL2R, particularly at the time of clinical exacerbations (Lai et

al 1993, Colver et al 1989). Untreated Graves' disease is associated with elevated sIL2R levels, which fall significantly by the third month of carbimazole treatment (Weryha et al 1991).

In the autoimmune rheumatic diseases serum sIL2R levels are usually increased, and frequently the levels are related to disease activity. Studies in SLE have shown raised concentrations of serum sIL2R; moderately elevated in inactive SLE, but significantly higher in active SLE defined clinically or by hypocomplementaemia (Wolf and Brelsford 1988, Semenzato et al 1988, Tokano et al 1989). In systemic sclerosis sIL2R levels are raised even in patients with limited cutaneous disease (Kantor et al 1992), but the highest levels are associated with generalized disease and with mortality (Degiannis et al 1990). In polymyositis Wolf and Baethge (1990) documented elevated sIL2R prior to treatment, with levels falling rapidly on treatment. Tokano et al (1992) also reported raised sIL2R levels in polymyositis and dermatomyositis, particularly prior to treatment but also in a majority of patients already on corticosteroid treatment.

Several studies have shown raised serum sIL2R in RA (Keystone et al 1988, Semenzato et al 1988, Campen et al 1988, Symons et al 1988b, Wolf et al 1992, Racadot et al 1992, Barrera et al 1993, Polisson et al 1994). In most of these studies, as well as that of Rubin et al (1990c), sIL2R levels were correlated with disease activity, but Keystone

et al (1988), Racadot et al (1992), Crilly et al (1993) and Polisson et al (1994) did not find a correlation with disease activity. Synovial fluid concentrations of sIL2R have also been shown to be elevated in RA (Keystone et al 1988, Symons et al 1988b, Wolf et al 1992, Cope et al 1992). The first three of these studies also demonstrated that synovial fluid levels of sIL2R were significantly higher than serum levels in RA.

In "non-autoimmune" arthritis such as OA and gout, serum and synovial fluid levels of sIL2R have been found to be similar to the serum levels of healthy controls (Keystone et al 1988, Symons et al 1988b, Semenzato et al 1988, Campen et al 1988). Wolf et al (1992) did find higher serum sIL2R in OA than in healthy controls but the control group was considerably younger than the OA patients.

In children with all types of JCA, serum sIL2R is raised, but levels are highest in systemic onset disease (Fassbender et al 1992). Psoriatic arthritis is also associated with elevated serum sIL2R, and clinical response to treatment is accompanied by a fall in these levels (Salvarani et al 1992a).

6.1.4 Serum sIL2R in PMR/GCA

Salvarani et al (1992b) found elevated serum sIL2R levels in 21 patients with PMR/GCA prior to steroid treatment. Although the control group consisted of blood donors and was therefore presumably not matched for age, the degree of

elevation in pre-treatment PMR/GCA was great (mean approx. 840 U/ml, SD approx. 660 U/ml) compared with the controls (mean 275 U/ml, SD 85 U/ml, p=0.002), so this elevation is probably meaningful. However, this study also reported persistently elevated sIL2R levels in 10 PMR/GCA patients followed for 6 months in clinical remission on prednisolone treatment. By this stage the mean sIL2R level had fallen to 413 (±152) U/ml, compared with 901 (±437) U/ml before treatment and this fall was significant (p=0.02). So, the finding that these 6-month levels were still significantly higher than the blood donor control group (mean 275 ±85 U/ml, p=0.02) may be misleading, because of the relatively small difference and the lack of age-matching.

6.2 SUBJECTS AND METHODS

6.2.1 Patients and controls

Serum concentrations of sIL2R were measured in 42 PMR/GCA patients (30 female). Their ages ranged from 51 to 87 years (median 71.5 years). 24 had PMR alone, 7 had GCA alone and 11 had both at some stage. Temporal artery biopsy showed active arteritis in 6 patients. Pre-treatment serum was available from 41 patients, and also from 33 clinical relapses in 17 patients.

Age- and sex-matched controls were obtained as described in Chapter 1, section 1.3.1. Serum sIL2R was also measured in 12 patients with active RA, 11 patients with febrile illnesses (as described in section 4.2.1) and 5 patients

with multiple injuries (as described in section 5.2.1). For these younger patient groups age-matched controls also included some laboratory staff.

In addition serial serum samples were available for analysis from one healthy volunteer before, during and after a 10 day course of prednisolone. Prednisolone (enteric coated) was taken in a once daily dose of 20mg for 3 days, followed by 10mg for 3 days and 5mg for 4 days. 3 serum samples were obtained before prednisolone administration, 7 samples during the course and 3 samples afterwards.

6.2.2 Technical methods

Serum samples were separated from the clot within 4 hours of venesection, and stored at -70^{o}C. Prior to assay the samples had undergone no more than one freeze-thaw cycle. EDTA plasma samples available on the same patients were not suitable for use with this particular ELISA, hence only serum samples were analysed.

Serum sIL2R was measured using the Cellfree Interleukin-2 Receptor Test Kit (T Cell Sciences, Cambridge, Massachusetts). In this ELISA, 50μl samples of serum were assayed in duplicate in 96-well plates precoated with mouse monoclonal antibody to human sIL2R. At the same stage, the second monoclonal antibody conjugated to horseradish peroxidase was added to the wells. (The second murine monoclonal antibody was directed against a second, distinct, epitope on the human sIL2R molecule). After 3 hours

incubation at room temperature on a rotator set, the wells were washed. Bound enzyme was then identified by adding the substrate (o-phenylenediamine) and incubating for 30 minutes at room temperature. After stopping the reaction with 2N sulphuric acid, the absorbance at 490nm was read and compared to the provided standards in serum (ranging from 0 to 3200 U/ml). The plate reader and computer software were as described in section 4.2.4. The detection limit of the assay was 50 U/ml, and the coefficient of variation within and between assays was <6% for 3 standard concentrations.

6.2.3 Statistical methods

The Mann-Whitney test was used for comparison of patient data with control data. Wilcoxon's rank sum test was used for paired comparisons within the PMR/GCA group. To examine the relationships between sIL2R and other laboratory parameters, Spearman's rank correlation coefficient was used.

6.3 RESULTS

6.3.1 Serum sIL2R concentration in PMR/GCA at presentation

Before treatment, serum sIL2R levels were raised in PMR/GCA at 476 U/ml, compared with 366 U/ml in controls (p=0.031). Figure 6.1 illustrates this and the subsequent fall in levels on treatment. At the time of disease suppression there was a trend for sIL2R to be lower than controls but this did not reach statistical significance (median 327 U/ml, p=0.060).

6.3.2 Serum sIL2R in relapses of PMR/GCA

Prior to relapses of PMR/GCA, serum sIL2R levels were significantly reduced at 301 U/ml compared with controls ($p=0.0005$), suggesting an effect of the prednisolone itself. At relapse there was a slight rise of sIL2R levels to a median of 341 U/ml. This rise was not significant and the levels were still lower than controls ($p=0.044$). Figure 6.2 illustrates this data in relapses.

Analysis of further samples from 11 patients during prolonged remission on prednisolone also showed a trend for serum sIL2R levels (at 290 U/ml) to be lower than controls, although in this small group this difference did not reach statistical significant ($p=0.109$). Within these prolonged remissions there was no evidence that higher doses of steroids were associated with a lower level of sIL2R, with not even a trend in this direction.

6.3.3 Serum sIL2R in one volunteer taking prednisolone

Figure 6.3 illustrates the effect on serum sIL2R of a 10 day course of prednisolone in a healthy volunteer. From day 3 of the prednisolone course to the third day after the course, sIL2R levels were lower than the baseline values. This seems to illustrate that the prednisolone was lowering sIL2R with a delay in effect of a few days. As the volunteer data was drawn from only one individual, the conclusions drawn from it must be limited, but it does seem

to confirm the suggestion in the PMR/GCA patients that corticosteroid treatment suppresses sIL2R below normal levels.

6.3.4 Serum sIL2R and age

The relationship between serum sIL2R concentration and age was investigated from all the control data available. The age span covered was 37-90 years. There was a non-significant trend towards increasing sIL2R with age (r_s=0.212, 0.1>p>0.05).

6.3.5 Relationship of sIL2R to other laboratory parameters in PMR/GCA

Serum sIL2R concentration showed no significant correlation with ESR, CRP or plasma IL1ß. There was however a significant correlation between serum sIL2R and plasma IL6 concentration at presentation of PMR/GCA (r_s=0.518, p<0.005). No such correlation was apparent in relapses.

Thirty three of these PMR/GCA patients also had pretreatment data available on activated T cells, defined by the expression of DR antigen on T cells. (see Chapter 7, section 7.A.2.2.) Although the absolute numbers of activated T cells measured in this way were not raised in PMR/GCA, there was a weak correlation between the numbers of activated T cells and the serum sIL2R levels (r_s=0.294, 0.025<p<0.05). Activated T cells as a percentage of total lymphocytes did show a non-significant trend to be raised in PMR/GCA (See Chapter 7, Tables 7.1 and 7.2).

6.3.6 Serum sIL2R in RA, in trauma and in febrile illness

In 12 patients with active RA the median sIL2R level was 687 U/ml (interquartile range 411-908 U/ml) which was significantly higher than matched controls (p=0.011). Although there was an obvious trend for these results in RA to be higher than in pre-treatment PMR/GCA (where the median sIL2R level was 476 U/ml), the small numbers of RA patients meant that this difference did not reach statistical significance (p=0.093).

In 5 trauma cases the median serum sIL2R level was 808 U/ml (full range 308-2277 U/ml). 5 age-matched controls had a median sIL2R level of 210 U/ml (full range 192-264 U/ml). Despite the small groups here, the trauma cases had significantly higher levels than the controls (p=0.009), although not significantly higher than the pre-treatment PMR/GCA patients.

In 11 patients with febrile illness there was a median sIL2R level of 678 U/ml (interquartile range 451-1174 U/ml). This was significantly higher than matched controls (p=0.004), and significantly higher than pre-treatment PMR/GCA patients (p=0.011).

6.4 DISCUSSION

This study shows that serum sIL2R levels are raised in PMR/GCA compared with age-matched controls. This confirms the impression given by the study of Salvarani et al

(1992b), despite the limitation of that study in that controls were not properly age-matched.

On prednisolone treatment it appears that sIL2R levels are suppressed even below normal values. This was not evident in the data from Salvarani et al (1992b); on the contrary, in that study, the sIL2R level remained slightly, but significantly, higher than controls in 10 PMR/GCA patients followed for 6 months on treatment. However, as the controls were probably much younger than the patients in that study, this would account for the apparently raised values in patients. My finding of subnormal sIL2R levels on corticosteroids are supported by other studies. *In vitro* corticosteroids suppress IL2R expression (Boumpas et al 1991), and in some leprosy patients corticosteroid treatment reduces sIL2R below control values (Tung et al 1987).

From subnormal sIL2R levels on prednisolone treatment, my data showed no significant rise of sIL2R in relapses of PMR/GCA. In fact, overall these levels remained significantly lower than the age-matched controls. Some individual relapses, particularly on no or low prednisolone, did show an obvious rise in sIL2R but, in general, sIL2R levels do not appear to be useful in confirming relapse of PMR/GCA on treatment. It is interesting that Wolf and Baethge (1990) found that, in all 4 of their patients with relapses of polymyositis on prednisolone, sIL2R levels did rise prior to, or simultaneously with, muscle weakness and/or elevated creatine kinase levels.

The elevated sIL2R level at presentation of PMR/GCA, and its suppression on prednisolone, suggests that this disease, like RA and SLE, is associated with increased immune activation. Although **total** activated T cells were not increased in my patients, activated T cells as a **percentage** of total lymphocytes did show a non-significant trend to be raised (see Chapter 7, Tables 7.1 and 7.2). Dasgupta et al (1989) showed, by dual staining for CD8 and DR, that the percentage of activated CD8+ cells was increased, whereas activated CD4+ cells were not increased. They suggested that these activated CD8+ cells might be responding to a causative antigen and mediating the pathogenic effects in PMR/GCA. Although the initial trigger for the observed immune activation remains a matter of speculation, the elevated circulating sIL2R levels shown here do confirm that there is increased activation of the immune system. This is likely to play a part in the pathogenesis of the disease, and indeed the role of corticosteroids in suppressing both the sIL2R levels and the clinical disease does support a link between the two.

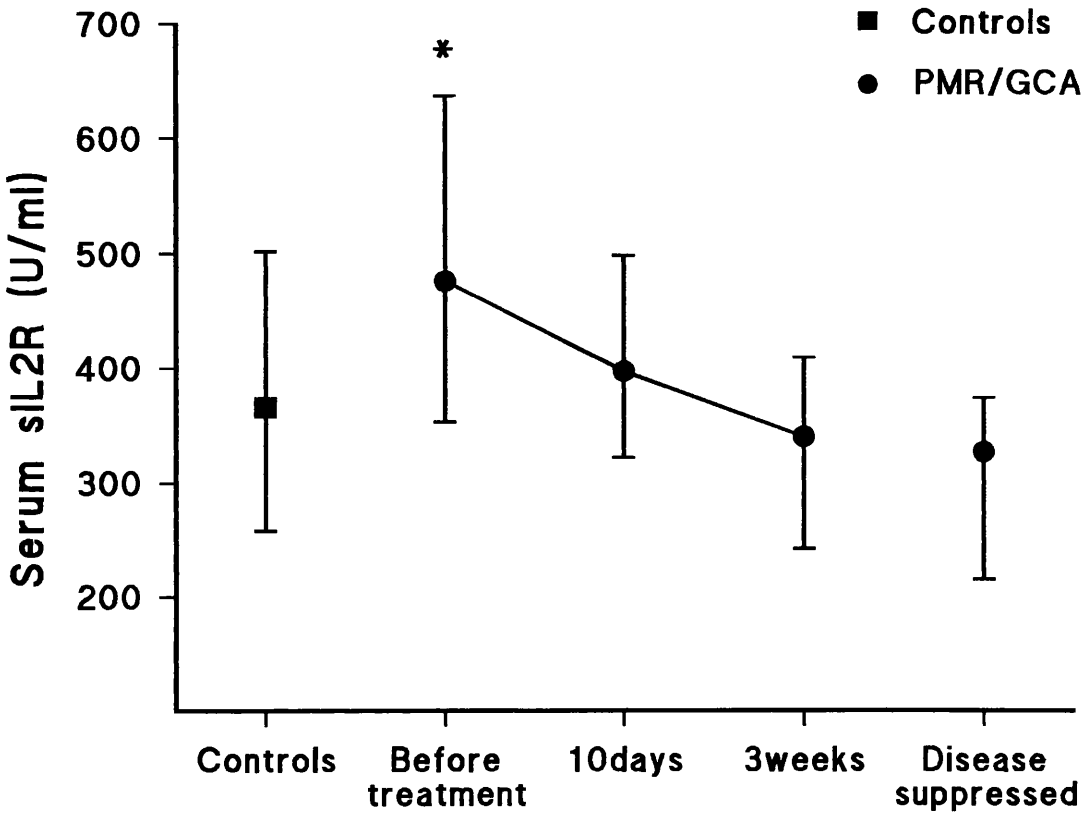

Figure 6.1 Serum levels of soluble IL2 receptors in 41 PMR/GCA patients, before treatment and during early prednisolone treatment. ("Disease suppressed" represents the earliest sample where symptoms were controlled on treatment, which was usually at 3 weeks or earlier.) Results expressed as medians + interquartile ranges. *=$p<0.05$ for the comparison with controls.

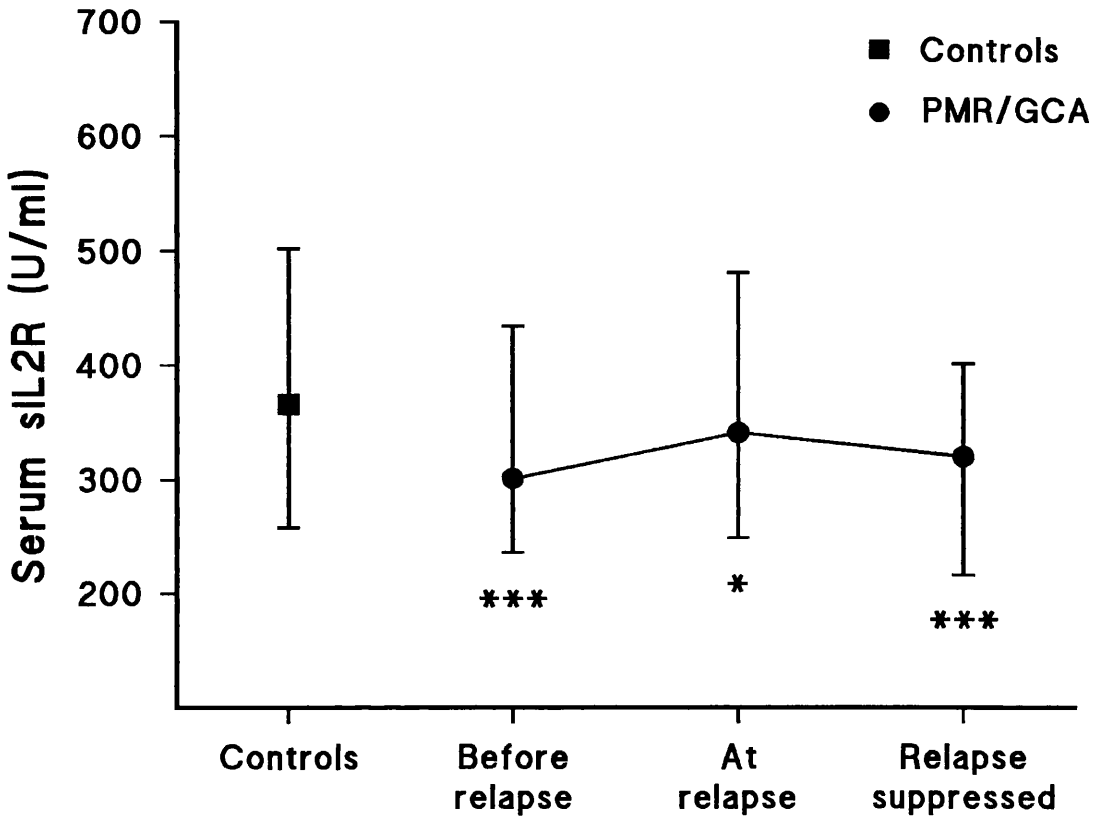

Figure 6.2 Serum levels of soluble IL2 receptors in 33 clinical relapses of PMR/GCA occurring in 17 patients. Results expressed as medians + interquartile ranges. ***=p<0.001, *=p<0.05, for the comparison with controls.

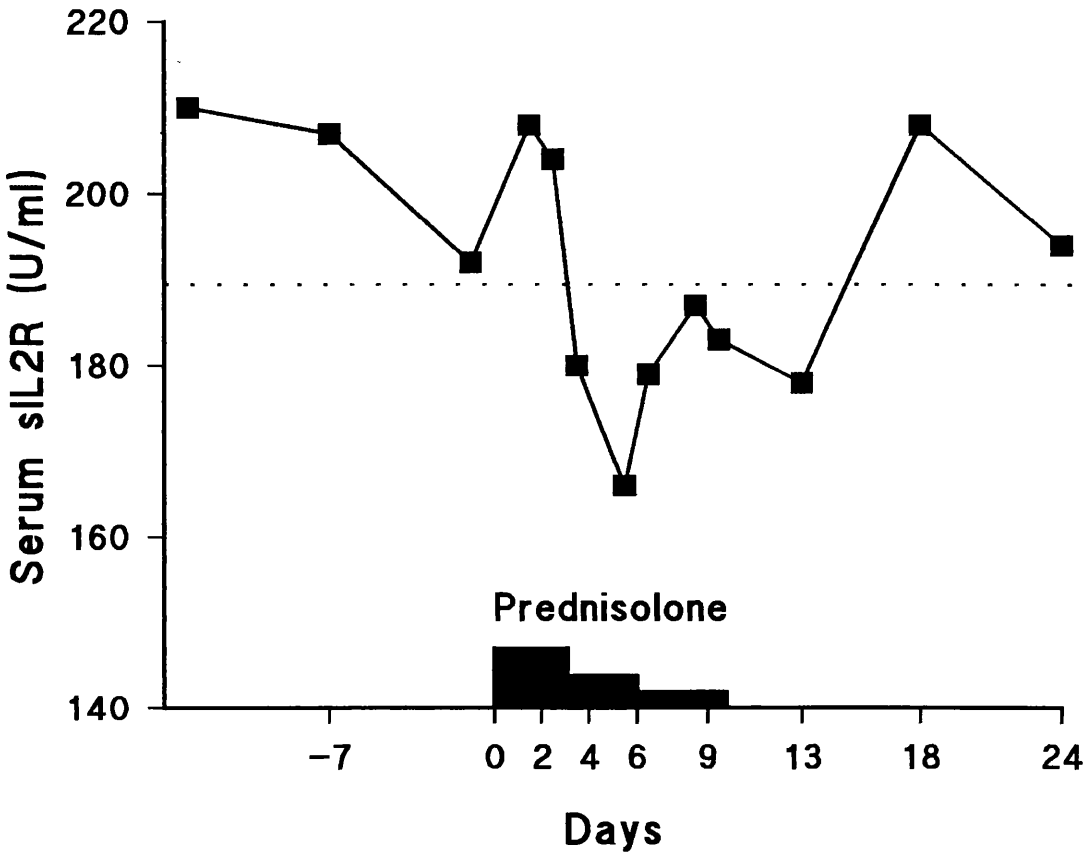

Figure 6.3 Serum levels of soluble IL2 receptors in a healthy volunteer before, during and after 10 days prednisolone administration. The dotted line separates those sIL2R results between day 3 of the prednisolone course and the third day after the course, from the higher levels seen during the rest of the period.

CHAPTER 7

CIRCULATING LYMPHOCYTE SUBSETS
A. IN PMR/GCA, AND
B. IN HEALTHY VOLUNTEERS DURING PREDNISOLONE ADMINISTRATION

7.A. CIRCULATING LYMPHOCYTE SUBSETS IN PMR/GCA

7.A.1 INTRODUCTION

There have been several studies of circulating T cell subtypes in PMR/GCA with differing results. In GCA prior to treatment, Andersson et al (1988) found normal numbers of CD8+ and CD4+ cells, and Banks et al (1983) documented normal helper:suppressor ratios. Other studies in PMR and GCA have found a decreased percentage of CD8+ cells (Macchioni et al 1993, Elling et al 1990, Dasgupta et al 1989, Elling and Elling 1985, Chelazzi and Broggini 1984, Benlahrache et al 1983) whilst Dasgupta et al (1989), Elling et al (1990) and Macchioni et al (1993) also reported reduced absolute numbers of CD8+ cells.

All of the studies apart from the negative study by Andersson et al (1988) used mononuclear cells separated on a Ficoll-Hypaque density gradient. This method selectively decreases the CD8+ subset, leading to a significantly lower percentage of CD8+ cells compared with the whole blood lysis method (Renzi and Ginns 1987). This artefact would not necessarily affect control samples and patient samples to

the same extent, so could distort results. Patient CD8+ cells might have intrinsic differences from control cells affecting their migration on a density gradient and might well also have differences due to delay in processing compared with control cells. A marked fall in %CD8+ cells and %CD4+ cells has been shown in blood stored for 24 hours when the Ficoll-Hypaque method is used but not with the whole blood lysis method (Ashmore et al 1989). Furthermore, a delay of more than 6 hours before processing blood samples results in a considerable fall in the absolute numbers of lymphocytes counted by automated haematology counters (Bird AG 1990). Such delay might occur more often with patient samples than control samples, particularly in multicentre studies where patient blood samples may be transported from other hospitals for analysis.

The diurnal variation in lymphocyte numbers produces a nadir in numbers of lymphocytes, T cells, CD4+ and CD8+ cells around 10.00 am to 12.30 pm (Ritchie et al 1983, Levi et al 1988). Obviously if patient samples and control samples were not taken at the same time of day this would confound results.

There is evidence of a fall in CD8+ cells with age, both in absolute numbers and as a percentage of lymphocytes (Ligthart et al 1985, Nagel et al 1981) so controls should be matched for age, which is not addressed in some of the previous studies (Macchioni et al 1993, Andersson et al 1988, Banks et al 1983).

In some of the previous studies the PMR/GCA patients were already taking prednisolone at the time of T cell subset analysis (Elling and Elling 1985, Benlahrache et al 1983). It has been shown that circulating lymphocyte subsets change in response to a single dose of corticosteroid. Tonnesen et al (1987) and ten Berge et al (1984) reported reduced T cells, OKT4+ cells and OKT8+ cells 2 to 6 hours after administration of corticosteroids in healthy volunteers. After 4 weeks corticosteroid treatment in idiopathic thrombocytopenic purpura (ITP), Ferari et al (1991) found **increased** numbers of T cells, CD4+ and CD8+ cells. Hence corticosteroid administration can alter lymphocyte subtypes in different ways depending on the timing of the sample.

To clarify the conflicting data from previous studies, this study was designed to eliminate these variables.

7.A.2 SUBJECTS AND METHODS

7.A.2.1 Patients and controls

Thirty six patients with PMR/GCA (26 female) were assessed before, during and after treatment with prednisolone. Ages ranged from 51 to 87 years (median 70 years). 23 patients had PMR alone, 6 had GCA alone and 7 had both at some stage. Temporal artery biopsy was positive in 4 cases.

These patients had blood samples taken between 10 am and 2 pm, prior to treatment and subsequently after 10 days, 3

weeks, 6 weeks, 3 months of treatment and thereafter every 3 months until treatment could be discontinued. Venesection was always carried out at the same time of day and **before** the daily prednisolone dose. Treatment for PMR was begun with prednisolone 10-20 mg daily, or for GCA with prednisolone 40 mg daily. Prednisolone was prescribed as the enteric-coated preparation and as a once daily dose in the morning. Subsequently dosage was reduced according to clinical disease activity.

Controls were matched for age and sex and were recruited as described in Chapter 1. Control samples were collected between 10 am and 2 pm, to correspond to the time of venesection in patients.

7.A.2.2 Technical methods

All patients and controls were seen at Addenbrooke's Hospital and all blood samples were processed within 6 hours of venesection. Total white count and lymphocyte count were measured using routine techniques in the haematology department at Addenbrooke's Hospital. T cell subtypes were analysed by flow cytometry using a whole blood lysis technique. Total T cells were measured using anti-CD3 (Leu 4). Activated T cells were those CD3+ cells which co-expressed HLA-DR. CD4+ cells were defined using Leu 3, CD8+ cells using Leu 2. NK cells were $CD3^-$ expressing CD16/56 (Leu 11c+19). All monoclonal antibodies were purchased from Becton Dickinson (Oxford, UK) from the Simultest range. Aliquots of blood were incubated with

antibody pairs for dual staining for 15 minutes at room temperature. Erythrocytes were lysed using FACS lysing solution (Becton Dickinson) and leucocytes were fixed with 0.5% formaldehyde. Cells were analysed on the day of processing using a Becton Dickinson FACscan, and Simulset software. The lymphocyte gate was set by the software where possible but otherwise was set manually. Subsequent analysis excluded the non-lymphocyte events. Quality control using standard cell preparations revealed coefficients of variation of 2-6% for T cells, T cell subtypes and NK cells, and up to 8% for B cell enumeration.

7.A.2.3 Statistical methods

Pre-treatment values for lymphocyte subsets in the 36 PMR/GCA patients were compared with controls. In addition, to see if patient selection was a critical factor, the patients with biopsy-proven GCA and/or the most severe symptoms of PMR were considered separately. This group of 13 was compared with controls and with the remaining group of 23 patients with less severe GCA/PMR.

Statistical analysis consisted of the Mann Whitney test to compare groups, and paired analysis by the Wilcoxon test to examine within-patient changes in T cell subsets.

7.A.3 RESULTS

7.A.3.1 PMR/GCA cases overall

Considering all 36 PMR/GCA patients prior to prednisolone

treatment (Table 7.1); lymphocytes, T cells and T cell subtypes were all normal compared with controls.

After 10 days and 3 weeks of prednisolone treatment, absolute numbers of lymphocytes, T cells and CD4+ cells had risen significantly, while CD8+ cells as a percentage of lymphocytes had fallen significantly (Table 7.1).

For CD8+ cells these changes are also shown in Figure 7.1, continuing up to 2 year follow-up.

7.A.3.2 Severe PMR/GCA

Considering the severe PMR/GCA group separately (Table 7.2); this group had reduced lymphocyte and reduced CD8+ numbers, before treatment, compared with controls. However CD8+ cells as **percentage** of the lymphocytes were not reduced.

The subgroup of patients with biopsy-proven GCA was too small for separate statistical analysis (n=4), but their CD8+ counts were similar to the severe PMR subgroup (as absolute numbers and as a percentage of lymphocytes).

7.A.4 DISCUSSION

The results of this study do not confirm the conclusions of some previous studies that %CD8+ cells are reduced in GCA/PMR before treatment (Elling et al 1990, Dasgupta et al 1989, Chelazzi and Broggini 1984) but agree with the studies of Andersson et al (1988) and Banks et al (1983) in finding no abnormality prior to treatment. The significant fall in

%CD8+ cells after the commencement of prednisolone treatment suggests that the corticosteroid itself may have been responsible for this fall. This raises the possibility that the descriptions of lowered %CD8+ cells in untreated PMR/GCA may be due to the inadvertent inclusion of patients already on corticosteroid treatment. In practice it is often difficult to recruit PMR/GCA patients before treatment, as any delay in corticosteroid therapy may lead to irreversible complications such as blindness (Ross Russell 1959, Bengtsson and Malmvall 1982). In my study several patients seen rapidly for their first assessment and assumed to have received no corticosteroids, had actually received the initial dose of prednisolone from the general practitioner, and therefore were excluded from the study. This clinical history must be diligently sought. Multicentre studies may be particularly prone to include treated patients, in that it is more difficult at a distance to ensure that no treatment has been given before the initial blood sample.

Some differences in study results in PMR/GCA might be due to patient selection. In this study, raised ESR and CRP were not required. This might result in the cases being less florid than other series although, in fact, 34/36 patients did have either a raised ESR or CRP. I have addressed this problem by separately analysing the data from the most severe cases of PMR together with the cases of biopsy-proven GCA. Although absolute numbers of CD8+ cells were lowered in this group, this was part of a slight overall lymphopenia

and there was no selective depletion of CD8+ cells. This suggests that studies incorporating only the most severe cases of PMR/GCA may be more likely to demonstrate a lowered lymphocyte count and therefore a lowered absolute CD8+ count. However CD8+ cells as a **percentage** of lymphocytes do not seem to be influenced by disease severity. It is therefore unlikely that patient selection accounts for the difference between this study and other studies showing a reduced **percentage** of CD8+ cells.

The differing results from studies of CD8+ cells in PMR/GCA raise doubts about whether CD8+ cells are actually reduced in these patients. The design flaws mentioned could distort data and lead to an erroneous conclusion that CD8+ cells are reduced in PMR/GCA either as a percentage of lymphocytes or as absolute numbers. Published results may also be distorted in favour of an abnormality, in that negative studies showing no such abnormality are less likely to be published (Easterbrook et al 1991, Dickersin et al 1992, Rennie and Flanagin 1992).

The finding of lowered %CD8+ cells only after prednisolone treatment was commenced suggests that this may be an effect of the corticosteroid rather than the disease. To investigate this further a study of prednisolone administration in healthy volunteers was conducted and this is described in Section 7.B.

7.B. CIRCULATING LYMPHOCYTE SUBSETS IN HEALTHY VOLUNTEERS DURING PREDNISOLONE ADMINISTRATION

7.B.1 INTRODUCTION

The study described in section 7.A suggested that prednisolone treatment increases numbers of circulating lymphocytes, T cells and CD4+ cells, while decreasing the percentage of CD8+ cells and NK cells. These changes were evident by 10 days after the commencement of prednisolone. To distinguish the effects of the corticosteroid from the effects of the disease, it is necessary to know what happens to circulating lymphocyte subsets in healthy individuals taking corticosteroids.

Some of the short-term effects of corticosteroids on lymphocyte subsets have been documented in volunteers. Yu et al (1974) and Fauci (1976) showed lymphopenia maximal 4-6 hours after a single dose of corticosteroid, given to healthy volunteers. With the advent of monoclonal antibodies and flow cytometry, ten Berge et al (1984) documented reduced T cells, particularly OKT4+ cells, at 6 hours following a single dose of prednisolone in normal volunteers compared with controls. They also showed that by 24 hours this effect had disappeared with a slight "rebound effect", i.e. OKT4+ and OKT8+ cells were slightly increased by 24 hours, though this was not statistically significant. This study did not look at the effects of longer-term

administration of prednisolone.

Similarly Tonnesen (1987) infused cortisol into healthy volunteers for 5 hours and showed reduced lymphocytes, OKT3+, OKT4+ and OKT8+ cells by 2 hours compared with controls. These changes persisted 15 minutes after the cortisol infusion was discontinued, but were not monitored after that time.

Hogevold et al (1991) gave high dose methylprednisolone preoperatively and 4 hours and 12 hours after total hip replacement. After this short duration of corticosteroids, they showed reduced total T cells and helper and suppressor cells, compared with control operated patients, at 20 hours (=8 hours after the last dose of methylprednisolone).

The effects on T cells of longer term corticosteroid administration have not been documented in healthy humans, although changes occurring in patients treated with corticosteroids have been described. Ferrari et al (1991) reported increased lymphocytes, including increased absolute numbers of T cells, CD4+ and CD8+ cells after 4 weeks of corticosteroid treatment in ITP patients. The percentage of CD4+ and CD8+ cells was not significantly changed. In that study therefore the longer term effects of corticosteroid seem to be quite different from the acute effects a few hours after a single dose.

Fauci (1975) described the effects of 7 days cortisone administration in guinea pigs, as well as the acute effects

of a single dose of hydrocortisone. At both stages there was a fall in lymphocyte and T cell numbers. The observation of reduced lymphocytes and T cells 4 hours after the hydrocortisone corresponds to the acute effects seen in human volunteers (Yu et al 1974, Fauci 1976, ten Berge et al 1984, Tonnesen et al 1987). However, the 7 day effect in Fauci's guinea-pigs (Fauci 1975) are at odds with the effects in Ferrari's ITP patients, where after 4 weeks of corticosteroids lymphocytes and T cells had risen (Ferrari 1991). The difference between these studies might be a species effect, or the effects of corticosteroids in patients might be modified by the disease process itself. The effects of corticosteroids seen in my study of PMR/GCA patients (section 7.A) correspond to those in Ferrari's patients, suggesting that the guinea pig data cannot be extrapolated to humans.

The nature of the acute changes in the few hours after a single dose of corticosteroid has been well documented in the work described above (Yu et al 1974, Fauci 1976, ten Berge et al 1984, Tonnesen et al 1987, Hogevold et al 1991). However there is little information about the effects of longer term corticosteroid administration in humans, and in particular there is no published study of these longer term effects in the absence of disease. A study was therefore carried out on the effects of prednisolone administration in healthy volunteers.

7.B.2 SUBJECTS AND METHODS

7.B.2.1 Subjects and corticosteroid regime

Twelve healthy volunteers were recruited from senior medical staff. Eight were male and the ages ranged from 31 to 50 years. None had contraindications to corticosteroid administration and all gave informed consent.

A pilot study in one volunteer showed the acute post-dose effects on T cell subsets to be maximal around 7 hours after a dose of 20mg prednisolone enteric coated (EC). In the longer term, different effects were seen which were maximal after 3-4 days prednisolone administration. A 3-day period of corticosteroid treatment was therefore used in the whole volunteer group and blood tests were taken for 4 days.

Each volunteer had baseline blood samples taken at 9 am and then took prednisolone EC 20 mg daily orally at 9 am for 3 days. Further blood samples were taken at 7 hours after the initial dose and then at 24, 48 and 72 hours (i.e. 24 hours after the latest prednisolone dosage, to avoid the acute post-dose effects). In addition, 4 volunteers had blood samples at 55 hours, i.e. 7 hours after the third dose of prednisolone.

7.B.2.2 Technical methods

All blood samples were processed within 6 hours of venesection. Total white cell counts and lymphocyte counts were measured using routine methods in the haematology

department at Addenbrooke's Hospital. T cells and NK cells were analysed as described in section 7.A.2.2, with the exception of CD4+ and CD8+ cells which were defined by dual staining using anti-CD3 (Leu 4) together with Leu 3 or Leu 2. In addition, B cells were measured using anti-CD19.

7.B.2.3 Statistical methods

The paired data for 0 and 7 hours and for 0 and 72 hours were analysed using Wilcoxon's rank sum test. Spearman's rank correlation coefficient was used to examine the relationship between age and effect of prednisolone on %CD8+ cells.

7.B.3 RESULTS

The changes in lymphocyte subsets during prednisolone administration are shown in Table 7.3.

7.B.3.1 Absolute cell counts: early and late changes

The absolute numbers of lymphocytes, T cells, CD4+, CD8+ and B cells had all fallen significantly by 7 hours after the first dose of prednisolone, and by contrast had all risen significantly higher than baseline numbers at 72 hours (i.e. 24 hours after the third dose of prednisolone). This contrast is shown graphically for lymphocytes, T cells, CD4+ and CD8+ cells in Figure 7.2.

7.B.3.2 Percentage cell counts: early and late changes

As a percentage of total lymphocytes, CD4+ cells fell at 7 hours then rose significantly (Table 7.3). The percentage

of CD8+ cells did not change significantly at 7 hours but was significantly lowered at 72 hours. However, this change was small. An unexpected finding was the more pronounced fall in %CD8+ cells by 72 hours in the older compared with the younger volunteers. This is illustrated in Figure 7.3. Spearman's rank correlation coefficient was -0.599 (two-tailed $p < 0.05$). No correlation was seen, in this study, between age and the baseline percentage of CD8+ cells. No substantial difference in the T cell changes was observed between the male and female subjects but the number of females was too small for separate statistical analysis.

7.B.3.3 Combined acute and late effects, at 55 hours

Values of lymphocyte subsets at 55 hours (i.e. 7 hours after the 3rd dose of prednisolone) are shown in Table 7.3 although the data is drawn from only four subjects. These values are intermediate between the 7 hour and 72 hour values as might be expected.

7.B.4 DISCUSSION

7.B.4.1 The effects of corticosteroids on lymphocyte subsets

This study of the effects of prednisolone on circulating lymphocyte subsets describes new findings. It demonstrates that the effects of continuing prednisolone administration beyond 24 hours in healthy volunteers results in effects which contrast markedly with the changes described in the first few hours after a single dose of prednisolone. These

longer term effects are consistent with the changes described in section 7.A in patients treated with prednisolone for PMR/GCA. Ferrari et al (1991) showed a similar rise in absolute numbers of lymphocytes, T cells and CD4+ cells in ITP patients treated with corticosteroids for four weeks, but they did not find a significant difference in the percentage of CD4+ or CD8+ cells.

Seven days corticosteroid administration in guinea pigs seems to produce different effects from those seen in these human studies. Fauci (1975) found a fall in lymphocytes and T cells in guinea pigs which persisted from the acute stage to 7 days. Human and guinea pig lymphyocytes share a resistance to the cell lysis caused by corticosteroid treatment in some other species (Boumpas et al 1991), but it appears from this study that there are certain differences between human and guinea pig lymphocytes in their response to long-term corticosteroids.

The changes we have demonstrated 7 hours after the first dose of prednisolone have confirmed the findings in the controlled studies of ten Berge et al (1984) and Tonnesen et al (1987). These authors described decreased absolute numbers of lymphocytes, T cells, CD4+ and CD8+ cells a few hours after a single dose of corticosteroid. In addition, we have shown a short-term fall in the percentage of CD4+ cells.

Circadian variation of lymphocyte subsets has been

demonstrated (Ritchie et al 1983, Levi et al 1988). Ritchie et al described peak numbers of T cells, CD4+ and CD8+ cells at 10 pm with an inverse relationship to plasma cortisol levels. However Levi et al found the peak of T cells and CD4+ cells around 2 am, with no relationship to plasma cortisol levels, even allowing for possible delayed hormonal action. So the role of endogenous cortisol in the diurnal changes in T cell subsets is not clear. It is perhaps not surprising that such physiological effects, if present, may be less pronounced than the pharmacological effects of corticosteroids.

In Cushing's disease absolute numbers of T cells are greatly decreased (Shohat et al 1979). This does not necessarily conflict with the finding of raised T cells numbers in volunteers and in PMR/GCA patients during long-term prednisolone. In Cushing's disease the high circulating level of cortisol is sustained and may be equivalent to the acute post-dose effects we have seen, (i.e. a fall in T cells) but persisting. In contrast to the sustained cortisol levels in Cushing's disease, daily administration of prednisolone produces fluctuating blood levels, and the pre-dose samples used in our studies would contain low levels of corticosteroids. At this stage, the longer term effects of the corticosteroids would be evident, in the absence of the post-dose effects.

What are the possible mechanisms for the changes shown in this study? The acute reduction in circulating lymphocytes

and T cells after a dose of prednisolone is too rapid to be due to an effect on lymphocyte proliferation. The absence of fever and rigors, as seen when lymphocytes are depleted using monoclonal antibody treatments, suggests that cell lysis is not involved. The most likely explanation appears to be alteration in lymphocyte trafficking, with sequestration in the lymphoid organs. In guinea-pigs this has been shown to be due to redistribution to the bone marrow (Fauci 1975). In the longer term, the rise in circulating lymphocytes and T cells probably also reflects redistribution of the lymphocytes, this time in the reverse direction, however other mechanisms cannot be ruled out.

7.B.4.2 The interaction of age and corticosteroid effects

The results of this investigation suggest that the older subjects had a more pronounced fall in %CD8+ cells with prednisolone, and in fact the youngest subjects actually had a slight rise in %CD8+ cells. There was no age-dependent difference in baseline %CD8+ cells in our study, although a decrease has been described in subjects over 60 years old (Nagel et al 1981) and over 75 years (Ligthart et al 1985). The numbers in our study are small and the age range is narrow (31-50 years), but if it is only older subjects who respond to prednisolone with a decease in the percentage of CD8+ cells, this would account for the observation of a fall in %CD8+ cells in PMR/GCA patients on prednisolone treatment, whose age ranged from 51-87 years (median 70 years), while Ferrari et al (1991) found no significant

change in %CD8+ cells in ITP patients with prednisolone treatment, where the age range was 15-57 years (median 32 years).

One possible mechanism for a change in corticosteroid responsiveness with age might be changing numbers of corticosteroid receptors. Armanini et al (1992) have described reduced corticosteroid receptors on mononuclear leucocytes in aged subjects (62-97 years), but in vitro glucocorticoid sensitivity does not seem to be related to numbers of corticosteroid receptors (Smith KA et al 1977). Further studies are required in volunteers and patients over a wide range of ages to clarify the T cell effects of corticosteroids in different age groups.

7.B.4.3 The role of corticosteroids in the T cell changes described in PMR/GCA

The contrasting short-term and longer term effects of corticosteroids demonstrated here indicate the importance of controlling for corticosteroid effects in all studies of lymphocyte subsets. Those studies in which PMR/GCA patients were already receiving prednisolone treatment at the time of the initial analysis of T cell subtypes (Arnold et al 1993, Elling and Elling 1985, Benlahrache et al 1983) cannot give us useful information on CD8+ numbers in PMR/GCA itself. In those studies reporting reduced CD8+ cells in PMR/GCA before treatment (Elling et al 1990, Dasgupta et al 1989, Chelazzi and Broggini 1984), these results could also be misleading if patients were inadvertently included after starting

prednisolone. Blood tests taken a few hours after the initial dose of prednisolone would be expected to show lowered absolute numbers of CD8+ cells, while in later samples %CD8+ cells would be lowered.

During follow-up of treated PMR/GCA, the timing of blood samples relative to the daily dose of prednisolone will govern the results obtained. In the study of PMR/GCA described in section 7.A, the T cell subsets were measured **prior** to the daily dose of prednisolone and hence there was a trend for long-term elevation of absolute numbers of CD8+ cells and a fall in %CD8+ cells for some months (Figure 7.1). The volunteer study described here confirms these changes on prednisolone (Figure 7.2 and Table 7.3). Studies reporting prolonged lowering of %CD8+ cells (Elling and Elling 1985, Dasgupta et al 1989) or absolute numbers of CD8+ cells (Dasgupta et al 1989) in treated PMR/GCA may also be demonstrating the effects of prednisolone, rather than the disease, depending on the timing of blood sampling in relation to prednisolone dose.

In interpreting the results of lymphocyte subset analysis, in diseases treated with corticosteroids, it is vital to consider the role of the treatment itself. The design of future studies should control for the effects of corticosteroids as well as for circadian variation of lymphocyte subsets.

Table 7.1

Lymphocytes and subsets in controls and PMR/GCA patients before treatment and after 3 weeks prednisolone treatment. Results expressed as medians, in absolute numbers and as % of lymphocytes.

	Controls n=36	PMR/GCA before treatment n=36 (p for comparison with controls	PMR/GCA After 3 weeks treatment n=34 (p for comparison with pre-treatment)
Lymphocytes			
($x10^9$/l)	1.67	1.47 (NS)	2.19 (p=0.005)
Total T cells			
($x10^9$/l)	1.10	1.18 (NS)	1.59 (p=0.007)
(%)	68.5	71 (NS)	71.5 (NS)
CD4+ cells			
($x10^9$/l)	0.75	0.78 (NS)	1.05 (p=0.002)
(%)	45	48 (NS)	50.5 (NS)
CD8+ cells			
($x10^9$/l)	0.49	0.44 (NS)	0.49 (NS)
(%)	28	28 (NS)	25 (p=0.006)
CD4:CD8 ratio	1.59	1.74 (NS)	1.92 (p=0.006)
Activated T			
($x10^9$/l)	0.12	0.11 (NS)	0.13 (NS)
(%)	6.5	8 (NS)	6 (NS)
NK cells			
($x10^9$/l)	0.23	0.22 (NS)	0.17 (NS)
(%)	13	15 (NS)	8 (p=0.027)

TABLE 7.2

Lymphocytes and subsets in the severest PMR/GCA cases compared with controls and with less severe PMR/GCA. (Severe PMR/GCA = biopsy-proven GCA and/or clinically severe PMR). Results expressed as medians, in absolute numbers and as % of lymphocytes.

	Controls n=36	Severe PMR/GCA before treatment n=13 (p for comparison with controls)	Less Severe PMR/GCA before treatment n=23 (p for comparison with severe)
Lymphocytes			
($x10^9$/l)	1.67	1.42 (p=0.048)	1.72 (NS)
Total T cells			
($x10^9$/l)	1.10	1.05 (NS)	1.24 (NS)
(%)	68.5	71 (NS)	71 (NS)
CD4+ cells			
($x10^9$/l)	0.75	0.71 (NS)	0.80 (NS)
(%)	45	50 (NS)	47 (NS)
CD8+ cells			
($x10^9$/l)	0.49	0.36 (p=0.037)	0.48 (NS)
(%)	28	27 (NS)	30 (NS)
CD4:CD8 ratio	1.59	1.90 (NS)	1.58 (NS)
Activated T			
($x10^9$/l)	0.12	0.14 (NS)	0.11 (NS)
(%)	6.5	9 (NS)	8 (NS)

TABLE 7.3

The effects on circulating lymphocyte subsets of prednisolone EC 20mg o.d. for 3 days, in healthy volunteers (n=12). Results expressed as medians. P values given are for the two-tailed comparison with baseline data, (Wilcoxon's rank sum test), ** = $p<0.01$, * = $p<0.05$, NS = not significant).

	Baseline	7 hours	24 hours	48 hours	(55 hrs) (n=4)	72 hours
Lymphocytes						
($x10^9/l$)	2.09	1.02**	2.53	2.65	(2.35)	3.39**
Total T cells						
($x10^9/l$)	1.59	0.58**	1.84	2.00	(1.71)	2.54**
(%)	72.5	61 *	75.5	76	(70.5)	75 NS
CD4+ cells						
($x10^9/l$)	1.06	0.36**	1.30	1.42	(1.01)	1.61**
(%)	45.5	36 *	48	49.5	(43.5)	51.5*
CD8+ cells						
($x10^9/l$)	0.58	0.41**	0.67	0.59	(0.69)	0.83**
(%)	26.5	29 NS	25	24	(25.5)	24.5*
CD4:CD8 ratio	1.83	1.45 NS	2.00	2.10	(1.95)	2.10**
Activated T (n=9)						
($x10^9/l$)	0.11	0.07 NS	0.17	0.13	-	0.14 NS
(%)	4	6*	6	7	-	6 NS
NK Cells						
($x10^9/l$)	0.32	0.35 NS	0.33	0.23	(0.45)	0.34 NS
(%)	16.5	20*	12.5	9.5	(16)	9.5**
B Cells						
($x10^9/l$)	0.23	0.15*	0.27	0.34	(0.32)	0.53**
(%)	10.5	13*	12.5	13	(12.5)	14.5**

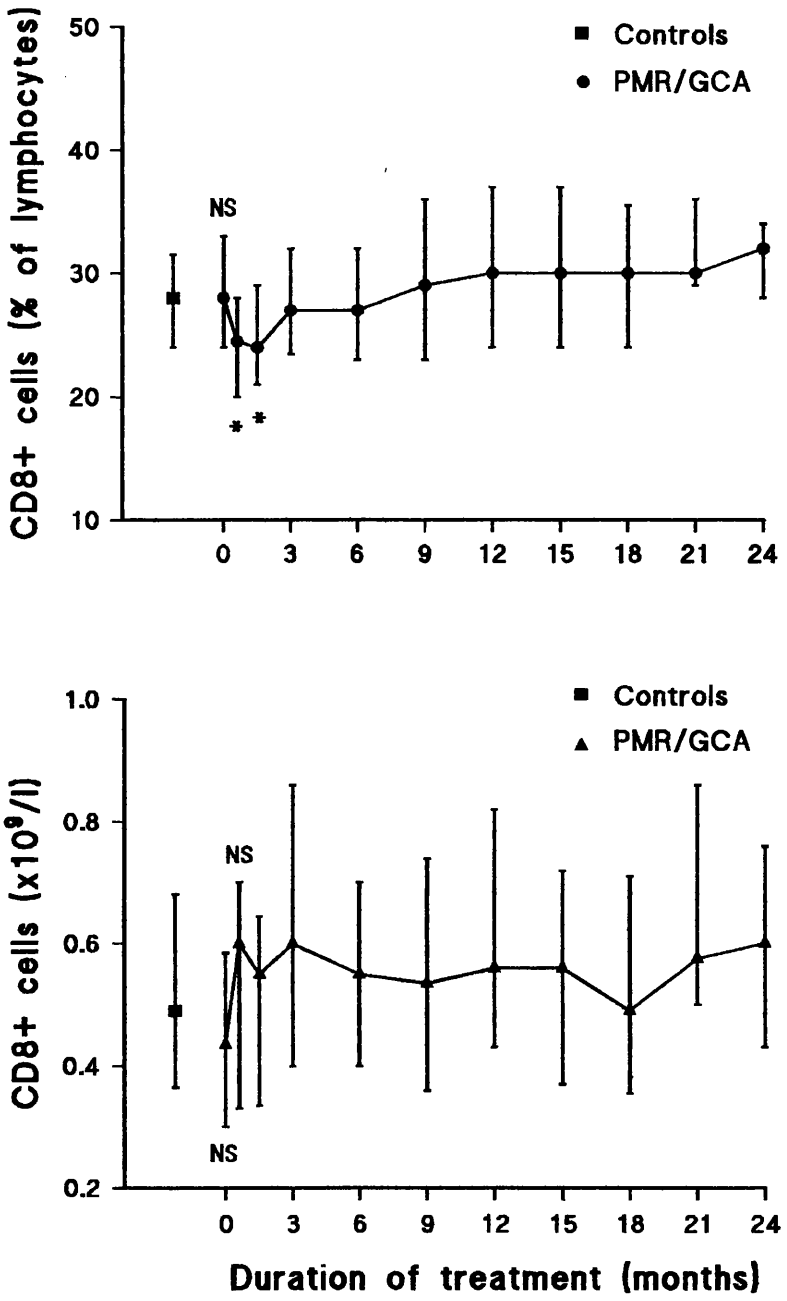

Figure 7.1 CD8+ cells in controls and PMR/GCA patients before and during prednisolone treatment, after 10 days, 6 weeks, 3 months and 3-monthly thereafter. (3 week data points omitted for clarity: for 3 week data see Table 7.1) Venesection was performed before the daily prednisolone dose. Results expressed as % of lymphocytes (top) and as absolute numbers (bottom), medians + interquartile range. * = $p<0.05$, NS = not significant, for the comparison with controls.

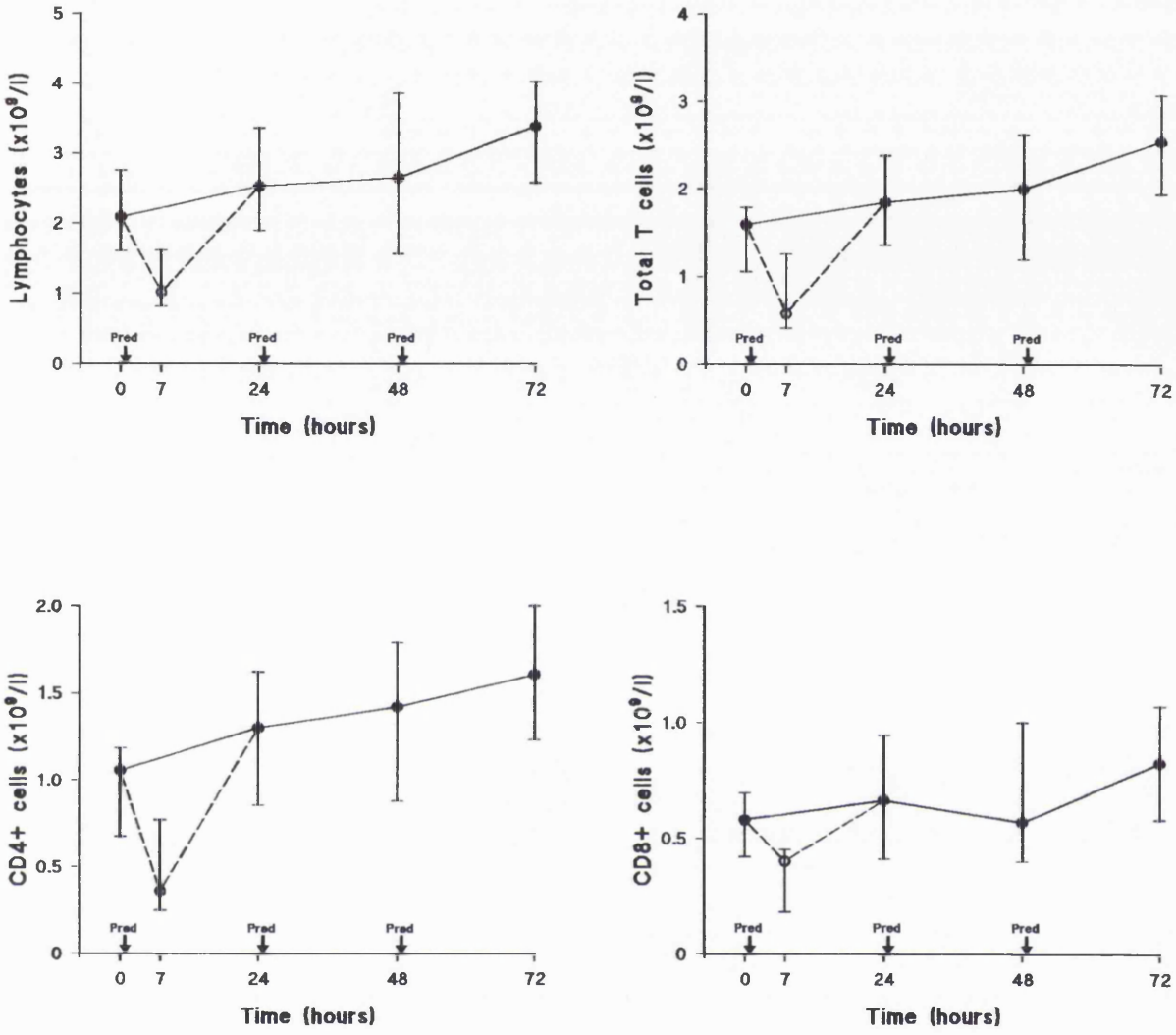

Figure 7.2 Absolute numbers of circulating lymphocytes and subsets in 12 healthy volunteers in relation to doses of prednisolone EC 20mg (↓). Results expressed as medians + interquartile range.

- ● - in blood samples taken immediately prior to the next prednsilone dose and 24 hours after the previous dose.
- o - in blood samples taken 7 hours after the initial dose of prednisolone.

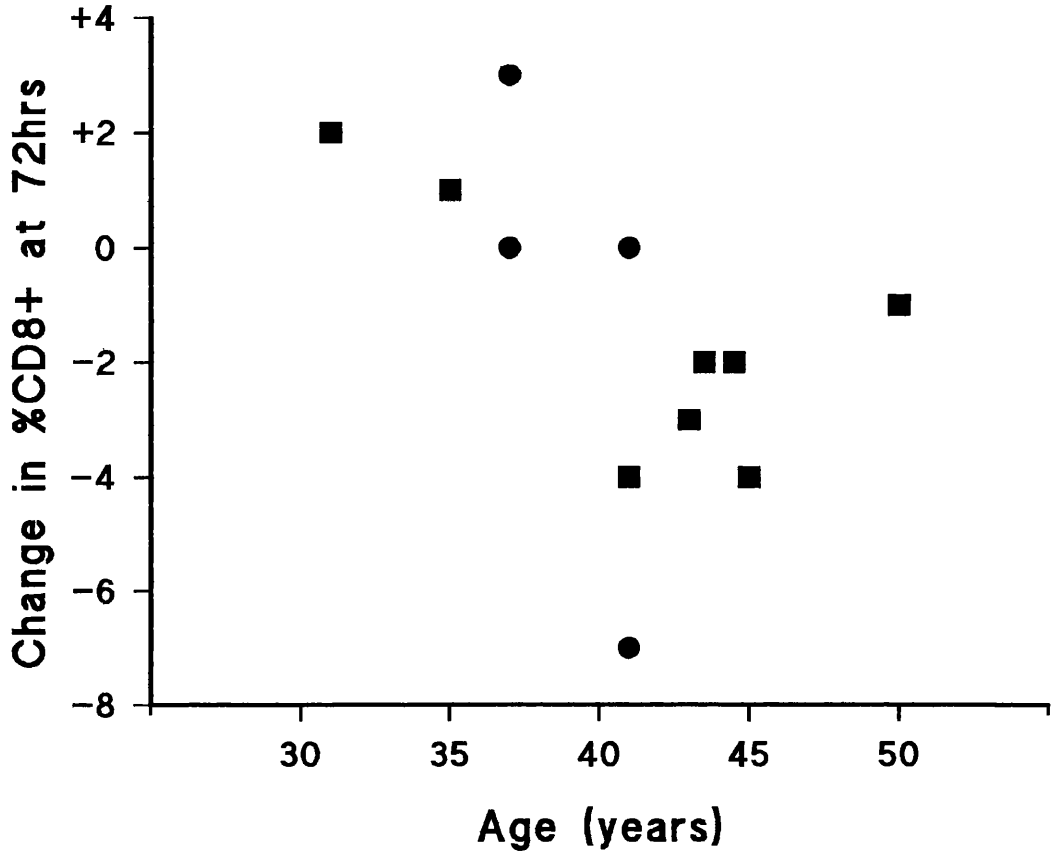

Figure 7.3 Change in %CD8+ cells after 72 hours prednisolone administration in 12 healthy volunteers, according to age. ■ = males, ● = females. Change in % CD8+ cells is expressed as a percentage of lymphocytes. Spearman's rank correlation coefficient = −0.599 (two−tailed $p < 0.05$).

CHAPTER 8

USEFUL INVESTIGATIONS IN THE MANAGEMENT OF PATIENTS WITH PMR/GCA

8.1 USEFUL INVESTIGATIONS IN DIAGNOSING PMR/GCA

The traditional investigation, ESR, remains a useful investigation in confirming a clinical diagnosis of PMR or GCA. However this study supports the finding of others that a normal ESR may be found in PMR/GCA. This has been shown even in the presence of a positive biopsy for GCA.

This study has demonstrated that CRP is usually raised in PMR/GCA at presentation, but is also raised (≥6mg/l) in a quarter of elderly controls. A normal CRP has not yet been reported in biopsy-proven GCA, but this test has only recently been widely available. It does seem from this study that clinical PMR/GCA may present with a normal CRP.

ACT was raised (>0.64g/l) in over 90% of PMR/GCA patients at presentation in this study, but some 40% of elderly controls also had ACT concentrations above this level. Therefore a raised ACT result would not necessarily confirm a diagnosis of PMR/GCA, but a **normal** ACT result would tend to weigh against a diagnosis of PMR/GCA.

Only one out of 42 patients had ESR, CRP and ACT all normal at presentation. Obviously, the more investigations that

are carried out, the more likely it is that one of these will be abnormal, so a combination of investigations has limitations in confirming the diagnosis of PMR/GCA. However when used in the opposite way, i.e. to question the diagnosis, then a combination of normal ESR, CRP and ACT would make the diagnosis of PMR/GCA unlikely.

Although plasma IL1ß and serum sIL2R were both significantly raised in PMR/GCA patients before treatment, there was considerable overlap with control subjects so these investigations would not be useful in the diagnosis of the individual patient. In the case of IL1ß measurements there is the additional limitation that the levels in PMR/GCA are often below the reliable limit of sensitivity of the assay. Even with newer ELISAs for IL1ß this would continue to be a problem, with lower limits of sensitivity only as low as 1.7pg/ml (CLB, Amsterdam).

Serum IL6 levels measured by ELISA were not helpful in diagnosis. A non-significant trend for IL6 to be elevated at presentation suggests this investigation is limited by the lack of sensitivity of ELISAs. Bioassays on the other hand are limited by their labour-intensiveness. In fact the production of CRP, which correlated with IL6 levels in this study, was amplified compared with IL6 levels and hence CRP was a more useful investigation.

This study did not confirm the finding of some groups that CD8+ cells were reduced in PMR/GCA. Whatever the reasons

for the difference in results between studies, the variation suggests that CD8+ cell enumeration is not likely to be a clinically useful test in the diagnosis of PMR/GCA.

8.2 MARKERS OF DISEASE ACTIVITY IN PMR/GCA

8.2.1 The nature of "disease activity"

In considering "disease activity" in PMR/GCA there seem to be two separate aspects. Firstly there is the overt clinical activity due to inadequate suppression with corticosteroid treatment. Although this type of disease activity may be obvious, at other times the non-specific nature of some of the symptoms of PMR/GCA may make it difficult to diagnose such relapses. Hence any investigations which were markers of this type of disease activity would be useful in the management of patients.

Secondly there is the "underlying" disease activity beneath currently adequate corticosteroid suppression of overt disease activity. In general in PMR/GCA, this underlying disease activity will gradually decrease over 2 to 3 years until no corticosteroid suppression is required.

Of course, overt clinical activity also indicates continuing underlying disease activity, but a lack of overt clinical activity may mean two possible things: either that the current corticosteroid dose is adequate to suppress overt clinical activity, or that there is no underlying disease activity remaining. Hence, any investigations helping to

identify underlying disease activity in PMR/GCA would be useful in guiding the reduction in corticosteroid dose. The aim would be to avoid giving unnecessarily high doses of prednisolone, to minimise side-effects, while ensuring adequate suppression of underlying disease activity, so that clinical relapses do not occur with their troublesome symptoms and risk of visual loss.

8.2.2 Confirming relapses of PMR/GCA

This study has shown that ESR is not usually helpful in confirming a clinical impression of relapse. In addition to demonstrating a normal ESR (<30mm/h) in 88% of relapses of PMR/GCA, I have documented one case of relapse in biopsy-proven active GCA where ESR was only 18mm/h at the time of sight-threatening arteritis. This illustrates the importance of not relying on a raised ESR to confirm a clinical impression of relapse in PMR/GCA, although unfortunately many doctors still do.

CRP is barely more helpful than ESR in confirming relapses. Half of the patients in the study had CRP ≥6mg/l during relapses of PMR/GCA, but almost a quarter of elderly controls in this study also had a CRP of this level.

Plasma IL1ß levels may be useful in the future for confirming clinical relapses of PMR/GCA. Overall, IL1ß concentrations in this study were raised at relapse but only at low levels relative to the sensitivity of the assay. If assay sensitivities can be substantially improved, this

investigation may become useful in monitoring PMR/GCA patients, but at present this is not a useful test. IL6 and sIL2R levels were also not helpful in confirming relapses of PMR/GCA, with no clear rise in levels, while sIL2R also has the complicating factor of reduced levels due to prednisolone itself.

8.2.3 Assessing underlying disease activity of PMR/GCA during corticosteroid treatment

This study has provided the first evidence that ACT may be useful in assessing underlying disease activity of PMR/GCA during prednisolone treatment. The concept of indicators of underlying disease activity in PMR/GCA has been addressed in other studies. Monteagudo et al (1994) have shown the presence of antibodies to intermediate filaments (AIF) in 72% of PMR/GCA patients before treatment, followed by a gradual reduction in the prevalence of these antibodies as the disease "burnt itself out". This group suggested that this investigation might be a convenient way of determining the underlying activity of PMR. Rather than being a quantitative indicator of underlying disease activity, the **disappearance** of circulating AIF would be an indicator of "burnt out" disease. This could be useful, albeit at a late stage, in accelerating the reduction of the corticosteroid dosage in such "burnt out" PMR/GCA. In another study, Dasgupta et al (1989) suggested that there is a persisting "underlying immunoregulatory deficit" in PMR/GCA for over 1 year, as indicated by persistently low CD8+ cell counts. However I have not found a persistence of low **absolute**

counts of CD8+ cells, and the low percentage of CD8+ cells I have found appears to be due to the prednisolone treatment itself, as confirmed in volunteers. Hence my study does not support the use of CD8+ enumeration as an indicator of underlying disease activity in PMR/GCA.

If ACT does indicate persisting underlying disease activity in PMR/GCA, then it is likely to be a very useful investigation in the management of this disease. My work has suggested that after 12 months treatment of PMR/GCA, the ACT level is helpful in distinguishing those patients who are more likely to relapse at a later date. I am now in the process of verifying this data in a new group of patients, after which I propose to test whether the ACT level at 12-18 months can be used to tailor the prednisolone reduction to suit the patient.

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Circulating T cell subtypes in polymyalgia rheumatica and giant cell arteritis: variation in the percentage of CD8+ cells with prednisolone treatment

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Abstract

***Objectives*—Some reports have described a decreased percentage of circulating CD8+ cells in patients with polymyalgia rheumatica and giant cell arteritis (PMR/GCA) before treatment and persisting for some months during treatment with corticosteroids. Other studies have found no such changes. There are overt methodological variations between these studies and there may also be hidden differences, such as the timing of blood samples. The purpose of this study was to investigate T cell subtypes in patients with PMR/GCA while controlling for variables known to affect T cells.**

***Methods*—Circulating T cell subsets were measured in 36 patients with PMR/GCA before and during treatment with prednisolone. Blood samples during treatment were taken before the daily dose of prednisolone. The whole blood lysis method was used followed by flow cytometry.**

***Results*—Compared with controls, CD8+ cells were not reduced before treatment in patients with PMR/GCA ($0{\cdot}44\times10^9$/l; 28% of lymphocytes). CD4+ cells were also normal ($0{\cdot}78\times10^9$/l; 48% of lymphocytes). During treatment with prednisolone total T cells increased from 1·18 to $1{\cdot}59\times10^9$/l and CD4+ cells increased from 0·78 to $1{\cdot}05\times10^9$/l. The percentage of CD8+ cells decreased on treatment from 28 to 25%.**

***Conclusions*—This study does not confirm the finding of some groups that the percentage of circulating CD8+ cells is reduced in patients with PMR/GCA before treatment. It does show that the percentage of CD8+ cells decreases during treatment with corticosteroids. This needs to be considered when designing studies of lymphocyte subsets in diseases treated with corticosteroids.**

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There have been several studies of circulating T cell subtypes in patients with polymyalgia rheumatica and giant cell arteritis (PMR/GCA) with differing results. In GCA before treatment, Andersson *et al*[1] found normal numbers of CD8+ and CD4+ cells, and Banks *et al*[2] documented normal ratios of helper to suppressor cells. Other studies in patients with PMR and GCA have found a decreased percentage of CD8+ cells,[3–7] although Dasgupta *et al*[4] and Elling *et al*[3] also reported reduced absolute numbers of CD8+ cells.

All of the studies apart from the negative study by Andersson *et al*[1] used mononuclear cells separated on a Ficoll-Hypaque density gradient. This method selectively decreases the CD8+ subset, leading to a significantly lower percentage of CD8+ cells than the whole blood lysis method.[8] This artefact would not necessarily affect samples from controls and patients to the same extent, so could distort the results. Patient CD8+ cells might have intrinsic differences from control cells affecting their migration on a density gradient and might well also have differences due to a delay in processing compared with control cells. A marked decrease in the percentage of CD8+ cells and CD4+ cells has been shown in blood stored for 24 hours when the Ficoll-Hypaque method is used, but not with the whole blood lysis method.[9] Furthermore, a delay of more than six hours before processing blood samples results in a considerable decrease in the absolute number of lymphocytes counted by automated haematology counters.[10] Such a delay might occur more often with patient samples than control samples, particularly in multicentre studies where patient blood samples may be transported from other hospitals for analysis.

The diurnal variation in lymphocyte numbers produces a nadir in the number of lymphocytes, T cells, CD4+, and CD8+ cells around 1000[11] to 1230 hours.[12] Obviously, if patient and control samples are not taken at the same time of day, this would confound results.

There is evidence of a decrease in CD8+ cells with age, both in absolute number and as a percentage of lymphocytes,[13 14] so controls should be matched for age, which is not addressed in some of the previous studies.[1 2]

In some of the previous studies the patients with PMR/GCA were already receiving prednisolone at the time of T cell subset analysis.[6 7] We have seen changes in T cell subtypes occurring with prednisolone in normal volunteers.[14a] Blood samples taken seven hours after a single 20 mg dose of prednisolone have a lowered absolute number of CD8+ cells, whereas the effect of longer term treatment with prednisolone is to increase the absolute numbers of CD8+ cells. These effects parallel the changes in total lymphocyte numbers. CD8+ cells as a

percentage of lymphocytes do not change significantly in the first few hours, but with longer term prednisolone treatment this percentage decreases significantly. Hence corticosteroid treatment alters the T cell subtypes depending on the timing of the sample.

To clarify the conflicting data from previous work, this study was designed to eliminate these variables.

Patients and methods

Thirty six patients with PMR/GCA were assessed before, during, and after treatment with prednisolone. Ages ranged from 51 to 87 years (median 70 years). Diagnosis was made according to the clinical criteria of Jones and Hazleman.[15] Increased erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) were not used as inclusion criteria, as these and other laboratory variables were to be studied, but in fact 34 of 36 patients did have an ESR above 30 mm/h or a CRP above 6 mg/l, or both. Serum creatine kinase and protein electrophoresis were normal in all patients with PMR.

These patients had blood samples taken between 1000 and 1400 hours, before treatment, and subsequently after 10 days, three weeks, six weeks, and three months of treatment, and thereafter every three months until treatment could be discontinued. Venesection was always carried out at the same time of day and before the daily prednisolone dose. Treatment for PMR was begun with prednisolone 10–20 mg daily, or for GCA with prednisolone 40 mg daily. Prednisolone was prescribed as the enteric coated preparation and as a once daily dose in the morning. Subsequently the dose was reduced according to clinical disease activity.

Controls were matched for age and sex and consisted of healthy volunteers and patients with osteoarthritis. Ethical committee approval was obtained to approach the controls for blood tests. Control samples were collected between 1000 and 1400 hours.

All patients and controls were seen at Addenbrooke's Hospital and all blood samples were processed within six hours of venesection. The total white blood cell count and lymphocyte count were measured using routine techniques in the haematology department at Addenbrooke's Hospital. T cell subtypes were analysed by flow cytometry using a whole blood lysis technique. Total T cells were measured using antibodies to CD3 (Leu 4). Activated T cells were those CD3+ cells which coexpressed antibodies to HLA-DR. CD4+ cells were defined using Leu 3 and CD8+ cells using Leu 2. Natural killer cells were CD3– expressing CD 16/56 (Leu 11c+19). All monoclonal antibodies were purchased from Becton Dickinson (Oxford, United Kingdom) from the Simultest range. Aliquots of blood were incubated with antibody pairs for dual staining for 15 minutes at room temperature. Erythrocytes were lysed using FACS lysing solution (Becton Dickinson) and leucocytes were fixed with 0·5% formaldehyde. Cells were analysed on the day of processing using a Becton Dickinson FACScan and Simulset software. This software analyses the lymphocyte gate and corrects the subsequent analysis for non-lymphocyte events.

Pretreatment values for lymphocyte subsets in the 36 patients with PMR/GCA were compared with controls. In addition, to see if patient selection was a critical factor, the patients with biopsy-proved GCA or the most severe symptoms of PMR, or both, were considered separately. This group of 13 was compared with controls and with the remaining group of 23 patients with less severe GCA/PMR.

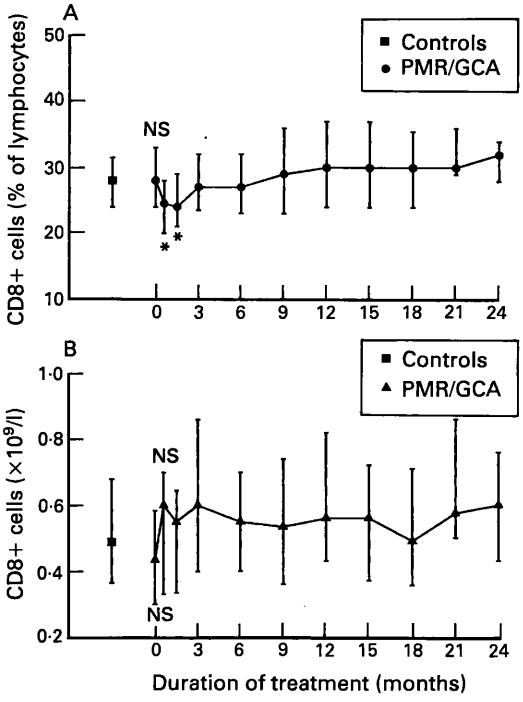
Statistical analysis consisted of the Mann-Whitney test to compare groups and paired analysis by the Wilcoxon test to examine within patient changes in T cell subsets.

Results

In all 36 patients with PMR/GCA before prednisolone treatment, lymphocytes, T cells, and T cell subtypes were all normal compared with controls (table 1).

After 10 days and three weeks of prednisolone treatment the absolute numbers of lymphocytes, T cells, and CD4+ cells had increased significantly, whereas CD8+ cells as a percentage of lymphocytes had decreased significantly (table 1).

The two year follow up of CD8+ cells is shown in the figure.



*CD8+ T cells in controls and patients with polymyalgia rheumatic and giant cell arteritis (PMR/GCA) before and during treatment with prednisolone, after 10 days, six weeks, three months, and every three months thereafter (three week data points omitted for clarity; for three week data see table 1). During treatment venesection was carried out before the daily dose of prednisolone. Results expressed as percentage of lymphocytes (A) and as absolute numbers (B), medians plus interquartile range. * $p<0·05$ and NS=not significant compared with controls.*

Table 1 Lymphocytes and lymphocyte subtypes in controls and patients with polymyalgia rheumatica and giant cell arteritis (PMR/GCA) before treatment and after three weeks of treatment with prednisolone. Results expressed as medians, in absolute numbers, and as a percentage of lymphocytes

Parameter measured	*Controls (n=36)*	*Patients with PMR/GCA before treatment (n=36)**	*Patients with PMR/GCA after three weeks of treatment (n=34)†*
Lymphocytes ($\times 10^9$/l)	1·67	1·47 (NS)	2·19 (p=0·005)
Total T cells			
($\times 10^9$/l)	1·10	1·18 (NS)	1·59 (p=0·007)
(%)	68·5	71 (NS)	71·5 (NS)
CD4+ cells			
($\times 10^9$/l)	0·75	0·78 (NS)	1·05 (p=0·002)
(%)	45	48 (NS)	50·5 (NS)
CD8+ cells			
($\times 10^9$/l)	0·49	0·44 (NS)	0·49 (NS)
(%)	28	28 (NS)	25 (p=0·006)
CD4+:CD8+ ratio	1·59	1·74 (NS)	1·92 (p=0·006)
Activated T cells			
($\times 10^9$/l)	0·12	0·11 (NS)	0·13 (NS)
(%)	6·5	8 (NS)	6 (NS)
NK cells			
($\times 10^9$/l)	0·23	0·22 (NS)	0·17 (NS)
(%)	13	15 (NS)	8 (p=0·027)

*Values in parentheses are p values compared with controls.
†Values in parentheses are p values compared with pretreatment results.

Table 2 Lymphocytes and lymphocyte subtypes in the patients with most severe polymyalgia rheumatica and giant cell arteritis (PMR/GCA) compared with controls and with patients with less severe PMR/GCA. Results expressed as medians, in absolute numbers, and as a percentage of lymphocytes

Parameter measured	*Controls (n=36)*	*Patients with severe PMR/GCA before treatment (biopsy proved GCA or severe PMR, or both) (n=13)**	*Patients with less severe PMR/GCA before treatment (n=23)†*
Lymphocytes ($\times 10^9$/l)	1·67	1·42 (p=0·048)	1·72 (NS)
Total T cells			
($\times 10^9$/l)	1·10	1·05 (NS)	1·24 (NS)
(%)	68·5	71 (NS)	71 (NS)
CD4+ cells			
($\times 10^9$/l)	0·75	0·71 (NS)	0·80 (NS)
(%)	45	50 (NS)	47 (NS)
CD8+ cells			
($\times 10^9$/l)	0·49	0·36 (p=0·037)	0·48 (NS)
(%)	28	27 (NS)	30 (NS)
CD4+:CD8+ ratio	1·59	1·90 (NS)	1·58 (NS)
Activated T cells			
($\times 10^9$/l)	0·12	0·14 (NS)	0·11 (NS)
(%)	6·5	9 (NS)	8 (NS)

*Values in parentheses are p values compared with controls.
†Values in parentheses are p values compared with patients with severe PMR/GCA.

We considered the group with severe PMR/GCA separately (table 2): this group had reduced lymphocyte and reduced CD8+ numbers before treatment compared with controls. CD8+ cells as a percentage of the lymphocytes were not reduced, however.

The subgroup of three patients with biopsy-proved GCA was too small for separate statistical analysis but their CD8+ counts were similar to the subgroup with severe PMR (as absolute numbers and as a percentage of lymphocytes).

Discussion

The results of this study do not confirm the conclusions of some previous studies that CD8+ cells are reduced in patients with GCA/PMR before treatment[3–5] but agree with the studies of Andersson *et al*[1] and Banks *et al*[2] in finding no abnormality before treatment. Our previous finding that the percentage of CD8+ cells decreases significantly on treatment with prednisolone illustrates the importance of ensuring that initial samples are obtained before treatment is begun. In practice, this is often difficult, as any delay in the treatment of PMR/GCA may lead to irreversible complications such as blindness.[16] Hence recruiting untreated patients is not easy. We found that several patients seen rapidly for their first assessment and assumed to have received no corticosteroids had actually received the initial dose of prednisolone from their family doctor, and therefore were excluded from our study. This clinical history must be diligently sought and the magnitude of the effect of a single dose of corticosteroids appreciated.

Our work in normal volunteers[14a] has shown that the effects of prednisolone on lymphocyte subsets occur in two phases. The initial effects (maximum at about seven hours after a 20 mg dose of enteric coated prednisolone) include a pronounced decrease in the absolute numbers of lymphocytes, total T cells, CD4+, and CD8+ cells. The effects of longer term prednisolone treatment are almost the opposite of the early effects—that is, at 72 hours the absolute numbers of total T cells, CD4+, and CD8+ cells are markedly increased, with a decrease in the percentage of CD8+ cells. These later effects begin to develop by 24 hours after the first dose of prednisolone. Before this study only acute lymphopenia (particularly of CD4+ cells) had been shown after a single dose of prednisolone in volunteers.[17 18] The same effect has been assumed by some to occur in longer term treatment with corticosteroids, whereas our study in normal volunteers shows that the later changes contrast markedly with the acute effects. Those studies in PMR/GCA where patients were already receiving prednisolone at the time of the initial analysis of T cell subtypes[6 7] cannot give us useful information about CD8+ numbers in PMR/GCA per se. In those studies reporting a reduced percentage of CD8+ cells in PMR/GCA before treatment,[3–5] the inadvertent inclusion of patients after even a single dose of prednisolone might render these results similarly misleading. Multicentre studies may be particularly prone to this factor, in that it is more difficult at a distance to ensure that no treatment has been given before the initial blood sample.

During follow up of treated PMR/GCA, T cell subtypes will again be critically altered by the interval since the previous dose of prednisolone. In this study T cells during treatment were measured before the daily dose of prednisolone, and this is reflected in the long term increase of CD8+ numbers seen in the figure, though this did not reach statistical significance. Other studies have not referred to the timing of the blood samples. If blood had been taken a few hours after a prednisolone dose, then the absolute numbers of CD8+ cells would be lowered, and if blood had been taken about 24 hours after a dose of prednisolone then CD8+ cells as a percentage of lymphocytes would be lowered.[14a] Hence studies reporting prolonged lowering of CD8+ cells in PMR/GCA may be showing the effects of prednisolone rather than the effects of PMR/GCA per se.[4 6]

Some differences in the results of studies in patients with PMR/GCA might be due to patient selection. In our study, an increased ESR and CRP were not required (as these tests were also being investigated). This might have resulted in our patients being less florid than other series, though, in fact, 34 of 36 patients did have either an increased ESR or CRP. We have addressed this problem by separately analysing the data from the most severe cases of PMR together with the cases of GCA proved by biopsy. Although absolute numbers of CD8+ cells were lowered in this group, this was part of a slight overall lymphopenia and there was no selective depletion of CD8+ cells. This suggests that studies incorporating only the most severe cases of PMR/GCA may be more likely to show a lowered lymphocyte count and therefore a lowered absolute CD8+ count. CD8+ cells as a percentage of lymphocytes, however, do not seem to be influenced by disease severity. It is therefore unlikely that patient selection accounts for the difference between this study and other studies showing a reduced percentage of CD8+ cells.

The differing results from studies of CD8+ cells in patients with PMR/GCA raises doubts about whether CD8+ cells are actually reduced in these patients. We have illustrated design flaws which could distort data and lead to an erroneous conclusion that CD8+ cells are reduced in PMR/GCA. Published results may also be distorted in favour of an abnormality of CD8+ cells, in that negative studies showing no such abnormality are less likely to be published.[19–21] We therefore conclude that the case that CD8+ cells are lowered in patients with PMR/GCA is not proved.

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α1-ANTICHYMOTRYPSIN, C-REACTIVE PROTEIN AND ERYTHROCYTE SEDIMENTATION RATE IN POLYMYALGIA RHEUMATICA AND GIANT CELL ARTERITIS

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SUMMARY

Forty-four patients with polymyalgia rheumatica and/or giant cell arteritis (PMR/GCA) were followed from presentation, through remissions and relapses for a median duration of 36 months. Clinical disease activity, ESR, CRP and α1-antichymotrypsin (α1-ACT) were measured.

Before treatment ESR, CRP and α1-ACT were all significantly raised, compared with age- and sex-matched controls.

On clinical remission with prednisolone treatment, ESR and CRP fell to control levels but α1-ACT behaved quite differently, remaining raised for 18 months or until prednisolone treatment could be withdrawn. At 18 month follow-up of PMR/GCA, an α1-ACT level of ≤0.7 g/l was associated with a reduced risk of subsequent relapse ($P = 0.006$).

At clinical relapse during treatment, ESR was not raised compared with controls, and CRP, although significantly higher than controls ($P = 0.015$), remained less than 6 mg/l in the majority of patients.

The three laboratory investigations were, therefore, of limited value in confirming relapses of PMR/GCA during prednisolone treatment, but α1-ACT may be useful as an indicator of underlying disease activity and hence as a guide to the speed that the prednisolone dosage should be reduced.

KEY WORDS: α1-Antichymotrypsin, Erythrocyte sedimentation rate, C-Reactive protein, Polymyalgia rheumatica, Giant cell arteritis.

AT presentation of polymyalgia rheumatica and/or giant cell arteritis (PMR/GCA), ESR and CRP are usually raised [1–6], although this is not always the case [7–12]. Other acute phase proteins have also been shown to be elevated at presentation of PMR/GCA, namely orosomucoid (= α1-acid glycoprotein) and haptoglobin [13, 14], serum amyloid A protein (SAA), fibrinogen and α1-antichymotrypsin (α1-ACT) [14]. The value of haptoglobin and fibrinogen in the acute phase response is limited as they may be reduced by consumption [15, 16]. SAA is so sensitive that even the common cold causes a rise in the serum concentration [16, 17], which limits its value in confirming more significant inflammation. Orosomucoid is said to be elevated by corticosteroid therapy [18] and therefore may not be suitable for monitoring PMR/GCA once treatment has begun. α1-ACT does not have these disadvantages. Like CRP it begins to rise by 6–8 h after an inflammatory stimulus and reaches a peak at 2–3 days [18]. α1-ACT has a longer half-life than CRP and its biosynthesis may also continue longer after the inflammatory stimulus. Hence, in chronic diseases, a combination of CRP and α1-ACT may give complementary information [19, 20]. Both these tests are easily performed [21], and it has been recommended that they be available in all teaching hospitals [16].

Microheterogeneity of α1-ACT may be useful in diagnosing PMR/GCA, in that crossed immunoaffinity electrophoresis shows a relative decrease in ConA reactive fractions of α1-ACT in active PMR/GCA [22, 23]. However at present this test is not routinely available.

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During clinical relapses of PMR/GCA on treatment, ESR and CRP often remain normal [5, 7, 24, 25]. α1-ACT has been described as having no correlation with disease activity at various stages of PMR/GCA [14], and has also been found to remain raised after disease suppression in both PMR/GCA [14] and RA [20].

We have studied ESR, CRP and α1-ACT levels in PMR/GCA, particularly during remission and relapses on treatment, to compare the roles these investigations might have in the management of PMR/GCA.

SUBJECTS AND METHODS

Forty-four patients with PMR/GCA were followed from presentation through remissions and relapses, for a period of 6 to 54 months (median 36 months). Their ages ranged from 51 to 87 yr (median 71 yr) and 31 were female. Diagnosis was made according to the criteria of Jones and Hazleman [2] excluding the requirement for a raised ESR or CRP. Twenty-six patients had PMR alone, seven had GCA alone, and 11 had both at some stage. Treatment was with prednisolone at an initial daily dose of 10–20 mg for PMR and 40 mg for GCA. This dose was maintained for 1 month and then reduced as described in standard guidelines [26], modified according to clinical progress.

Blood samples for ESR and sera for storage were taken before treatment and during routine follow-up. In addition, 23 of the patients had a total of 49 symptomatic relapses documented, requiring an increase in prednisolone dosage. Blood samples were taken during and after these relapses, but laboratory results were not known at the time of diagnosing a relapse.

Forty-four age- and sex-matched controls were

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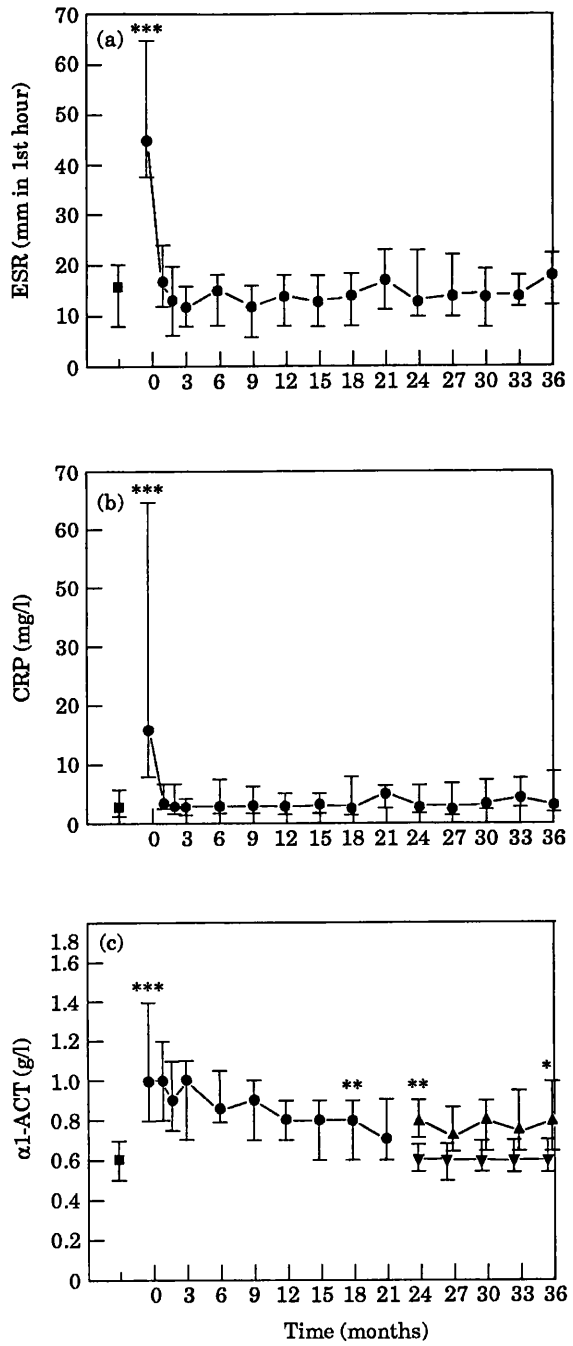

FIG. 1.—Three-year follow-up of (a) ESR, (b) CRP, and (c) α1-antichymotrypsin (α1-ACT), in polymyalgia rheumatica/giant cell arteritis (PMR/GCA). Results expressed as medians + interquartile ranges. From 24 months, α1-ACT results have been divided into those from patients still requiring prednisolone treatment and patients successfully off treatment. ***$P<0.001$, **$P<0.01$, *$P<0.05$, for the comparison with controls, Mann–Whitney test. ■, Controls; ●, PMR/GCA; ▲, PMR/GCA on treatment; ▼, PMR/GCA off treatment.

drawn from healthy volunteers, attenders at a geriatric day hospital and outpatients with OA. In addition, 12 younger volunteers (healthy senior medical staff) took prednisolone 20 mg daily for 3 days, and sera were stored for α1-ACT measurement. In a pilot study, one of these healthy volunteers took prednisolone for 9 days and sera were stored.

ESR was measured by the Westergren method. CRP was measured on stored sera, using a Beckman rate nephelometer and Beckman reagents. Results were reported to the nearest 1 mg/l. α1-ACT was measured on stored sera available from 42 of the patients as well as 42 controls and the 12 healthy volunteers taking prednisolone. The analysis for α1-ACT was carried out in the Department of Clinical Biochemistry at Addenbrooke's Hospital by immunoturbidimetry. This assay is routinely available here. Results in the PMR/GCA patients and their matched controls were reported only to the nearest 0.1 g/l, while in the healthy volunteers taking prednisolone, results were available to the nearest 0.01 g/l.

RESULTS

ESR, CRP and α1-ACT were all significantly raised prior to treatment (Fig. 1). Of the 44 patients, six had ESR <30 mm/h before treatment, nine patients had CRP <6 mg/l and three had α1-ACT <0.65 g/l. Using a combination of ESR and CRP, four patients had both results normal before treatment, but addition of α1-ACT left only one patient in whom all three results were normal.

ESR and CRP had fallen to normal levels at 10 days but α1-ACT remained significantly elevated for 18 months in PMR/GCA overall ($P<0.01$ throughout) [Fig. 1(c)]. From 24 months α1-ACT results are shown separately for patients still requiring prednisolone treatment, and in this group α1-ACT remained significantly higher than controls (median 0.8 g/l, $P = 0.005$, $n = 21$ on prednisolone at 24 months, and median 0.8 g/l, $P = 0.011$, $n = 11$ on prednisolone at 36 months).

Although α1-ACT is said not to be elevated by corticosteroid therapy [18], the persistence of high levels of α1-ACT in clinically suppressed PMR/GCA raises the possibility of this being an effect of the corticosteroid treatment. To investigate this possibility, α1-ACT levels have been measured daily in 12 healthy volunteers before, during and after 3 days prednisolone administration. The median α1-ACT in this group was 0.53 g/l before prednisolone, and 0.56 g/l after 2 and 3 days of prednisolone 20 mg daily. This small difference was significant over 3 days ($P = 0.04$, Friedman's test) but is too small to account for the high levels of α1-ACT persisting in PMR/GCA patients. We cannot exclude a greater elevation of α1-ACT levels with more prolonged prednisolone administration, but in this group of volunteers α1-ACT seemed to have reached a plateau between 2 and 3 days. A pilot study in one volunteer showed a rise of only 0.05 g/l after 9 days prednisolone administration, reaching a maximum between 3 and 4 days.

In relapses of PMR/GCA [Fig. 2(a)], ESR was not significantly greater than controls, and only six out of 49 relapses were accompanied by an ESR ≥30 mm/h. CRP in relapses was significantly higher than controls [Fig. 2(b)], but only 24 out of 49 relapses were accom-

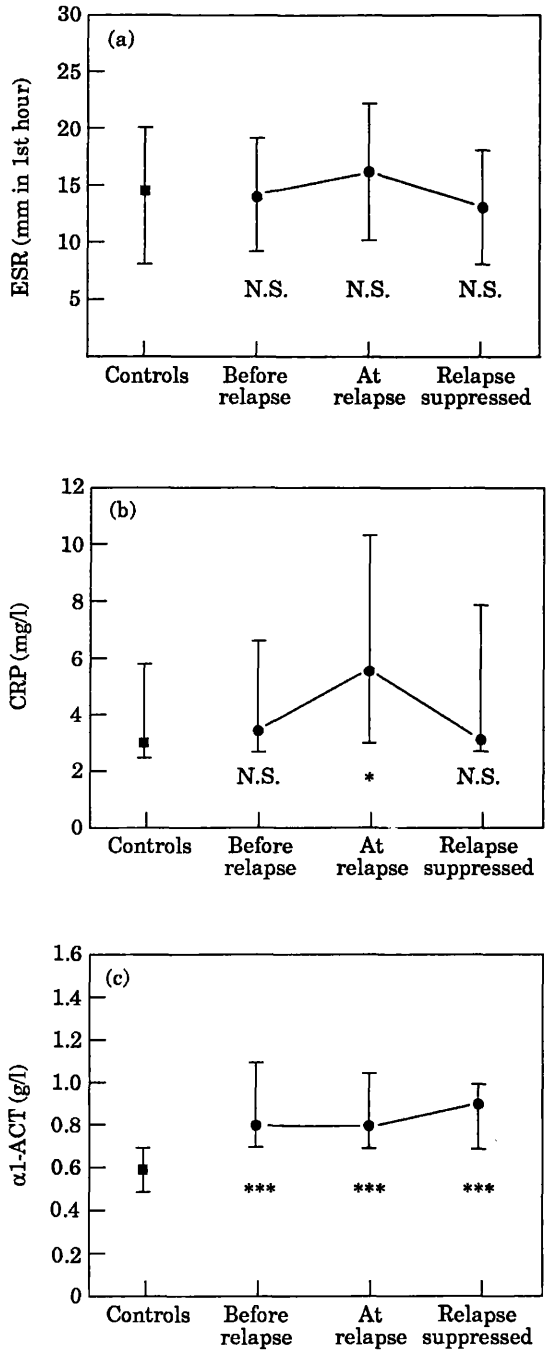

FIG. 2.—(a) ESR, (b) CRP, and (c) α1-antichymotrypsin (α1-ACT), in relapses of polymyalgia rheumatica/giant cell arteritis (PMR/GCA). Results expressed as medians + interquartile ranges. ***$P<0.001$, *$P<0.05$, N.S. = not significant, for the comparison with controls, Mann–Whitney test. ■, Controls; ●, PMR/GCA.

panied by a CRP ≥6 mg/l. α1-ACT levels were already raised prior to relapses [Fig. 2(c)] and rose no further during relapse.

In one relapse, active GCA was demonstrated on temporal artery biopsy despite an ESR of only 18 mm/h and CRP of 7.8 mg/l. The patient, who had previously had only symptoms of 'pure' PMR, developed amaurosis fugax after 9 months of treatment, whilst taking prednisolone 6.25 mg daily. The normal ESR and barely raised CRP were obtained the next day, before the dose of prednisolone had been increased, and the positive histology was obtained only a few hours after the blood tests. It is interesting that α1-ACT was elevated, at 1.0 g/l, 2 months prior to this relapse, and remained at 1.0 g/l at the time of relapse.

The persistence of elevated α1-ACT levels in PMR/GCA, particularly in patients continuing to require treatment, raises the possibility that α1-ACT levels might predict the clinical course of PMR/GCA. α1-ACT at presentation did not correlate with the subsequent duration of prednisolone treatment required nor with the number of relapses occurring before treatment was successfully stopped. However α1-ACT levels from 12 months onwards did correlate with both these parameters. (Spearman's rank correlation coefficient ranged from 0.3 to 0.5, $P<0.05$ for all.)

Table I shows the prognostic value of the α1-ACT level having fallen to 0.7 g/l or less. By 18 months this significantly predicted a reduced risk of subsequent relapse. The criterion of α1-ACT ≤0.7 g/l on two successive occasions did not add specificity and reduced the sensitivity as a prognostic indicator. Likewise, considering the *fall* in α1-ACT level was no more useful than the absolute level. Using a threshold value of ≤0.8 g/l was less reliable than ≤0.7 g/l as an indicator of reduced risk of subsequent relapse, but it was useful at an earlier stage, reaching statistical significance at 12 months ($P = 0.048$).

One explanation of an apparent prognostic value of α1-ACT at 18 months, would be that it simply reflected clinical disease activity over the previous few months, which in turn was the real predictor of the likelihood of future relapse. In fact, this is not the case, as α1-ACT levels were no different in those with and without a relapse in the preceding 6 months. Neither was there an association between relapse in the previous 6 months and subsequent relapse. However, perhaps not surprisingly, relapse at some stage before 18 months was associated with an increased likelihood of relapse after 18 months ($P = 0.039, \chi^2$).

DISCUSSION

We have shown that α1-ACT in PMR/GCA behaves in an entirely different way from CPR and the ESR, in that the circulating level of α1-ACT remains raised long after clinical suppression of the disease by corticosteroid treatment. From our data in healthy volunteers,

TABLE I
Subsequent relapse rates in patients grouped according to serum α1-antichymotrypsin (α1-ACT) concentration at particular stages of follow up

	Follow-up in months				
	12	15	18	21	24
Subsequent relapse rate if α1-ACT ≥0.8 g/l	13/18	14/19	13/17	9/12	8/12
Subsequent relapse rate if α1-ACT ≤0.7 g/l	9/18	7/16	5/17	8/22	7/22
P for the difference between the two groups	0.171	0.071	0.006**	0.031*	0.050

it would seem that corticosteroids themselves would not cause raised α1-ACT levels to the extent seen in these PMR/GCA patients. However we cannot rule out the possibility that the biosynthesis of α1-ACT is increased by corticosteroids in PMR/GCA patients to a greater extent than in healthy volunteers. The median daily prednisolone dosage in the PMR/GCA patients on treatment was only 5 and 2.5 mg at 18 and 24 months respectively. It is unlikely that these doses would account for the elevation of α1-ACT levels from 0.6 to 0.8 g/l, when much higher doses in healthy volunteers produced such a small effect.

Our results also indicate that the persistent elevation of α1-ACT in PMR/GCA is not simply a prolonged response to previous relapse. Rather, it seems to reflect underlying disease activity not clinically evident during prednisolone treatment but predisposing to subsequent clinical relapse.

When using a laboratory test in the management of the individual patient, it is useful to have a threshold value distinguishing between disease and normality. The normal range for α1-ACT in our hospital laboratory is 0.33–0.64 g/l [21]. In this series of PMR/GCA patients, an α1-ACT level of ≤0.7 g/l seemed to indicate a reduced risk of subsequent relapse, but this was significant only after 15 months. Even at this stage α1-ACT ≤0.7 g/l did not guarantee freedom from subsequent relapse so although the test is an aid to clinical management it is not a substitute for clinical diagnosis of relapse. Although the useful threshold value is referred to here as 0.7 g/l, test results at the time of the study were available only to one decimal place, so the threshold value is actually 0.75 g/l.

The finding that ESR and CRP are not elevated in the majority of relapses supports the assertion of Kyle and Hazleman [27] and Mason and Walport [28] that these investigations are no substitute for clinical assessment in diagnosing relapses of PMR/GCA. However, in practice, the ESR is used by many general practitioners and hospital physicians to diagnose relapses [2, 28, 29]. Chakravarty *et al.* [29] found that two-thirds of such doctors would increase prednisolone in response to a raised ESR in the absence of symptoms of PMR/GCA, while very few considered increasing the corticosteroid dosage on the basis of symptoms alone. Our case of biopsy-proven GCA relapse with normal ESR and near-normal CRP highlights the importance of not depending on these blood tests to diagnose relapses.

A possible mechanism for the lack of an acute phase response in many relapses of PMR/GCA would be down regulation of this response in chronic inflammation [15]. This has been demonstrated in uveitis, where a much smaller acute phase response is seen in patients who have had several previous attacks despite clinical evidence of similar inflammation [30].

Although serum α1-ACT does not rise at the time of a clinical relapse of PMR/GCA, the previous α1-ACT result(s) might help to confirm the diagnosis of relapse by indicating the likelihood of relapse in a particular patient. This would obviously be convenient, as this result would be available at the time the patient was seen with a suspected relapse. For instance, in our biopsy-proven GCA relapse α1-ACT had remained high up to this time and therefore would have helped to confirm relapse immediately. However, α1-ACT used in this way would be an adjunct to clinical assessment and not a replacement for it. It may be possible to use α1-ACT in a pro-active way and divide patients into a 'fast stream' and a 'slow stream' for prednisolone reduction. This division could be made on the basis of α1-ACT ≤0.8 g/l at 12 months and ≤0.7 g/l at 18 months. Such a study is planned in a new series of patients to see whether knowledge of α1-ACT levels can reduce the total prednisolone dose without increasing the relapse rate.

Although α1-ACT measurement is not currently available in most hospitals, it is technically simple and cheap and could therefore be more widely available. Indeed Thompson *et al.* [16], reporting for the Association of Clinical Biochemists, have recommended that it be available in all teaching hospitals. They stress its usefulness in 'situations where a wider time window is required' than that given by CPR measurement. Our results suggest that the long-term follow up of PMR/GCA is one such situation where ACT measurement maybe useful.

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Effects of single dose compared with three days' prednisolone treatment of healthy volunteers: contrasting effects on circulating lymphocyte subsets

G D Pountain, M T Keogan, B L Hazleman, D L Brown

Abstract

***Aims*—To investigate the effects of longer term corticosteroid treatment on circulating lymphocyte subsets.**

***Methods*—Prednisolone (20 mg daily) was given to 12 healthy volunteers in a single morning dose for three days. Circulating lymphocyte subsets were measured by flow cytometry after whole blood lysis.**

***Results*—Seven hours after the first dose of prednisolone there was a significant fall in absolute numbers of lymphocytes, T cells, CD4+ and CD8+ cells, and B cells. The percentage of T cells fell significantly, due to a fall in percentage of CD4+ cells. In contrast to the seven hour findings, at 72 hours there was a significant rise in absolute numbers of lymphocytes, T cells, CD4+, CD8+, and B cells. This trend was already apparent by 24 hours. The percentage of CD4+ cells was significantly raised at 72 hours, while that of CD8+ cells had fallen significantly. The percentage of natural killer cells had fallen at 72 hours; that of B cells remained increased at 72 hours.**

***Conclusions*—These findings show that corticosteroid treatment causes significant changes in lymphocyte subsets, and that such changes must be considered when designing studies of lymphocyte subsets during illness.**

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In a study of T cell subsets in polymyalgia rheumatica and giant cell arteritis (PMR/GCA)[1] we observed that once prednisolone treatment had been started, total numbers of T cells rose and the percentage of CD8+ cells fell significantly. Lymphocyte subsets were normal before treatment compared with age matched controls. This suggested that the prednisolone, not the PMR/GCA, was responsible for the T cell changes. We therefore decided to investigate further the effects of prednisolone treatment, using healthy volunteers.

Some of the short-term effects of corticosteriods on lymphocyte subsets have been documented. Yu[2] and Fauci[3] showed lymphopenia to be maximal 4–6 hours after a single dose of corticosteroids given to healthy volunteers. With the advent of monoclonal antibodies and flow cytometry, ten Berge *et al*[4] documented reduced T cells, particularly OKT4+ cells, at six hours after a single dose of prednisolone given to normal volunteers compared with controls. They also showed that by 24 hours this effect had disappeared with a slight "rebound effect"—OKT4+ and OKT8+ cells were slightly increased by 24 hours, though this was not significant. This study did not look at the effects of longer term administration of prednisolone.

Similarly, Tonnesen *et al*[5] infused cortisol into healthy volunteers for five hours and showed reduced lymphocytes, OKT3+, OKT4+ and OKT8+ cells by two hours compared with controls. These changes persisted 15 minutes after the cortisol infusion had been discontinued, but were not monitored after that time.

Hogevold *et al*[6] gave high dose methylprednisolone preoperatively and four and 12 hours after total hip replacement. After this short duration of corticosteroids, they showed reduced total T cells and helper and suppressor cells, compared with control patients, at 20 hours (= eight hours after the last dose of methylprednisolone).

The effects on T cells of longer term corticosteroid administration have not been documented in healthy people, although changes occurring in patients treated with corticosteroids have been described. Ferrari *et al*[7] reported increased lymphocytes, including increased absolute numbers of T cells, CD4+ and CD8+ cells, after four weeks of corticosteroid treatment in idiopathic thrombocytopenic purpura (ITP). The percentages of CD4+ and CD8+ cells were not significantly changed. In this study, therefore, the longer term effects of corticosteroid seem to be quite different from the acute effects a few hours after a single dose.

Fauci[8] described the effects of seven days of cortisone administration in guinea pigs, as well as the acute effects of a single dose of hydrocortisone. At both stages there was a fall in lymphocyte and T cell numbers. The observation of reduced lymphocytes and T cells four hours after the hydrocortisone corresponds to the acute effects seen in human volunteers.[2–5] But the seven day effects in Fauci's guinea pigs[8] were at odds with the effects in Ferrari's patients with ITP, where after four weeks of corticosteroids lymphocytes and T cells had risen.[7] The difference between these studies might be a species effect, or the effects of corticosteroids in patients might have been modified by the disease process itself.

Although the nature of the acute changes

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Effects on circulating lymphocyte subsets of prednisolone EC 20 mg for three days in healthy volunteers (n = 12)

	Baseline	7 hours	24 hours	48 hours	(55 hours) (n = 4)	72 hours
Lymphocytes (×10⁹/l)	2·09	1·02**	2·53	2·65	(2·35)	3·39**
Total T cells (×10⁹/l)	1·59	0·58**	1·84	2·00	(1·71)	2·54**
CD4+ cells (×10⁹/l)	1·06	0·36**	1·30	1·42	(1·01)	1·61**
CD8+ cells (×10⁹/l)	0·58	0·41**	0·67	0·59	(0·69)	0·83**
Activated T cells (×10⁹/l) (n = 9)	0·11	0·07 NS (p = 0·75)	0·17	0·13	—	0·14 NS (p = 0·063)
NK cells (×10⁹/l)	0·32	0·35 NS	0·33	0·23	(0·45)	0·34 NS
B cells (×10⁹/l)	0·23	0·15*	0·27	0·34	(0·32)	0·53**
T cells (% of lymphocytes)	72·5	61*	75·5	76	(70·5)	75 NS
CD4+ cells (% of lymphocytes)	45·5	36*	48	49·5	(43·5)	51·5*
CD8+ cells (% of lymphocytes)	26·5	29 NS	25	24	(25·5)	24·5*
Activated T cells (% of lymphocytes) (n = 9)	4	6*	6	7	—	6 NS
Natural killer cells (% of lymphocytes)	16·5	20*	12·5	9·5	(16)	9·5**
B cells (% of lymphocytes)	10·5	13*	12·5	13	(12·5)	14·5**
CD4+ *CD8+ ratio	1·83	1·45 NS	2·00	2·10	(1·95)	2·10**

(Results expressed as medians with two tailed p values for the comparison with baseline data (Wilcoxon's rank sum test); **p < 0·01; *p < 0·05; NS = not significant.)

in the few hours after a single dose of corticosteroid has been documented in the work described above,[2-6] there is little information about the effects of longer term corticosteroid administration in people, and in particular we were unable to find any study of these longer term effects in the absence of disease. We therefore studied a group of healthy volunteers taking prednisolone.

Methods

Twelve healthy volunteers were recruited from among senior medical staff. Eight were men and the ages ranged from 31 to 50 years. None had contraindications to corticosteroid administration and all gave informed consent.

A pilot study in one volunteer showed the acute postdose effects on T cell subsets to be maximal around seven hours after a dose of 20 mg prednisolone enteric coated (EC), while in the longer term maximal effects were seen after three to four days of prednisolone administration. We therefore used a three day period of corticosteroid treatment in the volunteer group with blood tests taken over four days.

Each volunteer had baseline blood samples taken at 0900 hours and then took prednisolone EC 20 mg daily orally at 0900 hours for three days. Further blood samples were taken at seven hours after the initial dose and again at 24, 48, and 72 hours—24 hours after the latest prednisolone dose, to avoid the acute postdose effects. Four volunteers also had blood samples taken at 55 hours—seven hours after the third dose of prednisolone.

All blood samples were processed within six hours of venesection. Total white cell counts and lymphocyte counts were measured using routine methods in the haematology department at Addenbrooke's Hospital. T cell subsets were analysed by flow cytometry using a whole blood lysis technique. Total T cell numbers were measured using anti-CD3 (Leu 4). Activated T cells were those CD3+ cells which coexpressed anti-HLA-DR. CD4+ cells were defined by dual staining with anti-CD3 (Leu 4) and anti-CD4 (Leu 3), while CD8+ cells were defined using anti-CD3 with anti-CD8 (Leu 2). Natural killer cells were CD3- expressing CD16/56 (Leu 11c + 19). B cells were measured using anti-CD19. All monoclonal antibodies were purchased from Becton Dickinson (Oxford, England) from the Simultest range. Aliquots of blood were incubated with antibody pairs for dual staining for 15 minutes at room temperature.

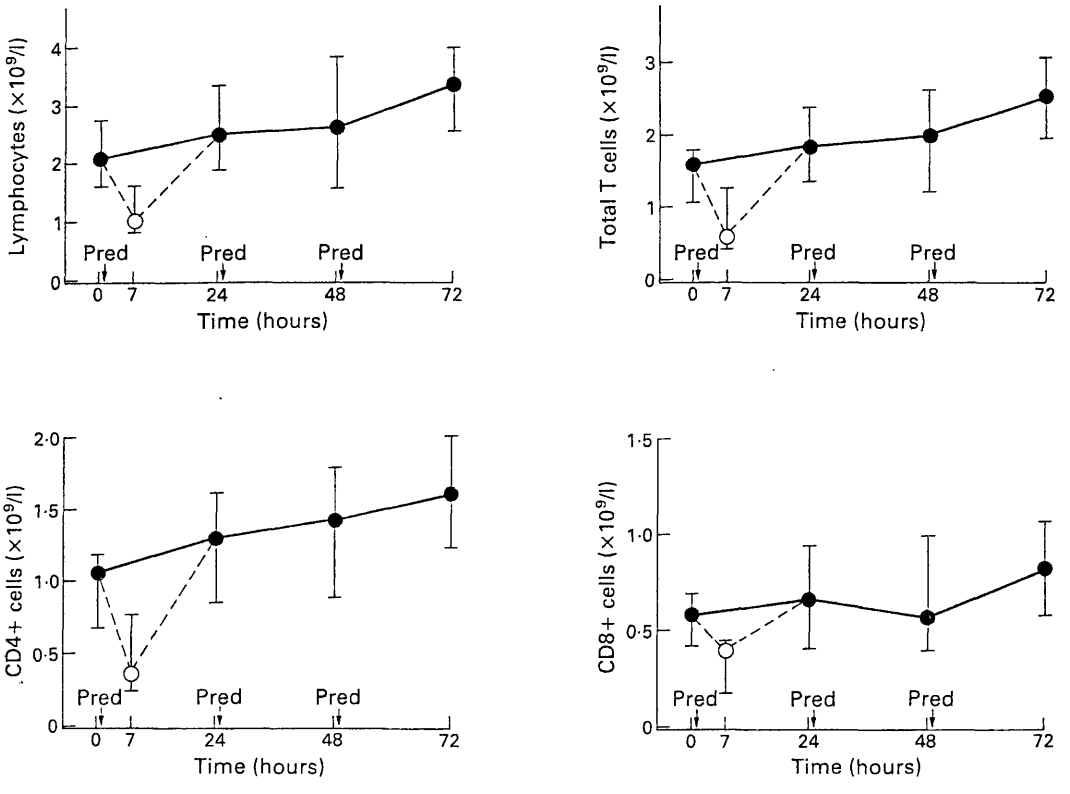

Figure 1 Lymphocytes and subsets in 12 healthy volunteers, in relation to doses of prednisolone EC 20 mg (↓): ●—in blood samples taken immediately before the next prednisolone dose and 24 hours after the previous dose; ○—in blood samples taken 7 hours after the initial prednisolone dose. Results expressed as medians + interquartile range.

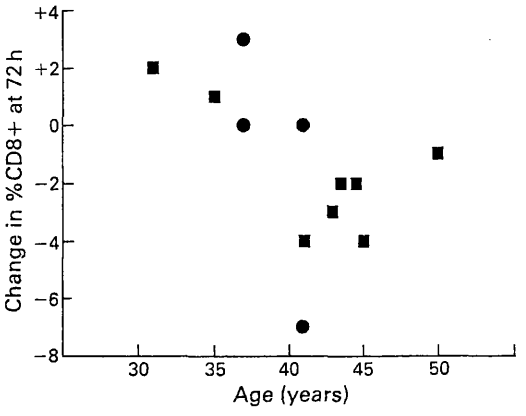

Figure 2 Change in percentage of CD8+ cells after 72 hours of prednisolone in 12 healthy volunteers, according to age. ■ = men, ● = women. Change in percentage of CD8+ cells expressed as percentage of lymphocytes. Spearman's rank correlation coefficient = −0·599 (two-tailed $p < 0·05$).

Erythrocytes were lysed using FACS lysing solution (Becton Dickinson) and leucocytes were fixed with 0·5% formaldehyde. Cells were analysed on the day of processing using a Becton Dickinson FACscan, and Simulset software.

The paired data for nought and seven hours and for nought and 72 hours were analysed using Wilcoxon's rank sum test.

Results

The changes in lymphocyte subsets during prednisolone treatment are shown in the table. The absolute numbers of lymphocytes, T cells, CD4+, CD8+ and B cells had all fallen significantly by seven hours after the first dose of prednisolone, and by contrast had all risen significantly higher than baseline numbers at 72 hours (24 hours after the third dose of prednisolone) (fig 1).

As a percentage of total lymphocytes, CD4+ cells fell at seven hours, then rose significantly (table). The percentage of CD8+ cells did not change significantly at seven hours but was significantly lowered at 72 hours. This change was small. An unexpected finding was the more pronounced fall in the percentage of CD8+ cells by 72 hours in the older compared with the younger volunteers (fig 2). The Spearman rank order correlation coefficient was −0·599 (two-tailed test, $p < 0·05$). No correlation was seen in this study between age and the baseline percentage of CD8+ cells. No substantial difference in T cell changes was observed between men and women, but the number of women was too small for separate statistical analysis.

Values of lymphocyte subsets at 55 hours (seven hours after the third dose of prednisolone) are shown in the table, although the data were drawn from only four subjects. These values were intermediate between the seven hour and 72 hour values as might be expected, but the net effects were closer to the longer term effects—that is, the acute postdose effects are partially masked.

Discussion

This study of the effects of prednisolone on circulating lymphocyte subsets describes new findings. We have shown that the effects of continuing prednisolone administration beyond 24 hours in healthy volunteers results in effects which contrast strongly with the changes described in the first few hours after a single dose of prednisolone. These longer term effects were consistent with the changes we have observed in patients treated with prednisolone for PMR/GCA.[1] Ferrari *et al*[7] showed a similar rise in absolute numbers of lymphocytes, T cells, and CD4+ cells in patients with ITP treated with corticosteroids for four weeks, but they did not find a significant difference in the percentage of CD4+ or CD8+ cells.

Seven days of corticosteroid given to guinea pigs seem to produce different changes from those seen in studies in man. Fauci[8] observed a fall in lymphocytes and T cells in guinea pigs which persisted from the acute stage to seven days. Therefore, there seems to be a species difference in the effects of long term corticosteroids on lymphocytes and subsets.

The changes we have shown seven hours after the first dose of prednisolone have confirmed the findings in the controlled studies of ten Berge *et al*[4] and Tonnesen *et al*.[5] These authors described decreased absolute numbers of lymphocytes, T cells, CD4+ and CD8+ cells a few hours after a single dose of corticosteroid. We have also shown a short term fall in the percentage of CD4+ cells.

Circadian variation in lymphocyte subsets has been shown.[9,10] Ritchie *et al*[9] described peak numbers of T cells, CD4+ and CD8+ cells at 2200 hours with an inverse correlation to plasma cortisol concentrations. Levi *et al*,[10] however, found the peak of T cells and CD4+ cells around 0200 hours, with no association with plasma cortisol values, even allowing for possible delayed hormonal action. So the role of endogenous cortisol in the diurnal changes in T cell subsets is not clear. It is perhaps not surprising that such physiological effects, if present, may be less pronounced than the pharmacological effects of corticosteroids.

In Cushing's disease absolute numbers of T cells are greatly decreased.[11] This does not necessarily conflict with our finding of raised T cells numbers in volunteers and in patients with PMR/GCA patients during long term prednisolone.[1] In Cushing's disease the high circulating concentration of cortisol is sustained and may be equivalent to the acute postdose effects we have seen (a fall in T cells), but persistent. In constrast to the sustained cortisol concentration in Cushing's disease, daily administration of prednisolone produces fluctuating blood concentrations, and the pre-dose samples used in our studies would contain low corticosteroid concentrations. At this stage the longer term effects of the corticosteroids would be evident, in the absence of the postdose effects.

Possible mechanisms for the changes are debatable. The acute reduction in circulating lymphocytes and T cells after a dose of prednisolone is too rapid to be attributable to an effect on lymphocyte proliferation. The

absence of fever and rigors, as seen when lymphocytes are depleted using monoclonal antibodies, suggests that cell lysis is not involved. The most likely explanation seems to be a change in lymphocyte trafficking, with sequestration in the lymphoid organs. In guinea pigs this has been shown to be due to redistribution to the bone marrow.[8] In the longer term, the rise in circulating lymphocytes and T cells probably also reflects redistribution of the lymphocytes, but other mechanisms cannot be ruled out.

Our results suggest that the older subjects had a more pronounced fall in percentage of CD8+ cells with prednisolone, and in fact the youngest subjects actually had a slight rise in the percentage of CD8+ cells. There was no age dependent difference in baseline percentage of CD8+ cells in our study, although a decrease has been described in subjects over 60 years old[12] and over 75 years.[13] The numbers in our study were small and the age range was narrow (31–50 years), but if only older subjects respond to prednisolone with a decrease in the percentage of CD8+ cells, this would account for the observation of a fall in percentage of CD8+ cells in patients with PMR/GCA receiving prednisolone treatment, whose age ranged from 51–87 years (median 70 years).[1] Ferrari *et al*[7] found no significant change in CD8+ cells in patients with ITP receiving prednisolone treatment, where the age range was 15–57 years (median 32 years). One possible mechanism for the changing responsiveness to corticosteroids with age might be the changing numbers of corticosteroid receptors. Armanini *et al*[14] have described reduced corticosteroid receptors on mononuclear leucocytes in aged subjects (62–97 years), but in vitro glucocorticoid sensitivity does not seem to be related to numbers of corticosteroid receptors.[15] Further studies are required in volunteers and patients over a wide range of ages to clarify the T cell effects of corticosteroids in different age groups. We are now undertaking these studies.

The contrasting short term and longer term effects of corticosteroids shown in this study indicate the importance of controlling for corticosteroid effects in addition to circadian variation in all future studies of lymphocyte subsets.

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