

Blood lymphocyte subsets in rats with adjuvant arthritis

A Franch, C Castellote, M Castell

Abstract

Objectives—To determine the phenotype of peripheral blood lymphocytes during the time-course of adjuvant arthritis (AA) to detect alterations that could be involved in the pathogenesis of the arthritic process.

Methods—Phenotype analysis was performed on days 7, 14, 21, 28, 42, 56 and 70 after arthritis induction using monoclonal antibodies to CD5, CD4 and CD8 subsets, and flow cytometry. The proportion of activated lymphocytes and lymphocytes was also assessed with monoclonal antibodies to IL-2R (CD25), to Ia antigen and by polyclonal antibodies to rat Ig.

Results—Adjuvant arthritis produced leukocytosis with neutrophilia. Rats with AA showed a marked increase in the number of both CD4⁺ and CD8⁺ cells. The ratio CD4/CD8 decreased because the rise in CD8⁺ cells was more pronounced than the increase in CD4⁺ cells. Changes in lymphocyte counts showed two well-defined periods: the first, from day 14 to day 28, during which the inflammation of the joints reached a maximum and changes in lymphocyte subsets were more pronounced, that is, there was a threefold increase in CD8⁺ lymphocytes over normal counts, and the second, from day 42 to day 70, in which modified parameters improved considerably but remained different from controls.

Conclusion—Alterations were detected in the phenotype of peripheral blood lymphocytes in AA, which provides an additional marker of disease activity.

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Adjuvant arthritis (AA) is a well recognised model of rheumatoid arthritis, developed in 1956 by Pearson.²⁷ It has been extensively used to study the pathogenesis of inflammatory arthritis and to assess the action of anti-inflammatory and anti-rheumatic drugs. AA is induced in rats by injection of heat-killed mycobacteria and is characterised by a severe polyarthritis. The exact antigen that develops this experimental model is still unknown although T-cell-mediated mechanisms are involved in its pathogenesis.³¹ Antibodies against autologous components have been found in sera of animals with adjuvant arthritis.^{6 7 14} Although cell immune mechanisms are involved in AA, little is known about

the quantitative relationship of lymphocyte subpopulations in adjuvant arthritic rats.

The aim of this study is to determine the phenotype of peripheral blood lymphocytes during the time-course of adjuvant arthritis to detect alterations that could be involved in the pathogenesis of the arthritic process.

Materials and methods

Female Wistar rats (Charles River, Barcelona) aged 10 weeks and weighing 180-200 g were housed in cages with free access to commercial pellet chow (UAR-A-04^R) and water ad libitum. Temperature (21 ± 1°C) and light cycle (on from 8:00 to 20:00 hours) were controlled. Housing conditions were clean but not pathogen free. Animals were left for two weeks to acclimatise to the housing conditions before the experiments began.

INDUCTION AND EVALUATION OF ARTHRITIS

AA was induced by a single subplantar injection (0.1 ml) of heat-killed *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI) suspended in mineral oil (5 mg/ml) in the right hind-paw. A group of untreated animals matched by age and sex to arthritic animals were used as healthy controls. The severity of AA was quantified by the volume increase in the left hind-paw. On day 0 (before induction) and subsequently, 7, 14, 21, 28, 42, 56 and 70 days after adjuvant injection, the animals were weighed, and left- and right-hind-paw volumes were measured using a mercury plethysmometer (Ugo Basile, Milan). On day 14, those rats whose left-hind-paw volume increase exceeded the average left-hind-paw volume increase of the control group plus two standard deviations, were selected as arthritic rats.

BLOOD SAMPLES

At 7, 14, 21, 28, 42, 56 and 70 days after arthritis induction rats were anaesthetised with ether, and blood samples (5 ml) were taken, by cardiac puncture, into heparin-coated sterile Venoject vacuum tubes. Blood samples were taken from 8 healthy control animals and 10 arthritic rats between 8.00 and 9.00 am.

CELL COUNTS

The number of leukocytes from each rat was determined using a counting chamber (Kova, ICL Scientific), and differential analysis of every sample was performed on staining blood smears.

Unitat de Fisiologia,
Facultat de Farmàcia,
Universitat de
Barcelona, Barcelona,
Spain

A Franch
C Castellote
M Castell

Correspondence to:
Dr A Franch,
Unitat de Fisiologia, Facultat
de Farmàcia, Av Joan XXIII
s/n, 08028 Barcelona, Spain.

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CELL SEPARATION, LABELLING AND FLOW CYTOMETRY

Rat mononuclear cells were isolated, labelled and analysed by flow cytometry as described previously.¹³ Briefly, rat mononuclear cells were isolated by Ficoll-Isopaque gradient centrifugation, using the method of Boyum as modified by Davidson and Parish,⁹ adapted to rat ($d = 1.09 \times 10^3 \text{ kg/m}^3$). Viability of each lymphoid cell suspension was assessed using acridine orange-ethidium bromide staining.

Mononuclear cells ($0.5-1 \times 10^6$) were incubated with monoclonal antibodies to CD5 (OX19) as T lymphocyte marker (purchased from Sera-Lab);⁸ CD4 (W3/25) as T helper/inducer marker;³³ CD8 (OX8) as T suppressor/cytotoxic marker;⁴ Ia molecule (OX6) as marker of B cells and activated T cells;²⁴ and CD25 (OX39) as marker of activated T cells²⁶ (these monoclonal antibodies were kindly provided by Dr A Williams, Oxford, UK). FITC-conjugated goat F(ab')₂ anti-mouse IgG antibodies (Sigma) were added to stain mouse monoclonal antibodies. B lymphocytes were also directly labelled with FITC-conjugated rabbit anti-rat immunoglobulins (Dako). Labelled cells were fixed in 1% paraformaldehyde in saline solution and stored in the dark at 4°C until analysis. All samples were analysed within 6 days of lymphocyte labelling, but determinations performed after 21 days did not reveal loss of positive fluorescence staining over time.

The percentage of fluorescent lymphocytes was determined using a FACScan flow cytometer (Becton Dickinson, Mount View, CA). Analysis gates were set in such a way that T or B lymphocytes were selected.¹³ Results for each lymphocyte subset are expressed as a percentage of positive cells compared with total lymphocytes and as the number of cells per litre of blood compared with lymphocyte count.

Statistical analysis was performed by means of an ANOVA of linear models¹⁹ considering the following causes of variation: arthritis (induced animals versus healthy control animals) and time (day of the study).

Results

HIND-PAW INFLAMMATION AND BODY WEIGHT

The injected right-hind-paw volume increases in induced animals were significantly higher than control from the first day of induction (from day 7 to day 70, $p < 0.0001$) (fig 1A). Left-hind-paw volume increases, which represent the systemic inflammation, were not detected until 10–14 days after induction. On day 14 the incidence of arthritis was 64/70. The maximum inflammation was seen on days 21 and 28. There was a slight decrease in systemic inflammation on days 42, 56 and 70, but without reaching reference values (from day 14 to day 70; $p < 0.0001$). From day 14, two well-defined phases were noted: a first period or period 1, from day 14 to 28, during which inflammation of the joints reached a maximum, and a second phase or period 2, from day 42 to 70, in which values decreased, although they remained higher than controls.

Animals in which arthritis had been induced also gained less weight in the first period after induction (fig 1B), which was significantly lower than controls on days 14–42 ($p < 0.005$). In advanced phases, arthritic weight increases tended to return to reference values and body weight was restored at the end of the period studied. The evolution of both hind-paw inflammation and body weight increases showed significant differences between period 1 (days 14–28, maximum systemic inflammation) and period 2 (days 42–70, tendency to control values) ($p < 0.005$).

LEUKOCYTE COUNT AND LEUKOCYTE DIFFERENTIAL ANALYSIS

From day 7 post-induction there was leukocytosis (fig 2A), which was more pronounced in period 1 than in period 2 ($p < 0.0001$).

In relation to leukocyte differential analysis, neutrophil and lymphocyte percentages are shown in fig 2B. The arthritic process did not modify the percentages of eosinophils or monocytes, while basophils were virtually absent. In contrast, from day seven AA induction produced an increase in neutrophil percentage (about 300% in relation to control values on days 14–28, $p < 0.0001$) and a reduction in lymphocyte percentage (30% decrease in relation to control values on days 14–28, $p < 0.0001$). Both types of leukocyte

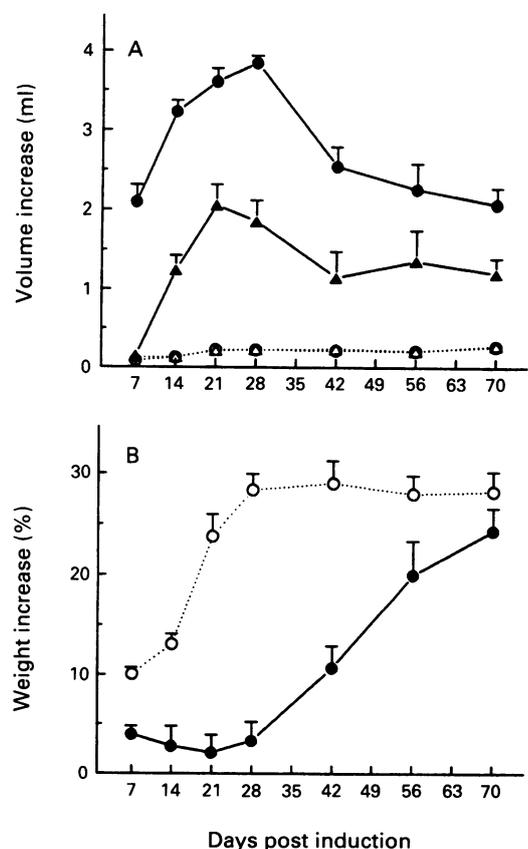


Figure 1 A) Evolution of right (circles) and left hind-paw (triangles) volume increases (ml) in arthritic (filled) and healthy (hollow) animals; B) Evolution of body weight increase in arthritic (●) and healthy (○) animals. Each point represents the mean (SEM) of 8–10 animals.

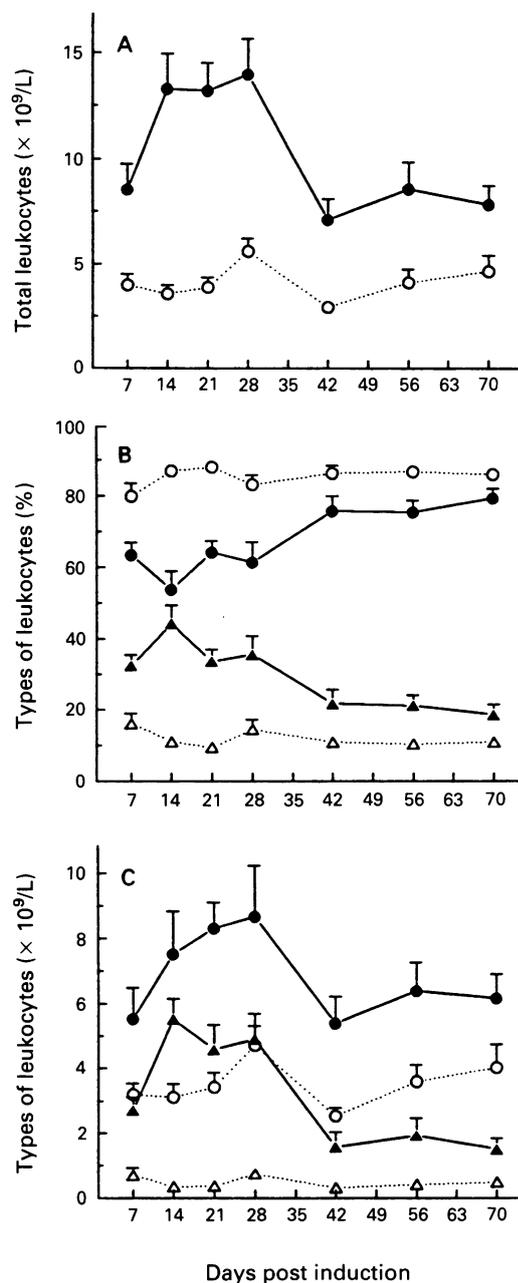


Figure 2 Leukocyte count and leukocyte differential analysis: A) Number of leukocytes in healthy (○) and arthritic (●) rats. Percentage (B) and absolute number (C) of lymphocytes (circles) and neutrophils (triangles) in healthy (hollow) and arthritic (filled) animals. Each point represents the mean (SEM) for 8–10 animals.

followed the two well-defined periods in the evolution of arthritis and the values in these phases were significantly different ($p < 0.0001$).

Time-courses of absolute numbers of neutrophils and lymphocytes are shown in fig 2C. Neutrophils increased significantly in arthritic animals, up to 10 fold in period 1 ($p < 0.0001$), while in period 2 the increase was only 4 fold ($p < 0.01$). Although the percentage of lymphocytes diminished in AA, their absolute number increased significantly, to double the control values, throughout the study ($p < 0.05$). There was a significant difference between the two periods of the arthritic process in the time-course of neutrophil and lymphocyte numbers ($p < 0.005$).

SUBSETS OF LYMPHOCYTES

Results of B-lymphocyte analysis are shown in fig 3. B-cells were determined in two ways: FITC-conjugated anti-Ig and anti-Ia monoclonal antibody. The results obtained with these two labels were similar, and only results for Ia⁺ cells are reported. AA induction increased the absolute number of B lymphocytes during period 1 of systemic inflammation ($p < 0.005$), but their percentage was not modified.

The absolute number of CD5⁺ cells (fig 3) increased throughout the study ($p < 0.001$); the increase was more pronounced in period 1 (maximum systemic inflammation) than in period 2 (slight remission of inflammation), the differences between them being significant ($p < 0.01$). No difference in CD5⁺ cell percentages was observed between arthritic and control group.

The absolute number of CD4⁺ cells (fig 3) increased in the peripheral blood of adjuvant arthritic rats ($p < 0.001$ compared with control values) in a biphasic pattern, following hind paw inflammation ($p < 0.01$, difference between periods). The percentage of CD4⁺ subset was not modified by the arthritic process.

Absolute numbers of CD8⁺ cells (fig 3) were considerably higher in arthritic animals during period 1 ($p < 0.0001$, compared with healthy control values); they later diminished but did not reach reference values. The percentage of CD8⁺ cells was significantly higher in arthritic rats, than in healthy animals, on day 14 post-induction ($p < 0.05$). The percentage remained high throughout the maximum inflammation period or period 1 ($p < 0.0001$). There was a positive correlation between the percentage of CD8⁺ cells and systemic inflammation measured in left-hind-paw ($r = 0.3321$, $p < 0.01$). In the last phase studied no difference in CD8⁺ lymphocyte percentage, in relation to healthy animals, was observed. CD4/CD8 lymphocyte ratio (fig 4) was significantly lower in arthritic rats than in healthy rats on days 21 and 28 post-induction ($p < 0.025$), reaching reference values in the last period.

To quantify activated lymphocytes the anti-IL2R MAb was used. The percentage of CD25⁺ cells was always less than 2% (background value), which indicated the absence of these cells from the blood of arthritic animals.

Discussion

This study describes the time-course of phenotypes of peripheral blood lymphocytes in arthritic rat blood for 70 days after arthritis induction. Two well-defined periods can be established in the arthritic process: the first, from day 14 to day 28 post-induction is characterised by the onset and establishment of systemic inflammation and loss of body weight, and the second, from day 42 to day 70, which corresponds to the chronic phase of the inflammation in which a tendency toward reference values was observed. Rats with AA showed a marked increase in the number of both CD4⁺ and CD8⁺ lymphocytes. The ratio CD4/CD8 decreased because the rise in CD8⁺

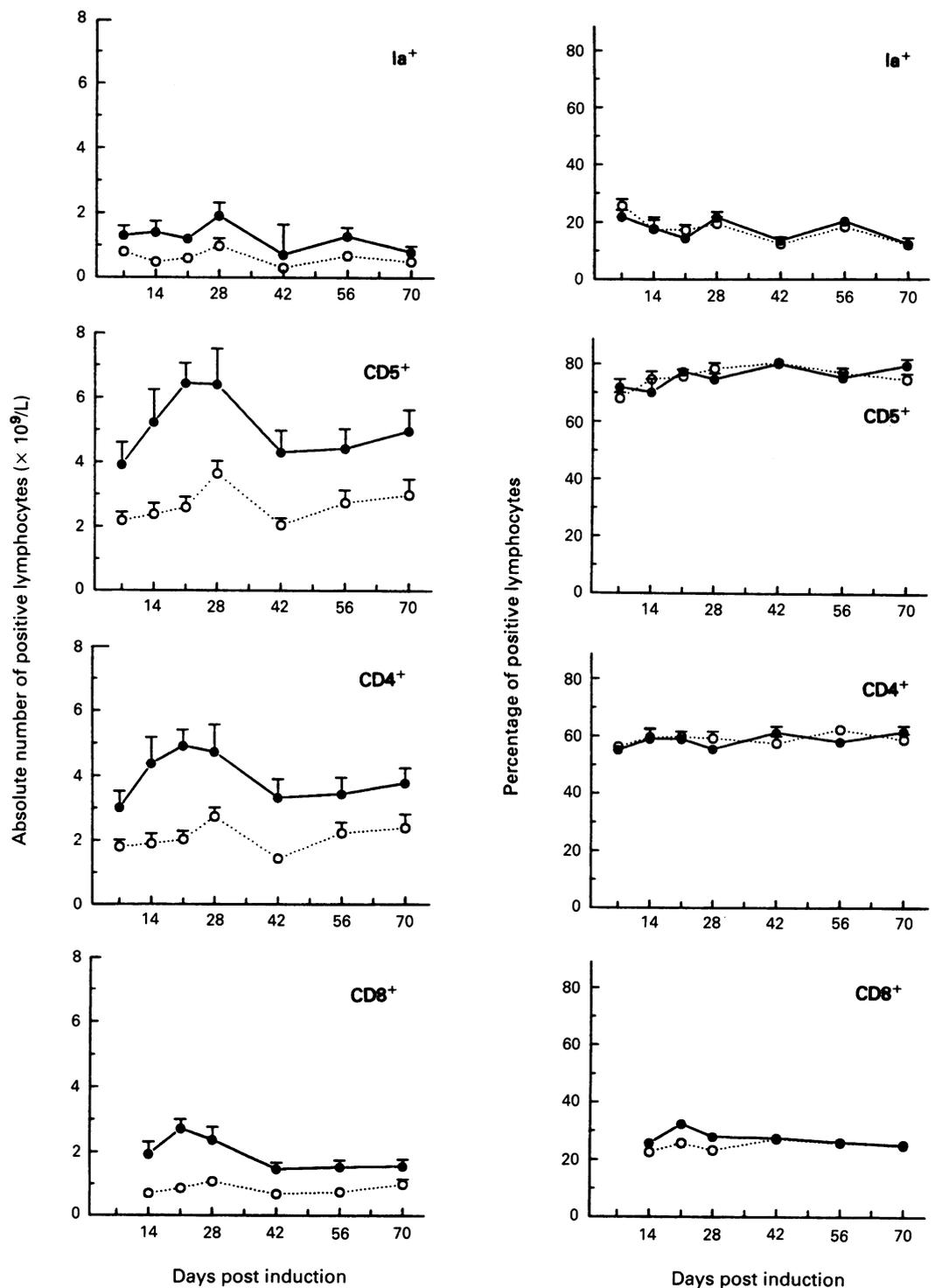


Figure 3 Absolute number and percentage of Ia⁺, CD5⁺, CD4⁺ and CD8⁺ lymphocytes in healthy (○) and arthritic (●) rats. Each point represents the mean (SEM) for 8–10 animals.

cells was more pronounced than the increase in CD4⁺ cells. The CD8 counts during the time-course of adjuvant arthritis followed the same pattern as the degree of inflammation measured as hind paw swelling. During the period in which inflammation of the joints reaches a maximum CD8⁺ cell counts increase threefold. However, when articular swelling diminished, CD8⁺ values improved considerably although they were still higher than controls.

Adjuvant arthritis is a well-known model of rheumatoid arthritis. However, to our knowledge, there have been very few studies on

the phenotype of circulating lymphocytes in AA. The remarkable shift in phenotype that we have detected previously, probably because it is more easily appreciated when absolute counts are considered.

The CD8 molecule is present in cytotoxic and suppressor lymphocyte subsets; thus it is not possible to attribute the percentage increase in CD8⁺ cells to a determinate function. An increase in CD8⁺ subset has been reported by Kaufmann²⁰ in human mycobacterial infectious diseases, and was attributed to cytotoxic activity. Although AA is

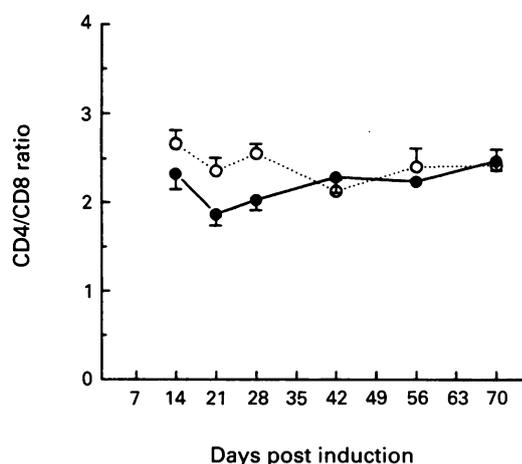


Figure 4 CD4/CD8 lymphocytes ratio in healthy (○) and arthritic (●) animals. Each point represents the mean (SEM) for 8–10 animals.

induced by heat-inactivated mycobacteria, a similar mechanism could be suggested. On the other hand, the increase in CD8⁺ cells might be due to a T-cell bearing gamma-delta receptors, some of which are CD8⁺¹⁷ and recognise mycobacterial determinants.^{3 29}

Although alterations in the number and percentage of CD8⁺ lymphocytes in AA has not been described before, the role of CD8⁺ lymphocytes in this experimental model was studied by Larsson *et al.*,²³ who observed that depletion of the CD8⁺ subset before AA induction did not modify the arthritic process. Other authors, however,^{1 21 22} have related this subset to susceptibility and development of AA. Moreover, Binderup² has shown that Con-A-induced T-suppressor cell activity decreased in rats with AA, and this reduction was found to coincide with the development of the polyarthritic lesions. In a recent study on arthritic rat synovial tissue we observed a marked increase in CD8⁺ cells in lining and subintimal layer, without quantitative fluctuations in CD4⁺ cells (Pelegri *et al* submitted). Further, a functional study of the CD8⁺ cells, the number of which increase in AA, is required to determine whether there is an increase in the T-cytotoxic subset (as has been described in clinical mycobacterial diseases²⁰) or whether there is an increase in the T-suppressor subset, but their activity is reduced.

If we compare our results with those reported for human RA, some similarities emerge. Thus the number of CD4⁺ cells has been shown to increase³² but also to remain normal.^{10 15} CD8⁺ cell number was seen to be similar to^{10 12} or lower than^{11 18 32} healthy reference values. Other studies do not report any difference in CD4/CD8 ratio in patients with RA.^{10 25} Regarding blood T-lymphocyte function, contradictory results have also appeared (reviewed in reference 28).

On the other hand, by means of anti-Ia and anti-CD25 MAbs, activated lymphocytes were not detected in arthritic rats, although they have been described in human RA.^{18 25 30} In AA, activated T cells have been reported only in arthritic synovia.²³

Our total and differential leukocyte study in adjuvant arthritis also revealed leukocytosis with a high percentage of neutrophils and a high number of lymphocytes. Our data confirm and complete those of Glenn *et al.*,¹⁶ who reported leukocytosis, neutrophilia and an increase in the number of lymphocytes on day 21 post-induction. These changes agree with results reported by Carlson *et al.*⁵ on day 49 post-induction, who also found an increase in monocyte percentage. These fluctuations in white blood cells are probably secondary to the inflammatory process present in AA.

In summary, we have detected alterations in the phenotype of peripheral blood lymphocytes in AA and its correlation with the inflammatory process, which provides an additional marker of disease activity. The specificity of this expanded population of CD8 cells remains unknown. Studies in which peripheral blood lymphocytes and the lymphocytes infiltrating the synovium are characterised in parallel may indicate what proportion of these CD8⁺ are involved in pathogenesis.

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