

Polymorphonuclear leucocyte function and previous yersinia arthritis: enhanced chemokinetic migration and oxygen radical production correlate with the severity of the acute disease

PÄIVI KOIVURANTA-VAARA,^{1,2} MARJATTA LEIRISALO-REPO,³
AND HEIKKI REPO¹

From the Department of Bacteriology and Immunology, University of Helsinki; the ²Finnish Red Cross Blood Transfusion Service, Helsinki; and the ³Second Department of Medicine, Helsinki University Central Hospital, Finland

SUMMARY Polymorphonuclear leucocyte (PMN) functions (migration in vitro, chemiluminescence, O₂ production, binding of chemotactic peptide, and aggregation) were studied in HLA-B27 positive patients with previous yersinia arthritis (YA). PMNs of patients whose disease had been severe showed chemokinetic and chemiluminescence responses significantly higher than the PMNs of those with a mild disease. The results support the view that enhanced PMN function contributes to inflammatory symptoms in patients with YA.

Key words: HLA-B27, chemotaxis, chemiluminescence, superoxide.

Both reactive arthritis, such as enteroarthritis following an infection caused by *Yersinia enterocolitica*, and ankylosing spondylitis are associated with the histocompatibility antigen HLA-B27.¹ The mechanism of this association and the pathogenesis of the HLA-B27 linked diseases remain unknown, but exaggerated inflammatory responsiveness may be involved.^{2,3} This is supported by the findings that polymorphonuclear leucocytes (PMNs) of HLA-B27 positive subjects, with or without yersinia arthritis (YA), and also those of patients with ankylosing spondylitis show higher chemotaxis in vitro than do PMNs of HLA-B27 negative healthy subjects.^{4,5} In addition, zymosan activated sera from HLA-B27 positive subjects stimulate PMN motility in vitro more than do such sera from HLA-B27 negative subjects.⁶ The relevance of these findings to the in vivo conditions has been confirmed by means of a skin chamber technique.⁷

To see whether a correlation can be found between enhanced PMN responsiveness and the severity of YA we compared PMN functions in two

groups (severe acute disease v mild acute disease) of HLA-B27 positive patients with previous YA.

Patients and methods

PATIENTS

Twenty four HLA-B27 positive patients with previous YA from a follow up study⁸ were assigned to two groups on the basis of their clinical characteristics during the acute phase of the disease (Table 1). The mean intervals between the acute phase of YA and the time of the present study were 8.5 years (range 5-14) in group I and 8.6 years (5-11) in group II. Two patients, one in each group, were studied simultaneously, and the pairs were matched for age and sex (three pairs were matched only for age). At the time of the present study one patient in each group was taking indomethacin (75-100 mg/day), the others were free of symptoms and signs of active disease.

CELLS

Buffy coat cells for the migration assays were separated from heparinised venous blood by dextran sedimentation. PMNs (95-99% pure) for other assays were separated from the buffy coat cells by

Table 1 Clinical characteristics of the patients in group I (severe acute phase) and group II (mild acute phase) during the acute phase of yersinia arthritis†

	Group I‡	Group II‡
Number of patients	12	12
Sex: men/women	6/6	5/7
Age in years at the onset of the disease, mean (range)	30.2 (17-45)	33.7 (22-54)
Number of involved joints, mean (range)	7.1 (1-15)	4.2 (2-13)
Duration of symptoms in months, mean (range)	5.0 (2-12)	3.2 (1-5)
Laboratory findings		
<i>Yersinia enterocolitica</i> agglutinins, geometric mean (range)§	2148 (160-10 240)	1328 (320-10 240)
Highest erythrocyte sedimentation rate, mm/h, mean (range)	101 (41-148)*	54 (16-80)
Highest white blood cell count, 10.0×10 ⁹ /l, mean (range)	12.4 (10.8-16.5)**	7.6 (6.2-10.9)
Extra-articular symptoms		
Total number of patients affected	9*	3
Iritis/conjunctivitis	5	1
Balanitis/pyuria	6	2
Keratoderma blenorrhagica	1	—
Erythema nodosum	—	1

†The patients in group I showed at least two, and the patients in group II only one (or none) of the following clinical characteristics: (a) highest erythrocyte sedimentation rate ≥ 110 mm/h; (b) highest white blood cell count $\geq 10.0 \times 10^9/l$; (c) presence of extra-articular manifestations.

‡The significance of the difference between the two groups is indicated as follows: * $p < 0.005$, ** $p < 0.001$.

§Maximal *Yersinia enterocolitica* agglutinins expressed as reciprocals of dilutions.

density gradient centrifugation⁹ followed by hypotonic lysis of the erythrocytes.

NEUTROPHIL STIMULATING AGENTS

Zymosan activated serum (ZAS) was prepared from pooled normal human serum (NHS) as previously described.¹⁰ *N*-Formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma Chemical Co, St Louis, Missouri. For opsonisation, 20 mg of zymosan particles (Sigma) in 1 ml of NHS were incubated at 37°C for 30 min, washed twice, and resuspended in 1.4 ml of phosphate buffered saline (PBS).

MEMBRANE FILTER ASSAY

The leading front modification¹¹ of the Boyden chamber technique¹² was applied according to Wilkinson.¹³ The attractants and reference solutions were used above and below the filters as follows: HBSS/HBSS (Hanks's balanced salt solution) in studies of spontaneous locomotion, 0.2% HSA/0.2% HSA (human serum albumin, AB Kabi, Stockholm, Sweden), 10⁻⁸ M fMLP/10⁻⁸ M fMLP, 10⁻⁹ M fMLP/10⁻⁹ M fMLP, and 12% ZAS/12% ZAS in studies of chemokinesis, and 0.2% HSA/10⁻⁸ M fMLP, and 3% ZAS/12% ZAS in studies of chemotaxis. All fMLP dilutions were made in HBSS containing 0.2% HSA. The filters were incubated for 45 min at 37°C. The coefficient of variation was 2-18%.

AGAROSE ASSAY

PMN migration under agarose was determined as described earlier.¹⁰ In studies of spontaneous locomotion the agarose medium contained 0.2% HSA. In studies of chemokinesis and chemotaxis the attractants were 10⁻⁸ M and 10⁻⁹ M fMLP and 12% ZAS, and 5×10⁻⁷ M fMLP and ZAS respectively. The coefficient of variation was 5-20%.

CHEMILUMINESCENCE ASSAY

PMNs (2.0×10⁶) in 100 µl of PBS were kept at 37°C for one minute and mixed with 100 µl of 10⁻⁵ M fMLP or 100 µl of zymosan particles, and with 200 µl of the luminol solution (10 µg/ml in PBS; Fluka AG, Buchs SG, Switzerland). The recording periods were 8 min for fMLP and 13 min for zymosan. Both the area under the chemiluminescence curve (cm²) and the height of the curve as millivolts (mV) at one minute intervals were determined.

O₂ PRODUCTION ASSAY

The method of Pick and Mizel¹⁴ was used as described¹⁵ to determine O₂ production of PMNs. fMLP (10⁻⁶, 10⁻⁷, and 10⁻⁸ M) was used as the stimulus. Incubation periods were 15, 30, and 60 min. The coefficient of variation was 7-25%.

f-Met-Leu-(³H)Phe BINDING ASSAY

Binding was assayed largely as described by Williams *et al.*¹⁶ Fifty microlitres of f-Met-Leu-(³H)Phe (New England Nuclear, Boston, Massachusetts;

specific activity 48.3 Ci/mmol (1.787 TBq/mmol)) in 30, 60, 120, 160 nM concentrations, 50 µl of unlabelled fMLP (240 µM), or incubation buffer,¹⁶ and 50 µl of PMNs (90.0×10⁶/ml) were incubated at 37°C for 12 min. Ice cold buffer (2 ml) was added, and the suspensions rapidly filtered through glass fibre filters (Gelman Sciences Inc, Ann Arbor, Michigan). The filters were washed with 10 ml of ice cold buffer, dried, and their radioactivity measured. Specific binding in duplicate filters was calculated by subtracting the non-specific binding (the amount of binding not inhibited by 80 µM unlabelled fMLP) from the total binding of radiolabelled fMLP, and expressed as fmol/10⁶ PMNs. The coefficient of variation was 1–20%.

AGGREGATION ASSAY

A standard platelet aggregometer (Payton Associates, Buffalo, New York) was applied as described by Craddock.¹⁷ Light transmission limits were calibrated with cell suspensions (10.0×10⁶ and 5.0×10⁶ PMNs/ml) of each patient. An aliquot of 0.3 ml of the PMN suspension (10.0×10⁶/ml) was added to a siliconised cuvette containing a siliconised stirring

bar (900 rpm). After two minutes 5 µl of fMLP (10⁻⁵ M) or HBSS was added, and the changes in light transmission were recorded for five minutes. Both the area (cm²) under the curve and the maximal change (cm) in light transmission were determined. All experiments were done in duplicate. The coefficient of variation was 1–10%.

STATISTICAL METHODS

Comparisons of the clinical characteristics between the two groups were performed with Student's *t* test (age, laboratory findings; geometric means were used in comparing *Yersinia enterocolitica* agglutinin titres), χ^2 test with Yates's correction (sex distribution, extra-articular manifestations), Kolmogorov-Smirnov test (duration of symptoms), or Wilcoxon rank sum test (number of involved joints). In all other comparisons the paired sample *t* test was used.

Results

MIGRATION

In the membrane filter assay the group I PMNs showed significantly higher chemokinetic migration in ZAS than did the group II PMNs (Table 2, Fig. 1A). Also the chemotactic migration towards ZAS was higher in group I, but the difference was not significant (*p*<0.10). No clear differences were found

Table 2 The mean distances of spontaneous, chemokinetic, and chemotactic migration in two groups of HLA-B27 positive patients with previous yersinia arthritis. Group I: severe acute phase, group II: mild acute phase

	Distance of migration‡	
	Group I	Group II
<i>Membrane filter assay†</i>	<i>(µm, mean (SD))</i>	
Spontaneous locomotion	18 (3)	17 (2)
<i>Chemokinesis:</i>		
0.2% HSA	51 (5)	51 (8)
12% ZAS§	75 (16)**	61 (12)
10 ⁻⁸ M fMLP	52 (11)	54 (13)
10 ⁻⁹ M fMLP	54 (11)	50 (12)
<i>Chemotaxis:</i>		
12% ZAS	62 (9)	58 (10)
10 ⁻⁸ M fMLP	61 (13)	64 (18)
<i>Agarose assay†</i>	<i>(mm, mean (SD))</i>	
Spontaneous locomotion	1.0 (0.3)	0.9 (0.3)
<i>Chemokinesis:</i>		
12% ZAS	1.4 (0.3)	1.4 (0.4)
10 ⁻⁸ M fMLP§	3.1 (0.6)*	2.9 (0.7)
10 ⁻⁹ M fMLP	1.8 (0.6)	1.7 (0.6)
<i>Chemotaxis:</i>		
ZAS	2.0 (0.5)	1.9 (0.4)
5×10 ⁻⁷ M fMLP	3.3 (0.4)	3.1 (0.6)

†Abbreviations: HSA=human serum albumin; ZAS=zymosan activated serum; fMLP=*N*-formyl-methionyl-leucyl-phenylalanine. ‡The significance of the difference between the two groups (paired sample *t* test) is indicated as follows: **p*<0.05, ***p*<0.01. §Detailed data are presented in Fig. 1.

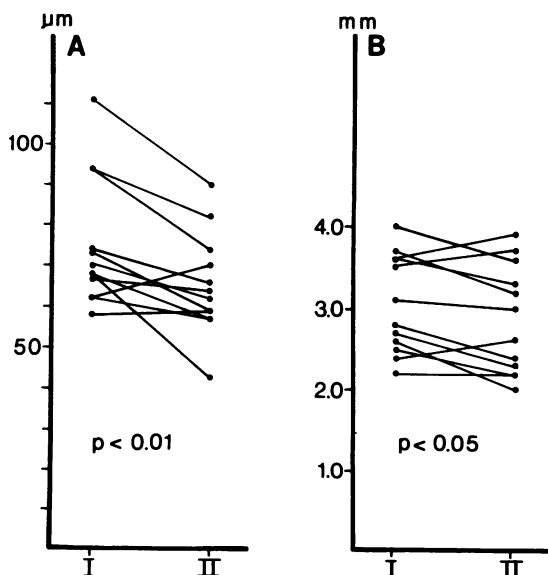


Fig. 1 Chemokinetic migration of PMNs (I, severe acute phase; II, mild acute phase). (A) 12% ZAS in the membrane filter assay; (B) 10⁻⁸ M fMLP in the agarose assay.

in spontaneous locomotion (in HBSS), chemokinetic locomotion (in HSA or fMLP), or chemotactic locomotion induced by fMLP (Table 2).

In the agarose assay the group I PMNs showed higher chemokinetic migration in fMLP at the optimal 10^{-8} M concentration (Table 2, Fig. 1B). Chemotactic migration towards fMLP was higher in nine out of 12 pairs, but the difference was not statistically significant. No major differences were found in spontaneous locomotion, or chemokinetic or chemotactic locomotion induced by ZAS (Table 2).

We also tested the patients' sera for their ability to stimulate chemokinetic locomotion of the cells of one healthy blood donor by means of the membrane filter assay. No significant differences were found between the two groups tested in parallel using fresh sera, heated (56°C, 30 min) sera, ZAS, and heated ZAS (data not shown).

CHEMILUMINESCENCE

The shape of the curves was the same in both groups, with a single peak 1–2 min or 5–7 min after adding fMLP or zymosan respectively (Fig. 2). That only one peak was found with fMLP was probably because the cells had been kept at room temperature for two to three hours before testing.¹⁸ The group I PMNs tended to display higher responses to both zymosan and fMLP (Fig. 2). In studies with zymosan the mean areas (SD) under the curves and the mean heights (SD) of the maximal responses in groups I and II were 52.5 (18.0) and 44.1 (16.9) cm² (Fig. 3A) and 131 (52) and 105 (38) mV respectively. The differences between the two groups were not statistically significant. In studies with fMLP the areas under the curves were significantly larger in group I than in group II ($p < 0.02$, Fig. 3B), the mean areas being 43.0 (15.1) v 30.0 (11.7) cm². The mean

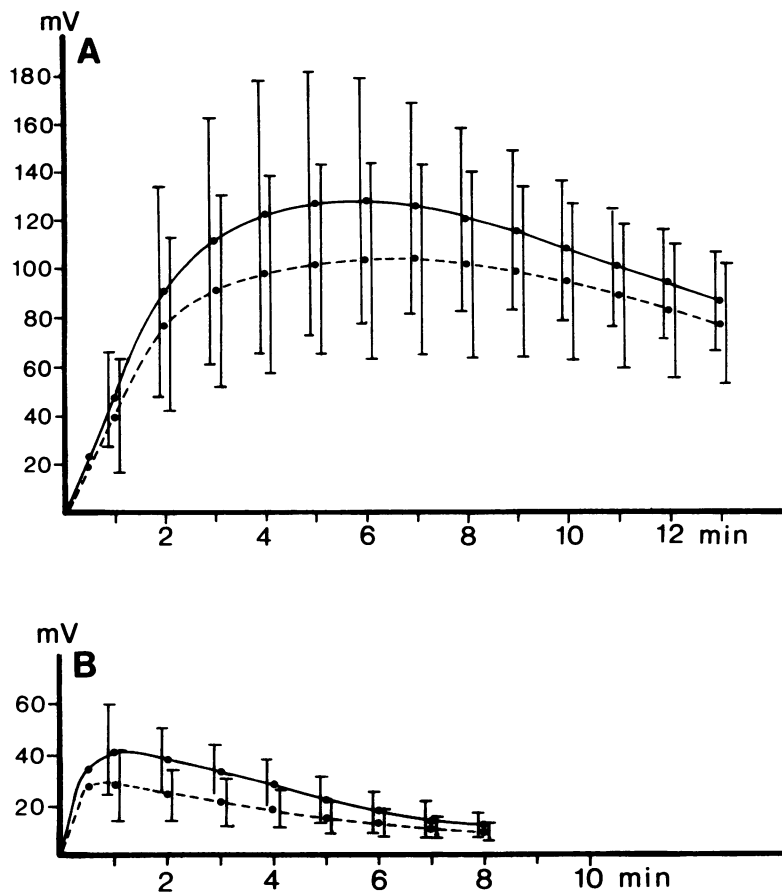


Fig. 2 Chemiluminescence responses of PMNs to (A) zymosan and (B) fMLP. The curves are drawn through points representing the mean response of the whole group ($n=12$ in A; $n=11$ in B) at each time point. Solid line, severe acute phase (group I); dashed line, mild acute phase (group II).

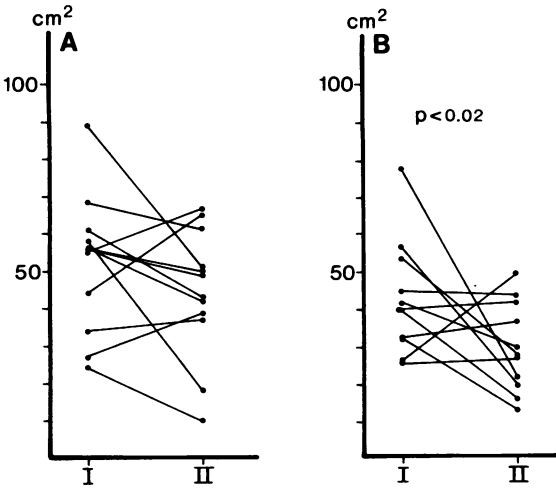


Fig. 3 Chemiluminescence responses (areas under the curves) of PMNs to (A) zymosan and (B) fMLP. I, severe acute phase; II, mild acute phase. In one patient the response to fMLP could not be studied because of a low yield of PMNs.

the two groups, the mean (SD) responses in groups I and II being 24.2 (3.9) and 25.7 (5.7) cm, and 28.2 (5.0) and 28.6 (6.4) cm² respectively.

heights of the maximal responses to fMLP were 44 (20) and 31 (18) mV in groups I and II respectively (not statistically significant).

PRODUCTION OF O₂⁻

The maximum response was achieved with 10⁻⁶ M fMLP during a one hour incubation. There were no statistically significant differences between groups I and II in the maximal responses (mean (SD): 41.1 (26.2) v 50.6 (25.9) nmol/mg of PMN protein respectively), or in the responses recorded with any fMLP concentration at any time point.

f-Met-Leu-(³H)Phe BINDING

The binding reached a plateau between 40 nM and 80 nM fMLP. The amount of f-Met-Leu-(³H)Phe bound at 80 nM was considered to represent the total binding capacity. The PMNs of group I bound somewhat more radiolabelled fMLP (mean (SD): 108 (35) fmol/10⁶ PMNs) than group II PMNs (89 (27)), but the difference was not statistically significant (Fig. 4).

AGGREGATION

The tracings of the aggregation responses to 10⁻⁵ M fMLP were of uniform shape in both groups. They reached a peak in one to two minutes and showed slow disaggregation thereafter. Neither the maximal responses (Fig. 5) nor the areas under the curves showed statistically significant differences between

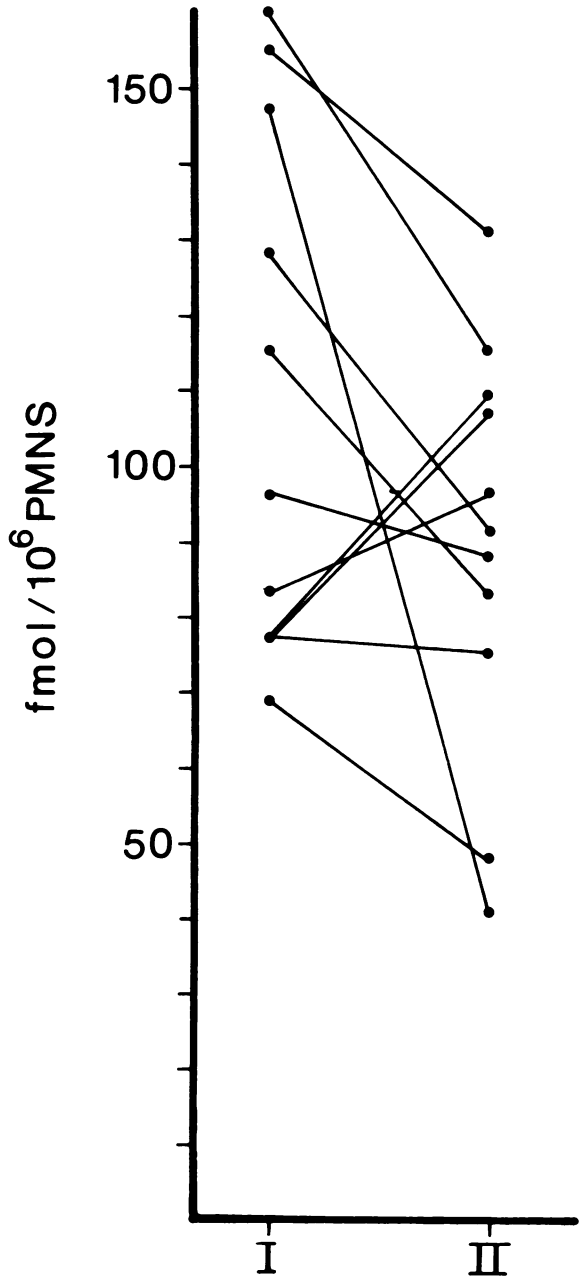


Fig. 4 Specific binding of 80 nM f-Met-Leu-(³H)Phe by PMNs. I, severe acute phase; II, mild acute phase. In one patient, binding could not be studied because of a low yield of PMNs.

Discussion

We have studied several parameters of PMN function in two groups of HLA-B27 positive patients

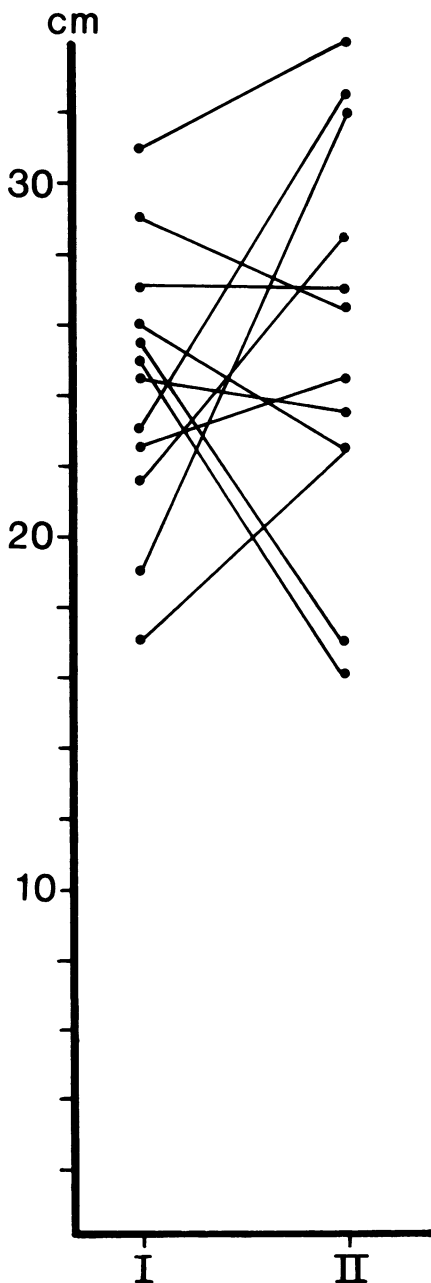


Fig. 5 Maximum aggregation responses of PMNs. I, severe acute phase; II, mild acute phase.

with previous YA. The results indicate that both migratory and oxygen radical producing capacities of PMNs from patients who had had severe YA (high erythrocyte sedimentation rate, high white blood cell count, high prevalence of extra-articular manifestations) were higher than in patients whose disease had been mild.

To migrate under agarose PMNs may have to deform less than in the filter,¹⁹ and their distances of migration are 50- to 100-fold greater. We therefore used the two assays in parallel. Group I PMNs showed enhanced chemokinesis both under agarose and in filter, though towards different attractants. The reason for the discrepancy is not known, but it may involve differences in the character of the two methods.

In the chemiluminescence assay the group I PMNs tended to display higher responses to both zymosan and fMLP, whereas spectrophotometric assessment of O_2^- production showed no differences between the groups. The latter technique, however, detects only reduction of cytochrome *c* sensitive to superoxide dismutase, whereas in the chemiluminescence assay combined actions of O_2^- , hydrogen peroxide, and myeloperoxidase on the oxidation of luminol can be measured.²⁰

The capacity of the cells to bind radiolabelled fMLP can be taken as an estimate of the amount of fMLP receptors on the PMNs. The group I neutrophils bound slightly more fMLP than the group II cells.

We found no difference between the two groups in the aggregation assay, which measures the adherence of PMNs to each other. PMN aggregation and adhesion to surfaces, however, may be separate cell functions,^{21 22} and it might be interesting to compare the groups using an assay measuring the adherence of PMNs to surfaces, such as endothelial cell monolayers.

In conclusion, the results show that among HLA-B27 positive patients the severity of the acute YA correlates with enhanced PMN function. The interval between the acute phase of the disease and the present study was five to 14 years, and thus the cells seem to retain their high responsiveness even in the absence of clinically active disease. At present it cannot be concluded with certainty whether the high PMN responsiveness is genetically determined or secondarily acquired. Still, the exaggerated PMN function may contribute to inflammatory tissue injury in YA³ and, evidently, also in other HLA-B27 associated diseases such as ankylosing spondylitis.²³ It remains to be seen whether the long term prognosis of the patients with enhanced PMN function (group I in the present study) is different from that of group II patients.

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