

Polymorphonuclear leucocyte motility in men with ankylosing spondylitis

C T PEASE, M FENNELL, AND D A BREWERTON

From the Department of Rheumatology, Westminster Hospital, London

SUMMARY The polymorphonuclear leucocyte (PMN) response to a chemotactic or chemokinetic stimulus is enhanced in men with ankylosing spondylitis (AS). This effect does not parallel the severity of disease activity or the size of the acute phase response, and it is independent of non-steroidal anti-inflammatory drug treatment. Polymorph function is normal in HLA-B27 positive brothers of probands with AS and in other HLA-B27 positive individuals in the absence of disease. Polymorph motility is also normal in patients with psoriasis vulgaris or Crohn's disease, indicating that enhanced PMN motility is not a non-specific consequence of all inflammatory disorders.

Key words: chemotaxis.

Healthy individuals with HLA-B27 are more likely to develop ankylosing spondylitis (AS) than B27 negative subjects and are also prone to develop a reactive arthritis after exposure to certain bacteria. The fundamental mechanisms involved for susceptibility to these disorders are unknown.

Previously, it has been reported that the polymorphonuclear leucocyte (PMN) response to certain chemotactic substances is enhanced in men with AS,^{1,2} HLA-B27 positive men with yersinia arthritis,^{3,4} and in healthy HLA-B27 positive controls.¹⁻³ Thus polymorphs from HLA-B27 positive individuals may inherit an enhanced ability to respond to certain chemotactic stimuli. It has been proposed that such hyperresponsive PMNs would accumulate in large numbers at sites of inflammation and may be responsible for an excessive inflammatory response.⁵ Clinically, patients with either yersinia arthritis or sexually acquired reactive arthritis tend to have more severe disease if they are HLA-B27 positive.^{6,7} The situation is less clear with AS because of the difficulty in recognition and diagnosis of mild cases, but it is known that acute uveitis occurs less frequently in patients with AS who do not have B27.⁸ The purpose of this study was to investigate further the relation between PMN migration, HLA-B27, and AS.

Subjects and methods

PATIENTS AND CONTROLS

Forty men with classical AS were investigated. Thirty seven were HLA-B27 positive, three B27 negative. Their ages ranged from 22 to 72 years (mean 43.5) and duration of disease ranged from three to 52 years (mean 19). Drug treatment was not discontinued before the investigation. At the time of testing no patient or control had symptoms or signs of an acute bacterial or viral infection.

Seventeen men with AS attending an outpatient clinic at Westminster Hospital had healthy brothers willing to participate. All of the probands and 10 of the 17 brothers were B27 positive. The probands and controls were examined, and the ranges of chest expansion and spinal movement recorded. Three of the probands with AS had HLA identical brothers, two of whom on examination were proved to have AS. Thus these two pairs were analysed separately. The remaining 15 healthy brothers had no symptoms to suggest AS or uveitis, and examination was normal. An x ray examination of the spine was not performed routinely. Two brothers had been x rayed previously; the films were reviewed and found to be normal. The mean age of the probands was 43 years (range 22–61) and of the healthy brothers 43 years (18–64). Each pair was tested concurrently with a control matched for age and sex. Haemoglobin (Hb), erythrocyte sedimentation rate (ESR), and C reactive protein (CRP) values differed

significantly between probands and brothers. The probands had the following values: Hb 109–162 g/l (mean 146 g/l), ESR 2–49 mm/h (mean 16 mm/h), CRP 6–50 mg/l (mean 15 mg/l); and the values for the brothers were Hb 135–178 g/l (mean 158 g/l), ESR 1–6 mm/h (mean 2 mm/h), CRP—all values below 6 mg/l. Ten healthy HLA-B27 positive individuals with neither history nor signs of AS or family history of AS were used as an additional control group for the brother study.

The B27 negative controls were members of staff of St Stephen's and Westminster Hospitals. They were selected using the following criteria: male, aged between 20 and 60 years, no joint disease, no family history of AS, and no current drug treatment. All subjects were tissue typed, and only those without HLA-B27 were included in the investigation.

DISEASE ACTIVITY

Each subject's disease activity was assessed at every visit. The amount of back pain was recorded on a four point scale; peripheral joint involvement was noted and graded on a similar scale; the duration of early morning stiffness was recorded in minutes; and the need for drug treatment and its effectiveness were recorded. From this data, and from previous knowledge of the patient, the activity of disease was classified in three groups: 1. Active—Significant trunk pain or active peripheral arthritis with morning stiffness in excess of one hour. Regular drug treatment required. 2. Probably active—Patients in whom an acute episode had settled incompletely or in whom significant morning stiffness worsened if drug treatment was discontinued. 3. Inactive—Symptomless, or mild back pain with less than 20 minutes' morning stiffness. Drugs taken irregularly or not at all.

Blood was taken at each visit for ESR and CRP estimations, but these values were not used in the clinical assessment of disease activity.

METHODS

Polymorphonuclear leucocytes were separated from heparinised blood by a Hypaque-Ficoll separation procedure.⁹ Cells were washed three times with minimum essential medium (MEM) supplemented with 1% albumin and were then resuspended in MEM at a concentration of 3.2×10^7 PMNs/ml. After the final wash PMN viability was approximately 98% when measured by trypan blue dye exclusion.

Agarose plate method

The procedure followed that described by Repo.¹⁰ Two wells were cut 2.5 mm apart in an agarose gel with a template and a 3 mm skin biopsy punch (Steifel Lab (UK), Slough, UK). To assess unstimu-

lated migration 2.5×10^{-3} PMNs suspended in MEM were placed in one well and MEM in the opposite well. To measure directed PMN migration 7 μ l *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) at a concentration of 10^{-7} mol/l or 7 μ l zymosan activated pooled serum (ZAS=90% serum+zymosan suspension 10 mg/ml) was placed opposite the cell well. Plates were incubated in a humidified incubator at 37°C for two and a half hours and then the plates were fixed and stained. The leading two cell distance was measured. Each experiment was performed in triplicate.

Microdroplet technique

The microdroplet technique for measuring PMN motility has been described by McCoy *at al.*¹¹ Polymorphs obtained from heparinised venous blood were suspended in MEM at a concentration of 3.2×10^7 PMN/ml of MEM. Agarose (Marine Colloids Inc) at a concentration of 0.8% w/v was autoclaved, and while still molten, placed in a water bath at 37°C. An equivalent volume of double strength MEM+1% human serum albumin was added to the agarose. Equal volumes of the agarose-MEM mixture and of the PMN suspension were mixed and 2 μ l droplets of the mixture placed in the centre of a cooled 96 well microtitre plate (non-tissue cultured—Sterilin). The droplets required a few minutes to solidify, after which 100 μ l of MEM or the test substance (FMLP/serum/ZAS) was carefully added to each well. Use of a 96 well microtitre plate meant that five concentrations of each substance could be tested simultaneously. The plates were incubated at 37°C for three hours and then transferred to a fridge at 4°C until read. Cell migration was assessed by measuring the distance of the outermost cell from the edge of the droplet in four directions, each experiment being performed in duplicate.

STATISTICS

The data were analysed by the Wilcoxon rank sum test.

Results

POLYMORPH MOTILITY IN AS

Blood was obtained from 30 HLA-B27 positive men with AS and 30 B27 negative controls matched for age and sex, and their PMN motility was compared using the agarose plate techniques. The results for unstimulated polymorph motility (random) were similar both for men with AS and control subjects. Directed polymorph motility, stimulated by FMLP or ZAS, was significantly increased in patients with

AS compared with the control group (Wilcoxon $p < 0.001$ and $p < 0.02$ respectively). The mean (SD) results obtained for men with AS were unstimulated migration 1.02 (0.32) mm; FMLP 2.23 (0.43) mm; ZAS 1.56 (0.4) mm. The results for control subjects were unstimulated migration 0.93 (0.24) mm; FMLP 1.95 (0.5) mm; ZAS 1.41 (0.31) mm.

Assessment of PMN migration towards FMLP, by use of the chemotactic differential (directed motility minus unstimulated motility), gave similar results (Wilcoxon $p = 0.001$). Polymorph motility results for the three B27 negative patients with AS were similar to those obtained for the B27 positive patients (results not presented).

EFFECT OF AS DISEASE ACTIVITY ON PMN MOTILITY

Polymorph motility results, obtained using the agarose plate technique, for 20 patients with active AS were compared with those obtained for 18 patients with clinically inactive AS. No significant difference in PMN motility was recorded for unstimulated migration or for the response to ZAS or FMLP. Yet when the results for patients with either active AS or inactive AS were compared with the results for the controls, who had been tested concurrently, a significant difference was observed in response to either ZAS ($p < 0.05$, $p < 0.05$ respectively) or FMLP ($p < 0.02$, $p = 0.001$ respectively). The mean (SD) results were unstimulated migration—active AS 1.02 (0.36) mm, control 0.90 (0.25) mm; inactive AS 0.89 (0.19) mm, control 0.82 (0.18) mm. ZAS results—active AS 1.52 (0.44) mm, control 1.32 (0.33) mm; inactive AS 1.5 (0.46) mm, control 1.33 (0.35) mm. Figure 1 shows the results for FMLP.

Retrospectively, the agarose plate results for each patient with AS were matched with their CRP results obtained on the same day. This enabled us to compare the PMN response to FMLP from 10 patients with active AS and high CRP concentrations and 10 patients with inactive AS and normal CRP concentrations. No correlation could be observed between the CRP concentration and PMN responses to FMLP (data not illustrated).

POLYMORPH MOTILITY IN RELATION TO DRUG TREATMENT

To assess whether non-steroidal anti-inflammatory drugs taken orally could influence PMN motility in vitro PMN migration was measured using the agarose plate technique before and immediately after a two week course of naproxen (500 mg twice a day) in 11 men with AS. No drugs, except paracetamol, were permitted for one week before the drug trial. In men with AS both unstimulated and

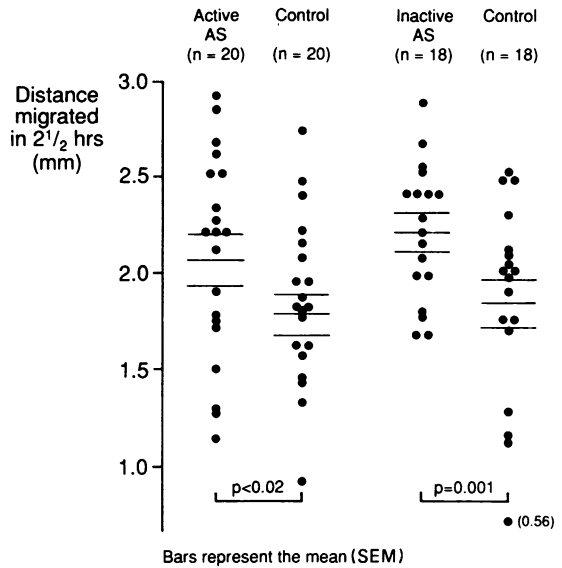


Fig. 1 Polymorph motility results for men with active or inactive ankylosing spondylitis (AS) compared with results obtained for the B27 negative controls. The method used was the agarose plate technique and the chemoattractant was *N*-formyl-methionyl-leucyl-phenylalanine (10^{-7} mol/l).

directed polymorph motility were unaffected by two weeks of oral naproxen. The results for the control population, who had been tested in parallel with the AS group, but who had not received non-steroidal anti-inflammatory drug treatment, were similar on both occasions.

Baseline results (mean (SD)): unstimulated migration—AS 0.95 (0.18) mm, control 1.03 (0.26) mm; FMLP—AS 2.23 (0.32) mm, control 2.12 (0.45) mm; ZAS—AS 1.47 (0.32) mm, control 1.51 (0.23) mm. Results after two weeks: unstimulated migration—AS 0.98 (0.18) mm, control 0.9 (0.2) mm; FMLP—AS 2.08 (0.27) mm, control 1.88 (0.29) mm; ZAS—AS 1.35 (0.34) mm, control 1.36 (0.36) mm.

THE BROTHER STUDY

The agarose plate technique was used to assess PMN motility in 15 HLA-B27 positive men with AS, their healthy brothers (eight HLA-B27 positive), 15 unrelated B27 negative controls, and, in addition, 20 other controls, of whom half were B27 positive.

Unstimulated PMN motility was similar in all groups. The mean (SD) results for men with AS were 0.86 (0.22) mm, healthy brothers 0.79

(0.23) mm, B27 negative controls 0.84 (0.22) mm, and in the second control group of 10 B27 positive controls 0.63 (0.14) mm and 10 B27 negative controls 0.66 (0.21) mm.

Figure 2 shows the results obtained for migration towards FMLP (10^{-7} mol/l). A significant difference was noted between the probands with AS and the group of 15 brothers (Wilcoxon $p < 0.02$). No statistical difference was found to exist between the B27 positive or B27 negative brothers or controls. The results for PMN migration towards ZAS were similar, with a significant difference noted between the probands with AS and the 15 brothers (Wilcoxon $p = 0.003$). The mean (SD) results for migration to ZAS were AS men 1.48 (0.43) mm, brothers 1.24 (0.4) mm, B27 negative controls 1.28 (0.37) mm,

and in the second control group B27 positive controls 1.12 (0.32), B27 negative controls 1.09 (0.28) mm.

SERUM EXPERIMENTS

Preincubation of PMNs with serum

Polymorphs from nine patients with AS and nine controls were preincubated for 30 minutes with MEM, 10% AS serum, or control serum before the standard agarose plate chemotaxis assay. Unstimulated PMN motility was not affected by preincubation with serum (Table 1). Polymorph migration towards either FMLP or ZAS was depressed after preincubation of PMNs in serum (either homologous or autologous) when compared with the results obtained after preincubation in MEM alone. Preincubation of

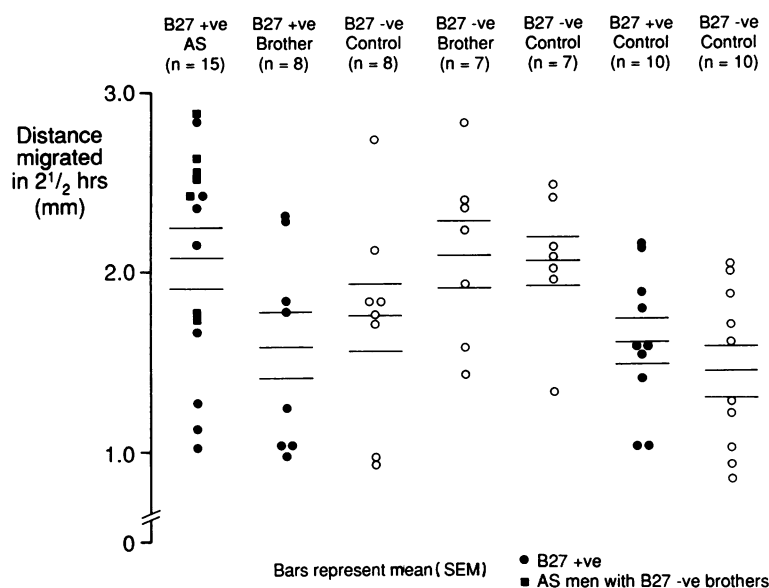


Fig. 2 Polymorph motility results for men with ankylosing spondylitis (AS), their healthy brothers (HLA B27 positive and B27 negative), and B27 positive controls compared with the results obtained for B27 negative controls matched for age and sex. The method used was the agarose plate technique and the chemoattractant was *N*-formyl-methionyl-leucyl-phenylalanine (10^{-7} mol/l).

Table 1 Effect on polymorph migration of preincubation for 30 minutes in 10% control serum or 10% serum from men with ankylosing spondylitis and comparison with the results of preincubation in minimum essential medium alone. * Results are mean (SD)

| | AS PMNs† (n=9) preincubation with: | | | Control PMNs (n=9) preincubation with: | | |
|---|---------------------------------------|-------------|---------------|---|-------------|---------------|
| | MEM† | AS serum | Control serum | MEM | AS serum | Control serum |
| Unstimulated migration (mm) | 0.82 (0.15) | 0.88 (0.3) | 0.92 (0.35) | 0.87 (0.28) | 0.82 (0.33) | 0.80 (0.33) |
| Zymosan activated sera (90% sera+zymosan) (mm) | 1.47 (0.4) | 1.29 (0.38) | 1.29 (0.48) | 1.35 (0.42) | 1.04 (0.42) | 1.2 (0.47) |
| FMLP† (10^{-7} mol/l) (mm) | 2.12 (0.54) | 1.87 (0.63) | 1.81 (0.72) | 1.85 (0.69) | 1.66 (0.67) | 1.6 (0.69) |

*The polymorph motility was measured by the agarose plate technique.

†AS=ankylosing spondylitis; PMNs=polymorphonuclear leucocytes; MEM=minimum essential medium; FMLP=*N*-formyl-methionyl-leucyl-phenylalanine.

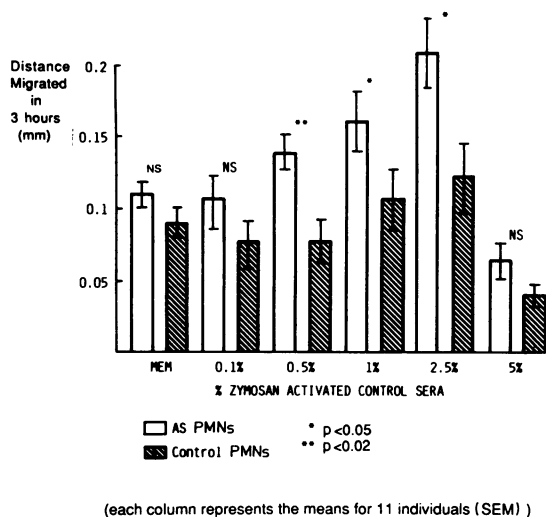


Fig. 3 Comparison of the results obtained with the microdroplet technique for polymorphs from men with ankylosing spondylitis (AS) and from B27 negative controls. Zymosan activated control serum was used as the chemoattractant. MEM=minimum essential medium.

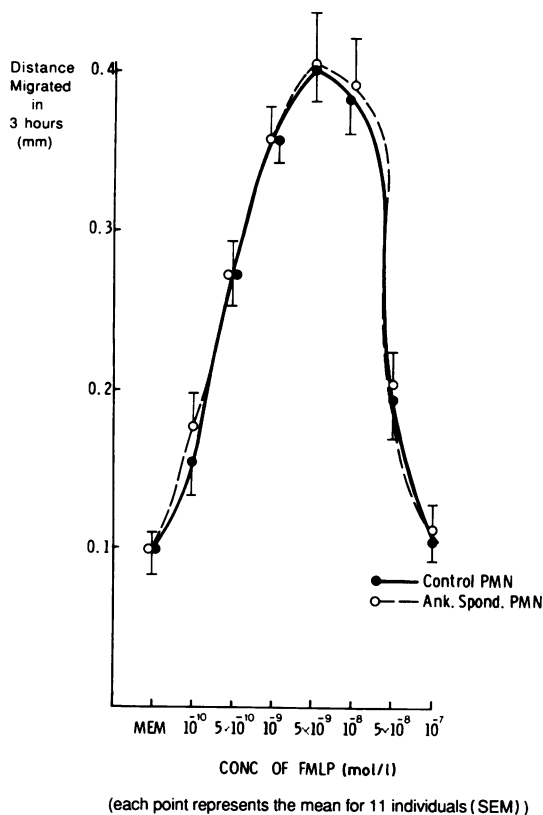


Fig. 4 Comparison of the results obtained with the microdroplet technique for polymorphs from men with ankylosing spondylitis and from B27 negative controls. N-formyl-methionyl-leucyl-phenylalanine (FMLP) was used as the chemoattractant.

control PMNs in AS sera or control sera did not enhance the PMN response to either FMLP or ZAS.

Chemokinetic PMN motility in patients with AS and in controls

The PMN response to different uniform concentrations of serum, ZAS, or FMLP was studied in 11 men with AS and 11 controls using the microdroplet technique. When unactivated serum was used in the supernate AS cells were more motile than control PMNs, but only at a 1% serum dilution ($p < 0.05$) (date not illustrated). This result was, therefore, of doubtful significance. Figure 3 illustrates the results for zymosan activated sera. The distances migrated by AS cells were significantly greater than control PMNs for several different concentrations of ZAS. But the AS and control PMN responses to seven different concentrations of FMLP were identical (Fig. 4).

Discussion

This study has shown that the PMN response to two chemotactic substances is enhanced in men with AS. In contrast with earlier reports,¹⁻³ this exaggerated PMN response was not found in healthy B27 positive brothers of men with AS or in a second

group of unrelated healthy B27 positive people. This PMN response was independent of disease activity, unrelated to the size of the acute phase response, and unaffected by non-steroidal anti-inflammatory drug consumption. In both this and our earlier studies^{1,2} the PMN response to a chemotactic substance was increased in HLA-B27 positive men with AS. This last result, plus the normal results obtained in the B27 positive control population, make a direct association between enhanced PMN motility and HLA-B27 less likely. It is possible that a genetic factor other than B27 may be associated with the enhanced PMN response seen in B27 positive men with AS. In two separate studies involving patients with Crohn's disease¹² and psoriasis vulgaris,¹³ however, two diseases commonly associated with the development of AS in B27 positive patients, no abnormality of PMN motility was apparent.

Enhanced directed PMN motility is thus likely to occur as a consequence of the disease state and not as a genetically determined response. The lack of any association between PMN migration and disease activity in patients with either AS, Crohn's disease,¹² or psoriasis vulgaris¹³ shows that PMN hypermotility is not a non-specific response common to all inflammatory disorders. Preincubation of PMNs with either AS or control sera did not enhance PMN motility: in both situations migration was mildly impaired. This impairment of motility is more likely to be due to enhanced PMN adhesion than to deactivation of PMN responsiveness to C_{5a} because PMN migration to FMLP was similarly impaired. No serum factor capable of enhancing PMN migration was detected. Enhanced PMN migration is therefore a cellular phenomenon and not a serum mediated event. In contrast with our results, Repo *et al* reported both a cellular and a serum abnormality in B27 positive individuals.¹⁴

In a uniform concentration of FMLP the PMNs from patients with AS or from controls responded in an identical fashion to all the concentrations tested (Fig. 4). Yet, under the same experimental conditions on the same 96 well plate marked differences in migration were observed in response to ZAS. Even at low concentrations of ZAS the AS PMNs were more motile than those of the controls (Fig. 3). The AS PMN response to FMLP appears to depend on the experimental technique used to measure it. When exposed to a gradient of FMLP (on the agarose plate) the AS PMN response is enhanced in comparison with the control PMN result, whereas in a uniform concentration of FMLP (as on the microdroplet plate) it is identical to the control result. One explanation for this paradox may be that the different experimental techniques measure different aspects of PMN motility. This would not explain, however, why AS PMN motility is enhanced in comparison with the control result when exposed to either a gradient or uniform concentration of ZAS. Alternatively, the differing response of AS PMNs to ZAS and FMLP may represent an abnormality at the cellular level. No single receptor on the PMN appears to be crucial in inducing this response. Enhanced PMN migration is a phenomenon which can be elicited by several different chemotactic stimuli—namely, FMLP, ZAS—or by the use of chemotactic factors liberated by live bacteria.¹² An investigation of the number and sensitivity of receptors on AS PMNs for C_{5a} or FMLP would be of interest.

In contrast with our results, Mowat and Baum did not detect any abnormality in PMN function in either 14 patients with AS or 10 with Reiter's disease.¹⁵ Similarly, Baum reported that PMN

motility was normal in five patients with AS.¹⁶ Both these studies used the Boyden chamber technique, with a long incubation period, and PMNs were suspended in 50% plasma as opposed to MEM.^{15 16} These technical differences make it difficult to compare our results with theirs. Results similar to ours have been reported in yersinia arthritis^{3 4} and Behçet's disease,^{17 18} yet this phenomenon has not been reported in rheumatoid arthritis,^{15 19} systemic lupus erythematosus,²⁰ or scleroderma.²¹ Behçet's disease, though not associated with HLA-B27, is associated with HLA-Bw51.²² An enhanced PMN response to a chemotactic substance has been reported to occur as a temporary phenomenon in several different acute infections.^{23 24} The evidence to support a bacterial or viral infection in the pathogenesis of AS remains controversial, however.²⁵ In reactive arthritis the finding by Keat *et al* of chlamydial elementary bodies in synovium from patients with sexually acquired reactive arthritis, and the persistence of IgA anti-yersinia antibodies in serum of patients with yersinia arthritis,^{26 27} suggest that bacterial products may persist for much longer than previously imagined. Investigation of PMN motility in patients with overt chronic sepsis would be valuable. Polymorphs from patients with AS, Reiter's disease,¹² or yersinia arthritis³ demonstrate a small but definite increase in motility in response to stimulation by chemotactic factors when compared with PMNs from the control population. This abnormality of PMN function is not part of a generalised PMN defect because PMN adherence, phagocytosis, and superoxide production seem unaffected.^{2 28} The normal results obtained for healthy B27 positive siblings of probands with AS indicate this PMN abnormality is likely to be acquired and not 'inherited'. We could not confirm an association between PMN function and HLA-B27. The lack of any correlation between disease activity, disease severity, or the magnitude of the acute phase response and the PMN response to the chemotactic factors implies that this phenomenon is not a consequence of an inflammatory state but a direct product of the disease process.

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