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Neutrophil functions, spondylarthropathies and HLA-B27: a study of 43 patients

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ABSTRACT. **Objectives**. *Several hypotheses have been proposed regarding the role of HLA-B27 antigen in the pathogenesis of the spondylarthropathies.*

Methods. *We studied some neutrophil functions* in vivo *in patients affected by ankylosing spondylitis or by reactive arthritis, with or without HLA-B27, and in healthy control subjects.* In vivo *neutrophil migration was investigated by Senn's skin window technique. An adhesion assay was also conducted and superoxide production was measured in circulating and migrating neutrophils after different stimuli.*

Results. *Neutrophil migration* in vivo *was higher in the HLA-B27 positive patients than in the controls, while no difference was found between the HLA-B27 negative patients and controls. Our data showed an increased response to formyl-methionyl-leucyl-phenylalanine by circulating neutrophils in the patients with ankylosing spondylitis, both HLA-B27 positive and negative, in comparison with all the other subjects.*

Conclusions. *Our results revive the question of the role of HLA-B27 in the regulation of neutrophil migration; the reported* in vivo *priming of circulating neutrophils seems to be related to ankylosing spondylitis rather than to HLA-B27.*

Key words: neutrophil, spondylarthropathies, HLA-B27.

Introduction

Several hypotheses have been proposed regarding the role of HLA-B27 antigen in the pathogenesis of spondylarthropathies. Some authors have pointed out that stimulated polymorphonuclear neutrophil (PMN) chemotaxis is enhanced in HLA-B27 positive patients with ankylosing spondylitis (AS) or Yersinia reactive arthritis (1-5). On these grounds it has been suggested that an increased PMN accumulation at the sites of inflammation may amplify the injury to osteotendinous structures typical of spondylarthropathies (6). However, the literature reports discordant data about PMN chemotaxis in HLA-B27 positive healthy subjects (2-4).

Different results have also been reported in studies on superoxide production from circulating PMN in patients with spondyloarthropathies. Some authors (7-10) detected no difference in superoxide production between PMN from B27 positive and negative subjects when stimulated by formyl-methionyl-leucyl-phenylalanine (fMLP). El Abbouyi *et al.* (11) reported a low O_2-production in response to opsonized zymozan in the PMN from patients with AS; in this study, however, the patients were not selected on the basis of the presence of HLA-B27. Finally, Wendling *et al.* (12) reported an increase in the phagocyte oxidative metabolism functioning of the PMN after stimulus with fMLP and opsonized zymozan in patients suffering from AS, compared with controls. There was no difference between HLA-B27 positive and negative patients.

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We studied some PMN functions *in vivo* in patients affected by AS or by reactive arthritis with or without HLA-B27, and in healthy control subjects. *In vivo* PMN migration was investigated by Senn's skin window technique; an adhesion assay and a determination of superoxide production after different stimulations of the circulating and migrating PMN were also performed.

The goal of this work was to study PMN migration *in vivo,* and PMN activity in cells from blood and from an experimental inflammatory exudate to elucidate their possible relationship with either the HLA-B27 antigen or the disease.

Subjects and methods

Patients and controls. We studied: 14 HLA-B27 positive patients affected by AS (11 M/ 3 F, mean age 30.1 ± 8.6 yrs, range 20 - 50 yrs); 10 HLA-B27 positive patients affected by reactive arthritis (8 M/ 2 F, mean age 33.5 ± 12.5 yrs, range 20 - 52 yrs); 9 HLA-B27 negative patients affected by AS (7 M/ 2 F, mean age 35.3 ± 7.4 yrs, range 25 - 47 yrs); 10 HLA-B27 negative patients affected by reactive arthritis (7 M/ 3 F, mean age 33.3 ± 6.7 yrs, range 23 - 43 yrs); and 20 sex- and age-matched control subjects. The patients were evaluated during a 4-year period (1989-1993) at the Institute of Special Medical Pathology of the University of Verona. All the patients were in an active phase of their disease; the PMN study was carried out during the diagnostic exam after an adequate pharmacological wash out.

All the patients fulfilled the classification criteria for spondylarthropathies (13). The diagnosis of AS was made according to the modified 1984 New York criteria (14). The reactive arthritis group included 4 subjects with classic Reiter's syndrome (2 HLA-B27 positive and 2 negative), 4 subjects with arthritis following Salmonella infection (1 HLA-B27 positive and 3 negative), 3 subjects with arthritis following Campylobacter infection (1 HLA-B27 positive and 2 negative), 2 subjects with arthritis following Yersinia infection (HLA-B27 positive) and 7 subjects with arthritis following Chlamydia infection (4 HLA-B27 positive and 3 negative).

Salmonella, Campylobacter and Yersinia were cultivated from the stools and Chlamydia from smears of patients suffering from reactive arthritis.

The spondylarthropathy was considered to be active based on the following criteria: persistent low back pain, functional limitation of the spine for AS (by cervical flexion, thoraco-lumbar flexion-distention from C-7 to the iliac crest, Schober's test and lateral-flexion); peripheral arthritis; an erythrocyte sedimentation rate higher than 30 mm/h; and C reactive protein higher than 1 mg/dl for reactive arthritis.

HLA typing was not determined in healthy control subjects.

Reagents. The chemotactic peptide fMLP, zymosan and phorbol myristate acetate (PMA) were purchased from Sigma Chemical Company (St. Louis); cytocrome C from Boerhringer (Mannheim, Germany); and the purified serum bovine albumin and human albumin from the Behring Institut (Marburg, Germany). Percoll was from Pharmacia, Uppsala. Sterile 96-well flat-bottomed microtiter plates (Linbro type) were from Flow Laboratories. Hank's balanced salt solution (HBSS) and reagents were of the highest purity available.

Zymosan, opsonized with a pool of normal human sera (STZ) as described by Metcalf *et al.* (15), was stored in aliquots at -20°C. In order to avoid contamination, a possible cause of artifactual activation or priming of the cells, sterile apyrogenic solutions and disposable plasticware were used in all experiments, which were carried out whenever possible under a laminar flow hood. Reagents were prepared using pyrogen-free water or 0.9% NaCl solutions.

Cell preparation. Neutrophils were obtained from blood and from skin window exudates of subjects who had given their informed consent. Blood neutrophils were prepared from 40 ml of ethylene diamine tetraacetate-anticoagulated blood by centrifugation over Percoll gradients (15). Cells were finally suspended in HBSS containing 5 mM glucose and 0.2% human serum albumin (HGA) and kept at room temperature until used. A few minutes before use, 100x concentrated solutions of $CaCl_2$ and $MgSO_4$ were added to the cell suspensions to the final concentration of 0.5 mM and 1 mM respectively.

Exudate neutrophils were isolated according to the method described by Senn (16), with modifications (17). The volar surface of the nondominant forearm was disinfected with ether and an abrasion of 1 cm^2 was obtained with a rotating sterile abrasive cylinder operated by a milling cutter (minidrill, Saint Julien en Genevois, France).

The abrasion did not cause bleeding as only the epidermis was removed and the wet, transuding surface of the derma was exposed. A bell-shaped, sterile, disposable plastic skin chamber with a circular adhesive base (FAR Italia, Verona, Italy) was placed on the skin abrasion and fixed with fenestrated sticking plaster. The chamber has a 5 mm-wide hole at the top equipped with a plug. One ml of autologous serum was then injected into the chamber and twenty-four hours later the exudate was collected by aspiration. The exudate cells were then centrifuged at 1200 rpm, washed twice with phosphate buffered saline (PBS) and finally suspended in HGA and kept at room temperature until used. Before use, the cell suspensions were supplemented with $CaCl_2$ and $MgSO_4$ as described above for the blood cells.

Oxidative metabolism assay. Superoxide anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome C (18) in a microplate assay system. The microplate assay for O_2- production was performed according to previously published methods (19), with the following modifications. The wells were coated with fetal bovine serum as described (19), and then were supplemented with 25 µl of 0.6 mM cytochrome C in HGA containing 0.5 mM $CaCl_2$ and 1 mM $MgSO_4$ (HGACM) and with 25 µl of the stimulants diluted in HGACM at a concentration exactly 4 times higher than that required in the assay. This was done because each agent was diluted 4 times in the final incubation mixture (see below). Each plate was then brought to 37°C, and 50 µl of the neutrophil suspension (2 x 10^5 cells), pre-warmed at 37°C, were added to each well using a multichannel pipette. The plates were incubated at 37°C for the required time. The plates were then rapidly transferred into a microplate reader (Reader 400 SLT Labs Instruments) and the reduction of cytochrome C was measured at 550 nm using 540 nm

as the reference wavelength and 0.037 optical density units as the standard for 1 nmole of reduced cytochrome C (19).

Adhesion assay. For the adhesion measurements, the plates were transferred to an automatic washer (Easy Washer 2, SLT Lab Instruments) and subjected to two washing cycles with PBS at room temperature. The adherent cells were quantitated by measuring the membrane enzyme acid phosphatase, and the percentage of adhesion was calculated on the basis of a standard curve obtained with known numbers of neutrophils (19).

Statistics. Statistical analysis was carried out using the non-parametric Wilcoxon test.

Results

Table I shows the results for PMN migration in patients and in controls. The migration was 66.12 ± 6.45 x 10^6 PMN/cm^2/24 hrs in healthy subjects; in HLA-B27 positive patients the migration was higher: 93.04 ± 19.13 x 10^6 PMN/cm^2/24 hrs in patients with AS, and 86.13 ± 8.5 x 10^6 PMN/cm2/24 hrs in patients affected by reactive arthritis ($p < 0.001$ for both groups versus controls). In HLA-B27 negative patients the migration was not different from the controls.

Table II reports data on superoxide production by circulating and migrating neutrophils, under basal conditions and after specific stimuli (fMLP, STZ or PMA). Exudate cells from both controls and patient groups were primed to the response to fMLP but not to the response to STZ or PMA. The factor specificity of the priming phenomenon has been previously noted by others (20) and by us (17, 21). Circulating neutrophils from patients with AS (both HLA-B27 positive and negative) showed O_2- production after fMLP to be higher than in the controls. The other values were not different from the control subjects.

Table III shows the results for adhesion of circulating and migrating neutrophils under basal conditions and after stimuli. There was no difference between normal subjects and patients.

Table I. *In vivo* neutrophil migration (x 10^6 neutrophils/cm^2/24 hours) in controls and in patients affected by ankylosing spondylitis (AS) or reactive arthritis (ReA). Means were compared by the non-parametric Wilcoxon test.

Controls	66.12 ± 6.45	
AS HLA-B27+	93.04 ± 19.13	$p < 0.001$
AS HLA-B27-	62.5 ± 5.82	p = NS
ReA HLA-B27+	86.13 ± 8.5	$p < 0.001$
ReA HLA-B27-	61.27 ± 5.24	p = NS

Table II. O_2- production (nmoles/10 minutes/10^6 cells) by resting and fMLP-, STZ- and PMA-stimulated neutrophils from blood (B) and skin window exudates (SW) in controls and in patients affected by ankylosing spondylitis (AS) or reactive arthritis (ReA). Means were compared by the non-parametric Wilcoxon test.

	None	fMLP (10^{-7} M)	STZ (0.1 mg/ml)	PMA (10 ng/ml)
Controls				
B	0.6 ± 0.5	7.8 ± 1.7	10.1 ± 3.5	23.5 ± 5.2
SW	0.8 ± 0.6	21.9 ± 5.2	10.3 ± 3.4	24.4 ± 5.6
AS HLA-B27+				
B	0.6 ± 0.6	11.9 ± 2.8*	10.7 ± 3.7	22.3 ± 4.4
SW	0.8 ± 0.6	24.5 ± 6.1	10.7 ± 3.4	23.9 ± 3.9
AS HLA-B27-				
B	0.4 ± 0.5	11.5 ± 1.9*	9.3 ± 2.2	23.4 ± 2.3
SW	0.7 ± 0.5	21.9 ± 3.5	9.5 ± 2.3	23.9 ± 2.2
ReA HLA-B27+				
B	0.4 ± 0.5	6.9 ± 2	9.4 ± 3.6	22.4 ± 2.3
SW	0.7 ± 0.3	20.3 ± 7.9	9.7 ± 1.9	22.8 ± 3.1
ReA HLA-B27-				
B	0.5 ± 0.5	7.2 ± 2.1	9.1 ± 2.1	23.1 ± 1.6
SW	0.5 ± 0.4	19.6 ± 5.4	9.3 ± 2.6	22.7 ± 2.1

*$p < 0.001$

Table III. Adhesion (% of total cells) by resting and by fMLP-, STZ- and PMA-stimulated neutrophils from blood (B) and skin window exudates (SW) in controls and in patients affected by ankylosing spondylitis (AS) or reactive arthritis (ReA). Means were compared by the non-parametric Wilcoxon test.

	Resting	fMLP (10^{-7} M)	STZ (0.1 mg/ml)	PMA (10 ng/ml)
Controls				
B	5.6 ± 3	21.4 ± 5.8	21.8 ± 8.2	51.8 ± 3.8
SW	13.4 ± 6.9	25.3 ± 7.7	27.1 ± 9.4	51.6 ± 4.8
AS HLA-B27+				
B	5.8 ± 3	23.2 ± 7.2	21.7 ± 4.9	51.3 ± 2.7
SW	12.6 ± 5.1	28.2 ± 6.9	26.9 ± 4.9	51 ± 3.9
AS HLA-B27-				
B	5.26 ± 2.9	21.9 ± 3.5	21.4 ± 4.4	51.9 ± 3.6
SW	13.5 ± 4.7	27.9 ± 3.6	26.5 ± 6.5	52.3 ± 4.1
ReA HLA-B27+				
B	5.7 ± 2.6	21.9 ± 5.8	22.8 ± 6.1	53.2 ± 5.3
SW	12.8 ± 3.9	30.1 ± 7.9	28.1 ± 5.9	52.8 ± 6.8
ReA HLA-B27-				
B	5.1 ± 2.2	20.6 ± 4.2	20.9 ± 2.9	51.6 ± 4.7
SW	11.9 ± 3.9	27.7 ± 4.4	28.6 ± 5.8	51.7 ± 4.5

Discussion

In patients suffering from HLA-B27 positive spondylarthropathies, alterations in PMN function have been described. In 1980 Lierisalo *et al.* (4) suggested that HLA-B27 might influence the responsiveness of PMN to chemotactic stimulus. Pease *et al.* in 1984 (2) found that directed motility was increased in subjects with HLA-B27 or AS when compared to the B27 negative controls. This study suggested that AS and the presence of B27 are both associated with increased PMN motility. In 1989, Pease *et al.* reported a study on HLA-B27 positive and negative AS patients, their healthy brothers (HLA-B27 positive and negative) and unrelated HLA-B27 positive and negative controls (3). Their study showed that the PMN response to two chemotactic substances was enhanced only in men with AS with or without HLA-B27, and the authors concluded that PMN abnormality is acquired as a direct result of AS, rather than being inherited. These studies were all carried out *in vitro*, while Koiuvanta-Vaara *et al.* (5) described enhanced neutrophil migration *in vivo* in HLA-B27 positive subjects.

Discordant data concerning PMN respiratory burst activity in B27 positive patients have been reported: Miller & Russel (7), Tertti (8, 10) and Repo *et al.* (9) detected no differences in superoxide production or chemiluminescence response between PMN from B27 positive and negative subjects when stimulated by fMLP or opsonized zymosan. El Abbouyi *et al.* (11) reported low O_2- production in response to opsonized zymozan in the PMN from a patient with AS; in this study the patients were not selected on the basis of the presence of HLA-B27. Wendling *et al.* (12) reported an increase in the phagocyte oxidative metabolism function of PMN after stimulus with fMLP or opsonized zymosan in AS patients compared with controls. There was no difference between HLA-B27 positive and negative patients.

To our knowledge the Senn's skin window technique has never been used, even though it permits one to evaluate *in vivo* the PMN migration and to obtain leukocytes from an inflammation focus.

The accumulation of neutrophils at the sites of infection and inflammation is accompanied by modifications in the activity of the cells. Previous investigations showed that exudate neutrophils are metabolically primed, being more responsive to fMLP stimulus than blood neutrophils (17, 20-26) and showing increased adherence in the absence of stimulants (27).

We studied PMN migration *in vivo*, and superoxide production and the adhesion of PMN from blood and from an experimental inflammatory exudate, in order to determine their possible relationship with either the HLA-B27 antigen or the disease. Our results revealed a significant increase of PMN migration in HLA-B27 positive patients affected by spondylarthropathies in comparison with the HLA-B27 negative patients and the control subjects. No differences were observed between the patients with AS and reactive arthritis, both in the HLA-B27 positive and negative subgroups. The HLA-B27 negative patients also did not differ from the controls. Our *in vivo* data are thus in agreement with previous *in vitro* (2-4) and *in vivo* (5) observations.

The increased PMN migration in HLA-B27 positive patients might suggest a correlation with this histocompatibility antigen, independently of the related disease. The increased PMN accumulation at sites of inflammation might be one of the mechanisms responsible for the expression and progression of the spondylarthropathies, as hypothesized by some authors (6).

Moreover, the increased PMN accumulation might be related to the disease severity. Nevertheless, in our patients the differing PMN migration associated with the presence or absence of HLA-B27 antigen was not correlated with the incidence of extra-articular features, such as ocular involvement.

In the functional assays on isolated cells we found:

a) that the comparison of superoxide production between circulating and migrating PMN confirmed previous findings: the migrating cells are primed in their response to fMLP. In patients no alteration of this priming phenomenon in skin window PMN was found.

b) an increased response to fMLP by circulating PMN from the patients with AS, whether HLA-B27 positive or negative, in comparison with all the other subjects. In AS patients only circulating PMN (and not migrating PMN) presented an increased metabolic response to fMLP, perhaps linked to the hypothesized long permanence of bacterial products in the blood of these subjects (28, 29). It is possible that various chemical substances of exogenous (bacterial products) or endogenous (cytokines) origin can exert a priming action on the circulating PMN of AS patients, with a consequent exposition of receptors to fMLP, as observed in the cells migrating to inflammation foci (21). Since it is known that different cytokines can have different and even opposite effects on leukocytes, it would be of interest in future studies to determine whether patients affected by AS express a different pattern of inflammatory cytokines with respect to patients with reactive arthritis.

c) the adhesion assay showed no alterations, indicating that the presence of the B27 antigen and AS have no effect on other PMN structures, such as the membrane integrin molecules or cytoskeleton proteins, which are involved in adhesion functions.

In conclusion, our results bring into the question the role of HLA-B27 in the regulation of PMN migration. The reported *in vivo* priming of circulating PMN seems to be related to AS and not to HLA-B27.

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