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Stimulation of whole blood cultures in patients with ankylosing spondylitis by a mitogen derived from *Mycoplasma arthritidis* (MAS) and other mitogens

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Abstract In this study we compared cytokine production and cell proliferation of immunocompetent cells derived from patients with ankylosing spondylitis (AS) to those from healthy blood donors using a whole blood assay. To this end, blood cell cultures were stimulated with the superantigens MAS (Mycoplasma arthritidis supernatant) and staphylococcal enterotoxin B (SEB) and the plant lectins phytohaemagglutinin (PHA) and concanavalin A (Con A). The number of white blood cells (WBC) and lymphocyte subsets were also determined. Cell proliferation and levels of interferon-γ (IFN-γ), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were measured after stimulation with the different mitogens. An ELISA test was used to analyse supernatant cytokine levels. Individuals with AS showed significantly lower IFN-γ concentrations and markedly lower cell proliferation rates with all tested mitogens than healthy controls, while there was no significant difference in IL-6 synthesis. IL-1β levels were slightly impaired in the patient group, but only blood cell cultures stimulates with MAS showed a statistical significance. Furthermore, there was a significant elevation of leucocytes and lymphocytes in patients with AS resulting in higher numbers of CD4-positive cells, which implies a higher CD4:CD8 cell ratio. CD19- and CD8-positive cells were not significantly distinct compared to healthy controls. This deviation in cytokine levels and cell proliferation points to a suppression of T lymphocytes. A disturbed T-lymphocyte function may play a part in the pathogenesis of AS.

Key words Ankylosing spondylitis · MAS · Cytokines · Whole blood assay

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Introduction

Ankylosing spondylitis (AS) is a systemic rheumatic disorder involving entheses and joints, especially in and around the spine. In the final stage the spine will be completely fixed. Although genetic and environmental influences are considered to play a role in the aetiopathogenesis [1], there is still no generally accepted explanation regarding the factors that cause the disease. It has been suggested that a defect in immunological tolerance resulting in activation and expansion of self-antigen-specific T and B lymphocytes, as well as in the production of autoantibodies and cytokines, might be involved in the pathogenesis [2]. More recently, the discovery of bacterial superantigens and the clarification of their mechanism have had a profound influence on the study of autoimmune diseases. Superantigens exert their effects on T cells by ligating the T-cell receptor (TCR) to class II MHC molecules expressed on other cells, such as B cells and monocytes, without antigen processing. The activation of the T cells is dependent only on the V_β chain and not on other variable elements of the TCR [3]. The recognition of superantigens by T cells may have several different consequences, including proliferation and expansion or the induction of anergy or even cell death [4]. However, the factors that determine which of these alternative reactions occur are poorly understood.

It is known that the superantigen MAS (Mycoplasma arthritidis supernatant) derived from the supernatant of cultures of *Mycoplasma arthritidis* triggers inflammatory arthritis in mice [5]. *Mycoplasma arthritidis* is a natural pathogen of rodents that can become established as a chronic infection in the joints. Histopathologically, the subsequent development of an inflammatory arthritis re-

sembles rheumatoid arthritis in humans [6]. In this context, a link between the superantigen MAS and AS has been suggested by Seitz et al. [7, 8]. MAS is known to activate T cells and monocytes in rats, mice and humans [9–12]. The present study was undertaken to investigate whether, compared to healthy controls, patients with AS show a different immune response in the whole blood assay after stimulation with the superantigens MAS and staphylococcal enerotoxin B (SEB) and plant lectins such as phytohaemagglutinin (PHA) or concanavalin A (Con A). Further, it was of interest to evaluate whether there were differences in the white blood cell count or lymphocyte subpopulations.

Patients and methods

We studied 21 patients with AS (mean ± SD age: 42 ± 14 years) from the Centre for Rheumatology at Bad Bramstedt, Germany, and 29 healthy blood donors (mean ± SD age: 47 ± 16 years) from the Institute of Immunology and Transfusion Medicine, University of Lübeck Medical School, Germany. AS patients consisted of 16 male and 5 female individuals who fulfilled the New York criteria for definite AS [13]. Rheumatoid factor was negative in all blood samples. The mean duration of disease was 12.5 years; in addition, eight patients showed an asymmetric oligoarthritis or uveitis. Patients received nonsteroidal anti-inflammatory drugs (NSAID) only, except for four patients who were also treated with sulphasalazine. Any therapy with corticosteroids was discontinued at least 4 weeks prior to the study. Control individuals consisted of 19 male and 10 female blood donors.

Flow cytometry and immunofluorescence

White blood cell count, lymphocyte count and the expression of T- and B-cell surface antigens with monoclonal antibodies was determined by using flow cytometry (Epics Profile II, Coulter Electronics GmbH, Krefeld, Germany). Monoclonal antibodies were obtained from Coulter Immunology. T4-RD1/T8-FITC antibodies were used for the differentiation of CD4- and CD8-positive cells, and B1-FITC antibodies, for CD19-positive cells. Antibody labelling was performed in whole blood samples preincubated with monoclonal antibody. Cell preparation of the blood samples was accomplished by using an immunology work station (Coulter-Q-Prep, Coulter Electronics GmbH, Krefeld, Germany).

Cell cultures

In order to study the induction of cytokines and cell proliferation we used the whole blood technique as described by Kirchner et al. [14]. Heparinized blood was taken from patients and blood donors in 10-ml syringes (15 IU Li-heparin/ml blood, Sarstedt Monovetten, Nümbrecht, Germany). The blood was mixed 1:10 with RPMI 1640 medium (Biochrom KG, Berlin, Germany) supplemented with 2 m*M* L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco GmbH, Karlsruhe, Germany). The blood suspension was distributed in 0.9-ml aliquots in test tubes (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) to which mitogens were added at appropriate concentrations in aliquots of 0.1 ml. The contents of the tubes were mixed and distributed to duplicate wells of round-bottomed 96-well plates (Falcon, Becton Dickinson Labware, N.J., USA).

MAS was obtained from cultures of *Mycoplasma arthritidis* ATCC 14124, Jasmin strain, and partially purified as described by Homfeld et al. [11]. The same preparation was used in all experiments. SEB, PHA and Con A were purchased from Sigma (Deisenhofen, Germany). Blood cell cultures were incubated at 37 °C in 5% CO_2 and 95% humidity. Supernatants were harvested after 6 days for measurement of the cytokine concentrations.

Determination of cytokines in the supernatant

The concentration of the cytokines was determined in a quantitative enzyme-linked immunosorbent assay (ELISA). Noncommercially available ELISA for interferon-γ (IFN-γ) was kindly provided by Hoffmann La Roche (Basel, Switzerland). Interleukin-1β (IL-1β) and interleukin-6 (IL-6) ELISAs were obtained from Biermann (Bad Nauheim, Germany). The microtitre plate was coated with a murine monoclonal antibody against IFN-γ, IL-β or IL-6. The standard cytokines used were recombinant human IFN-γ, IL-1β and IL-6 included in the respective ELISA test kit. Samples and standards were pipetted into the wells. After washing, a horseradish peroxidase-linked polyclonal antibody specific for IFN-γ, IL-1β or IL-6 was added to the wells. Following a washing to remove unbound antibody-enzyme reagent, a substrate solution was added. The colour intensity of the enzymatic indicator reaction was measured photometrically at 450 nm in an ELISA plate reader (Anthos Labtec, Salzburg, Austria).

Proliferation assay

The proliferation assays were performed in triplicate in wells of sterile round-bottomed 96-well plates in 100-µl samples. Blood cell cultures were incubated as described in the section Cell cultures. After 6 days the cells were pulsed with 37 kBq/well ^{3}H-thymidine (Amersham Buchler, Braunschweig, Germany) and harvested after 4 h on glass fibre filters. Incorporated radioactivity was determined by liquid scintillation counting.

Statistical analysis

Mean ± SD were determined for each group. Patient and control populations were compared and analysed for statistically significant differences using the Mann-Whitney *U* test.

Results

Cell subsets in blood samples from patients and healthy blood donors

Each blood sample was examined for white blood cell count, lymphocyte count, CD19, CD4 and CD8 subsets and the CD4:CD8 ratio. As shown in Table 1, the numbers of leucocytes and lymphocytes were significantly higher in the blood samples from patients than in blood samples from

Table 1 Leucocyte and lymphocyte count and lymphocyte subsets in blood samples from healthy controls and patients with ankylosing spondylitis. Mean values and standard deviation (SD) are shown

	Controls (n=29)	Patients (n=21)	
Leucocytes	5.0 ± 1.5[a]	6.4 ± 1.8	(P<0.01)
Lymphocytes	1.5 ± 0.6	2.0 ± 0.7	(P<0.01)
CD4	45.0 ± 7.3[b]	49.7 ± 9.2	n.s.
CD8	24.5 ± 7.5	23.5 ± 7.7	n.s.
CD19	10.0 ± 3.3	11.7 ± 5.6	n.s.
CD4:CD8	2.2 ± 0.9[c]	2.5 ± 1.4	n.s.

[a] Cell number per nanolitre ± SD
[b] Percentage of positive cells ± SD
[c] Ratio ± SD

Table 2 Production of interferon-γ (IFN-γ) in whole blood cultures from patients and controls after stimulation with different mitogens and 6 days of incubation. Mean values and standard deviation are shown (*MAS* Mycoplasma arthritidis supernatant, *SEB* staphylococcal enterotoxin B, *PHA* phytohaemogglutinin, *Con A* concanavalin A)

Stimulus	IFN-γ (pg/ml ± SD)		
	Controls ($n=29$)	Patients ($n=21$)	
MAS 1:100	720 ± 681	411 ± 565	($P<0.05$)
SEB 100 ng/ml	4986 ± 1149	3842 ± 1605	($P<0.05$)
PHA 5 µg/ml	2301 ± 1334	1672 ± 1256	n.s.
Con A 5 µg/ml	1899 ± 1309	501 ± 632	($P<0.001$)
Median control	0	0	

Table 3 Interleukin-1β (IL-1β) synthesis in whole blood cultures of patients and controls after stimulation with MAS, SEB and PHA

Stimulus	IL-1β (pg/ml ± SD)		
	Controls ($n=25$)	Patients ($n=17$)	
MAS 1:100	775 ± 483	540 ± 334	($P<0.05$)
SEB 100 ng/ml	124 ± 69	78 ± 77	n.s.
PHA 5 µg/ml	56 ± 47	44 ± 36	n.s.
Medium control	0	0	

Table 4 Interleukin-6 (IL-6) synthesis in whole blood cultures of patients and controls after stimulation with MAS, SEB and PHA

Stimulus	IL-6 (pg/ml ± SD)		
	Controls ($n=25$)	Patients ($n=17$)	
MAS 1:100	1660 ± 812	1748 ± 925	n.s.
SEB 100 ng/ml	599 ± 402	402 ± 363	n.s.
PHA 5 µg/ml	862 ± 404	1043 ± 605	n.s.
Medium control	0	0	

controls. Furthermore, we found an elevated CD4:CD8 ratio in the patient group caused by a higher number of CD4-positive cells, but the difference did not reach statistical significance. CD19- and CD8-positive cells were only slightly different in patients and controls.

Decreased production of immunostimulatory cytokines in patients with AS

In previous experiments we have examined the optimum concentration for each mitogen to achieve highest cytokine values in the whole blood assay, which for MAS is 1% diluted in RPMI medium, for SEB, 100 ng/ml, and for PHA and Con A, 5 µg/ml final concentration. Kinetic profiles have shown maximal concentrations of all assayed cytokines in the supernatant after 6 days of culture, as reported earlier [12].

Table 5 Cell proliferation in whole blood cultures from patients with ankylosing spondylitis and healthy controls after stimulation with the superantigens MAS and SEB

Stimulus	^{3}H-Thymidine incorporation (cpm ± SD)		
	Controls ($n=29$)	Patients ($n=21$)	
MAS 1:100	2678 ± 1673	2054 ± 1852	n.s.
SEB 100 ng/ml	30417 ± 9085	23539 ± 8328	($P<0.05$)
Medium control	86 ± 53	94 ± 56	n.s.

In contrast to the control group, patients with AS showed remarkably reduced IFN-γ synthesis after stimulation with all tested mitogens. Statistical significance was achieved by stimulating with MAS, SEB and Con A (Table 2). We also observed an impaired release of IL-1β protein in the activated blood cell cultures of the patient group. Differences were statistically significant in cultures stimulated by MAS (Table 3). Production of IL-6 protein failed to demonstrate a statistically significant difference. Interestingly, blood cell cultures stimulated by MAS and PHA showed higher levels of IL-6 in the supernatant of the patient group than of controls (Table 4). In both groups, MAS was a better inducer of IL-1β and IL-6 than SEB and PHA in optimum concentrations.

Cell proliferation in whole blood cultures from patients with AS and healthy controls

Cell proliferation was examined as a further parameter of immunological activity in whole blood cultures stimulated with the superantigens MAS and SEB. As shown in Table 5, the proliferative response was clearly lower in the patient group than in healthy individuals. Cultures incubated without nitrogen did not show any essential difference between both groups. Statistical significance was achieved in blood cell cultures exposed to SEB.

Discussion

There is strong evidence for the involvement of humoral and cell-mediated immunity in the pathogenesis of AS, though investigations on abnormalities in immune cell numbers in peripheral blood [15–17], cytokine production [7, 8, 18] and occurrence of certain antibodies to different microbes [2, 19, 20] show conflicting results. It should be considered that there is a wide diversity of presenting symptoms in AS, and clinical expression varies a lot [21]. It is probable that immunological and other biological parameters show a similar variety in that rheumatic disorder, further influenced by anti-inflammatory drug therapy. Disease activity, progression and prognosis are difficult to define in AS and inflammatory parameters such as C-reac-

tive protein (CRP), other acute phase proteins and erythrocyte sedimentation rate (ESR) correlate poorly with clinical parameters such as pain, stiffness and discomfort [18, 22].

In our study the numbers of patients with abnormal results for ESR and CRP were only 6 and 4, respectively, although 16 of the patients needed regular treatment with NSAIDs. Although the cellular parameters were within normal ranges in patients and controls, our results clearly demonstrated that leucocytes and lymphocytes in the peripheral blood were significantly increased in the patient group. Furthermore, the CD4:CD8 cell ratio was markedly elevated due to a higher number of circulating CD4-positive cells. The percentage of B cells (CD19) and CD8-positive T cells were not significantly distinct between patients and controls. This finding may reflect a general recruitment of immune cells with a preference for CD4-positive T cells.

We used the whole blood technique to obtain the best physiological conditions, when comparing the immune cell functions such as cytokine production and cell proliferation. Methods for separating peripheral blood mononuclear cells (PBMC), such as Ficoll-Hypaque separation, have previously been found to alter the composition of T-cell subsets and decrease cell proliferation and cytokine production [23–25].

Disturbances in the cytokine network and the T-cell activation pathways have been found to play a role in the development of inflammatory rheumatic conditions [26], although the cause is still not known. The idea that superantigens may be an important factor in the pathogenesis of such diseases is supported by several studies [2, 4, 27]. MAS has been shown to exert a suppressive effect on lymphocyte function in vivo. In the mouse model a suppression of T-cell responses in MAS-injected mice following stimulation by MAS and other mitogens, such as plant lectins, has been observed [28]. A similar in vitro anergy in peripheral T lymphocytes has been described in the mouse model for SEB [29]. Furthermore, MAS not only influences T-cell functions, but also increases monocyte activity by enhancing the production of IL-6 and IL-1β [11, 12, 30].

A major observation made in the present study was that IFN-γ synthesis and the proliferative response to T-cell mitogens were significantly lower in patients with AS than in healthy controls. This impaired lymphocyte function was demonstrated in vitro by stimulation with the superantigens MAS and SEB and the plant lectins PHA and Con A. Interestingly, we observed no significant differences in IL-6 production and only a slightly impaired IL-1β synthesis after stimulation by SEB and PHA, although a significant difference was seen after stimulation by MAS. IL-4 inhibits IL-1β secretion by human monocytes [31], and it has been suggested that the suppression of T lymphocyte proliferation induced by MAS may be associated with an enhancement of IL-4 production [30]. However, we did not observe any difference in IL-4 synthesis between patients and controls (data not shown). IL-1β and IL-6 are often expressed together, but there are several examples of discoordinated regulation [32]. We propose that the decreased level of IL-1β might be a result of impaired IFN-γ production in the whole blood culture. IFN-γ is known to have macrophage-activating properties and to enhance IL-1 synthesis [33].

In conclusion, we did not observe any unusual reactivity to the superantigen MAS compared to other mitogens in patients with AS. We showed a significantly elevated lymphocyte count and differences in lymphocyte subsets accompanied by a significantly impaired T-cell function in those patients. It remains to be established whether lymphocyte suppression in AS is caused by MAS or other superantigens.

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