The Immune Response to Autologous Bacteroides in Ankylosing Spondylitis Is Characterized by Reduced Interleukin 10 Production

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ABSTRACT. ***Objective.*** Ileocolitis is a recognized feature of ankylosing spondylitis (AS) and is likely to play a role in the pathogenesis of AS, in conjunction with the normal intestinal microbiota. In order to investigate the host immune response in AS, we measured cytokines in tissue culture following exposure of peripheral blood mononuclear cells (PBMC) to autologous colonic bacteria.

Methods. Twenty-one patients with AS and 21 matched controls were recruited. Subjects in the AS group were assessed clinically. *Bacteroides* species belonging to the *B. fragilis* group were selectively cultured from stool samples and paired with blood samples from each participant. Ten cultures of autologous *Bacteroides* were randomly selected from cultures grown from the fecal specimens of each of the 21 patients with AS and 21 controls. These were then tested for reactivity with PBMC and the cytokines produced by proliferating lymphocytes [interleukin 10 (IL-10), IL-17, interferon-γ, tumor necrosis factor-α] were measured in cell culture supernatants. Differences between groups were analyzed using censored normal regression analysis.

Results. The patients with AS had severe active AS with Bath AS Disease Activity Index 5.5 (± 1.6) and C-reactive protein (mg/l) 13.8 (± 12.2) (mean ± standard deviation). IL-10 concentrations in *ex vivo* assay supernatants were lower in the AS group compared with controls ($p = 0.047$). There were no statistically significant differences between the groups for other cytokines.

Conclusion. In AS, reduced IL-10 production in response to stimulation with autologous *Bacteroides* cultures may represent a mechanism by which intestinal inflammation develops and persists, a situation analogous to inflammatory bowel disease. (J Rheumatol First Release Feb 15 2009; doi:10.3899/jrheum.080964)

Key Indexing Terms:
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In ankylosing spondylitis (AS) there is evidence that an environmental trigger, originating in the gastrointestinal tract, is an etiological factor. This has been demonstrated in an animal model, where rats transgenic for human HLA-B27 have been shown to develop a disease characterized by inflammation of the spine and joints and colitis[1]. However, if these rats are derived under germ-free conditions they remain healthy. Subsequent colonization of the gut with normal commensals, particularly *Bacteroides vulgatus*, leads to the development of disease in these rats[2]. These observations indicate that interactions between a dysfunctional immune system and the bowel microbiota are fundamental aspects of the pathogenesis of AS. Further, in human populations, a strong association with Crohn's disease[3] is well established and ileocolitis has been observed in two-thirds of patients with AS[4].

We have shown previously that the proportions of the major bacterial phylotypes comprising the fecal microbiota did not differ between subjects with AS and healthy controls[5]. Moreover, populations of *B. vulgatus*, a bacterium shown to promote colitis in HLA-B27 transgenic rats[6], did not differ in size between patients with AS and controls. We therefore postulated that the nature of the host immune response to autologous bowel bacteria may be of greater importance than the presence or absence of particular bacterial species. To investigate this, we measured the cytokine profile produced by peripheral blood mononuclear cells (PBMC) from patients with AS when exposed to the cells of autologous *Bacteroides*.

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MATERIALS AND METHODS

Ethical approval was obtained from the Lower South Regional Ethics Committee.

Twenty-one patients fulfilling the Modified New York Criteria for definite AS[7] and 21 age- and sex-matched controls were recruited to the study. Patients with AS and controls were excluded from the study if they were pregnant, or had a current infection, a history of inflammatory bowel disease (IBD) or malignancy. In addition, controls were excluded if they had a history of arthritis, uveitis, or psoriasis.

All patients were assessed clinically using the following instruments identified as core measures. Disease activity and severity was assessed using the Bath AS Indices: Disease Activity Index, BASDAI; Functional Index, BASFI; Global Assessment, BAS-G; and Metrology Index, BASMI[8]. The C-reactive protein (CRP), 44-joint peripheral tender and swollen joint count, and Maastricht AS Enthesitis Score (MASES) were also recorded[9]. Treatment with nonsteroidal antiinflammatory drugs (NSAID) and disease modifying antirheumatic drugs (DMARD) was recorded.

Patients and controls were assessed on a single occasion and provided a single blood sample and fecal sample within 1 week of the clinical assessment.

Bacteroides cultures. Fecal samples were collected from patients with AS and controls and cultured within 2 h of collection. All subsequent manipulations were carried out in an anaerobic glovebox. Homogenates (10% w/v) were prepared and diluted in 10-fold steps to 1×10^{-8} using brain-heart infusion broth supplemented with vitamin K and hemin (BHIS)[10]. One hundred-milliliter aliquots of dilutions were spread on plates of Bacteroides-Bile-Aesculin agar (which selects for members of the *B. fragilis* group, including *B. vulgatus*). The plates were incubated anaerobically for 2 days at 37°C. Ten bacterial colonies were picked from BHIS plates showing discrete colonies. Culture media used in the study had been prereduced before use for 24 h in an anaerobic glovebox. BHIS broth cultures of the 10 pure cultures obtained from each of the subjects were used separately to obtain *Bacteroides* cells by centrifugation. The cells were washed twice in sterile 0.85% sodium chloride solution and the bacterial suspensions were standardized spectrophotometrically to 1×10^9 CFU per ml. The bacterial cells were heat-killed (80°C, 30 min) and the suspensions were stored at –80°C until use[11].

Preparation and use of PBMC. Twenty-milliliter volumes of heparinized blood were obtained from patients with AS and controls. PBMC were purified from the blood by centrifugation through a Ficoll-Hypaque gradient (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The cells were cultured at 37°C in a modified atmosphere incubator (5% carbon dioxide) in flat-bottomed microtiter plates using Iscove's modified Dulbecco medium containing insulin, transferrin, bovine serum albumin, penicillin/streptomycin, and 2-ME. Triplicate cultures of PBMC from each patient at 2×10^5 per well were separately exposed to each of their 10 autologous *Bacteroides* suspensions at 2×10^6 cells per well for 5 days. Negative controls contained PBMC and culture medium, whereas positive controls contained PBMC and staphylococcal enterotoxin A (SEA; 50 ng per well). Culture supernatants were subsequently used to measure the concentrations of interleukin 10 (IL-10), IL-1ß, IL-17, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), using BioPlex Suspension Array methodology (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Mean values for the 10 different *Bacteroides* colonies selected from each individual and resultant cytokine profiles were analyzed using censored normal regression analysis, where allowance was made for censored values outside the limits of detection of the assay. No adjustments have been made for multiple testing.

RESULTS

The patient group was characterized by severe active AS, mean duration 18.4 years (Table 1).

Table 1. Clinical and demographic characteristics of the AS subjects and controls.

	Ankylosing Spondylitis (AS) Mean (± SD) or n (%)	Controls Mean (± SD) or n (%)
Age, yrs	44.9 ± 15.1 (range 19–75)	43.4 ± 16.9 (range 18–79)
HLA-B27 status, n positive	21 (100)	2 (9.5)
Sex, male	14 (66.6)	14 (66.6)
Current NSAID use	17 (81)	
Current DMARD use	4 (19)	
BASMI score	3.6 ± 2.4	
BASFI score	5.1 ± 1.8	
BAS-G score	4.7 ± 1.4	
BASDAI score	5.5 ± 1.6	
CRP, mg/l	13.8 ± 12.2	
n of patients with peripheral arthritis	7 (33)	
Peripheral tender joint count	1.8 ± 1.9	
Peripheral swollen joint count	0.7 ± 1.2	
MASES Enthesitis Index	2.78 ± 3.0	
Duration of morning stiffness, h	0.8 ± 0.8	
Extraarticular features	5 (24)	

SD: standard deviation; BASMI: Bath AS Metrology Index; BASFI: Functional Index; BAS-G: Patient global assessment; BASDAI: Disease Activity Index; MASES: Maastrict AS Enthesitis score; NSAID: nonsteroidal antiinflammatory drugs; DMARD: disease modifying antirheumatic drugs; CRP: C-reactive protein.

All patients with AS were HLA-B27-positive with radiographic evidence of sacroiliitis (Grade ≥ 2 bilateral or 3–4 unilaterally). It was noted that 19 of the 21 patients would qualify for treatment with a biologic agent using Australian criteria. None of the subjects were receiving such treatment, as it is unavailable for this indication in New Zealand.

Table 2 shows cytokine production following exposure of PBMC to autologous *Bacteroides* cells and to SEA 50 ng as positive control.

Significantly lower levels of IL-10 were produced following stimulation with autologous *Bacteroides* in the AS group. In contrast, both groups responded similarly to SEA stimulation. No significant difference between AS and control groups was noted with any of the other cytokines. There was no correlation between any clinical variable and cytokine levels.

DISCUSSION

We defined a group of patients with severe AS naive to biologic therapies. In comparison to healthy controls, we observed attenuated IL-10 production by the PBMC of patients with AS when exposed to autologous isolates of *Bacteroides* cells. This finding is of interest since IL-10 has been shown to play an important role in mucosal tolerance in IBD, a condition acknowledged to have both genetic and histopathological commonality with AS. This attenuation could not be attributed to a defect in IL-10 production by PBMC from patients with AS, as no significant difference

Table 2. Cytokine levels (pg/ml) measured following peripheral blood mononuclear cell (PBMC) stimulation with autologous *Bacteroides* cells and Staphylococcal enterotoxin (control stimulus).

Cytokine	Subjects	Mean, pg/ml	SE	SD	95% CI Lower	95% CI Upper	p
IL-10	AS	383	65.0	298	256	511	0.047
	Control	610	94.0	429	426	795	
IFN-γ	AS	1351	405	1857	556	2148	0.793
	Control	1510	450	2061	627	2394	
TNF-α	AS	6019	1125	5156	3808	8230	0962
	Control	6106	1447	6629	3263	8950	
IL-17	AS	12	4	18	4	19	0.198
	Control	6	2	11	1	10	
IL-10 SEA	AS	1079	171	784	723	1437	0.719
	Control	1166	166	758	821	1511	
TNF-α SEA	AS	20483	3440	15771	13920	27273	0.326
	Control	14082	2629	12049	9743	19163	
IL-17 SEA	AS	2058	591	2710	1064	3363	0.227
	Control	1737	722	3306	728	3223	

AS: ankylosing spondylitis; CI: confidence interval; SEA: staphylococcal enterotoxin A (50 ng per well). IL: interleukin; IFN: interferon; TNF: tumor necrosis factor. IFN-γ production from PBMC following superantigen (SEA) stimulation exceeded upper limits of quantification for the assay in all subjects and did not differ between patients with AS and controls.

was noted in IL-10 production following stimulation with superantigen (SEA) between subjects and controls, and no difference was noted in production of any other measured cytokines following *Bacteroides* stimulation.

Although there are in excess of 400 bacterial species in the human colon, *Bacteroides* species constitute up to 30% of the fecal microbiota[12]. Tolerance to this huge antigenic load is a vital function of the mucosal immune system in the intestinal tract.

In AS, evidence that the intestinal microbiota act as an antigenic stimulant to both colonic inflammation and arthritis is largely confined to animal models, specifically the HLA-B27 transgenic rat model. Differential colonization of gnotobiotic animals has shown that *B. vulgatus* induces the strongest proinflammatory response, significantly greater than that seen with *E. coli*[2,6]. It was for this reason that we chose members of the *B. fragilis* group (which includes *B. vulgatus*) as the source of antigenic stimulation in our study.

There is substantial evidence that the intestinal tract is in a constant state of controlled inflammation. Loss of this control is an important mechanism in the development of IBD[13]. Animal models reveal the importance of an intact immune system for this homeostatic process since cytokine gene deletions and altered T cell subsets in murine models result in the development of IBD-like syndromes[14,15].

IL-10 plays a critical role in such mucosal immune regulation in the gut, and this is demonstrated by observations in mouse models. Mice deficient for the IL-10 gene develop an enterocolitis. Local mucosal delivery of recombinant IL-10 by modified *Lactococcus lactis* seems effective in ameliorating colitis in IL-10-deficient mice[13]. The development of colitis is dependent on the presence of bowel bacteria, since IL-10-deficient mice do not develop colonic inflammation under germ-free conditions[16].

In humans, evidence of loss of tolerance to resident intestinal microbiota in IBD was demonstrated by Duchmann, *et al*[17]. In their study, PBMC and lamina propria cells from IBD patients with active colitis, colitis in remission, and normal controls were exposed to both autologous and heterologous colonic bacteria. PBMC of patients with active IBD strongly proliferated in response to autologous bacteria. PBMC of controls and those in remission did not, but did proliferate in response to heterologous bacteria. Further, although the activated PBMC produced high levels of IFN-γ and IL-12, there was no corresponding increase in IL-10.

The strong association between AS and Crohn's disease[3,18] and the consistent demonstration of ileocolitis in patients with AS have been noted earlier. In this context, the finding of a deficient IL-10 response to autologous *Bacteroides* in AS is intriguing.

Although we have demonstrated significantly lower IL-10 production in AS following stimulation of PBMC with autologous bacterial cultures, we can only speculate on the mechanism for this. Further, our cohort was selected such that all participants with AS had severe, active AS. This did not allow us to demonstrate any correlation between reduced IL-10 levels and disease activity, although further studies examining this would be of interest. A previous study investigated PBMC cytokine production in AS in response to a standard phytohemagglutinin stimulation, and correlated this with disease activity as measured by BASDAI. This demonstrated a linear increase in levels of IL-1ß and TNF-α with increasing BASDAI, and a similar linear decline in IL-10 production in association with disease activity ($p < 0.01$)[19].

One notable feature of IL-10 is its ability to induce regulatory T cell (T_{reg}) development. These suppressive cells are termed adaptive T_{reg} and they have been further characterized as Tr1 and Th3 cells[20,21]. These cells mediate their action predominantly through the production of antiinflammatory cytokines, IL-10 in the case of Tr1 cells and transforming growth factor-ß in the case of Th3 cells. Such cells vary in number in active IBD with a contraction of the peripheral blood T_{reg} pool and a moderate expansion in the inflamed intestinal mucosa. Investigation of the number of T_{reg} cells and their functional capacity in the gut mucosa, peripheral circulation, and synovial membrane would be worthy of consideration given our results.

We have shown reduced IL-10 production following PBMC stimulation with autologous *Bacteroides* species in patients with severe AS, compared with healthy controls. We suggest that this may be an important mechanism in the pathogenesis and persistence of inflammation in this condition. We further postulate that a loss of tolerance to numerically significant constituents of the intestinal microbiota may be mediated by this reduced immunoregulatory cytokine secretion. Extrapolation from current knowledge in IBD suggests that this may reflect reduced proliferation and activity of the adaptive T_{reg} cell responses in AS.

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