Behçet’s disease patients present high levels of deglycosylated anti-lipoteichoic acid IgG and high IL-8 production after lipoteichoic acid stimulation

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ABSTRACT

Objectives. Lipoteichoic acid (LTA), induces some of the clinical symptoms of Behçet’s disease (BD) in a rat animal model. These results led to the hypothesis that LTA may also trigger BD in humans. We investigated the humoral and cellular immune response against LTA and lipopolysaccharide (LPS) in patients with BD, and compared these responses with those of patients with active chronic oral ulcers (OU) and normal controls.

Methods. Samples were obtained from 12 active BD, 12 inactive BD, 12 active OU and 12 normal controls. Anti-LTA, anti-LPS antibodies levels and the capacity of immune complexes anti-LTA IgG-LTA to activate complement were studied. Exposed mannose residues in anti-LTA IgG were analyzed in the four groups. The interleukin-8 (IL-8) production by peripheral blood mononuclear cells cultures after LTA and LPS stimulation was also studied in all groups.

Results. The capacity to bind mannan binding protein (MBP) of anti-LTA IgGs was significantly higher in BD and active OU patients relative to normal controls (p < 0.001). However, only active BD patients generated significantly higher levels of C5a than controls (p < 0.0001). The IgGs purified from the sera of BD patients showed a high specificity for LTA from Streptococcus sanguis or Streptococcus faecalis. LTA also stimulates the secretion of IL-8 in peripheral blood mononuclear cells isolated from active BD patients. Anti-LPS IgA and IgG titers were significantly higher only in active OU patients relative to normal controls (p < 0.0018).

Conclusion. These results suggest a mechanism involving LTA from streptococci in the pathogenesis of BD.

Introduction

BD is an inflammatory disorder characterized by recurrent oral and genital ulcers, uveitis, and skin lesions (1-4). Several streptococcal species have been suggested as causative agents of BD and uveitis (5,6), although Streptococcus sanguis is common in the oral flora of BD patients (7). One streptococcal antigen, LTA, induces multiple abnormal biologic functions in lymphocytes (8) and anterior uveitis in a rat animal model (9), one of the systemic manifestations of BD in humans (5). Therefore, we hypothesized that LTA from streptococci may possibly be implicated in the pathogenesis of BD. For this purpose, we assessed the titers of total IgG, IgA, and their anti-LTA levels in BD, active chronic benign oral ulcers (OU), and healthy individuals.

In addition to evaluating the specificity of anti-LTA IgGs, their glycosylation status and their capacity to activate complement, we also assessed the immune response against LPS in these patients. Furthermore, because high serum levels of IL-8 are also detected in BD (10), we measured the IL-8 production by peripheral blood mononuclear cells (PBMC) isolated from these patients after LTA or LPS stimulation.

Patients and methods

Patients

The protocol was approved by the Institutional Review Boards of all participating centers. Serum samples were obtained from 24 BD patients fulfilling diagnostic criteria proposed by the International Study Group for BD (11). Twelve BD patients were active and the other twelve were inactive at the time of the study. Patients were considered inactive if they did not present any symptoms of the disease during the 3
previous months. Since high glucocorticoid doses may modify protein synthesis and protein glycosylation, none of the patients included in this study was administered doses greater than 10 mg of prednisone per day. Active BD patients were selected at the moment of diagnosis or clinical reactivation (most of them had active retinal vasculitis, 11/12). Five active BD patients were receiving maintenance treatment with steroid and colchicine, one was receiving steroids and cyclosporine. Four inactive BD patients were receiving colchicine and chlorambucil, and 7 patients were not treated with drugs at all. Blood samples were taken before initiation or dose augmentation of prednisone and immunosuppressive treatment. An additional group of 12 patients with active OU and 12 normal controls matched by age and sex were also included in this study. Because high levels of anti-LTA antibodies are common in patients with chronic gingivitis (12), all patients were thoroughly examined to rule out periodontal disease.

Antibodies
Alkaline phosphatase labeled secondary antibodies against human, rabbit and mouse IgG, human IgM and IgA, and horseradish peroxidase conjugated to protein G were purchased from Sigma (St. Louis, MO, USA). The mouse monoclonal antibody (Mab) to mannan binding protein (MBP) (clone 131-1) was purchased from Research Diagnostics (Flanders, NJ, USA).

Lipoteichoic acids and lipopolysaccharides
LTAs from S. sanguis, S. faecalis and S. aureus and LPS were purchased from Sigma. LTAs were further purified by hydrophobic chromatography on Octyl-Sepharose (13), producing LTAs essentially free of contaminant LPS (mean concentration < 0.005 µg LPS/mg LTA) as determined by the Limulus Amebocyte Lysate (LAL) method (14, 15).

Purification of human anti-LTA IgG
IgG was purified from 2 ml of sera of normal and BD individuals by a combination of chromatographies on Protein A Sepharose (16) and the conjugate LTA-ovoalbumin covalently attached to Sepharose. The second procedure involved a rinsing with 50 mM glycine-HCl, pH 3.2, to remove contaminant rheumatoid factor (RF), followed by addition of 50 mM Tris-HCl, pH 7.5 buffer containing 4 M guanidine-HCl to elute the high affinity anti-LTA IgG, followed by dialysis against buffer alone. The specificity of the IgG was assessed by immunoblotting techniques (19).

Enzyme-linked immunosorbent assays
Total IgA and IgG in serum were assayed by enzyme linked immunosorbent assays (ELISA) as previously described (20). Analyses of anti-LTA and anti-LPS Igs were determined in plates coated with S. sanguis LTA and E. Coli LPS. Serum samples (1: 5000 dilution) in PBS-Tween (200 l) were incubated at 37°C for 2h. The secondary antibodies as well as the conditions of the assay were similar to those described above (20). Calibration curves were constructed with anti-LTA or anti-LPS IgA and IgG purified from the serum of a single BD patient by affinity chromatography on LTA or LPS immobilized on Sepharose 4B, as described above. To avoid cross-reactivity between anti-LTA and LPS antibodies or viceversa, assays for anti-LTA antibodies were conducted in the presence of LPS (50 ng/ml). Similarly, assays for anti-LPS antibodies were conducted in the presence of LTA (50 ng/ml). Plates were then rinsed with PBS-Tween and incubated with 200 l of a solution containing specific anti-human IgA or IgG fragments of MBP (1 mg/ml p-nitrophosphatase for 1 h at 37°C, followed by rinsing with PBS-Tween and incubation with 200 l of alkaline phosphatase substrate (1 mg/ml p-nitrophosphatase) in 0.1 M glycine, 1 mM MgCl2, 1 mM ZnCl2, pH 10.4. Absorbance was monitored at 405 nm using an Anthos Labtec kinetic plate reader. Bound immunoglobulins were calculated from calibration curves constructed with purified human anti-LTA IgG, where specific values of absorbance at 405 nm are plotted versus concentrations of purified human IgA or IgG.

Binding of anti-LTA IgG to LTA by slot blot analysis
LTAs from S. sanguis, S. faecalis and S. aureus (10 µg) in 100 mM sodium carbonate, pH 9.6, were bound to nitrocellulose membranes using a Minifold II slot blotter (Schleicher & Schuell, Keene, NH). The slots were thoroughly rinsed with phosphate-buffered saline (PBS) solution and the membranes incubated with 3% BSA in PBS for 1 h at 22°C to block non-conjugated areas. Each serum (100 µl) in 5 ml PBS was singly incubated with membranes containing dots of the 3 LTAs overnight at 22°C, followed by 3 rinses in PBS for 10 min at 22°C an then incubation in 5 ml PBS containing alkaline phosphatase-conjugated anti-human IgG (1 µg/ml) for 60 min at 22°C. Detection was performed after incubation of the membranes with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitro blue tetrazolium (1 mM each) in 10 mM Tris-HCl, pH 8.5, containing 1 mM MgCl2 at 22°C. Densitometric analysis of the dots were performed with a Molecular Dynamics Storm 860 System (Amersham Biosciences, NJ, U.S.A.) using an Image Quantification software for WINDOWS according to manufacturer’s instructions.

Mannose binding protein assay
Plates coated with LTA were incubated with 200 ng of purified anti-LTA IgG. Plates were then washed twice with 50 mM glycine-HCl, pH 3.2, and 3 times with PBS-Tween and incubated in a 200 l final volume with increasing concentrations of mannose binding protein (MBP), purified according to the procedure described by Lu et al. (17), in 50 mM Tris-HCl, 150 mM NaCl, 20 mM CaCl2, pH 7.5, for 2 h at 37°C. The final concentration of MBP bound to the anti-LTA IgG (specific binding) was determined as previously described (21).

MBP binding to IgG by slot blot assays
MBP (5 µg/slot) was adsorbed to nitrocellulose using a Minifold II slot blotter as described above. The membrane was then incubated in PBS containing 3% BSA to block areas non-conjugated with MBP. Purified IgGs (100 µg) from
BD patients and normal controls were singly incubated with these membranes. After 1 h at 22°C the membranes were rinsed in PBS followed by incubation with alkaline phosphatase-conjugated anti-human IgG for 1 h at 22°C. Detection of the bound IgG was performed as described above.

**Assay of terminal carbohydrate residue of anti-LTA IgG**

Specific detection of terminal carbohydrate residues to anti-LTA IgGs was performed with a glycan differentiation kit purchased from Roche Diagnostics Corporation (Indianapolis, IN). Briefly, LTA from *S. sanguis* (10 µg) was first loaded to nitrocellulose membranes by slot blot adsorption. After blocking non-specific areas with 3% BSA in PBS, the membranes were singly incubated with IgGs purified from sera of BD patients or normal controls for 1 h at 22°C, followed by single incubation with digoxigenin-labeled peanut agglutinating (PNA) or galanthus nivalis agglutinin (GNA) which recognize the core disaccharide galactose \( β(1-3) N\)-acetylgalactosamine or terminal mannose \( α(1-3), α(1-6) \) or \( α(1-2) \) linked to mannose, respectively (18, 19). Bound lectins were assayed according to manufacturer’s instructions.

**Human complement C5a des Arg assay system**

The assay was performed in 96 well microtiter plates using purified anti-LTA IgG following to a previously reported protocol (21).

**Cytokine production by PMB**

Heparinized blood samples were collected from 8 active BD patients, 8 active OU patients, and 8 normal controls. Fresh PBM were stimulated with *S. sanguis* LTA and *E. coli* LPS (10 µg/ml) and incubated in an atmosphere of 5% CO\(_2\) and 95% O\(_2\) at 37°C for 24 h. Supernatants were harvested and frozen at -70°C until use (22). IL-8 was measured by an ELISA technique (Bender MedSystems, Vienna, Austria) according to manufacturer’s instructions.

**Statistics**

The statistical significance of differences between total IgS levels, anti-LTA IgA, and IgG, as well as the deglycosilated anti-LTA IgG levels were evaluated by means of Kruskal-Wallis analysis of variance. The specific comparisons within each group of variables were performed by means of the Bonferroni test.

**Results**

**Clinical manifestations and treatment of patients**

All demographics and clinical manifestations of patients whose sera were analyzed in this study are summarized in Table I. All BD patients had ocular involvement and recurrent oral ulcers.

**Total IgA and IgG concentrations of patients in all groups**

The median of total serum IgA titers were 1.58 mg/ml in patients with active BD, 1.52 mg/ml in patients with inactive BD, 1.86 in active OU, and 1.12 in normal controls. The differences between BD patients and controls and between active OU patients and normal controls were statistically significant (BD versus controls p < 0.002, OU ver-
sus normal controls p < 0.0001). The median of total serum IgG titers were 8.94 mg/ml in active BD, 8.42 mg/ml in inactive BD, 8.14 mg/ml in active OU, and 6.87 mg/ml in normal controls. Only active BD patients show significantly greater titers than normal controls. (active BD versus controls p < 0.004).

**Anti-LTA antibodies**

IgA and IgG from all groups of patients bind to LTA with similar affinity (data not shown). The median serum titers of anti-LTA IgA were 1.2 µg/mg IgA in active BD, 0.61 µg/mg IgA in inactive BD patients, 0.96 µg/mg IgA in active OU and 1.47 µg/mg IgA in normal controls (Fig. 1A). The ANOVA test did not detect an overall difference between the four groups. The median serum titers of anti-LTA IgG in active BD patients were 2.34 µg/mg IgG, 2.02 µg/mg IgG in active OU patients, 1.83 µg/mg IgG in inactive BD patients, and 2.02 µg/mg IgG in normal controls (Fig. 1B). Statistically, there was an overall difference in the IgG anti-LTA antibody levels between the four groups. Only patients with active BD had significantly higher levels than patients with inactive BD (p < 0.05).

**Specificity of the anti-LTA IgG reactivity**

Sera (100 µl) from five active BD patients were individually reacted with a dot blot containing bound LTA (10 µg) from *S. sanguis*, *S. faecalis* and *S. aureus* and assayed with an anti-human IgG antibody conjugated to alkaline phosphatase (Fig. 2A). A densitometric analysis of the blots (Fig. 2B) suggests that anti-LTA IgGs in the serum of these patients show a greater reactivity for LTAs from *S. sanguis* or *S. faecalis* than for LTA from *S. aureus*

**Binding of purified BD IgGs to immobilized MBP**

MBP was adsorbed to nitrocellulose membranes. Five active BD sera containing 2.70, 2.68, 2.32, 1.49 and 2.17 µg anti-LTA IgG/mg IgG, respectively, and five matched normal controls containing 1.81, 3.38, 2.30, 2.13 and 1.93 µg anti-LTA IgG/mg IgG, respectively, were individually purified by chromatography in protein A-Sepharose. Each blot was singly incubated with 100 µg of pure IgGs. The results (Fig. 3) suggest binding of the BD IgGs to MBP with different affinities, whereas binding of control IgGs to MBP is almost undetectable.

**C5 activation assay of anti-LTA antibody complexes**

The antigen-antibody complexes were also evaluated for their capacity to activate C5 to C5a. Statistically, there was an overall difference in the IgG anti-LTA antibody levels between the four groups (p<0.001). When differences between the four groups were studied, only patients with active BD generated significantly greater levels of C5a than controls (p < 0.0001) (Figure 5).

**Identification of exposed carbohydrate residues in IgGs from BD patients by a dot blot assay**

The results (Figs. 6A and 6B) show intense reactivity of the BD IgG with both PNA or GNA lectins, whereas the control (C) IgGs (Figs. 6A and 6B) showed decreased reactivity with these lectins. These data suggest that both galactose β(1-3) and mannose α(1-3, 1-6 or 1-2) are terminally exposed in the BD IgGs, thereby favoring the reactivity toward MBP described above.
OU patients, and 1.1 BD patients, 4.55 statistically significant (p < 0.01), as antibodies were 0.82 mal controls (Group 4).

LPS IgG were 0.76 measured by an ELISA technique. The median MBP binding capacity of specific anti-L TA IgG antibodies. MBP binding to specific anti-LTA IgG antibodies in the four groups was measured by an ELISA technique. The median concentrations of MBP bound to anti-LTA IgG antibodies were 0.82 µg MBP/µg IgG in active BD (Group 1), 0.49 µg MBP/µg IgG in inactive BD (Group 2), 0.52 µg MBP/µg IgG in active OU (Group 3), and 0.15 µg MBP/µg IgG in normal controls (Group 4).

Quantification of anti-LPS antibodies
The median serum titers of anti-LPS IgA were 1.31 µg/mg IgA in active BD patients, 3.33 µg/mg IgA in inactive BD patients, 4.55 µg/mg IgA in active OU patients, and 1.1 µg/mg IgA in normal controls (Fig. 7A). Specific differences were only detected between active OU and normal controls (p < 0.023). The median serum titers of anti-LPS IgG were 0.76 µg/mg IgG in active BD patients, 1.51 µg/mg IgG in inactive BD patients, 1.63 µg/mg IgG in active OU patients, and 0.38 µg/mg IgG in normal controls (Fig. 7B). The overall difference between groups was statistically significant (p < 0.01), as well as the difference between active OU and control patients (p < 0.0018).

IL-8 production by PBM after stimulation with LTA or LPS
These studies were performed on 8 active BD, 8 inactive BD, 8 active OU and 8 healthy individuals. High levels of IL-8 were detected in culture supernatants of non-stimulated PBM cells from BD patients when compared with secretion levels by cultured PMBs from patients of the other groups studied. The median values were 1877, 434, 398, and 478 pg/ml for active BD (Group 1), inactive BD (Group 2), active OU patients (Group 3), and normal controls (Group 4).

Discussion
LTA found in the cell membrane of streptococci is a potent immunogen (23). Since streptococci are frequently found in the oral flora of patients suffering from BD or active OU (7), we evaluated the titers of anti-LTA antibodies in their sera. Total IgAtiters in BD or active OU patients were greater than those of normal controls, but total IgG titers were greater only in active BD when compared with normal individuals. Our experiments suggest that the reactivity of anti-LTA IgGs from BD for LTAs from Streptococcus sanguis or Streptococcus faecalis are similar in dot blot analyses, whereas their reactivity for LTA from Staphylococcus aureus appears reduced, thereby suggesting streptococcal LTA as the immunogenic agent in these patients. These conclusions are supported by reports showing that pathogenic streptococcal strains are frequently identified in patients with BD (12, 24) or that antigens from Streptococcus sanguis show immunologic cross-reaction with heat shock proteins, and oral mucosa antigens from BD patients (25).

We found a greater binding of MBP to anti-LTA IgG of BD or active OU patients compared with normal controls, using ELISA or dot blot analyses. We also show that BD IgGs contain a larger proportion of exposed terminal mannosyl and galactosyl residues than those of normal controls. Furthermore, the complement activating capacity of
immune complexes between LTA and their IgG antibodies generated significantly greater levels of C5a only in active BD patients.

The differences in the complement activating capacity observed between active, inactive BD and active OU patients may be the result of differential glycosylation, which in addition to the one present in the Fc region, also affects the Fab sugars as a result of intervention by a number of factors, including cytokines, participating in differentiation and proliferation of B cells (26).

It is known that glycosylation can alter the ability of IgG to aggregate and form immune complexes, thereby affecting its interaction with the Fc receptor (27) or the binding affinity of the IgG molecule (28). Furthermore, circulating IgGs secreted with exposed terminal galactosyl or mannosyl carbohydrate residues are pathogenic in rheumatoid arthritis, where their titers correlate with the clinical severity of the disease (29, 30).

Oligosaccharide chains with nonreducing terminal mannosne in the IgG Fc region are ligands for the serum mannose binding protein (MBP), which associated to proteases like C1r and C1s lead to complement activation through the classical pathway (21). This is the case for deglycosylated IgGs, cross reactive with streptokinase and fibronectin commonly found in sera from rheumatoid arthritis patients, which bind MBP and have a greater complement activating activity than normal IgGs (31). Changes in glycosylation of anti-LTA IgGs of BD or active OU patients are also affecting their complement activating capacity but only in active BD patients. Although these data suggest a humoral immune response in the clinical manifestations of BD, its pathogenicity may be also expressed by other mechanisms.

Patients with BD show an increased T cell activity upon challenge with several peptides derived from the primary structure of heat shock protein 60, which are also able to induce uveitis in a rat animal model (32). Our experiments show that PBM in culture from active BD subjects spontaneously produce IL-8 at levels greater than those of inactive BD, active OU or normal subjects when stimulated with LTA. A previous study demonstrated that serum IL-8 levels were significantly greater in active BD than those of inactive BD or normal control subjects (33). Consistent with these results, our data show a strong correlation between IL-8 levels and disease. No differences in IL-8 production were observed in PBM cells from the three groups of patients stimulated with LPS, suggesting LTA as one causal agent of BD.

An increased expression of IL-8 by PMBs including monocytes, T lymphocytes, neutrophils, fibroblasts, endothelial cells, and epithelial cells has been previously reported for BD (10). It is known that T-cells can be stimulated by streptococcal antigens to produce IL-6 and IFN-γ (34). LTA binds and in some cases influences the function of polymorphonuclear leukocytes, lymphocytes, monocytes, and macrophages (35, 36) inducing maximal stimulation of TNF-α, IL-1β, IL-6, and IL-8 release from human monocytes (37, 38). Anti-LTA antibodies are known to induce not only an aggregation of LTA receptors in LTA-sensitized monocytes, but also enhance expression and secretion of pro-inflammatory cytokines (39).

The mechanisms involved in the in vitro stimulation are controversial. Two groups have recently proposed that LTA stimulates the in vitro secretion of these cytokines via interaction with Toll-like receptors 2 and 4 (40, 41). However, other groups have proposed an alternative mechanism in which

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**Fig. 7.** Serum titers of specific anti-LPS antibodies. The anti-LPS titers in the four groups were measured by an ELISA. (A) Median titers of anti-LPS IgAwere 1.31 µg/mg IgA in active BD, 3.33 µg/mg IgA in inactive BD, 4.55 µg/mg IgA in active OU and 1.1 µg/mg IgA in normal controls. (B) Median titers of anti-LPS IgG were 0.76 µg/mg IgG in active BD, 1.51 µg/mg IgG in inactive BD, 1.63 µg/mg IgG in active OU, and 0.38 µg/mg IgG in normal controls.

**Fig. 8.** IL-8 concentration in peripheral blood mononuclear cell cultures after stimulation with LTA or LPS. 24 hours after stimulation with S. sanguis LTA or E. coli LPS (10 µg/ml) among active BD (n = 8), inactive BD (n = 8), active OU and normal controls (n = 8). (A) Median levels of IL-8 after LTA stimulation were: 62425 pg/ml in active BD patients, 39940 pg/ml in inactive BD patients, 38630 pg/ml in active OU, and 39118 pg/ml in normal controls. (B) Median levels of IL-8 after LPS stimulation were: 47925 pg/ml in active BD patients, 48836 pg/ml in inactive BD patients, 50953 pg/ml in active OU, and 49732 pg/ml in normal controls.
LTA stimulates IL-8 production by monocytes in a CD14-dependent manner (42-44). Therefore, it is difficult to hypothesize which receptors may be involved in the LTA stimulated secretion of IL-8 by PMBs from BD patients in our study. Although anti-LTA antibodies are present in normal human sera (45), the local secretion of cytokines stimulated by LTA antibodies may be pivotal in the pathogenesis of BD (39).

In this context, the enhanced secretion of TNF-α or soluble TNF-α receptor in active BD patients plays a major role (46), because treatment of these patients with a monoclonal antibody to TNF-α results in remission of the disease (47, 48).

The immune response against LTA appears to be specific because titers of anti-LPS antibodies are similarly low in BD or healthy individuals, although all BD patients had active oral ulcers. Only the active OU patients show significantly greater anti-LPS antibody titers than normal controls, which may result from colonization by gram-negative bacteria of previously damaged mucosal cells, as it is common in patients with viral induced oral mucositis who show increased adherence of “abnormal” aerobic gram-negative bacilli to infected mucosal cells (49).

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