The Functional State of the Complement System in Leprosy

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Abstract. Ninety-one patients with different clinical forms of leprosy, 36 lepromatous (LL), 33 tuberculoid (TL), and 22 dimorphic (DL), and 31 healthy volunteer donors were included in this study. Total complement system (CS) activity was assessed by hemolytic methods, whereas individual components were quantified by the enzyme-linked immunosorbent assay. Under conditions allowing initiation of cascade by the classic pathway (CP) but not alternative pathway (AP) activation, significant CS consumption was detected only in sera from patients with LL. In this group of patients, C4 but not factor B (fB) or C3 was significantly reduced, whereas mannose-binding lectin (MBL) serum levels were significantly higher. These results indicate that the CP is involved in CS activation in patients infected with Mycobacterium leprae manifesting LL clinical form of leprosy. An association is likely between circulating immune complexes and MBL high serum levels for initiation of CS activation in patients with LL form of leprosy.

INTRODUCTION

Leprosy, an infectious disease caused by Mycobacterium leprae, an intracellular pathogen, affects skin and nerves.1,2 The clinical manifestations correlate with the immune response to the pathogen.3,4 At one end of the spectrum, the tuberculoid form (TL) is characterized by a limited self-curing disease with few bacteria and local expression of Type 1 cytokines characteristic of a strong Th1 immunity. In contrast, patients with lepromatous leprosy (LL) present clinically with disseminated skin lesions characterized by a high number of bacilli, expression of Type 2 cytokine profile typical of a strong Th2 immunity, and suppression of Th1 cellular immunity.4 The intermediate subtype consists of the borderline leprosy clinical forms (BL), which include patients presenting clinical conditions between the extreme conditions of TL and LL leprosy. The BLs are immunologically unstable, and disease manifestations may shift toward either pole.5 Along the chronic course of leprosy, sudden increases in immune activity may occur, leading to expression of either Type 1, the reversal reaction (RR), or Type 2, the erythema nodosum lepromus (ENL). Although RR is caused by an acute exacerbation of Th1 immune reaction, ENL is a predominant Th2 immune complex–mediated disease.6

Once M. leprae interact with macrophages or with Schwann cells to which it causes damage,1,2,4 bacterial heterologous and host autologous antigens are released. On preferential stimulation of the Th 2 immune response as in the LL form of leprosy, antibodies are formed and combine with the antigens, and the antigen-antibody complexes act as potent activators of both complement system (CS) and macrophages. Accordingly, mycobacterial antigen-rich immune complexes have been isolated from sera of LL.7,8 It is well documented that CS plays a pivotal role in preventing precipitation,9 inducing solubilization and elimination of immune complexes.10

The CS is made up of > 30 plasma and cell surface proteins that play a crucial role in host defense against infection. Activation of CS results in opsonization of pathogens and immune complexes, recruitment of leukocytes, inflammation, and cell lysis. The three major pathways of complement include the classic pathway (CP), which is usually initiated by antigen–antibody complexes followed by complement C1 activation; the alternative pathway (AP), which is initiated by spontaneous hydrolysis of complement component C311,12, and the mannose-binding lectin pathway (MBLP), which is activated by recognition of certain microbial polysaccharides.13 All three pathways converge at the C3 activation step, leading to the generation of opsonins, inflammatory peptides, and formation of the membrane attack complex.

Experiments performed with sera from leprosy patients indicated that CS can be activated by circulating immune complexes isolated from these patients.14 Whole M. leprae bacilli incubated with human serum activate the CS, became opsonized by C3 fragments, and can be recognized by CR1 and CR3 complement receptors and subsequently phagocytosed by human monocytes.15 These in vitro experiments suggest that in vivo activation of CS could be critical to control both the tissue bacillus load and the circulating immune complexes found in the LL leprosy form.

In this study, the total CS hemolytic activity and the levels of some of its individual components were evaluated in sera from leprosy patients. Additionally, the MBL serum levels were evaluated and compared with the controls. The MBL serum levels of patients presenting LL forms were lower than those of the other forms of the disease and the controls. Our results indicate that the total hemolytic activity of CS is lower in sera from patients with the LL form of leprosy and is caused by consumption of components belonging to CP.

MATERIALS AND METHODS

Patients. This study was performed on sera from 91 patients with definitive clinical and laboratory diagnoses of leprosy classified as follows: 36 patients with LL, 33 with TL, and 22 with DL, and 31 healthy volunteer donors. Table 1 includes the ratio of male to female in the different groups, the proportion of those tested that showed positive bacterial index on Ziehl-Neelsen (ZN) staining, and the mean and range of the ages of the different groups. Blood samples were collected before starting therapy regimens with the specific drugs, and sera were stored at ~70°C. Sera from 31 healthy donors, clinically free of infectious disease, were used as controls (C). For the hemolytic assays and determination of C4, C3, and fB, the group of study was made up of 78 patients (27 LL; 29 TL; 22
DL) and 25 healthy donors (C). For MBL assays, 78 patients were included: 36 LL; 33 TL, 7 DL, and 31 healthy donors. The Ethics Committee of the Faculdade de Medicina de Campos, Rio de Janeiro, Brazil, approved this study; patients and healthy volunteers were recruited after obtaining informed consent.

Complement hemolytic assay. Macroassay. Two hundred fifty microliters of sera from patients or controls was serially diluted (1/150 to 1/1,500) in GVB++ buffer (0.1% gelatin, 128 mmol/L NaCl, 0.15 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, and 1.8 mmol/L sodium barbital) and were added to tubes containing 150 μL 1.5 × 10^8 sheep erythrocytes (E⁺) previously sensitized with rabbit anti-E⁺ antibodies (E⁺A), also in GVB++ buffer, and the mixtures were incubated at 37°C for 1 hour. Similar E⁺A samples in 150 μL GVB++ buffer (to monitor spontaneous lysis) or water (100% hemolysis) were always run in parallel. The hemolytic reactions were stopped by the addition of 2 mL cold 0.15 mol/L NaCl, centrifuged at 4°C, 2,500 rpm, for 10 minutes, and the absorbance was read at 412 nm. The degree of lysis (y) at the 50% endpoint (CH50) of patient or control serum samples was calculated as the function of the optical density (OD) obtained in each serum dilution minus the sample OD from spontaneous lysis divided by the OD of 100% lysis minus sample OD times 100: y = (sample OD – spontaneous lysis OD/OD at 100% lysis – sample OD) × 100.

Microassay. Sera from leprosy patients or controls were serially diluted (1/150 to 1/1,500) in GVB++ buffer or in GVB-EGTAMg buffer (0.1% gelatin, 128 mmol/L NaCl, 1.8 mmol/L sodium barbital, 2 mmol/L MgSO₄, and 10 mmol/L EGTA). Fifty microliters of each serum dilution was mixed with 50 μL 1.5 × 10^8 E⁺A suspended in GVB++ for the CP assay or with unsensitized rabbit erythrocytes (E⁺) suspended in GVB-EGTAMg for the AP assay. Similar E⁺A or E⁺ samples in buffer (spontaneous lysis) or in water (100% hemolysis) were always included in the assays. The plates were incubated for 10 minutes at 37°C. The hemolytic reactions were stopped by the addition of 200 μL cold 10 mmol/L GVB-EDTA (0.1% gelatin, 128 mmol/L NaCl, 1.8 mmol/L sodium barbital, 20 mmol/L EDTA) to each well, the plates were centrifuged at 4°C, 2,500 rpm, for 10 minutes, and the absorbance of supernatant was read at 412 nm. Percentage of hemolysis was calculated and expressed as the number of hemolytic sites per erythrocyte.

Immunochemical assays. C3, C4, and fB CS components were quantified by ELISA using mouse mAbs anti-human C3 and anti-human fB or rabbit polyclonal anti-human C4 (Sigma, St. Louis, MO) antibodies as the first antibodies and peroxidase-labeled goat anti-mouse IgG (Caltag, Burlingame, CA) or goat anti-rabbit IgG (Sigma) as the second antibody. To assess indirect binding of human CS components fragments to activating particles, microtiter plates (Costar, Corning, NY) were coated with 50 μL purified human IgG (Aventis-Pasteur, Paris, France) at 20 μg/mL for C3 and C4 or 0.1% agarose for fB quantification. Non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), followed by the addition of serially diluted (1/8 to 1/1,024) serum samples from leprosy patients or control in GVB buffer (for C3 and C4 fragments binding) or GVB-EGTAMg (for fB fragment binding) followed by incubation at 37°C for 2 minutes. Each serum sample was tested in duplicate. After washing with PBS/0.05% Tween 20 (PB/T), bound C3b, C4b, or fBb was detected with the respective specific first antibodies. After washing as above, 40 μL of the appropriate peroxidase-coupled IgG (anti-mouse at 1/4,000 or anti-rabbit at 1/2,000) in the blocking solution was added and incubated for 1 hour at room temperature. Peroxidase activity was detected by adding 50 μL of a solution containing orthophenylenediamine (OPD) and hydrogen peroxide (H₂O₂). After 5 minutes, the reaction was stopped by the addition of 50 μL 3 N H₂SO₄, and the absorbance was measured at 492 nm. The mean values of C3, C4, and fB in sera from three healthy volunteers were used as standard in all assays. Results are expressed in micrometers per milliliter and calculated as the mean of the OD of each sample minus the average absorbance of the blank divided by the average absorbance of the standard sera minus the average absorbance of the blank, multiplied by 100. MBL in serum of patients and controls was determined by using the MBL mannan-binding ELISA oligomer kit 029 (Antibodyshop, Gentofte, Denmark). One hundred microliters of serum diluted 1:100, the standard for calibration, and an unrelated serum as negative control were added to each well in duplicate and incubated for 1 hour at room temperature. The wells were washed three times with washing buffer, 100 μL biotinylated anti-MBL antibody was added, and plates were incubated for 1 hour at room temperature. After washing, 100 μL streptavidin peroxidase was added to each well, and plates were incubated for 1 hour; washed, followed by the addition of specific substrate, and further incubated for 15 minutes at room temperature. Reactions were stopped, and absorbance was read at 450 nm. MBL concentrations were determined by plotting ODS against the calibration curve and expressed as nanograms per milliliter serum.

Statistical analysis. All data are expressed as the mean ± SEM. Statistical analysis was performed using the Kruskal-Wallis test. P < 0.05 was considered significant.

RESULTS

The functional state of the CS was analyzed in fresh sera from 78 patients with a clinical and laboratory diagnosis of leprosy. The patients group consisted of 27 with LL, 29 with TL, and 22 with DL leprosy clinical forms. Sera from 25 healthy donors were used as normal controls. The mean values ± SEM for CH₅₀ per milliliter in sera from patients with different clinical forms of leprosy, as evaluated by complement hemolytic assay of E⁺A under conditions allowing initiation of the CP, were 835.7 ± 154.9, 709.6 ± 107.6, and 784.0 ± 155.0 for TL, LL, and DL, respectively, compared

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* The proportion of those tested that showed positive bacterial index on ZN staining. ND, not done.

Clinical and demographic profile of patients with leprosy and controls

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with 841.1 ± 156.6 for sera from controls. A small but significant reduction of 15.6% (P < 0.05) was only detected in sera from patients with LL leprosy form compared with healthy donors. The distribution of quantified CH50 among the analyzed groups indicated that, in contrast to controls, the CH50 in LL patients, and to a smaller degree in DL patients, tends to concentrate below 800 CH50/mL serum (Figure 1).

Indications that the hemolytic activity of CS is lower in LL patients was further analyzed in a micro assay hemolytic test using E’sA under conditions allowing activation of the CP. The incubation time was decreased to 2 minutes, and the results are reported as the number of hemolytic sites Z per milliliter of undiluted serum. The values of Z per milliliter of undiluted serum were 414 ± 270 for TL, 286 ± 270 for LL, and 400 ± 270 for DL clinical forms compared with 529 ± 214 for normal donors (Figure 2A). The total hemolytic activity is lower in sera from LL patients (P < 0.032) compared with sera from TL and DL leprosy patients (P < 0.04), as well as compared with controls. When, however, E’s instead of E’sA erythrocytes were used to assay the hemolytic activity, under conditions allowing AP complement activation, no differences were observed among the sera from the three clinical forms and in controls (Figure 2B).

The individual complement components C4, C3, Iβ, and MBL were immunochemically evaluated using specific antibodies as detecting antibodies. C4 and C3 were quantified, allowing the serum samples to be activated by aggregated human IgG on 96-well microplates wells under conditions prevailing CP activation. The amounts of C4 were 88.8 ± 31.7 μg/mL in TL, 69.9 ± 26.3 μg/mL for LL, and 92.0 ± 34.9 μg/mL for DL compared with 95.2 ± 15.8 μg/mL for controls. A significant reduction of C4 (P < 0.008; Figure 3A) was detected only in sera from LL patients. C3 concentrations in sera from LL patients, although less compared with sera from leprosy patients with other leprosy clinical forms or health donors, were not significant (Figure 3B). Equally, the levels of native Iβ were not different (Figure 3C).

The distribution of MBL plasma levels detected in the leprosy patients and controls is shown in Figure 4. TL patients presented the lowest MBL mean values (498 ng/mL; N = 33) compared with those obtained in DL patients (2,494 ng/mL; N = 7) and LL patients (1,150 ng/mL; N = 36). The mean MBL plasma levels in controls were 500 ng/mL (N = 31).

**DISCUSSION**

In this study, we provided data showing that patients expressing the LL clinical form of leprosy, but not patients with other forms of this disease, present lower total hemolytic complement levels compared with controls. Reduced complement levels were only detected when the hemolytic complement activity in LL sera was assayed using antibody pre-coated E’s in GVB⁺⁺⁺ buffer, conditions directing C1, C4, and
C2 activation through the CP (Figures 1 and 2A). The indications that these complement components are the key initiators of the complement consumption in LL patients were reinforced by the findings that hemolytic activity reduction in LL sera was not observed when the assays were performed using Er as target in GVB-EGTAMg buffer, conditions in which C3 and fB initiator components of the AP are activated (Figure 2B) and that the observation that the amounts of native C4 (Figure 3A), but not native C3 and fB, are decreased as assayed by immunochemical methods using specific monoclonal antibodies as probes (Figures 3B and 3C).

Reduction in the total hemolytic complement activity in LL patients may be explained by the presence of circulating preformed immune complexes. Influence of MBL concentration in the serum complement reduction observed in LL patients cannot be ruled out, because the tissue lesions in these patients usually contain high amounts of bacilli. MBL, which acts as a pattern recognition molecule for a wide range of infectious agents, interacts with sugar moieties such as mannose, N-acetyl glucosamine, fucose, and glucose present on the surface of several microorganisms, leading to their phagocytosis and complement activation. The M. leprae cell surface, as that of other mycobacteria, is rich in 6-lipoarabinomannan (LAM), a mannose-containing carbohydrate. Although we have not directly addressed the MBL CP activation, coincidentally, patients with LL and DL presented the highest median values for MBL (1,150 and 2,494 ng/mL, respectively), which presented significantly lower hemolytic complement titers, as opposed to TL and controls that showed the lowest mean values for MBL (500 ng/mL for both), with normal hemolytic complement titers. The MBL serum levels found in the leprosy patients included in this paper are similar to those described in a cohort of patients from Paraná state, Brazil: TL patients presented the lowest MBL mean values compared with those with LL and DL forms.

Figure 3. A, Evaluation of active C4 in sera of patients with different clinical forms of leprosy: TL (N = 27), LL (N = 29), and DL (N = 22). Normal donors used as controls (N = 25). Samples of serum were added to microtiter plates containing immobilized aggregated human IgG and incubated under conditions allowing classic pathway activation. Active binding of C4b was measured by ELISA. The columns represent the arbitrary units (U/mL) of C4b fragment. The bars represent mean ± SEM. *Significant differences between patients and controls. B, Evaluation of active C3 in sera of patients with different clinical forms of leprosy: TL (N = 27), LL (N = 29), and DL (N = 22). Normal donors used as controls (N = 25). Samples of serum were added to microtiter plates containing immobilized aggregated human IgG and incubated under conditions allowing classic pathway activation. Active binding of C3 fragments was measured by ELISA. The columns represent the arbitrary units (U/mL) of C3 fragments. The bars are means ± SEM. C, Evaluation of active factor B in sera of patients with different clinical forms of leprosy: TL (N = 27), LL (N = 29), and DL (N = 22). Normal donors used as controls (N = 25). Samples of serum were added to microtiter plates containing immobilized agarose and incubated under conditions allowing alternative pathway activation. Active binding of factor fragment Bb was measured by ELISA. The columns represent the arbitrary units (U/mL) of Bb. The bars are means ± SEM.

Figure 4. MBL levels in sera from patients with different clinical forms of leprosy, TL (N = 33), LL (N = 36), and DL (N = 7), and normal donors (N = 31). Samples of serum were added to microtiter plates containing immobilized mannan, and active binding of MBL was measured by the mannan-binding ELISA oligomer kit 029 (Antibodyshop). MBL concentrations were determined by plotting ODs against the calibration curve and expressed as nanograms per milliliter serum. Boxes show the association between MBL serum levels and leprosy clinical forms. The horizontal lines within each box represent the mean. The lower and upper bounds represent the minimal (25%) and maximal (75%) values.

C2 activation through the CP (Figures 1 and 2A). The indications that these complement components are the key initiators of the complement consumption in LL patients were reinforced by the findings that hemolytic activity reduction in LL sera was not observed when the assays were performed using Er as target in GVB-EGTAMg buffer, conditions in which C3 and fB initiator components of the AP are activated (Figure 2B) and that the observation that the amounts of native C4 (Figure 3A), but not native C3 and fB, are decreased as assayed by immunochemical methods using specific monoclonal antibodies as probes (Figures 3B and 3C).

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ciency has been reported to confer partial protection against some intracellular pathogens, such as M. leprae and M. tuberculosis.

The higher serum C3 concentration (1.0–1.2 mg/mL) in comparison with trace amounts of the other complement components may explain the failure to observe C3 consumption in sera from patients with the LL clinical form. Remaining amounts of C3 in serum in which the initiator components C1, C2, and C4 were activated, even at low levels, are sufficient to organize C5 convertases enough to create effective C5b-C9 membrane attack complexes.11,12 Taken together, our results obtained from leprosy patients inhabiting Rio de Janeiro State in Brazil indicate that the CS consumption observed in LL patients is primarily caused by CP activation. The high MBL plasma levels in sera from LL patients suggest that MBLP can also be involved in CS activation by some mannan-rich M. leprae cell surface components.19 Published data on the serum complement profile in leprosy in India are controversial. One study, based on consumption of C1-inactivator, Clq, C3, C4, C5, C8, and C9, in sera from 30% to 50% of leprosy patients indicates that CS activation is initiated by the AP.24 The other study, based on the presence of fB breakdown product Ba in sera of leprosy patients, suggested that CS activation can also be initiated by the AP.25

Such discrepancies may be explained by differences in analyzed groups of leprosy patients, mainly in regard to regional origins and bacillary loads, by different M. leprae strains infecting both populations, and by differences in the methods used to identify the complement components. The presence of the fB breakdown products Ba in sera of patients with leprosy necessarily indicate primarily AP activation. Now it is well known that AP can also be initiated as an “amplification loop” when fixed C3b that is generated by the CP or MBLP pathway activation binds fB, resulting in conformational changes in fB that allow factor D to cleave it similarly to the AP initiation by a “tickover” process.26 Therefore, the presence of circulating the fB breakdown products Ba in sera of patients with leprosy necessarily indicate primarily AP activation. Now it is well known that AP can also be initiated as an “amplification loop” when fixed C3b that is generated by the CP or MBLP pathway activation binds fB, resulting in conformational changes in fB that allow factor D to cleave it similarly to the AP initiation by a “tickover” process.26 Therefore, the presence of circulating the fB breakdown products Ba in sera of patients with leprosy necessarily indicate primarily AP activation. Now it is well known that AP can also be initiated as an “amplification loop” when fixed C3b that is generated by the CP or MBLP pathway activation binds fB, resulting in conformational changes in fB that allow factor D to cleave it similarly to the AP initiation by a “tickover” process.26 Therefore, the presence of circulating the fB breakdown products Ba in sera of patients with leprosy necessarily indicate primarily AP activation. Now it is well known that AP can also be initiated as an “amplification loop” when fixed C3b that is generated by the CP or MBLP pathway activation binds fB, resulting in conformational changes in fB that allow factor D to cleave it similarly to the AP initiation by a “tickover” process.26 Therefore, the presence of circulating the fB breakdown products Ba in sera of patients with leprosy necessarily indicate primarily AP activation. Now it is well known that AP can also be initiated as an “amplification loop” when fixed C3b that is generated by the CP or MBLP pathway activation binds fB, resulting in conformational changes in fB that allow factor D to cleave it similarly to the AP initiation by a “tickover” process.26 Therefore, the presence of circulating the fB breakdown products Ba in sera of patients with leprosy necessarily indicate primarily AP activation.

Last, leprosy patients were classified according to the clinical forms of the disease. Patients exhibiting typical bacilli or by both is under study on our laboratory. Reprint requests: Therезa L. Kipnis, Laboratório de Biologia do Reconhecer, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense–Darcy Ribeiro, Av. Alberto Lamego, 2000 Parque Califórnia, CEP.28015-620, Campos dos Goytacazes, RJ, Brazil, Telephone: 5522-2726-1591, Fax: 5522-2726-1591. Edilbert P. Nahn Jr., Hospital M Ferreira Machado, Campos dos Goytacazes, RJ, Brazil.

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