Cytotoxicity of Human Serum for *Leishmania donovani* Amastigotes: Antibody Facilitation of Alternate Complement Pathway-Mediated Killing

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Mechanisms that mediate recovery from leishmanial infection have not been fully characterized but are generally believed to involve interactions between T lymphocytes and macrophages. A major role for serum-mediated effector mechanisms in the protection of humans from reinfection with *Leishmania*, however, has not been ruled out. In this report, amastigotes of *L. donovani* were incubated with dilutions of serum from normal subjects and from patients with kala-azar. Normal serum was cytotoxic for parasites at a dilution of ≥1:20. Cytotoxicity did not occur in the presence of EDTA, was abolished by heating serum to 56°C for 30 min, and was not diminished by prior adsorption of normal serum with parasites at 0°C. Killing proceeded normally in the presence of magnesium-ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid, however, and was fully effected by C2-deficient serum. These studies indicated that killing of amastigotes, unlike that of promastigotes, was mediated via the alternate pathway of serum complement. In further studies, cytotoxicity of normal serum was enhanced three-to fivefold by factors in patient serum. This enhanced cytotoxicity also proceeded via the alternate complement pathway. Factors that enhanced cytotoxicity were characterized as parasite-specific immunoglobulin G; they eluted with immunoglobulin G on column chromatography, were adsorbed by immobilized staphylococcal protein A, and were not removed from the parasite surface by extensive washing. Thus, infection of individuals with *L. donovani* resulted in the production of a new, qualitatively and quantitatively distinct immune mechanism directed against the amastigote form of the parasite, namely, antibody-directed, alternate complement pathway-mediated cytotoxicity. These results provide a mechanistic framework for a role of humoral factors in human resistance to reinfection with *L. donovani*.

The protozoan parasite *Leishmania donovani* causes a severe human disease known as visceral leishmaniasis, or kala-azar. Infection is acquired from sandfly bites after injection of the extracellular, promastigote form of the parasite into the skin. Promastigotes enter macrophages and convert to the intracellular, amastigote form. Amastigotes replicate and burst their host cell. Parasites are then released into the extracellular space and disseminate to liver, spleen, lymph nodes, and bone marrow. This dissemination is associated with the clinical syndrome of fever, hepatosplenomegaly, weight loss, and pancytopenia. Without specific antimicrobial therapy, patients die of infection; treated survivors, however, are immune to reinfection (12).

Mechanisms that mediate immunity to *Leishmania* spp. have not been fully characterized but are generally believed to involve interactions between T lymphocytes and macrophages. This belief is supported by certain phenomena that occur in infected patients and animal models: (i) human visceral leishmaniasis progresses despite extremely high titers of specific anti-parasite antibody; (ii) delayed cutaneous hypersensitivity to leishmanin, a preparation of Formalin-killed organisms, is depressed when visceral disease is active, but returns to responsive after successful treatment and recovery; and (iii) lymphoid cells, but not serum, adoptively transferred from immune mice can provide protection from subsequent intravenous or subcutaneous challenge with parasites (11). Despite these observations, a major role for serum-mediated effector mechanisms in protection of humans from reinfection with *L. donovani* has not been ruled out. Indeed, in a mouse system, immune serum markedly augments the protection against *Leishmania tropica* induced by adoptively transferred immune lymphoid cells (10). Moreover, processes that effect recovery from many established infections in humans may not necessarily be those that mediate resistance to reinfection. For example, chickenpox, caused by varicella-zoster virus, is usually a minor illness of healthy children. Infection can be prevented by transfer of immune serum, even in patients whose cell-mediated defenses are severely compromised (3). Once the viral infection is established, however, administration of immune serum does not affect the clinical course; furthermore, in individuals who lack adequate cellular immune responses, severe disease frequently ensues despite the presence of specific antibody (1). Thus, antibodies to varicella-zoster virus are sufficient to prevent acquisition of infection, but play little, if any, role in extinguishing established disease.

In this report, we show that factors present in high titer in sera of individuals with kala-azar significantly augment killing of *L. donovani* amastigotes by normal human serum. This killing is directed by specific antibody and mediated by the alternate complement pathway. These results provide a mechanistic framework for the role of humoral factors in human resistance to reinfection with *L. donovani*.
**MATERIALS AND METHODS**

Leishmania. *L. donovani*, Sudan strain 2-S, was propagated in Syrian hamsters (Charles River Laboratories, Wilmington, Del.). Amastigotes were harvested in RPMI 1640 with 25 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) buffer (GIBCO, Grand Island, N.Y.) from minced infected spleens after tissue homogenization in a Ten Broeck grinder and washed twice after centrifugation at 200 × g and twice after centrifugation at 2,000 × g (4). *Leishmania tropica major*, NIH 173, was propagated in infected foot pads of BALB/cJ mice, (Jackson Laboratories, Bar Harbor, Maine). Amastigotes were harvested as previously described (8), except that RPMI 1640 without serum was used throughout the harvest procedure. Parasites were stained with toluidine blue O and counted in a hemacytometer. Amastigotes (10^7 in 1.0 ml) were added to tubes and pelleted by centrifugation at 1,000 × g.

Serum. Serum previously collected from three patients who were chronically infected with *L. donovani* in Kenya was heated at 56°C for 30 min before testing. These sera had high titers (1:320 to 1:1280) of anti-leishmanial antibody detected by complement fixation with promastigotes as targets (data not shown; W. T. Hockmeyer, B. T. Welde, C. L. Sabwa, D. H. Smith, P. H. Rees, and P. A. Kager, Ann. Trop. Med. Parasitol., in press). Serum collected from normal volunteers was either used on the day of collection or stored at −70°C and thawed immediately before testing.

**Serum treatment of amastigotes.** Amastigotes were suspended in 0.6 ml of various dilutions of serum in RPMI 1640 and incubated at 37°C for 40 min. After incubation, amastigotes were washed twice in RPMI and suspended in 0.8 ml of assay medium (RPMI 1640, 10% heat-inactivated fetal bovine serum [GIBCO], 50 µg of gentamicin per ml). In certain experiments, amastigote suspensions were incubated with serum diluted with an equal volume of phosphate-buffered saline containing EDTA (10 mM final concentration) or MgCl2 (2.5 mM) and ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (10 mM), all at pH 7.4. To analyze the time course of cytotoxicity, we suspended amastigotes in 0.3 ml of RPMI and placed them at 37°C for 15 min. Then, 0.3 ml of diluted serum was added, and cultures were returned to the incubator. At appropriate intervals, ice-cold EDTA (10 mM final concentration) was added, and cultures were placed on ice before being washed twice with RPMI 1640.

**Estimation of parasite viability.** Analysis of amastigote killing required a reliable and accurate method to determine the viability of amastigote preparations. As detailed previously (5), we estimated viability by allowing amastigotes to convert to promastigotes at 26°C in the presence of hydroxyurea. Hydroxyurea permits transformation of amastigotes to enlarged, flagellated or nonflagellated forms but inhibits replication of promastigotes after conversion. Amastigotes were diluted 1:10 in conversion medium (1:1 Cunningham SM [2] medium/RPMI 1640–30% FBS–50 µg of gentamicin per ml) with 0.2 mg/ml hydroxyurea. After incubation at 26°C for 72 h, parasites were stained with toluidine blue O and counted in a hemacytometer. Amastigotes that did not convert remained small and rounded, in contrast to the larger, elongated conversion intermediates, which were easily distinguished from amastigotes after 72 h of incubation (6).

**Statistical analysis.** All data were analyzed for statistical significance by Student’s t test.

**RESULTS**

We have previously shown that certain factors in fresh normal human serum were strongly cytotoxic to amastigotes of *L. tropica* (5). These cytotoxic serum factors were generated during specific antibody-independent interaction of the parasite with components of the alternate complement pathway. We further showed that under identical conditions, amastigotes of *L. donovani* were substantially less susceptible to this complement-mediated cytotoxicity: cytotoxicity against *L. donovani* amastigotes required a higher concentration of normal human serum than that needed for *L. tropica*. Moreover, even at a 1:2 dilution, only half of the *L. donovani* amastigotes were killed, compared with 95% of *L. tropica* amastigotes (5). Experiments presented in Fig. 1 showed that the relatively weak cytotoxic activity of normal human serum against *L. donovani* amastigotes was significantly increased by addition of a 1:50 dilution of immune serum from patients with kala-azar. The cytotoxic activity of normal serum plus immune serum against *L. donovani* amastigotes was more than threefold greater than that of normal serum plus nonimmune control serum throughout the normal serum dose-response curve. The cytotoxic activity of normal serum with and without additional immune serum was abrogated by prior heating of normal serum at 56°C for 30 min.

This substantial enhancement by immune serum of normal human serum-mediated killing of *L. donovani* amastigotes
was further documented by analysis of the time course for cytotoxicity (Fig. 2). About 50% of the amastigotes were killed by factors in normal serum after 20 min of incubation at 37°C. Addition of immune serum significantly increased both the level and rate of cytotoxicity. At the plateau, one-fifth as many amastigotes survived in cultures with immune serum as in cultures with control serum; the rate of cytotoxicity was also increased fourfold in cultures with immune serum. Indeed, the rate and degree of cytotoxicity seen with L. donovani amastigotes incubated in normal serum plus immune serum were then comparable to those observed with L. tropica amastigotes cultured in normal serum alone (Fig. 2).

Activity in immune serum that increased normal serum-mediated cytotoxicity against L. donovani amastigotes was present in high titers (Fig. 3). In two separate experiments, dilutions of immune serum were added to concentrations of normal human serum that by itself effected minimal cytotoxicity (25% in Fig. 3A and 10% in Fig. 3B). Addition of immune serum increased this level of cytotoxicity three- to eightfold; the activity in immune serum was present through a 1:3,000 dilution. Titers of activity at least this high were found in sera of three different patients with kala-azar (data not shown). It should be noted that at the dilutions tested, i.e., ≥1:50, heat-inactivated immune serum by itself had no effect on amastigote viability.

The preceding experiments clearly show that factors in immune sera increased both the degree and the rate of cytotoxicity against L. donovani amastigotes by normal serum. The nature of these factors was clarified in further experiments. (i) Enhancing activity was not removed from the surface of amastigotes by washing; one-fourth of the concentration of normal serum was required to kill parasites incubated with immune serum compared with those incubated with control serum before washing and treatment with normal serum (data not shown). (ii) Factors coeluted with immunoglobulin G during gel chromatography (Fig. 4); in fact, activity was limited to the immunoglobulin G region of the column. (iii) Activity was removed by prior adsorption with staphylococcal protein A immobilized on Sepharose 4B. Immune serum adsorbed with protein A-Sepharose enhanced cytotoxicity of normal serum by only 4% at a 1:640 dilution. In contrast, the same concentration of unadsorbed immune serum or immune serum adsorbed with Sepharose alone enhanced killing by 47 and 46%, respectively. Immune serum (1 ml of a 1:10 dilution) was adsorbed three times with \(10^8\) amastigotes at 4°C in 5 experiments, with no change in dose-response of the serum activity (data not shown). Nevertheless, the experiments outlined above strongly suggested that the cytotoxicity-enhancing activity in immune serum was parasite-specific immunoglobulin G. Our failure to adsorb activity suggests that antibody may be of low affinity or does not require stable binding to the amastigote to enhance killing.

A previous report showed that the insect-associated, promastigote form of L. donovani was killed by normal serum (9). Killing was directed by natural, possibly cross-reacting antibody and was mediated by the classical complement pathway. This was not the case with L. donovani amastigotes. For example, cytotoxic activity against amastigotes was not removed from normal serum by prior adsorption with the parasite (Fig. 5). Cytotoxicity in normal serum was abrogated, however, by heating at 56°C for 30 min (Fig. 1), and was therefore likely to involve one or more complement components. This likelihood was examined further by analysis of the effects of chelating agents on serum-mediated cytotoxicity (Fig. 6). Cytotoxicity by normal serum with and
without immune serum was completely inhibited by EDTA but not by magnesium-ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid. These results suggested that the major pathway for cytotoxicity against *L. donovani* amastigotes by normal serum with or without immune serum was mediated by the alternate complement cascade. To confirm this hypothesis, we examined the cytotoxic effects of serum from C2-deficient patients on *L. donovani* amastigotes (Fig. 7). Serum from C2-deficient patients was cytotoxic to *L. donovani* amastigotes at a level comparable to that seen with normal serum; in both instances, cytotoxicity was increased fourfold by addition of a small amount of immune serum. Thus, normal human serum-mediated cytotoxicity against *L. donovani* amastigotes in the presence or absence of immune serum was effected via the alternate complement cascade.

**DISCUSSION**

Our data show that heat-stable factors in sera of patients with kala-azar markedly enhance the cytotoxicity of normal serum against amastigote forms of *L. donovani*. These observations require us to reconsider our concepts of the role of humoral factors in host immunity to *L. donovani*. Previous studies by Pearson and associates have shown that promastigotes, the insect-associated form of *L. donovani*, are killed by factors in normal human serum (9). Cytotoxicity, in that instance, was mediated by the classical complement cascade and was initiated by adsorbable factors, presumably cross-reacting, natural antibodies present in sera of normal subjects. The possible protective role of these cytotoxic but ubiquitous antipromastigote antibodies is unclear. It is possible that antibodies directed against the (usually) intracellular, amastigote form will be more protective than those directed against the promastigote form.

The studies described in this report enhance that possibility by documenting a novel interaction between *L. donovani* and factors in human serum. Killing of amastigotes, unlike that of promastigotes, is not mediated by the classical complement pathway; *L. donovani* amastigotes induce a low level of cytotoxicity mediated by direct activation of the alternate complement pathway. Moreover, in contrast to the antibody-directed killing of promastigotes, killing of amastigotes by normal serum is not mediated by adsorbable factors. Alternate pathway-mediated cytotoxicity, however, is significantly increased by antibodies that develop during natural infection. These findings document limited and nonprotective serum-mediated immune mechanisms directed against the amastigote form that are present in normal individuals. Infection of such individuals by *L. donovani*, however, generates in serum a new, qualitatively distinct immune response, i.e., antibody-directed, alternate complement pathway-mediated cytotoxic activity. Previously infected persons are also immune to reinfection (12). It is possible, then, that production of antibodies capable of enhancing serum cytotoxicity toward the amastigote stage is a crucial requirement for effective antiparasitic immunity. If so, strategies for vaccine development should be focused accordingly.
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FIG. 5. Cytotoxicity of adsorbed serum for *L. donovani*. Amastigotes were incubated for 40 min at 37°C with unadsorbed serum or serum that had been adsorbed five times with 5 x 10⁷ amastigotes at 0°C. Parasite viability was estimated by conversion to promastigotes. Curves for fresh serum differ significantly (P < 0.05) from control curve but do not differ from each other.

FIG. 6. Effect of complement inhibitors on killing of *L. donovani* amastigotes by human serum. Amastigotes (10⁷) were incubated at 37°C for 40 min with a 1:50 dilution of heat-inactivated normal (control) or immune serum plus a 1:20 dilution of fresh (unheated) or heat-inactivated normal serum in phosphate-buffered saline, 10 mM EDTA in phosphate-buffered saline, or 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid plus 2.5 mM MgCl₂ in phosphate-buffered saline, all at pH 7.4. The viability of treated amastigotes was estimated by conversion to promastigotes. The viability of the amastigotes incubated with fresh serum (with control or immune serum) in the presence of PBS or MgEGTA was significantly (P < 0.05) different from that of amastigotes in heat-inactivated serum or EDTA groups.

FIG. 7. Killing of *L. donovani* amastigotes by C2-deficient serum. Amastigotes (10⁷) were incubated with dilutions of fresh (unheated) normal or C2-deficient serum and a 1:50 dilution of heat-inactivated normal (control) or immune serum for 40 min at 37°C. The viability of treated amastigotes was estimated by conversion to promastigotes. The viability of the amastigotes was significantly less (P < 0.05) in the presence of normal and C2-deficient sera than in the presence of heated serum. Normal and C2-deficient serum did not differ from each other in cytotoxic effect.

Quite apart from considerations of vaccine development, one can raise innumerable questions regarding basic mechanisms of serum toxicity toward amastigotes. At the dilutions studied in this report, serum cytotoxicity toward promastigotes, in contrast to that toward amastigotes, is largely mediated by the classical complement pathway (9). This implies that transformation of the parasite from promastigote to amastigote stage dramatically alters its interactions with serum complement. No longer do natural antibodies mediate cytotoxicity; instead, amastigotes directly activate the alternate complement pathway. Moreover, antibodies present in the serum of infected patients markedly enhance cytotoxicity toward amastigotes; this enhancement is mediated exclusively via the alternate pathway. Studies with
chelating agents and C2-deficient serum clearly indicated that the classical pathway made no significant contribution toward antibody-directed serum killing of amastigotes. The characteristics of amastigotes or antibodies or both that lead to alternate rather than classical pathway activation and cytotoxicity remain unknown and are the subject of continued interest by our group.

More crucial questions address the mechanism by which antibody-facilitated alternate pathway-mediated killing occurs. Recent work indicates that antibodies, even minus the Fc portions, may focus and concentrate the interactions of alternate complement components with the cell wall and capsule of gram-negative bacteria. This influence of antibodies on the geometry of complement attachment to target may be more important than its influence on the rate of complement deposition in determining subsequent cytotoxicity by the membrane attack complex (7). The relationship of these observations to ours is not yet known. To facilitate antileishmanial cytotoxicity, must the entire antibody be present, or can fragments serve as well? Is rate, or geometry, more important in our system? Where in the process of complement, cell, and antibody interaction does enhancement of cytotoxicity toward amastigotes occur? How efficient is alternate pathway-mediated killing compared with the more familiar classical pathway-mediated process? Does preferential interaction of serum with amastigotes via the alternate pathway favor the parasite or the host? The answers to these questions will require substantial investigation. Such studies, however, should shed considerable light on the nature of the host-parasite relationship in this immunologically exceedingly complex disease.

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LITERATURE CITED