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Induction of complement-sensitivity in *Leishmania amazonensis* metacyclic promastigotes by protease treatment but not by specific antibodies

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During its life cycle, the protozoan parasite *Leishmania* has to differentiate into forms specialized in infecting vertebrate or invertebrate animals (Adler 1964). In this respect, leishmanias are similar to *Trypanosoma cruzi*, another trypanosomatid protozoan pathogenic to human beings (Brenner 1973). Thus, *Leishmania* promastigotes and *T. cruzi* epimastigotes multiply in the digestive tract of *Phlebotominae* and *Triatominae* insects, respectively. After a few days, *T. cruzi* epimastigotes differentiate into a mammal-infective stage, the metacyclic trypomastigote (mTry), acquiring the capacity to resist complement-mediated lysis when in contact with normal mammal sera (Nogueira et al. 1975). In a similar way, *Leishmania* promastigotes in the logarithmic phase of growth (logPro) differentiate into infective metacyclic promastigotes (mPro), also acquiring resistance to complement-mediated lysis (reviewed in Sacks 1989).

T. cruzi trypomastigotes, however, although resistant to complement-mediated lysis in normal sera, are readily lysed by antibodies (lytic antibodies) present in the sera of patients with Chagas' disease (Krettli et al. 1984). These antibodies have been associated both with infection activity and with immunity (Krettli et al. 1984).

On the basis of the many similarities between mTry and mPro, the possibility that *L. amazonensis* mPro might also be lysed by antibodies was investigated in the present study. Antibodies were obtained from *Leishmania*- or *T. cruzi*-infected patients (these antibodies react with several *Leishmania* promastigote antigens; Reed et al. 1987; dos Santos et al. 1987) and from *Leishmania*-immunized rabbits (a species normally resistant to infection by *Leishmania*). Diseases were diagnosed by the presence of specific antibodies in the sera as detected in an indirect immunofluorescence assay (Badaró et al. 1983) and by the direct demonstration of parasites at biopsy or xenodiagnosis. *Leishmania*-antibody titers (maximal serum dilutions leading to fluorescent parasites) ranged from 1:64 to 1:800. The titers of 100 normal control sera never exceeded 1:16. The sera from patients with Chagas' disease were shown to lyse *T. cruzi* mTry as described elsewhere (Krettli et al. 1984). Promastigotes in the stationary phase of growth (sPro), a proportion of which are mPro (Sacks 1989), were obtained from modified liver-infusion-tryptose (LIT; Difco Laboratories, Detroit, USA) cultures (Sadigursky and Brodskyn 1986) grown at 26° C and supplemented with 5% fetal calf serum. In cultures starting with 10^6 parasites/ml, a stationary phase of growth was reached after 6 days. The *Leishmania* isolate used was identified as *L. amazonensis* by a panel of species-specific monoclonal antibodies (Grimaldi et al. 1987).

The assay for lytic antibodies was similar to that described for *T. cruzi* (Krettli et al. 1984) and relied on the determination of the percentage of motile parasites remaining after a 30-min period of incubation at 37° C with fresh sera. At least 100 parasites were counted in a hemocytometer for each serum sample tested. Each immune serum specimen was tested in parallel with a normal sample of serum of the same species (human or rabbit) and with a sample of bovine fetal serum (Sigma Chemical Co., St. Louis, USA) as controls. To confirm that the parasites surviving the lytic process were indeed viable, on some occasions they were put back into fresh LIT medium, cultured for 48 h, and assessed for motility by light microscopy. No difference was observed in the viability of these parasites as compared with untreated promastigotes. In fact, the absence of flagellar motility has been shown to be a useful parameter to indicate the lethal effect of sera on *Leishmania* (Pearson and Steigbigel 1980).

As expected, virtually 100% of the promastigotes in the logarithmic phase of growth were susceptible to lysis in the presence of normal human sera (the mean percentage of parasites susceptible to lysis in 25 determinations with 17 sera was 99.4% ± 0.9%), whereas a proportion (ranging from 7% to 19%) of the stationary-phase promastigotes (sPro) was not affected by treatment with 7 fresh samples of normal human sera (Fig. 1).

Contrasting with the lytic activity of sera from patients with Chagas' disease against *T. cruzi* trypomasti-

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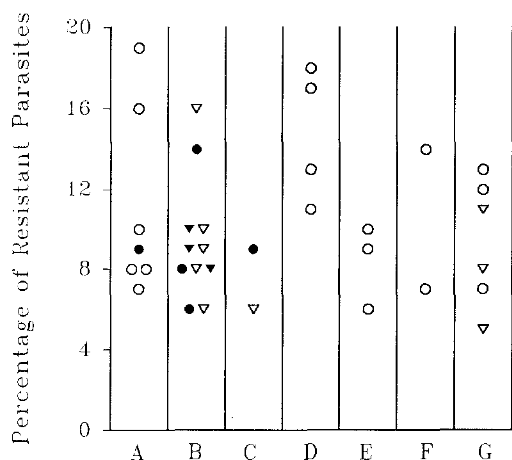

Fig. 1. Percentages of *Leishmania amazonensis* stationary-phase promastigotes (sPro) resistant to lysis in the presence of sera from healthy human beings (*A*), sera from patients with cutaneous leishmaniasis (*B*), mucocutaneous leishmaniasis (*C*), visceral leishmaniasis (*D*), or Chagas' disease (*E*), and sera from normal rabbits (*F*) and rabbits immunized with *Leishmania* sPro (*G*). Symbols represent data obtained for individual sera. The serum concentration in the parasite culture medium was 10% (*open symbols*) or 90% (*closed symbols*). (o, ●), Patients infected with unidentified *Leishmania* or with *Leishmania* species other than *L. amazonensis* or rabbits intravenously inoculated with six injections of live *L. amazonensis* sPro; (▽, ▼), patients infected with *L. amazonensis* or rabbits intravenously inoculated with six injections of live sPro and boosted with *L. amazonensis* sPro surface (glyco)proteins

gotes (data not shown; Krettli et al. 1984), sera from ten patients with cutaneous leishmaniasis, from two with mucocutaneous leishmaniasis, from four with visceral leishmaniasis, and from three with Chagas' disease had no similar activity against *L. amazonensis* metacyclic promastigotes, even when they were added at a 90% final concentration to the parasite suspension (the proportion of sPro left unaffected after treatment with these sera ranged from 6% to 18%; Fig. 1). This absence of lytic activity could not have been due to a possible species specificity of the lytic antibodies, since the parasites isolated from seven serum donors were identified as *L. amazonensis*, the species used in the lytic assay (Fig. 1).

Since parasite escape mechanisms could theoretically prevent the production of lytic antibodies in some animal species but not in others, we investigated the presence of these antibodies in the sera of *Leishmania*-immunized rabbits, which are naturally resistant to *Leishmania* infection.

Three rabbits were immunized with five weekly subcutaneous inoculations of live *L. amazonensis* sPro as described elsewhere (Adler et al. 1966). At 15 days after the last sPro inoculation, they were further immunized with an amphiphilic (mainly surface membrane) fraction obtained from sPro by semipurification with Triton-X114 (Sigma Chemical Co., St. Louis, USA) as described by Bordier (1981). A 1-mg dose of this immunogen, emulsified in complete Freund's adjuvant, was injected subcutaneously. This was followed by two fortnightly intramuscular boosters of 0.5 mg of the same amphiphilic fraction precipitated in alum.

Leishmania-antibody titers in all immunized rabbit sera (maximal serum dilutions leading to results above the mean plus two standard deviations of the values obtained in normal rabbit sera) were above 1:200000 as determined in an immunoradiometric assay (Pontes de Carvalho et al. 1986). Despite these high titers of specific antibodies, the immune sera from all three rabbits did not lyse sPro any better than did either the sera from two normal rabbits or the human sera (Fig. 1). This resistance of mPro to lysis by immune sera could derive from a failure of antibodies to interfere with mechanism(s) by which the parasite escapes the lytic activity of complement. Alternatively, *Leishmania* escape mechanisms might be so efficient as to altogether suppress the formation of these antibodies.

It is well accepted that cell-mediated mechanisms can control *Leishmania* infections (Garnham and Humphrey 1969; Mitchel et al. 1980). However, as promastigote infectivity has been associated with complement resistance (Sacks 1989), lytic antibodies could well confer immunity to *Leishmania*, provided that they could be elicited by immunointervention. Reexposure to promastigote antigens in endemic areas due to infected sand-fly bites could then perhaps function as natural boosters for the desirable immune response(s). Despite the observation reported herein, that sera from rabbits extensively immunized with sPro failed to lyse mPro, the possibilities discussed above should justify further studies on ways to interfere with the mechanism(s) utilized by mPro to escape from complement-mediated lysis.

The possibility that the complement resistance of mPro, like that of mTry (Kipnis et al. 1981), is trypsin-sensitive was also assessed. Pretreatment with 3000 BAEE units trypsin/ml RPMI medium (trypsin 1:250; Sigma Chemical Co., St. Louis, USA) for 15 min at 37° C markedly reduced the resistance of mPro to the

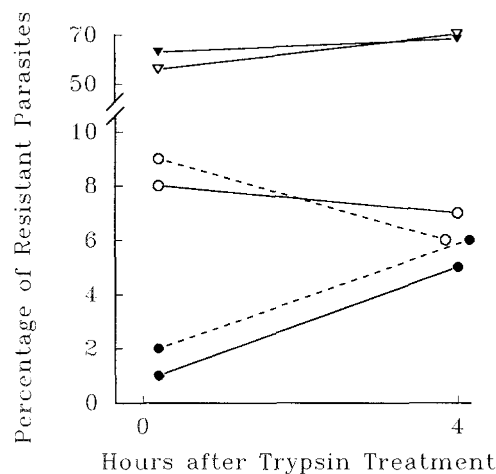

Fig. 2. Effect of pretreatment with trypsin on the complement resistance of *L. amazonensis* stationary-phase promastigotes. Parasites were preincubated for 15 min in RPMI medium in the presence (*closed symbols*) or absence (*open symbols*) of trypsin. The parasites were then washed by centrifugation and tested for complement resistance either immediately or after additional incubation in RPMI culture medium containing 1% bovine serum albumin for 4 h at 26° C. (o, ●), Percentages of motile parasites in the presence of fresh normal human serum; (▽, ▼), percentages of motile parasites in the presence of fetal calf serum. The *solid and broken lines* identify data obtained in two independent assays

lytic effect of fresh normal human sera (Fig. 2). The resistance recovered spontaneously after a 4-h period of incubation at 26° C (Fig. 2). The trypsin treatment by itself did not decrease the parasite motility as demonstrated by the finding that it had no effect on mPro when these were subsequently incubated with fetal calf serum in the place of fresh human serum (Fig. 2). These results suggest that a readily resynthesized surface (glyco)protein may play a role in complement resistance.

It has been reported that the complement resistance of mPro is due to release of the C5b-9 lytic complex from the parasite membrane, mediated by a lipophosphoglycan molecule (LPG) shown to be the main acceptor for C3 on the surface of both promastigote stages (Puentes et al. 1988, 1990). The present results, however, do not support the hypothesis that the difference in the complement resistance of mPro and logPro might be exclusively due to differences in their LPG (Puentes et al. 1988, 1990), as trypsin, of course, would not hydrolyze LPG. It is nonetheless possible, however, that the protease treatment could affect LPG by cleaving surface proteins or peptides associated with LPG and/or regulating its expression or conformation. Comparisons of LPG molecules from protease-treated parasites with those from untreated parasites should clarify this point.

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