INFECTION AND IMMUNITY, Aug. 1989, p. 2430–2433
0019-9567/89/082430-04$02.00/0

Vol. 57, No. 8

Altered Virulence and Vaccination Properties of *Leishmania* Parasites Grown in Infected Vaccinated Mice

REGINALD M. GORCZYNSKI

Departments of Surgery and Immunology, Mount Sinai Research Institute, University of Toronto, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada

Received 7 November 1988/Accepted 1 May 1989

BALB/c mice, which are normally highly susceptible to growth of *Leishmania mexicana* parasites in vivo, can be vaccinated with avirulent temperature-sensitive mutants of *L. mexicana* so that challenge with virulent organisms results in markedly diminished growth of the latter. Parasites extracted from the lesions which do appear in these mice are able to produce active infection in secondary hosts, although the rate of progression of these lesions is slower than that seen with the original virulent cloned organism. Interestingly, when irradiated parasites from the secondary hosts are themselves used to vaccinate naive BALB/c mice, less protection is seen than when irradiated virulent organisms from the initial infecting clone are used. These data suggest that when infection does take place in mice vaccinated with avirulent clones of parasite, the organisms which develop in lesions in these animals are substantially modified from those present in the initial infecting inoculum.

BALB/c mice are exquisitely sensitive to infection with *Leishmania mexicana*; infected animals develop nonhealing lesions which frequently metastasize to the abdominal viscera (10). However, these mice can be protected from such disease progression by a variety of means, including sublethal whole body irradiation (5), anti-idiotypic immunization (3), and prophylactic immunization with attenuated parasites or parasite antigens (1, 6, 11). In particular, intravenous immunization with irradiated virulent organisms (6) and intradermal vaccination with mutagenized, temperature-sensitive avirulent clones (1) have both been shown to retard significantly lesion development in animals subsequently infected with virulent organisms. The hypothesis, which is supported by biochemical studies (12, 14, 15), has been made (2) that carbohydrate moieties on parasite glycoproteins and glycolipids are of importance in the expression of protection-inducing epitopes.

In other parasite systems, there is evidence that immunogenic determinants expressed by infecting organisms change with time after infection (4, 11). It is not clear that this antigenic change is necessarily associated with the immune response mounted by the host against infection, since some change is seen, e.g., in African trypanosomes, in cultured parasites also. Nevertheless, the concept of antigenic variation has important implications for the possible development and application of widespread vaccination for parasitic disease. The goal of the current study was to address the issue of whether infection with a cloned virulent organism of mice vaccinated against *L. mexicana* using avirulent clones would lead to a change in the parasites subsequently harvested from these mice. The changes investigated were growth capacity in normal mice and ability to immunize normal mice against infection with virulent (parental) parasites.

MATERIALS AND METHODS

Mice. Eight-week-old BALB/c mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Mice were kept five per cage and allowed food and water ad libitum. All mice were entered into experiments at 10 weeks of age.

***Leishmania* parasites.** The cloned virulent strain of *L. mexicana* (R100282-99-2) used throughout has been described elsewhere (1–3). Avirulent clones of the parasite, selected after mutagenization with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and after selection for growth and infectivity in vitro at 19°C, are also described in an earlier communication (1). Parasites were grown in α-minimal essential medium with 10% heat-inactivated fetal calf serum–1% freeze-thawed human blood and harvested for infection when in the late log phase of growth (14), which has generally been 7 to 10 days after initiation of culture.

Mice were infected intradermally at the base of the tails with 10^6 parasites in 0.05 ml of phosphate-buffered saline, and lesion size was monitored at 5-day intervals with calipers. In some experiments, parasites were harvested from infected mice after homogenization of weighed amounts of tissue from the tails of those mice and culture of that tissue for 4 days at 28°C. At this time, the promastigote yield in the cultures was enumerated for comparison of parasite yield from vaccinated and nonvaccinated mice. Equivalent numbers of promastigotes from different cultures in the late log phase of growth were used to infect independent groups of mice.

Mice that were immunized by intravenous challenge with promastigotes received two injections, at 10-day intervals, of 10×10^6 irradiated (150 krads) washed promastigotes in 0.3 ml of phosphate-buffered saline. Immunized mice were tested for their resistance to infection by challenge with 10^6 virulent organisms injected into the base of the tails at 14 days after the last immunization.

RESULTS

Delayed growth of virulent *L. mexicana* in mice vaccinated with avirulent clones. In an earlier study, it was reported that mutagenized clones of *L. mexicana* (RLB-19-8-4 and RLB-19-12-2), which were selected to grow at 19°C, were unable to produce lesions in BALB/c mice, unlike the parental cloned virulent organisms (R100282-99-2) (1). In addition, mice prevaccinated with the avirulent clones showed a significantly decreased rate of growth of lesions after infection with the virulent parasite. Typical data for such a study are shown in Fig. 1.

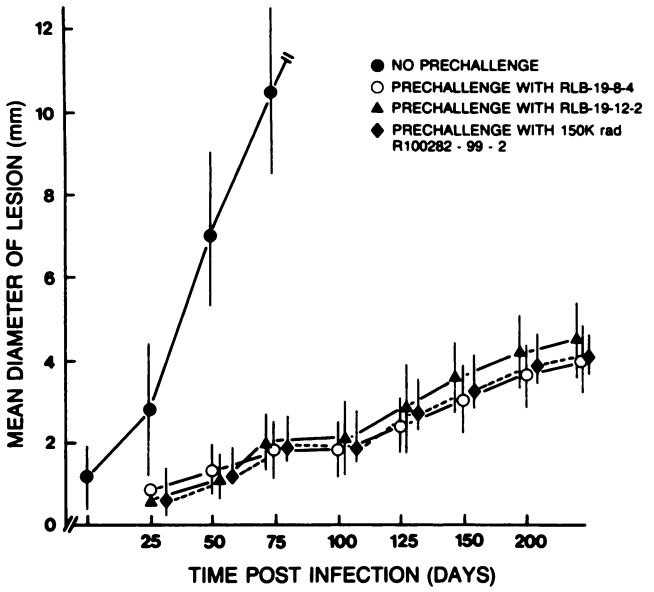

FIG. 1. Protection from infection with avirulent clones of *L. mexicana*. Groups of five BALB/c mice were inoculated in the tails with 10^6 promastigotes of the avirulent clones RLB-19-8-4 (○) or RLB-19-22-2 (▲) or with irradiated virulent R100282-99-2 (◆). A control group (●) received no such pretreatment. Twelve weeks later (no lesions visible in any mice), all three groups were infected with 10^6 promastigotes of the virulent clone R100282-99-2. Lesions were monitored at regular intervals thereafter. Data shown are arithmetic means (± standard error of the mean) for groups at different times postinfection with R100282-99-2.

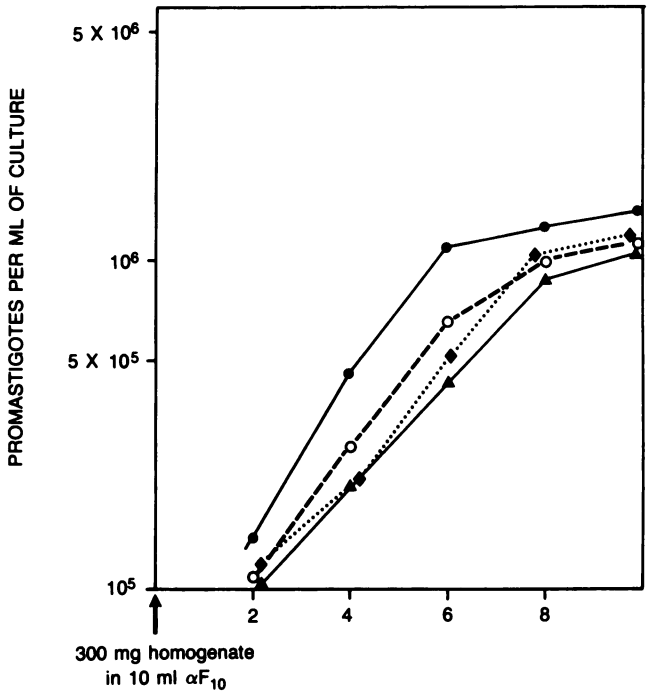

FIG. 2. In vitro growth characteristics of *L. mexicana* harvested from tail lesions of mice from the experiments shown in Fig. 1. A sterile tissue homogenate was made of tail lesions at 200 days postinfection (infected vaccinated mice, ○, ▲, or ◆) or at 60 days postinfection (infected nonvaccinated mice ●). All groups of mice had similar-sized lesions (approximately 8 to 10 mm in diameter). Homogenate (300 mg) was added to flasks containing 10 ml of αF10, and flasks were incubated at 28°C. Promastigote numbers per flask were enumerated at 2-day intervals.

Altered growth potential of virulent *L. mexicana* recovered from vaccinated mice. The low level of parasite growth seen in infected vaccinated mice (Fig. 1) could be due to sneaking through of growth of R100282-99-2. Alternatively, a modification in the parasite might have occurred in situ to render it resistant to mechanisms of growth control present in vaccinated mice (13). As an initial attempt to address this issue, the growth potential in normal animals of parasites harvested from the lesions of mice shown in Fig. 1 was studied.

Groups of five mice infected as described in the legend to Fig. 1 were used at 200 days postinfection (infected vaccinated mice) or at 60 days postinfection (infected nonvaccinated mice) as a source of tissue for homogenization and establishment of promastigote cultures (at 28°C) (see Materials and Methods). By choosing these different times postinfection, tissue was harvested from animals bearing similar-sized lesions (Fig. 1). At 4 days of culture, the promastigote parasite yield per group was evaluated. There was no reproducible difference among the cultures established from the three groups of vaccinated mice, which in general produced only 30 to 50% of the parasite numbers per gram of homogenized tissue compared with cultures prepared from nonvaccinated mice (Fig. 2).

Groups of five BALB/c mice were inoculated in the tails with 10^6 parasites from each of these four groups of cultures. The parasites were harvested in the late log phase as indicated in Materials and Methods. A control group received parasites (R11282-99-2) which had not been passaged through mice (either vaccinated or nonvaccinated) (Fig. 3, ■). Lesion size was monitored as described above, and typical data (one of three studies) are shown in Fig. 3.

Passage of R100282-99-2 through nonvaccinated mice apparently caused little perturbation in the growth characteristics on subsequent infection compared with growth of parasites maintained in culture throughout the study (Fig. 3, ■ versus ●). Interestingly, however, passage through mice vaccinated with virulent or avirulent clones of the parasite resulted in the recovery of virulent organisms which grew more slowly than did the parental stock of R100282-99-2 (see ▲, ◆, ○ in Fig. 3).

These data were not explainable in terms of in vitro growth characteristics of the in vivo-passaged parasites (15, 16) (Fig. 2). Thus, although the yields of parasites per gram of tissue were lower in infected vaccinated mice, the in vitro growth rates of those organisms were not significantly different from those of parasites harvested and cultured from infected nonvaccinated mice (Fig. 2, compare ● with ○, ▲, and ◆).

Changes in immunization potential, using parasites from infected vaccinated mice. As one last study series, the ability of organisms obtained from infected vaccinated mice (Fig. 1 through 3) to immunize a naive group of BALB/c mice from infection by R100282-99-2 was investigated. The protocol used was essentially that described earlier by Howard et al. (6). Irradiated (150 krads) promastigotes injected intravenously into the tail veins of adult mice have been found to diminish growth of a subsequent test challenge with parasites in the base of the tail.

Groups of vaccinated or nonvaccinated BALB/c mice were infected with R100282-99-2 as described in the legends for Fig. 1 and 3. After promastigotes were cultured from the lesions, these organisms were irradiated (150 krads) and

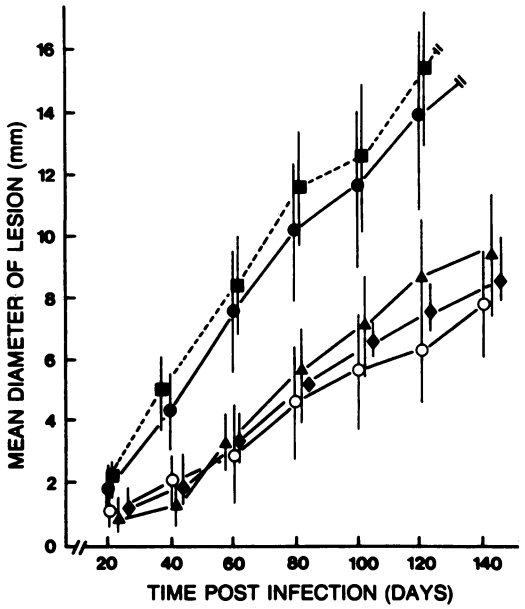

FIG. 3. Altered growth of parasites taken from infected vaccinated mice. Parasites were harvested from the tail lesions of groups of mice from the experiments shown in Fig. 1 after homogenization of tissue and culture at 28°C. At 96 h, promastigote numbers in the cultures were evaluated. Fresh groups of five BALB/c mice were infected in the tails with 10^6 promastigotes harvested from late-log-phase cultures (day 9). A control group (■) received parasites which had not been passaged through mice. Symbols are as shown in Fig. 1. Data shown are arithmetic means (± standard error of the mean) for lesion sizes per group at the times shown.

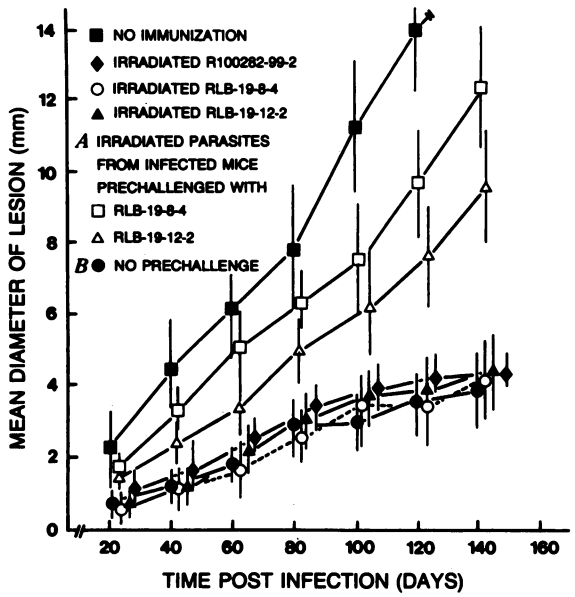

FIG. 4. Altered immunoprophylaxis using parasites from infected vaccinated mice. Data show growth of 10^6 virulent R100282-99-2 in untreated (■) BALB/c mice or in mice given two intravenous injections of 10×10^6 irradiated (150 krads) parasites, 10 days apart, the last 14 days before infection. In three groups of mice, the organisms used for immunization were obtained from infected vaccinated (□, △) and nonvaccinated (●) mice. Data points are mean lesion sizes ± standard error of the mean for five mice per group.

used to immunize groups of naive BALB/c mice given two intravenous injections at 10-day intervals. Control groups of mice were immunized with avirulent (150 krads of irradiation) or virulent parasites (nonpassaged in vivo). At 14 days after the last immunization, mice in these groups and in a control (nonimmunized) group were infected with 10^6 R100282-99-2 and lesion growth was monitored as before. Typical data (one of two studies) are shown in Fig. 4.

As described elsewhere (2, 6), intravenous immunization with irradiated (150 krads) avirulent or with virulent parasites protects BALB/c mice from subsequent infection with cloned virulent organisms of *L. mexicana* (Fig. 4, compare ■ with ▲, ○, or ◆). When R100282-99-2 was passaged through nonvaccinated mice, the organisms harvested from the lesions of these mice retained this protective capacity (Fig. 4, ●). However, if virulent parasites were given to vaccinated mice, the organisms harvested from the lesions had a diminished capacity to protect naive mice from infection (Fig. 4, □ and △).

When parasites obtained from R100282-99-2-infected, RLB-99-22-2-vaccinated mice were further passaged through RLB-99-22-2-vaccinated mice and when the tail lesions of these animals were used as sources of irradiated (150 krads) promastigotes for vaccination of naive mice, the diminished vaccination potential recorded in Fig. 4 was even more pronounced (e.g., Fig. 5, ◇ versus △). It should be noted that this serial study (Fig. 5) has to date been performed but once.

These data seem operationally to be analogous to the data reported earlier, which looked at the immunization potential of parasites grown in medium designed to decrease expression of N-linked glycoprotein epitopes on the parasite (2). Indeed, in a preliminary comparative analysis of the total N- and O-linked sugar content of late-log-phase cultures of R100282-99-2, RLB-99-22-2, and promastigotes obtained from one passage of R100282-99-2 through RLB-99-22-2-vaccinated mice (Fig. 4, △), the carbohydrate content (micrograms per 10^6 parasites) was 1.9 ± 0.3, 7.8 ± 1.2, and 0.7 ± 0.4, respectively. This finding is consistent with the earlier hypothesis that expression of N-linked sugars contributed significantly to the epitopes responsible for protective immunization (2).

DISCUSSION

There have been a number of reports which have analyzed the ability to immunize mice susceptible to parasite infection so that they become (relatively) resistant to such challenge. Included in these studies, for leishmaniasis, are those using attenuated organisms or biochemically purified material derived from them (1, 2, 6, 11, 12). The majority of studies, however, have addressed the issue of short-term immunoprotection and generally have shown decreased (but not absent) growth of parasites in vaccinated mice.

Given the data elsewhere which documents that antigenic variation is not uncommon in long-term host-parasite relationships (malaria [4] and trypanosomes [13]), it was of interest to ask whether similar antigenic change would occur in *L. mexicana* growing in vaccinated mice. Data shown in Fig. 3 to 5 indicate that the parasites derived from such animals, unlike the infecting organisms, produce a more slowly growing lesion in naive nonvaccinated mice and are less able to immunize naive mice for modulation of growth of the initial cloned virulent parasite used for infection.

Earlier studies indicate that parasite growth or immunogenicity may depend on the growth phase of the harvested

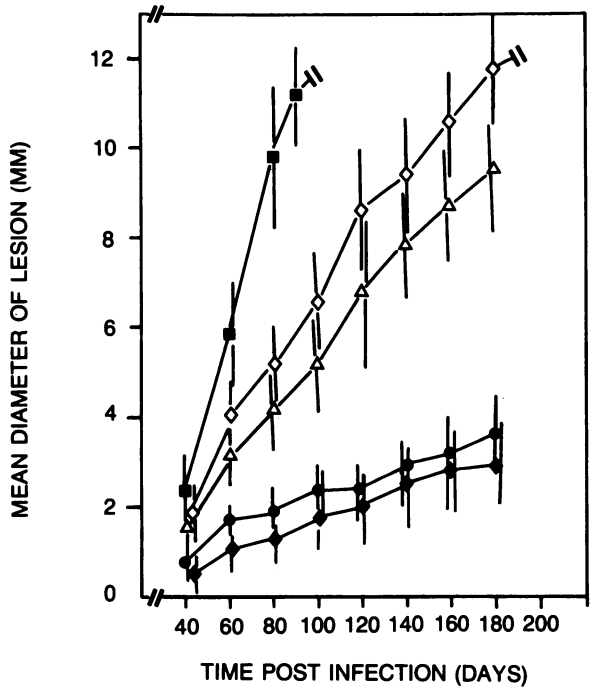

FIG. 5. Diminished immunoprophylaxis with irradiated parasites after two-time serial passage of R100282-99-2 through mice vaccinated with the avirulent clone RLB-99-22-2. All data points represent mean lesion sizes (± standard error of the mean) for five mice per group infected with 10^6 promastigotes of R100282-99-2. Control groups (■, ●) received, respectively, no pretreatment or were given two intravenous injections, 10 days apart, of 10×10^6 irradiated (150 krads) parasites from infected normal mice; the last injection was 14 days before infection. Another group (◆) was similarly vaccinated with 10×10^6 irradiated (150 krads) promastigotes maintained throughout in tissue culture. Groups were preimmunized respectively with irradiated (150 krads) parasites harvested from R100282-99-2-infected, RLB-99-22-2-vaccinated mice (△) or from RLB-99-22-2-vaccinated mice which had been infected with parasites obtained from the lesions of R100282-99-2-infected, RLB-99-22-2-vaccinated mice (◇).

cultured organisms (15, 16). Independent in vivo studies with promastigote challenge do not support this as a likely explanation of the data shown (see also Fig. 2). Other evidence implicates a role for glycolipid and glycoprotein molecules in the immunogenicity of this organism and implies that deliberate changes in N-linked oligosaccharide expression may alter immunogenicity (2, 12, 14, 15). Preliminary data, referred to above, also suggests that the parasites harvested from vaccinated mice, which seem to have a decreased capacity for protective immunization of naive animals, express on the order of twofold less N- and O-linked sugars. It seems too that protection against *Leishmania* infection in mice is a finely balanced state in which induction of Lyt1 T-helper cells outweighs activation of Lyt1 T-suppressor (inducer) cells (2, 3, 8, 9). One of the mechanisms mediating the interplay of T-cell subsets in infected vaccinated mice may relate to stimulation of different lymphokine secretory potentials (and thus altered macrophage activation [7]). The possibility exists that failure to immunize for protection (Fig. 4 and 5) may be due also to the evolution of parasites with increased expression of suppressor epitopes as well as, or rather than, decreased expression of helper epitopes. These important questions have yet to be addressed.

ACKNOWLEDGMENT

This work was supported by grant MA-5440 from the MRC of Canada.

LITERATURE CITED

1. **Gorczynski, R. M.** 1985. Immunization of susceptible BALB/C mice against *Leishmania braziliensis*. II. Use of temperature-sensitive avirulent clones of parasite for vaccination purposes. Cell. Immunol. **94**:11–20.
2. **Gorczynski, R. M.** 1986. Do sugar residues contribute to the antigenic determinants responsible for protection and/or abolition of protection in leishmania-infected BALB/C mice? J. Immunol. **137**:1010–1016.
3. **Gorczynski, R. M.** 1987. Immunization with leishmania-specific T-cell not B-cell lines or hybridomas can modulate the response of susceptible mice infected with viable parasites. J. Immunol. **139**:3070–3075.
4. **Hommel, M.** 1985. Antigenic variation in malaria parasites. Immunol. Today **5**:28–33.
5. **Howard, J. G., C. Hale, and F. Y. Liew.** 1981. Immunological regulation of experimental cutaneous leishmaniasis. IV. Prophylactic effect of sublethal irradiation as a result of abrogation of suppressor T-cell generation in mice genetically susceptible to *Leishmania tropica*. J. Exp. Med. **153**:557–568.
6. **Howard, J. G., S. Micklem, C. Hale, and F. Y. Liew.** 1982. Prophylactic immunization against experimental leishmaniasis. I. Protection induced in mice genetically vulnerable to fatal *Leishmania tropica* infection. J. Immunol. **129**:2206–2212.
7. **Liew, F. Y.** 1989. Functional heterogeneity of CD4 T cells in leishmaniasis. Immunol. Today **10**:40–45.
8. **Liew, F. Y., and J. S. Dhaliwal.** 1987. Distinctive cellular immunity in genetically susceptible BALB/C mice recovered from *Leishmania major* infection or after subcutaneous immunization with killed parasites. J. Immunol. **138**:4450–4456.
9. **Liew, F. Y., A. Singleton, E. Cillari, and J. G. Howard.** 1985. Prophylactic immunization against experimental leishmaniasis. V. Mechanism of the anti-protective blocking effect induced by subcutaneous immunization against *Leishmania major* infections. J. Immunol. **135**:2102–2107.
10. **Mitchell, G. F., J. M. Curtis, E. Handman, and I. F. C. McKenzie.** 1980. Cutaneous leishmaniasis in mice: disease patterns in reconstituted nude mice of several genotypes infected with *Leishmania tropica*. Aust. J. Exp. Biol. Med. Sci. **58**: 521–531.
11. **Mitchell, G. F., and E. Handman.** 1983. *Leishmania tropica major* in mice: vaccination against cutaneous leishmaniasis in mice of high genetic susceptibility. Aust. J. Exp. Biol. Med. Sci. **61**:11–23.
12. **Mitchell, G. F., and E. Handman.** 1985. T lymphocytes recognize leishmania glycoconjugates. Parasitol. Today **1**:61–64.
13. **Parsons, M., R. G. Nelson, and N. Agabian.** 1984. Antigenic variation in African trypanosomes: DNA rearrangements program immune evasion. Immunol. Today **5**:43–50.
14. **Rodrigues, M. M., M. T. Xavier, L. M. Previato, and M. A. Barcinski.** 1986. Characterization of cellular immune response to chemically defined glycoconjugates from *Leishmania mexicana* subsp. *amazonensis*. Infect. Immun. **51**:80–86.
15. **Sacks, D. L., S. Hieny, and A. Sher.** 1985. Identification of cell surface carbohydrate and antigenic changes between non-infective and infective developmental stages of *Leishmania major* promastigotes. J. Immunol. **135**:564–569.
16. **Sacks, D. L., and P. V. Perkins.** 1984. Identification of an infective stage of leishmania promastigotes. Science **223**:1417–1422.