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Advantages of Measuring Changes in the Number of Viable Parasites in Murine Models of Experimental Cutaneous Leishmaniasis

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Previously published studies of experimental cutaneous leishmaniasis in the mouse have relied almost exclusively on measuring changes in lesion size to follow the course of the infection. The purposes of the studies reported here were to develop a technique to quantitate the number of viable organisms in the tissues and to use the technique to follow the development and resolution of the primary infection as well as the development of acquired resistance to *Leishmania tropica* in a resistant (C3H/He) and a susceptible (BALB/c) mouse strain. It was found that individual *L. tropica* amastigotes derived from infected tissues would tranform to promastigotes and repeatedly divide to form discrete, countable colonies on rabbit blood agar. The plating efficiency was approximately 88%. Using the blood agar plating technique to quantitate the organism against time of the infection, we obtained data that suggest that acquired resistance develops in C3H/He mice earlier than is suggested by reduction in lesion size. In addition, although this resistance eliminates the parasites from the primary lesion in 10 weeks, 1,000 to 10,000 parasites persist for months in the lymph node draining the lesion site. In these studies, we found no evidence of acquired resistance in the susceptible BALB/c mice. The organism grows progressively, and the infection can disseminate to the spleen within 2 weeks. These studies illustrate the advantages of quantitating viable parasites in studies of immunity in cutaneous leishmaniasis.

With the use of inbred mouse strains, models of both resolving and progressive cutaneous leishmaniasis can be studied (2, 3, 14). In resistant mouse strains, data obtained thus far from other laboratories have been interpreted as supporting a major role for cell-mediated immunity in the resolution of *Leishmania tropica* infections. It has been shown, for example, that the lesions that develop in mice that have been thymectomized, irradiated, and reconstituted with bone marrow (7, 19) or that develop in congenitally athymic (nude) mice (13) appear to heal more slowly than comparable lesions in immunocompetent controls. In addition, lesions develop more slowly in animals adoptively immunized with cells (1, 20) but not with serum (20) taken from donor animals with healed lesions.

It is important to emphasize, however, that all published studies of experimental cutaneous leishmaniasis in the mouse have relied almost exclusively on measurements of changes in the diameter and swelling of the primary lesion which develops at the site of inoculation of the parasite as a means of following the course of the infection (8, 12, 17, 18, 25). To date, there has been only one attempt (17) to obtain quantitative data on the actual number of viable *L. tropica* parasites in host tissues during an infection. Obviously, until the relationship between lesion size and the number of parasites in the infected tissue is known, all of the previously published results must be interpreted with caution. This is because changes in the size of a lesion will also be dependent on the host response to the parasite infection. Thus, monitoring only the increase and decrease in size of the primary lesion may give inaccurate or even false impressions of the true progress of the disease, particularly if the host responsiveness has been experimentally enhanced by a prior immunization (20) or by treatment with adjuvants such as *Mycobacterium bovis* BCG (25) or *Corynebacterium parvum* (12). Similar problems in interpretation could arise if the host responsiveness were experimentally suppressed by exposure to ionizing radiation (8) or by treatment of the experimental host with immunosuppressive drugs (18).

The studies we describe here were designed to follow the number of viable *L. tropica* in a resistant and susceptible strain of mouse by measuring changes in the number of living para-

sites in the primary lesion which develops at the site of inoculation, as well as those in the draining lymph node and spleen. It was found that the number of viable parasites in the tissue does not always correlate with the size of the lesion. For example, in C3H/He mice, the onset of elimination of viable parasites in the lesion began before the lesion itself began to resolve. In addition, previously infected mice that were challenged at a distant site show a marked increase in local swelling at the challenge site despite the fact that the parasites were rapidly killed. These results illustrate the importance of measuring viable parasites rather than relying on the more subjective measurements of lesion size in studying immunity to cutaneous leishmaniasis.

MATERIALS AND METHODS

Mice. Specific-pathogen-free male C3H/He and BALB/c mice (8 to 10 weeks old) were used. The animals were obtained from the Trudeau Institute Animal Breeding Facility (Saranac Lake, N.Y.) and maintained under barrier-sustained conditions. The mouse colony is routinely monitored for the presence of pathogenic bacteria, including mycoplasmas, by standard bacteriological techniques. In addition, the colony is monitored for virus infections by serological tests (Mouse Virus Profile 80-211; Microbiological Associates, Bethesda, Md.). The mice used in these studies were free of known infectious bacteria and viruses.

Parasite. *Leishmania tropica major*, strain 173, was obtained from A. Ebrahimzadeh (Cornell University). The organism was originally isolated in Iran from a human cutaneous lesion. The parasite was maintained by passage in BALB/c mice, followed by in vitro passage in Grace Insect Medium (GIBCO Laboratories, Grand Island, N.Y.). Grace medium was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and 20% fetal bovine serum (Sterile Systems, Inc., Logan, Utah). This concentration of fetal bovine serum was the lowest concentration that would allow conversion of the amastigotes to promastigotes and that supported the sustained growth of the parasite in vitro up to a concentration of 10^7/ml. Growth in this medium was found to be comparable to the diphasic rabbit blood agar overlay medium (24).

The primary infections were initiated by injecting 50 μl of Grace medium containing 5×10^5 amastigotes subcutaneously into the hind footpad. The amastigotes used for infection were isolated from the footpads of BALB/c mice that had been infected 8 weeks earlier with 5×10^5 *L. tropica* promastigotes. In studies designed to compare the infectivity of amastigotes and promastigotes, groups of mice were infected with 5×10^5 amastigotes or 5×10^5 promastigotes, the latter derived from a log-phase, 2-day culture initiated with the same population of amastigotes. In the studies of the development of acquired resistance, animals were challenged with 2.5×10^6 amastigotes in the contralateral footpad.

Quantitation of viable parasites. At various times after infection, four or five animals per group were killed, and the spleen and popliteal lymph node draining the infected foot were aseptically removed and put into heavy-walled glass tubes containing 5.0 and 3.0 ml, respectively, of Grace medium. The infected foot was then rinsed with 70% ethanol, removed just below the heel, and cut into 10 pieces into 3.0 ml of medium. The tissues were then disrupted with the use of ice-cold, loose-fitting Teflon pestles. The homogenates were then appropriately diluted, and microscopic counts of intact amastigotes were made with standard hemacytometers.

We then applied a technique that capitalized on our finding that individual amastigotes of *L. tropica major* strain 173 transform and replicate to form discrete macroscopic colonies on blood agar plates. The medium used was similar to the rabbit blood agar that was described by Keppel and Janovy (10, 11). For our studies, however, the $CaCl_2$, KCl, and NaH_2PO_4 were omitted from the medium to achieve a more neutral pH (7.0 to 7.2). Therefore, it was not necessary to add NaOH, which in our hands, caused precipitation of some of the medium components. The medium consisted of a blood agar base (Tryptose Blood Agar Base; Difco Laboratories, Detroit, Mich.) which was supplemented with additional sodium chloride (3 g/liter), glucose (2.5 g/liter), and 14% defibrinated rabbit blood. The blood was heat inactivated at 56°C for 30 min and lysed with an equal volume of sterile distilled water and added to the agar base. Freshly poured plates were allowed to harden, and before they were used, the plates were dried to 37°C for 5 to 10 min to ensure slow, complete absorption of the inocula. The surfaces of the predried plates were inoculated with 50 μl of appropriate dilutions (estimated by microscopic counts) of the tissue homogenate so that between 30 and 300 intact amastigotes were plated. After the inocula were absorbed, the plates were inverted and incubated at 26°C. After 7 days, macroscopic colonies of *L. tropica* could be counted at 20× magnification with the aid of a dissection microscope.

Measurement of changes in lesion and organ size. The progress of the infections was also followed by measuring the changes in the thickness of the infected footpad as well as increases in the weight of the popliteal lymph node and spleen. All measurements of footpad swelling were made with a dial caliper (Oditest, 00-T; H. C. Kroplin Co., Hassen, Federal Republic of Germany) and compared with similar measurements of the contralateral footpad of the same animal as well as with the footpad thickness of uninfected mice of the same strain, sex, and age. Data on lesion size are expressed as the mean (± the standard error) of the increase in thickness (millimeters) of the inoculated footpads.

Since the amastigotes used to infect the mice were routinely obtained from the footpads of BALB/c mice, in studies in the C3H/He mice, it was necessary to control for footpad swelling, particularly in the reinfected animals, due to small amounts of BALB/c tissue debris possibly contaminating the inocula. Therefore an additional control experiment was included in these studies. At 0, 5, and 22 weeks of their primary infection, groups of C3H/He mice were inoculated with 50 μl of Grace medium containing 5×10^5 viable, allogeneic lymphocytes derived from the cervical lymph nodes of BALB/c mice. The difference in footpad swelling was compared daily for 7 days to the

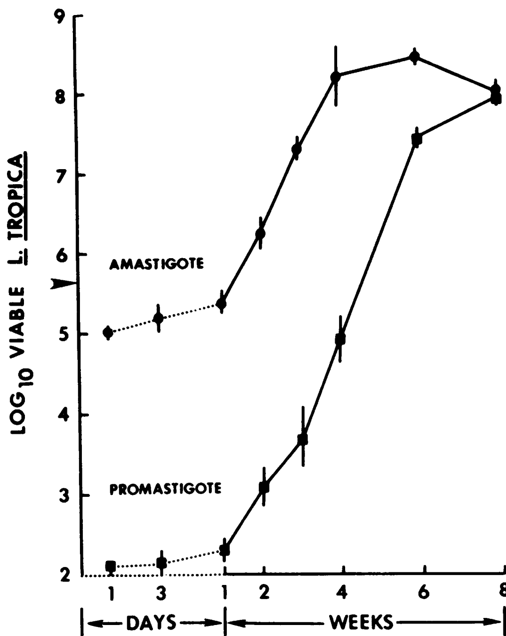

FIG. 1. Comparison of *L. tropica* infection in BALB/c mice initiated with 5 × 10^5 amastigotes or 5 × 10^5 log-phase promastigotes.

footpads of mice inoculated with medium alone. By 48 h, the footpads of all groups of mice were back to normal size, indicating no significant residual swelling in response to BALB/c antigens which would contribute to the swelling due to the host response to the parasitic infection.

RESULTS

Quantitation of the parasite. At concentrations of greater than 10^5 parasites per ml of footpad homogenate (3 × 10^5 per footpad), intact amastigotes were easy to identify and count. Such counts of intact amastigotes in tissue homogenates immediately after sacrifice were satisfactory for estimating the number of viable parasites present and the dilutions to plate on the rabbit blood agar.

In preliminary studies it was found that fresh, as well as commercially available, rabbit blood supported the growth and formation of discrete colonies of the parasites. Defibrinated blood obtained locally from five different rabbits along with two lots of commercially available blood (Pel-Freez Biologicals, Rogers, Ark., and Dutchland Laboratories, Denver, Pa.) were tested. The range in plating efficiency for inocula containing a known number of intact amastigotes was 78 to 108%. After 7 days at 26°C, *L. tropica* amastigotes had transformed to promastigotes and divided repeatedly to form discrete, transparent colonies which were from 0.1 to 0.2 mm in diameter. For convenience, commercial blood (Pel-Freez Biologicals) was chosen for use in the medium. Over the course of our studies, the plating efficiency of intact amastigotes was approximately 88%, and with this technique, as few as 100 viable parasites (CFU) could be detected in an organ.

Comparison of infectivity of amastigotes versus promastigotes. The first study was designed to compare the relative infectivity of *L. tropica* amastigotes with promastigotes in susceptible BALB/c mice (Fig. 1). It was found that within 24 h of infection with 5 × 10^5 amastigotes, about 20% of the inoculum could be recovered. During the first few days, the infection developed slowly, and thereafter, the parasite proliferated more rapidly in vivo, doubling in number every 48 h. However, in contrast to the animals infected with amastigotes, no viable parasites (less than 10^2) could be detected in the footpads of BALB/c mice 24 h after injection of 5 × 10^5 promastigotes. Only after 3 days were we able to detect any viable parasites in the animals infected with the promastigote form of the parasite. Even when animals were inoculated with 10^7 promastigotes, less than 10^4 viable parasites were found in the developing lesions at 7 days. However, regardless of the number of promastigotes injected, once the organisms began to proliferate in vivo, they did so at the same rate as that observed in animals infected with the amastigote form.

Because of the rapid loss of viable parasites immediately after inoculation of promastigotes and the length of time it took for the infection to develop even in the normal (nonimmune), susceptible BALB/c mouse, we chose to use amastigotes to infect animals in all subsequent experiments.

Primary infection in BALB/c mice. Figure 2 shows the complete course of the infection in BALB/c mice, including the changes in the number of viable organisms with time in the lesion, the lymph node draining the lesion, and the spleen. The infection was progressive. By 5 weeks, the lesions became purulent, and by 6 weeks, they contained greater than 10^8 viable parasites. Local tissue necrosis and atrophy progressed until at 8 weeks the feet began to fall off. During the development of the primary infection, the increase in lesion size occurred concomitant to the progressive increase in the number of parasites in the lesion.

The infection in the BALB/c mice rapidly disseminated to the spleen. Indeed, as early as 2 weeks after infection, parasites could be detected in the spleen, and by 10 weeks, the spleen and lymph node contained just as many viable parasites as had been observed in the lesion itself. Also at 10 weeks, metastatic lesions (swelling) began to appear in other footpads. Upon sacri-

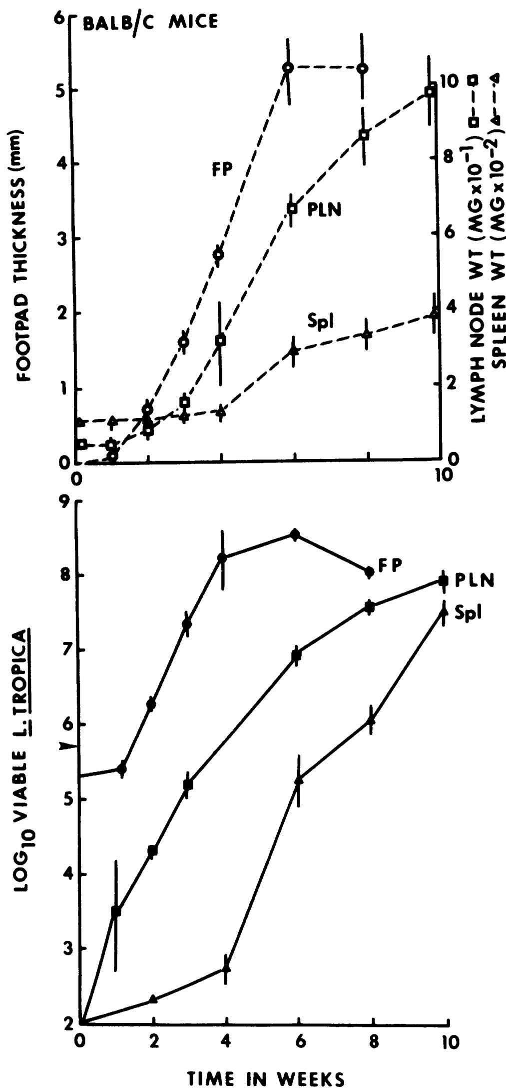

FIG. 2. Comparison of footpad (FP) swelling and changes in weight of the draining (popliteal) lymph node (PLN) and spleen (Spl) with the number of viable parasites after infection of BALB/c mice with 5×10^5 (arrow) *L. tropica* amastigotes.

fice, the gluteal and inguinal lymph nodes were also enlarged, and as many as 10^7 parasites could be detected in these organs as well as in the other footpads. The infected BALB/c mice began to die after 11 weeks of infection, apparently as a result of disseminated leishmaniasis; by 15 weeks, 84% (16 out of 19) of the animals that had not been sacrificed had died.

Primary infection in C3H/He mice. Data on the primary *L. tropica* infection in C3H/He mice are shown in Fig. 3. As was observed after inoculation of BALB/c mice, in the C3H/He mice, there was substantial loss in viable amastigotes within hours of inoculation. In fact, 4 h after infection, less than 20% of the inoculum could be recovered by plating the homogenate on blood agar plates (insert, Fig. 3). This drop in the number of viable amastigotes, however, could be predominantly due to the technique used to disrupt the tissue. This is because when a known number (10^6) of amastigotes was added to tubes containing normal C3H/He feet and the tissue was homogenized in the normal manner, only 42% of the inoculum was recovered as viable amastigotes.

However, in contrast to the infection in BALB/c mice, the parasites proliferated in vivo for only 2 weeks, after which the proliferation stopped and the number of viable parasites began to decline. The lesion was effectively sterile by 10 weeks after infection. No parasites were detected in the spleens of these animals by culturing on rabbit blood agar or by culturing 1.0 ml of the homogenate in 4.0 ml of Grace medium for 7 days. The spleen remained normal in size throughout the infection.

During the first 2 weeks of the infection in the C3H/He mice, when the parasite was proliferating, increases in footpad swelling and changes in

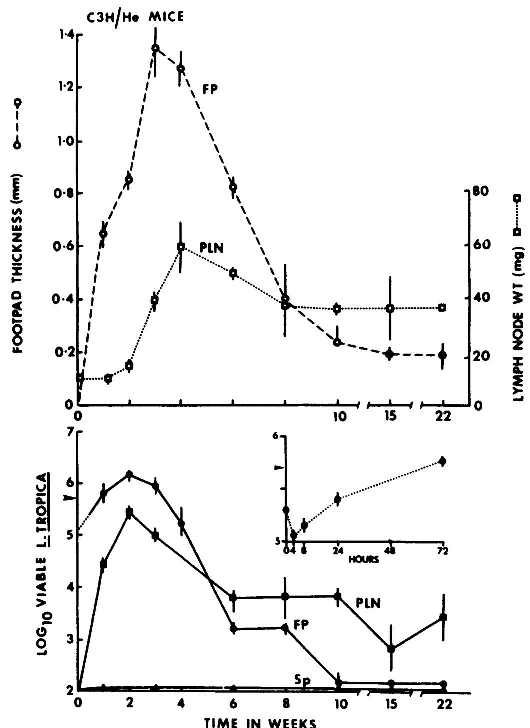

FIG. 3. Comparison of footpad (FP) swelling and changes in weight of the draining (popliteal) lymph node (PLN) and spleen (Spl) with the number of viable parasites after infection of C3H/He mice with 5×10^5 (arrow) *L. tropica* amastigotes.

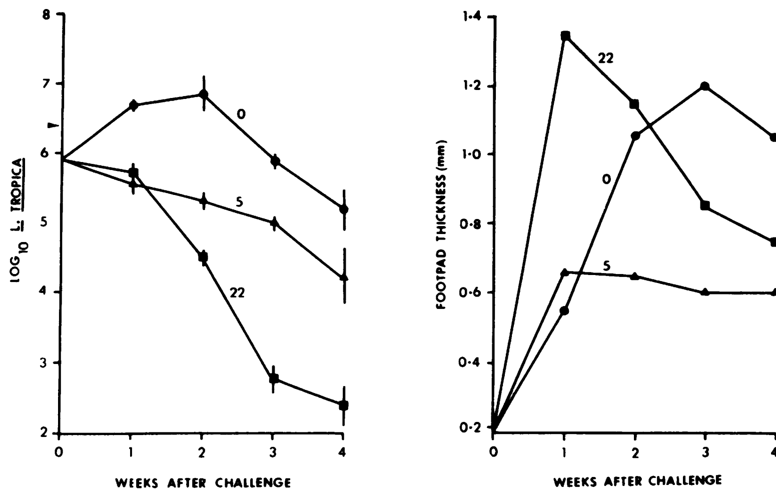

FIG. 4. Course of a challenge infection in terms of the changes in the number of viable parasites and footpad swelling after inoculation of C3H/He mice with 2.5×10^6 *L. tropica* amastigotes at 0, 5, and 22 weeks after a primary immunizing infection.

lymph node weight correlated with increases in the number of viable parasites present. However, the onset of elimination of viable parasites from the footpad and lymph node preceded the onset of healing of the lesion by 1 to 2 weeks. In addition, at 22 weeks, despite the fact that viable parasites had been eliminated from the primary lesion and the lesion had healed some 12 to 14 weeks earlier, 10^3 to 10^4 viable parasites were still present in the popliteal lymph node.

Resistance to challenge infection in C3H/He and BALB/c Mice. The decline in the number of viable organisms beginning at 2 weeks of infection in the resistant C3H/He mice (Fig. 3) suggested to us that an acquired host response was first being expressed at this time. It follows, therefore, that after 2 weeks, infected C3H/He mice should show increased resistance to reinfection. Similarly, the progressive growth and early dissemination of the parasite during the first weeks of the infection in the BALB/c mice suggested that no protective response was being effectively expressed in the susceptible strain. An experiment was therefore performed to determine whether the C3H/He mice that were still in the process of resolving a primary infection were resistant to reinfection. Groups of C3H/He as well as BALB/c mice were infected in the left hind footpad with 5×10^5 *L. tropica* amastigotes and then challenged in the contralateral footpad with 2.5×10^6 amastigotes 0, 5, or 22 (C3H/He only) weeks later. Changes in the number of viable parasites and changes in the swelling in the challenged footpad were followed for 4 weeks after challenge.

At 5 weeks, when the C3H/He mice were still in the process of resolving their primary infection, the C3H/He mice had developed some acquired resistance to reinfection (Fig. 4). The proliferation of the parasite seen in the control animals during the first 2 weeks after challenge did not occur, and the remaining organisms in the challenge inoculum were then gradually eliminated. When mice were challenged 22 weeks into the primary infection, not only was the initial proliferation of the parasite not seen, but the parasites in the challenged footpad were rapidly killed. By 4 weeks after challenge, few viable parasites could be detected at the site. It is important to point out that this progressive elimination of the parasite in the challenge infection occurred despite an abrupt increase in the size of the footpad.

No resistance to reinfection could be demonstrated when the BALB/c mice were challenged with 2.5×10^6 amastigotes at 5 weeks of their primary infection. Although by 2 weeks after challenge the amount of swelling observed in the BALB/c mice was the same as seen in the resistant C3H/He mice, the number of viable parasites had increased 30-fold (1.5 logs). In addition, the course of the secondary infection in the BALB/c mice was similar to that of a primary infection in terms of the progressive increase in the number of parasites at the site of inoculation.

DISCUSSION

These studies show that by counting colonies of *L. tropica* on blood agar plates inoculated with homogenates of infected tissue, it is possible to follow, quantitatively, the course of *L. tropica* infections in two contrasting mouse

models of cutaneous leishmaniasis. Although other investigators (6) have found that approximately 90% of *L. donovani* amastigotes found in tissue homogenates convert to promastigotes before replication in liquid media, counting amastigotes under the microscope may allow one to estimate the number of living parasites in the tissues only if they are present in sufficient numbers to be seen microscopically. Previously published studies (16, 17) have also proposed that if the division rate for a parasite in liquid culture is known, one can take the amastigote/promastigote transformation assay a step further to estimate the number of viable *Leishmania* cells in an organ even when none can be seen microscopically. This is done by seeding a known volume of tissue homogenate into liquid culture medium and then allowing the parasites present to transform and repeatedly multiply up to a concentration that is detectable by microscopic counting methods. The number of organisms originally added can then be estimated by linear extrapolation.

However, certain assumptions must be made to use extrapolation methods. For example, the strain of *Leishmania* used must be capable of sustained in vitro growth in liquid medium over several days. This property is apparently not common to all *Leishmania* species or strains (4). In addition, to confidently rely on the extrapolation methods, one must assume that the division rate for every organism in the tissue homogenate is the same and constant regardless of the immune status of the animal or the tissues from which the parasite was taken. Consideration must also be given to whether the sample is derived from a tissue that is relatively difficult to homogenize, such as skin, or from tissues more easily disrupted, such as lymph node or spleen (23), as well as to whether the sample is to be cultured for many days in the presence of large amounts of host cells and tissue.

The presence of host tissue in the culture does influence the growth rate of the organism. We have observed (unpublished) that when amastigotes are inoculated into liquid medium containing more than 40 mg of disrupted normal BALB/c spleen, the mean division time for the promastigotes in vitro is increased from 8.4 h to up to 16.4 h. In fact, when a whole spleen is disrupted and put into culture with *L. tropica* amastigotes, the resulting promastigotes can actually stop dividing completely after 3 to 4 days. Therefore, the kind and amount of host tissue present in liquid culture with the parasite can profoundly influence the reliability of extrapolation methods. We are convinced at this point that the adaption of the blood agar plating method to the quantitation of *L. tropica* provided a better method for quantitating the parasite. With this method, the parasites are not cultured for many days in the presence of large amounts of host tissue.

By following changes in the number of viable organisms with this method, we found that *L. tropica* promastigotes are much less infective than the amastigote form. In addition, we must add that the difference in infectivity is obvious as early as 24 h after inoculation. Whether this is due to the relative susceptibility of promastigotes to killing by serum or other host factors as has been proposed with *L. donovani* (9, 22) is now being studied.

Another, more important conclusion from the present studies is that knowledge of changes in the number of parasites during the infection is more meaningful than data on changes in lesion size. By quantitating viable parasites during the primary infection in C3H/He mice, it was found that the first evidence of the expression of acquired resistance was seen earlier than that indicated by the reduction in the size of the lesion itself. Obviously, the decrease in lesion size depends primarily upon the resolution of inflammation and edema and not only upon the reduction in the number of viable parasites. Furthermore, when C3H/He mice were challenged in the contralateral footpad later when all of the organisms in the primary lesion had been killed, there was marked swelling seen in the challenged footpad in spite of rapid elimination of the parasites. Thus, data on changes in lesion size as an indication of the state of resistance of the animals can be misleading.

Previous studies have shown that if one attempts to reinfect an animal after its primary lesion has healed, the lesion which appears at the challenge site develops and resolves rapidly (15, 20). However, by following changes in the swelling at the challenge site, under some circumstances, resistance can be demonstrated in immunized animals only if a sufficiently low challenge dose is used (20). With a higher challenge inoculum, the amount of swelling observed in the immunized animals is the same as that in the controls. Preston and Dumonde (20) concluded that their immunization protocol protected mice only against challenge with a low (10^4) number of organisms. However, our results show that in an immune animal, swelling can occur without an increase in the number of parasites and that by following changes in the number of viable parasites, resistance can be demonstrated to a challenge with five times as many organisms as were used to initiate the primary immunizing infection. We feel that only by following changes in the number of viable parasites during the primary and challenge infection can one follow with precision the development of acquired resistance in the host.

It was not clear from the previous studies of experimental cutaneous leishmaniasis whether protective immunity results in complete elimination of the viable parasites from the tissues. Our studies suggest that it does not. Acquired resistance results in the elimination of the parasites from the primary lesion and prevents the infection from disseminating to the spleen. However, it was found that some viable parasites persisted within the lymph node for at least 6 months, in spite of resistance to reinfection. In light of these data, it is tempting to speculate that the persistence of small numbers of *L. tropica* parasites in the lymph nodes draining the lesion site is actually responsible for maintaining a state of active immunity to reinfection. Studies are presently underway in an effort to determine why these organisms are not killed and what contribution the persisting organisms are making to the maintenance of an acquired state of resistance to reinfection.

The discussion of the development and maintenance of resistance hardly applies to the susceptible BALB/c mouse in which the progressive multiplication and dissemination of the parasite and lack of resistance to a challenge suggest that an acquired, protective immune response never develops or is overwhelmed by the parasite. It is well known that BALB/c mice are extremely susceptible to *L. tropica* infection (3, 5). However, based on quantitative data, the present studies contribute to our knowledge of the infection in this highly susceptible strain by documenting, quantitatively, the complete course of the infection including how soon (2 weeks) the infection can disseminate to the spleen. It is important to precisely follow the dissemination of the parasite not only to compare, as in the present studies, the infection in a resistant versus a susceptible mouse strain, but it is essential to know whether living parasites are present in lymph nodes and spleens of infected animals when these organs are used as sources of cells for adoptive immunization (14). Mitchell et al. (14) noted lesions on the abdominal walls of mice that had been adoptively immunized by an intraperitoneal injection of spleen cells from infected BALB/c mice. In their studies, the donor spleen cells probably contained living parasites. These authors appropriately point out that the effects that they observed may not be due to the passive transfer of immune cells alone.

A number of resistant mouse strains have been studied in efforts to develop animal models of the resolving form of human cutaneous leishmaniasis (2, 3, 20). In addition, the BALB/c mouse has been proposed as a model of diffuse disease, and a number of theories have already been proposed to explain the high degree of susceptibility shown by this mouse strain to *L. tropica* infection (8, 13, 21). Whatever the underlying mechanisms of resistance in mouse strains such as C3H/He or the reason(s) for susceptibility in BALB/c mice, the present study shows that these mechanisms are more appropriately and accurately studied by following the changes in the number of viable parasites in the host tissues during the infection.

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