Metacyclogenesis Is a Major Determinant of *Leishmania* Promastigote Virulence and Attenuation

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The in vivo virulence patterns of promastigote populations defined on the basis of agglutination by the lectin peanut agglutinin (PNA) were studied for various cloned lines of *Leishmania major*. Promastigotes derived from logarithmic-phase cultures, which were routinely 100% agglutinated at 100 µg of PNA per ml, were relatively avirulent for BALB/c mice. The relative virulence of stationary-phase promastigotes appeared to be attributable to the proportion of nonagglutinable (PNA⁻) promastigotes contained within these populations. Purification of PNA⁻ organisms from stationary cultures provided for each clone the most virulent inoculum, supporting the view that this change in lectin binding accurately reflects the development of infective metacyclic stage promastigotes. By studying this marker, we found that there was considerable variation in the degree to which different strains and clones underwent metacyclogenesis during growth. Examination of a reportedly avirulent *L. major* clone revealed that metacyclogenesis was unusually delayed and inefficient for this clone, but that those PNA⁻ promastigotes which could be recovered from late-stationary-phase cultures were virulent for BALB/c mice. The loss of virulence associated with frequent subculture could also be attributed to a drastic diminution in metacyclogenesis potential over time. A clone which yielded over 90% PNA⁻ promastigotes during growth within passage 1 generated fewer than 10% PNA⁻ promastigotes during growth by passage 94. Subcloning of late-passage attenuated promastigotes yielded a clone for which no PNA⁻ promastigotes could be generated during growth, and an infective population could not be derived from this clone. Thus, metacyclogenesis does not appear to be stable for even cloned lines of *Leishmania* promastigotes, and virulence comparisons between different strains and clones can be meaningfully made only if the metacyclic populations contained within the respective inocula are determined.

Recent observations have revealed that the life cycle of *Leishmania* spp. includes sequential development of invertebrate-stage promastigotes from a noninfective to an infective stage (4, 9, 13, 15, 18). This development can be demonstrated for promastigotes growing both within the phlebotomine midgut and within axenic culture (15, 16). Thus, promastigotes obtained during stationary phase of growth within the fly or culture are far more virulent for a susceptible vertebrate host than are their counterparts obtained during logarithmic phase. We and others (11) have chosen to term this infective promastigote stage metacyclic promastigotes, by analogy with the term used to denote the infective invertebrate stages of other hemoflagellates. Although the metacyclogenesis of *Leishmania* promastigotes is not accompanied by any obvious morphological change, we have been able to tentatively identify a biochemical surface marker for metacyclic promastigotes of *Leishmania major* (14). These organisms fail to be agglutinated by the lectin peanut agglutinin (PNA) at concentrations which agglutinate all noninfective promastigotes. These results indicate that metacyclogenesis involves changes in surface carbohydrates, and the lectin can be used to both quantitate and purify metacyclic *L. major* promastigotes from culture.

The loss of agglutination with PNA remains the only available phenotypic marker for *L. major* metacyclics or, for that matter, metacyclics of any *Leishmania* species. Since these conclusions were based on only in vitro observations involving a single *L. major* strain, we have in the present studies defined additional *L. major* strains and clones on the

basis of their agglutinability with PNA during growth and have examined the relative virulence of these promastigote subpopulations in vivo. We demonstrated that the loss of PNA agglutination is a generally accurate marker for *L. major* metacyclic promastigotes. More important, by employing this marker, we showed that the degree to which different *L. major* clones and strains undergo metacyclogenesis during growth can vary dramatically and that it is this difference which appears to account for the virulence and attenuation properties which these clones display.

MATERIALS AND METHODS

Mice. Female BALB/c mice (6 to 8 weeks old) were obtained from the Division of Research Sciences, National Institutes of Health, Bethesda, Md.

Parasites. The cloned isolates of *L. major* used in these studies were NIH/Friedlin (World Health Organization designation, MHOM/IL/80/Friedlin) and LRC-L137 (MHOM/IL/67/Jerico-II). Two clones derived from L137, designated V121 and A52 (7), were generously provided by E. Handman and T. Spithill, Walter and Eliza Hall Institute, Melbourne, Australia. Friedlin-strain promastigotes were also cloned as previously described (7) by limit dilution of promastigotes in NNN medium in flat-bottomed, 96-well tissue culture trays. The original cloned isolate of Friedlin strain was recloned after passage in BALB/c mice. Organisms were aspirated from footpad lesions, inoculated into Grace insect medium (GIBCO Laboratories, Grand Island, N.Y.), and cloned shortly after transformation to promastigotes. One clone was chosen for study and is designated V1. This clone was further subcloned after approximately 100 serial passages in vitro. The derived attenuated subclone chosen for study was

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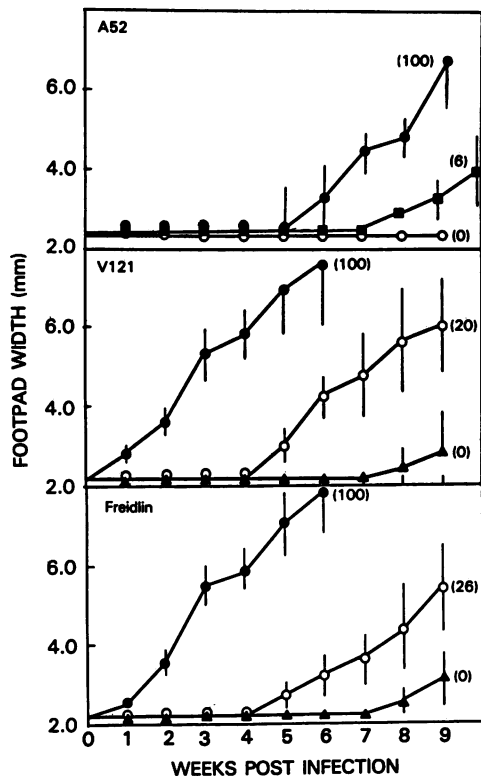
FIG. 1. Course of lesion development in BALB/c mice inoculated with log-phase (▲), day 6 stationary-phase (○), day 11 stationary-phase (■), or PNA⁻ purified (●) promastigotes from different clones of *L. major*. All mice were inoculated with 10⁶ promastigotes except mice inoculated with stationary-phase A52, which received 10⁷ promastigotes. Numbers in parentheses represent the percent PNA⁻ promastigotes contained within each inoculum. Results are expressed as mean footpad width ± 1 standard deviation of 5 or 6 mice per group.

designated A1. All parasites were maintained by serial passage of 1- to 2-day logarithmic-phase promastigotes at 26°C in Grace insect medium (GIBCO) containing 20% (vol/vol) heat-inactivated fetal bovine serum, 20 mM L-glutamine, 100 U of penicillin per ml, and 50 μg of streptomycin per ml.

Lectin-mediated agglutination. Agglutination assays using PNA (100 μg/ml; Vector Laboratories, Inc., Burlingame, Calif.) were performed as previously described (14) in 96-well, flat-bottomed microtiter plates that contained 2 × 10⁷ to 5 × 10⁷ parasites per ml. Equal volumes (50 μl) of the parasite suspension in Hanks balanced salt solution (HBSS) without NaHCO₃ and the lectin in HBSS were mixed and incubated at room temperature for 30 min. After gentle mixing, the numbers of single, unagglutinated promastigotes were determined by using a hemacytometer. For large-scale separation of PNA-agglutinated and unagglutinated promastigotes, the parasites were washed three times in HBSS and were suspended to 2 × 10⁸ to 5 × 10⁸/ml in HBSS with 100 μg of PNA per ml. After incubation for 30 min at room temperature, the suspension was centrifuged at 150 × *g* for 5 min, and the unagglutinated parasites remaining in the supernatant were then washed twice in HBSS and counted.

Mouse infections. BALB/c mice were inoculated subcutaneously in the left hind footpad with 10⁶ or 10⁷ promastigotes diluted in HBSS. Footpads were measured weekly with a direct-reading vernier caliper.

RESULTS

Metacyclogenesis and virulence. In Fig. 1, the in vivo virulence patterns of promastigote populations defined on the basis of PNA agglutination is shown. Promastigotes derived from logarithmic-phase cultures of a cloned isolate of Friedlin strain, which were 100% PNA⁺ at 100 μg of PNA per ml, were relatively avirulent for BALB/c mice. A promastigote inoculum derived from a day 6 stationary-phase culture, which contained 26% PNA⁻ organisms, produced detectable lesions by week 5. When PNA⁻ promastigotes were purified from these same stationary-phase cultures, this inoculum was extremely virulent, producing detectable lesions by the end of week 1, so that by week 7, the loss of the infected footpad had already occurred in most mice.

An identical pattern of virulence was observed with *L. major* clone V121 derived from parental isolate L-137 (Fig. 1). The relative numbers of PNA⁻ promastigotes contained in each inoculum prepared from logarithmic-phase, day 6 stationary-phase, or PNA⁻ purified populations determined the relative virulence of these promastigotes. Clone A52, also derived from L-137 and reported to be avirulent for mice (7), did not generate recoverable PNA⁻ promastigotes even when day 6 stationary-phase cultures were examined. Accordingly, these stationary-phase populations were avirulent for BALB/c mice (Fig. 1). For the stationary-phase populations, the inoculum was 10⁷ and the mice had no detectable lesions when they were examined up to 6 months after infection. Surprisingly, when day 11 late-stationary-phase cultures of A52 were examined, low numbers (6%) of PNA⁻ promastigotes were found, and an inoculum containing 10⁷ of this late-stationary-phase population produced progressive lesions in BALB/c mice beginning at week 9. An inoculum containing 10⁶ purified PNA⁻ A52 promastigotes was, as expected, more virulent, producing detectable lesions by week 5. In Fig. 2, the percentages of PNA⁻ promastigotes appearing during growth of V121 and A52 are compared in detail. PNA⁻ V121 promastigotes appeared by day 3 of culture, and optimal numbers (25%) were generated at days 6 and 7. In contrast to PNA⁻ V121 promastigotes, PNA⁻ A52 promastigotes did not appear until day 9 or 10, when considerable parasite death had begun to occur. The percentage of PNA⁻ A52 promastigotes which did appear in
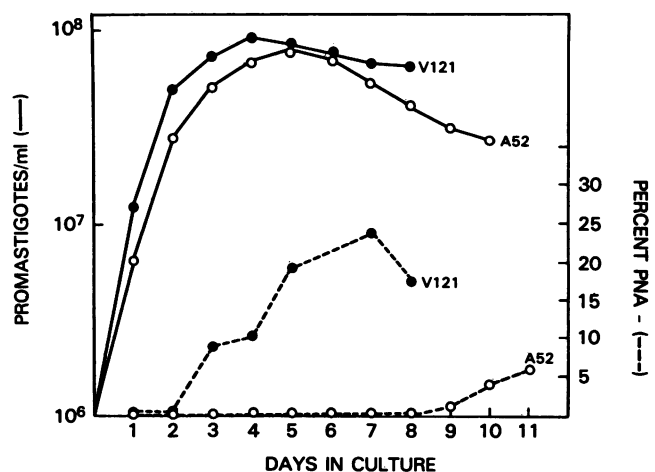

FIG. 2. Comparison of V121 and A52 promastigote growth and the percent unagglutinated PNA⁻ organisms found in promastigote populations derived from different points on the growth curve.
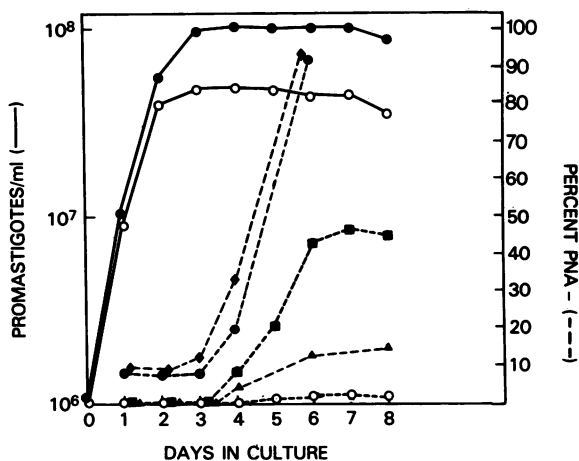
FIG. 3. Comparison of promastigote growth and the percent unagglutinated PNA⁻ organisms found in promastigote populations derived from different points on the growth curves of V1 passage 1 (●), V1 passage 24 (■), V1 passage 90 (▲), in vivo-passaged attenuated V1 (◆), and attenuated subclone A1 (○).

late-stationary-phase cultures remained low, at approximately 5%.

Metacyclogenesis and attenuation. We observed that the original clone of the *L. major* Friedlin isolate displayed decreased virulence after repeated passages in vitro and that the original virulence characteristics could be recovered by a single in vivo passage in BALB/c mice. To correlate these virulence changes with changes in metacyclogenesis, we recloned Friedlin promastigotes designated V1, derived from a footpad lesion and monitored their virulence behavior and metacyclogenesis during intervals after serial in vitro passages. The yield of PNA⁻ forms appearing during growth of passage 1 of clone V1 was extremely high (Fig. 3). By day 6, over 90% of passage 1 V1 promastigotes had transformed into PNA⁻ promastigotes. Surprisingly, by passage 24, the percentage of passage 1 V1 promastigotes had already decreased to 40%, and by passage 90, the percentage had dropped to approximately 10% (Fig. 3). Promastigotes derived from the delayed footpad lesions produced by attenuated late-passage V1 promastigotes recovered the PNA agglutination profile and virulence of the original V1 clone, generating over 90% PNA⁻ forms during growth. In contrast to this recovery, subcloning of passage 94 attenuated V1 promastigotes obtained in vitro yielded a clone, designated A1, which was unable to generate any PNA⁻ promastigotes during growth (Fig. 3).

The virulence patterns of early- and late-passage V1 and its attenuated subclone A1 are shown in Fig. 4. Day 6 stationary-phase promastigotes from passage 14 clone V1 were relatively virulent for BALB/c mice, producing detectable footpad lesions by week 2 postinfection. Stationary-phase promastigotes obtained from passage 94 V1 were significantly reduced in virulence; however, the parasites recovered from the delayed footpad lesions of these mice were selected to the extent that they displayed complete recovery of the virulence of the original V1 clone. Finally, stationary-phase promastigotes obtained from the A1 subclone appeared to be absolutely avirulent, failing to produce detectable lesions in BALB/c mice even when mice were examined up to 6 months postinfection. Thus, the dramatic differences in the metacyclogenesis potentials of these promastigote populations, all derived from a single

clone, were consistently reflected in their ability to initiate infection.

DISCUSSION

The growth of *Leishmania* promastigotes within the sandfly midgut or in axenic culture is accompanied by differentiation of some of these organisms into metacyclic-stage promastigotes which are uniquely adapted to survival within the vertebrate host. Although metacyclic promastigotes do not undergo any obvious morphological change, in our previous studies employing infections of mouse macrophages in vitro, *L. major* metacyclic promastigotes could be distinguished from noninfective promastigotes by their loss of agglutination at certain concentrations of the lectin PNA (14). By comparing virulence patterns in vivo of PNA-agglutinable and -nonagglutinable promastigotes, we were able in the studies reported here to confirm that this change is a generally accurate marker for metacyclic promastigotes of *L. major*. Furthermore, by studying this marker, we were able to compare the degrees to which metacyclogenesis occurred for different strains and clones during growth. We found that there was considerable variation in the efficiency of metacyclogenesis between different strains and clones and even within the same clone when promastigotes propagated for different lengths of time in culture were compared. These differences in metacyclogenesis appeared to completely account for the differences in virulence which the *L. major* clones displayed.

Differences in virulence between leishmanial promastigote strains and clones have been described repeatedly elsewhere (1–3, 5, 7, 10). These differences become difficult to interpret, since at the time of these studies, metacyclic promastigotes could not be distinguished from noninfective stages, and therefore the size of the effective inocula may not have been comparable. This point is most clearly demonstrated in the studies reported here involving two cloned lines, V121 and A52, which have been developed and studied extensively by Handman et al. (5, 7). Both clones were derived from the same parental isolate, yet one clone, V121, was reported to be virulent for BALB/c mice, while the other was avirulent. Using PNA agglutination as a marker, we found that the pattern of metacyclogenesis is very different for the two clones. V121 has a pattern which is typical of most *L*.
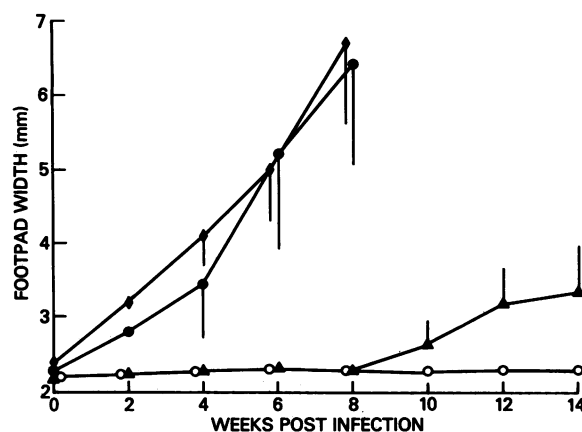

FIG. 4. Course of lesion development in BALB/c mice inoculated with 10⁶ purified PNA⁻ promastigotes from V1 passage 14 (●), V1 passage 94 (▲), in vivo-passaged attenuated V1 (◆), and attenuated subclone A1 (○). Results are expressed as mean footpad width ± 1 standard deviation of 5 or 6 mice per group.

major isolates we have studied. Metacyclic promastigotes begin to appear during growth on day 3 and reach optimal numbers (20 to 30%) by day 6. In contrast, the number of metacyclic promastigotes appearing during the growth of A52 is delayed until days 9 and 10 and remains extremely low. The reported avirulence of A52 is understandable, since it is unlikely that promastigotes would have been used from such late, ostensibly dying cultures. It is clear that the metacyclic promastigotes which do finally appear in A52 cultures are not derived from such a genetically distinct virulent subpopulation or contaminant, since promastigotes derived from lesions produced by metacyclic A52 promastigotes (i.e., selected by in vivo passage) retained the same pattern of low metacyclogenesis and virulence as the unselected A52 (data not shown). Whether poor metacyclogenesis is an artifact of the culture conditions used or whether it also may occur in nature is unknown. The data do point out, however, the importance of defining promastigote inocula with respect to the developmental stages they contain, since, for example, had logarithmic-phase V121 and late-stationary-phase A52 been compared, the opposite conclusions regarding virulence might have been drawn.

Clone A52 is similar in many respects to the attenuated Friedlin clone V1 after serial passage in vitro. The loss of virulence associated with prolonged in vitro cultivation is well described elsewhere (1, 6, 12, 17). Our observations suggest that attenuation can also be attributed to a drastic decline in metacyclogenesis when promastigotes are subcultured over long periods. In our studies, a clone which yielded over 90% PNA⁻ promastigotes during growth within passage 1, yielded by passage 24 only 40% PNA⁻ promastigotes and by passage 94 not more than 10% PNA⁻ forms.

The rapid decline in metacyclogenesis and virulence which accompanied frequent subculture occurred despite the use of cloned cells. As far as we are aware, this occurrence is a novel observation, and it indicates that the genetic factors which control metacyclogenesis and virulence are not necessarily stably expressed. Subcloning of the attenuated V1 promastigotes yielded a clone, A1, which had lost all capacity to generate metacyclic promastigotes during growth and, accordingly, had lost all capacity to initiate infection in BALB/c mice. The metacyclogenesis and virulence potential of the original V1 clone could be easily recovered by a single passage of the attenuated V1 clone in vivo. A similar enhancement of *Leishmania donovani* promastigote infectivity by several mouse passages was recently reported by Katakura and Kobayashi (8). We presume that after more-prolonged subculture of V1, in vivo selection would ultimately fail to yield infective organisms and the virulence of the original clone would be completely lost, much as it is for the attenuated A1 subclone. We suggest that the infectivity of promastigotes of a given clone or strain can be reproducibly maintained by culturing fresh or frozen stocks of in vivo isolates up to a rigorously defined growth phase prior to the preparation of each inoculum.

These studies provide a fundamental explanation for the virulence and attenuation patterns displayed by different leishmanial strains and clones. The studies emphasize the difficulties in drawing conclusions about the relative virulence of different leishmanial strains and clones based on the usual comparison of the virulence displayed by promastigotes obtained from culture. Just as meaningful conclusions regarding the relative virulence of two strains cannot be made by comparing the infectivity of amastigotes from one strain with promastigotes of another strain, comparisons based on the infectivity of different developmental stages of promastigotes will be equally difficult to interpret. Unfortunately, since metacyclogenesis can vary so extensively between different strains and even within a given clone cultured over time, comparisons based on the infectivity of promastigotes obtained even from an identical growth phase might also be inadequate. As we have demonstrated in these studies, virulence comparisons between different strains and clones of *L. major* can be more meaningfully made if the developmental stages contained within each inoculum are enumerated on the basis of their agglutinability with PNA. We expect that the identification of metacyclic promastigotes of *L. major* as well as that of other species will be refined as other molecular markers for their differentiation become available.

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