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Persistence of Virulent *Leishmania major* in Murine Cutaneous Leishmaniasis: a Possible Hazard for the Host

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The persistence of *Leishmania major* parasites in mice resistant to infection was investigated by the polymerase chain reaction and in vitro culture methods. Parasite-specific DNA was detected in the lymph nodes, spleens, bone marrow, and livers of C57BL/6 mice 1 year after their recovery from infection. Live parasites were also recovered from these tissues (except liver tissues) and were used to establish in vitro isolates. Pulsed-field gel electrophoresis, Southern blotting, and Western blot (immunoblot) analyses showed that these isolates retained the karyotype and the phenotype of the original inoculum, including the levels of expression of gp63 and lipophosphoglycan, the two major surface molecules of *Leishmania* species. More importantly, these isolates were virulent and induced fatal disease when injected into susceptible BALB/c mice. Persistence was shown to be a more general phenomenon, since several different strains of mice which were resistant to *L. major* infection also harbored persistent parasites. The implications for the etiology of human leishmaniasis in immunocompromised individuals such as AIDS patients are discussed.

Human leishmaniasis includes a spectrum of diseases ranging from cutaneous ulcers to a fatal visceral disease caused by intracellular protozoan parasites of the genus *Leishmania* (for a review, see reference 6). Infection usually results in a chronic disease which may last for several months or even years. However, at least in cutaneous leishmaniasis, strong, long-lasting, cell-mediated immune responses protect the host from subsequent infections. Yet, some manifestations of the disease are thought to be associated with the recurrence of a latent infection after the primary lesions have healed (20). This indicates that *Leishmania* parasites may persist even in immune hosts, a phenomenon that, though widely accepted, has not been thoroughly explored experimentally.

The persistence of pathogens in the presence of functional specific immunity is well documented for infectious agents as diverse as viruses (31) and helminths (26). Under circumstances of immunosuppression, these persistent pathogens may become a severe health hazard for the host. Indeed, reactivation of leishmaniasis has been suggested to occur in transplant recipients (14). More recently and perhaps of much greater concern are the reactivations of toxoplasmosis (24) and the cases of mainly visceral leishmaniasis (33) which have been documented in human immunodeficiency virus-infected individuals. In view of this, one could envisage an increased risk of recurrent leishmaniasis caused by persistent parasites in individuals cured of disease or asymptomatically infected who become immunosuppressed. Moreover, the number of individuals living in endemic areas carrying asymptomatic infections may be larger than previously thought, thus increasing the size of the population at risk (25, 35).

The purpose of this study was to examine in an animal model the extent of *Leishmania* persistence in immune hosts, i.e., resistant mice experimentally infected with *Leishmania major*. The focus was placed on the frequency of persistent infections in mice that had recovered from primary infections, identification of the organs most likely to contain persistent parasites, and characterization of these persistent *L. major* parasites. In addition, the persistent parasites' virulence and their ability to cause diseases in new hosts were examined.

MATERIALS AND METHODS

Parasites. The *L. major* strain LRC-L137 was originally obtained from the WHO Reference Centre for Leishmaniasis in Jerusalem, Israel. The virulent cloned line of LRC-L137, V121 (18), was used to infect mice. Promastigotes were grown in vitro on NNN medium as described previously (18) or in Schneider's *Drosophila* medium (GIBCO, Glen Waverley, Australia) supplemented with 10% fetal calf serum. Promastigotes from stationary-phase cultures were used for the infection of mice. Alternatively, parasites were passaged in vivo in CBA *nu/nu* mice, and amastigotes prepared as described by Glaser et al. (10) were used to infect mice.

Mice. All of the mice used in this study were bred at the animal-breeding facility of this institute under specific-pathogen-free conditions. At 6 to 8 weeks of age, they were transferred and used in conventional animal facilities.

Infection of mice with *L. major*. Unless otherwise stated, mice were infected by intradermal injection of 2×10^6 V121 amastigotes at the base of their tails. For comparison, 2×10^6 V121 promastigotes or promastigotes of persistent parasites isolated from cured C57BL/6 mice grown on NNN as described above were injected intradermally.

Isolation of persistent V121 parasites. Mice infected with 2×10^6 parasites were sacrificed at the indicated times after infection, and the inguinal and lower periaortic lymph nodes as well as the femuri (for bone marrow), spleen, liver, and kidneys were removed from each mouse. Single-cell suspensions of these organs were prepared in phosphate-buffered saline (PBS) (pH 7.4), overlaid onto NNN, and cultured at 26°C. Cultures were checked for the presence of flagellated parasites every 2 days.

Titration of *Leishmania*-specific antibodies in sera from infected mice. Mice infected with the persistent *L. major*

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parasites or with the original clone V121 were bled individually at biweekly intervals, and the sera were analyzed by enzyme-linked immunosorbent assay (ELISA). The 96-well U-bottom microtiter plates (Dynatech, Chantilly, Va.) were coated for 4 h at 4°C with a saline-soluble extract of V121 promastigotes (28) at a concentration of 25 μg of protein per ml. Serially diluted sera were added and incubated for 3 h at room temperature. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin M (IgM) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), goat anti-mouse IgG1 and IgG2a (Southern Biotechnology, Birmingham, Ala.), sheep anti-mouse Ig (Silenius, Melbourne, Australia), and 2,2'-azino-bis-(3-ethylbenzothiazoline-6 sulfonic acid) (Pierce, Rockford, Ill.). Titers are indicated as serum dilutions at which a signal above background was still obtained.

Western blot (immunoblot) analysis of parasite antigens. Stationary-phase promastigotes (cultured in Schneider's *Drosophila* medium) and amastigotes were washed three times in PBS and lysed by sequential freeze-thawing (three times at −70°C), sonication for 15 min, and boiling for 5 min in reducing sodium dodecyl sulfate (SDS) sample buffer (19). Thirty-five micrograms of protein from each sample was separated in SDS–10% polyacrylamide gels as described previously (19). The separated proteins were electroblotted onto nitrocellulose BA83 (Schleicher & Schuell, Dassel, Germany) and probed with affinity-purified rabbit antisera directed against *Leishmania* gp63 and with the monoclonal antibodies WIC79.3 and WIC108.3 directed against the lipophosphoglycan (LPG) (8).

Alternatively, V121 lesion amastigotes were purified from CBA *nu/nu* mice. A total of 5×10^8 amastigotes were lysed in 1 ml of a buffer containing 0.5% Triton X-100 (19), and the equivalent of 3×10^7 parasites were separated on SDS–10% polyacrylamide gels. Proteins separated on these gels were blotted onto nitrocellulose as described above and probed with different mouse sera normalized for their *Leishmania*-specific antibody titers. Antigens detected by these sera were visualized with horseradish peroxidase-conjugated goat anti-mouse IgG1 (Southern Biotechnology) and the horseradish peroxidase substrate 4-chloro-1-naphthol (Sigma, Castle Hill, Australia) or by autoradiography after incubation with ^{125}I-labeled protein G (Pharmacia, North Ryde, Australia).

Detection of parasites in host tissues by PCR. Single-cell suspensions were prepared as described above. Cells were washed in PBS and pelleted, and the cell pellet was lysed in 4 M guanidine–HCl (approximately 4×10^6 cells per ml of guanidine-HCl). Genomic DNA was purified from 1 ml of lysed cells with Geneclean silica matrix (Bio 101, La Jolla, Calif.) by following the manufacturer's instructions. The polymerase chain reaction (PCR) was performed on this DNA by using the following nested oligonucleotides specific for the glycoprotein gp63 gene: A1 (5' GATCGCAACGCAG GACGAGCTCATGGCG 3'), A2 (5' CACGACAGGGCAGT AGTCCATGAAGGCGG 3'), and B1 (5' CTTCAGCAAGG CCGAGGTGATGCCGTGGGG 3'), B2 (5' GAGGGACGG GTCCGTGAAGTACTGCCAGTA 3'). A fragment corresponding to 222 bp stretching between base pairs 1328 and 1530 of gp63 (5) was amplified by two rounds of PCR, each comprising 40 cycles of 2 min at 94°C, 1 min at 60°C, and 100 s at 72°C. The amplified products were analyzed by conventional agarose gel electrophoresis.

Southern blot analysis and karyotyping. Genomic DNA of promastigotes was prepared as described previously (40). One microgram of genomic DNA was digested to completion with the indicated restriction enzymes by using 15 to 20 U/μg of DNA. The digested DNAs were separated on 0.8% agarose gels and transferred to Hybond-N$^+$ membranes (Amersham, North Ryde, Australia). The Southern blots were then hybridized according to the method of Church and Guilbert (7) to ^{32}P-labeled probes specific for several cloned *Leishmania* genes, gp63 (5), P100.11E (40), Hsp70 (42), and PSA-2 (30).

The karyotypes of the different isolates were compared by pulsed-field gel electrophoresis. Briefly, chromosomal DNA was prepared as described previously (29) with a parasite concentration of 2×10^8/ml. Chromosomal DNA was separated on a 1% agarose gel with a contour-clamped homogeneous electric field mapper (Bio-Rad, Richmond, Calif.) under conditions described previously (29). The separated chromosomal bands were blotted onto Hybond-N$^+$ and hybridized with the probes under the conditions described above.

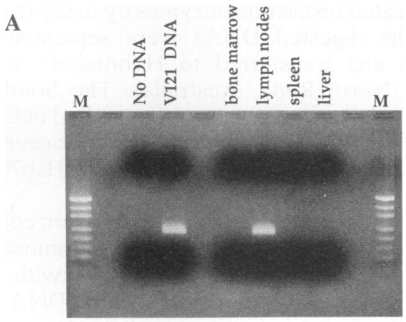
RESULTS

Detection and isolation of persistent *L. major* parasites. C57BL/6 mice were infected with a clone of *L. major* V121, and individual mice were analyzed 12 months postinfection. At this time point, no signs of ongoing infection were visible in any of the mice. Lymph nodes draining the original site of infection (inguinal and lower periaortic), spleens, bone marrow, livers, and kidneys were examined for the presence of parasites either by microscopic examination of cultures prepared from these organs or by detection of *Leishmania*-specific DNA sequences by using PCR and oligonucleotide primers specific for a sequence present in the gene of the major surface glycoprotein of promastigotes, gp63 (5).

Figure 1 summarizes the analysis of eight C57BL/6 mice 1 year after infection with *L. major*. Parasite-specific DNA was detected in DNA samples prepared from lymph nodes, livers, spleens, or bone marrow (Fig. 1B). An example of this analysis is given for one mouse in Fig. 1A, which shows a gp63-specific DNA fragment that was amplified from lymph node DNA. Half of the cells from these organs were cultured on NNN agar to determine whether the PCR result reflected the presence of live parasites. Live parasites were detected in lymph nodes and occasionally in spleens and bone marrow from these mice (Fig. 1B), thus confirming the results obtained by PCR. Culturing persistent parasites from livers, however, was not successful (Fig. 1B), even when *L. major* DNA was detected in this tissue, probably because of toxic metabolites from the liver cells. Although the results obtained by PCR and the culture assay did not correspond in every single case (Fig. 1B), subsequent experiments were performed by using the culture method, since its sensitivity for detecting parasites in lymph nodes, bone marrow, and spleens was comparable to or higher than that of PCR.

To analyze whether mice from other inbred strains could also become persistently infected with *L. major*, mice from six different strains were infected with 2×10^6 V121 amastigotes and sacrificed 7 months later. Some of the mouse strains used in this analysis were intermediately resistant to *L. major* infection (see Table 1) (16). Although these mice did not display overt lesions, the lymph nodes harvested from individuals of these strains were enlarged (data not shown). The highly resistant C57BL/6 and CBA mice, which at the time of analysis had been completely cured of their lesions and had normal lymph node cell numbers, were also found to harbor persistent parasites.

In summary, from a total of 23 mice of different strains



B

L. major detection

Tissue	PCR	culture
bone marrow	2	1
lymph nodes	3	6
spleen	1	1
liver	2	--
kidney	--	--

FIG. 1. Detection of persistent *L. major* parasites in host tissues 1 year after infection. (A) Detection of *L. major*-specific DNA in different host tissues from a single mouse by PCR with nested *L. major* gp63-specific oligonucleotides. DNA samples were prepared from a recovered C57BL/6 mouse 1 year after infection. V121 genomic DNA was used as a positive control. Ten independent control samples of DNA isolated from the same tissues of uninfected mice were all negative (not shown). The reaction mixture was analyzed on a 1.8% agarose gel. Lane M, molecular weight marker (Hpa2-digested pUC19 DNA). (B) Summary of *L. major* detection in tissues of eight C57BL/6 mice. Animals were sacrificed, and the specified organs were removed. Parasites were detected by PCR as shown for one example above and by culturing live promastigotes in aliquots of the same organs. All eight mice tested were shown to harbor parasites in one or both assays. Numbers refer to the numbers of positive mice in the respective assay. --, none.

TABLE 1. Detection of persistent *L. major* in tissues from different mouse strains

Strain	Susceptibility[b]	n/n_p[c]	No. of tissue samples harboring parasites[a]:		
			Lymph node	Spleen	Bone marrow
Ab/HL (Biozzi)	I	2/2	2	0	0
A.TL	I	3/3	3	3	0
A.TLab	I	2/2	1	1	0
B10.A (2R)	I	2/2	2	2	1
C57BL/6	R	8/8	6	2[d]	3[d]
C57BL/6 Ly5.1	R	3/3	3	1	0
CBA/N	R	3/3	3	0	0
Total		23/23	20	9	4

[a] Parasites were detected by culturing live *L. major* isolates from single-cell suspensions of the indicated tissues.

[b] Mice were graded according to their healing kinetics. I, intermediately resistant mice, with a proportion of individuals in a group not healed of initial lesions within 4 to 6 weeks after infection; R, resistant mice developing small lesions self-healing within 4 to 6 weeks after infection.

[c] n, number of individual mice examined; n_p, number of mice harboring detectable parasites.

[d] Cumulative data from culture and PCR analyses (Fig. 1).

analyzed, all proved to persistently harbor parasites in the absence of overt lesions. From the organs investigated, the lymph nodes draining the sites of the original inoculations were most consistently infected (Table 1).

Genomic characterization of persistent *L. major* parasites. Persistent parasites in an immune host would presumably have to escape the host's immune response. Strategies by which pathogenic organisms evade the host immune attack may involve molecular changes through mutational (34) or recombinational (4) processes. Therefore, the genetic relatedness of persistent and parental parasites was investigated. Seven *L. major* persistent isolates (L1 to L7), five of which were independently derived from individual mice (L1 to L5), were obtained from C57BL/6 mice infected 1 year prior to being analyzed. Isolates L5 to L7 were recovered from different organs of a single mouse, i.e., lymph nodes (L5), spleen (L6), and bone marrow (L7). These isolates were examined to determine whether they could be distinguished from the parent clone of *L. major* V121 by restriction fragment length polymorphisms for known *L. major* gene loci or by karyotype analysis.

Southern blot analysis with *Eco*RI- and *Pst*I-digested DNA from all five independent isolates (L1 to L5) showed identical hybridization patterns when blots were probed with radiolabeled probes specific for P100.11E, a reductase (40); the heat shock protein Hsp70 (42); and the promastigote surface antigen 2, PSA-2 (30) (data not shown). In contrast, in one isolate (L4), a restriction fragment length polymorphism was detected with a DNA probe specific for the major surface glycoprotein gp63 (5). Additional bands of 3, 3.3, and 3.2 kb were detected in DNA from L4 cut with *Pst*I, *Sal*I, and *Sma*I, respectively, by using a genomic probe for gp63 (Fig. 2).

The restriction fragment length polymorphism in L4 could be the result of a chromosomal alteration involving the gp63 locus. No difference between this isolate and V121 was detected in the chromosomal localization of gp63 (Fig. 3A). In addition, analysis of the karyotypes revealed no differ-

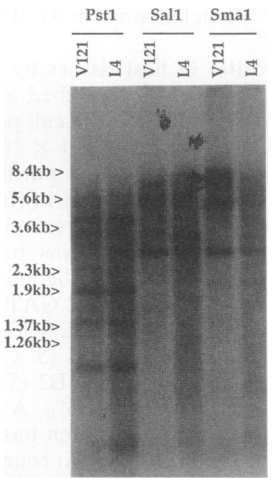


FIG. 2. Restriction fragment length polymorphism in the gp63 locus of persistent *L. major* isolate L4. Genomic DNA from the *L. major* clone V121 and that from the isolate L4 were digested with *Pst*I, *Sal*I, and *Sma*I, separated on 0.8% agarose gels, blotted onto Hybond-N$^+$, and hybridized to a genomic gp63 probe (5). Molecular sizes (in kilobases) are on the left.

ences in the size of chromosome bands as detected by pulsed-field gel electrophoresis in the completely independent isolates L1 to L5 of persistent *L. major* parasites and the parent clone, V121 (Fig. 3B). Therefore, persisting *L. major* V121 parasites appear to retain the genetic characteristics of the initial inoculum.

gp63 and LPG expression in persistent *L. major* parasites. The surface glycoprotein gp63 and LPG, the major antigenic components of the *L. major* promastigote surface, have been shown to be important for infectivity (17, 37). The restriction fragment length polymorphism detected in L4 might reflect a mutation leading to altered expression of gp63 by this isolate. The expression of gp63 and LPG was therefore investigated by Western blot analysis with monospecific polyclonal antisera or specific monoclonal antibodies.

gp63 was expressed by promastigotes of all isolates, including L4, at a level similar to that of V121 as detected by a gp63-specific rabbit polyclonal antiserum (Fig. 4A). In addition, amastigotes of L1 to L7 and V121 expressed gp63 reactive with the same antiserum at low levels, and no significant differences were detected between V121 and the persistent lines (data not shown). LPG expression was determined with the well-characterized monoclonal antibodies WIC108.3 and WIC79.3 (8, 22). The molecular weight distribution and amount of LPG displaying the epitope recognized by WIC79.3 varied in the promastigote cultures of the persistent isolates (Fig. 4B). Most isolates expressed WIC79.3-reactive LPG with a molecular weight higher than that of LPG expressed by V121 (Fig. 4B). LPG detected by WIC108.3, a monoclonal antibody recognizing a different epitope on LPG (15), was identical in all of the isolates, and the difference in molecular weight distribution between their LPGs and that of V121 was less evident than with WIC79.3 (Fig. 4C). Similarly, amastigotes of all isolates expressed LPG detected by WIC108.3 at the same level and with a molecular weight distribution identical to that of V121 amastigotes (Fig. 4D).

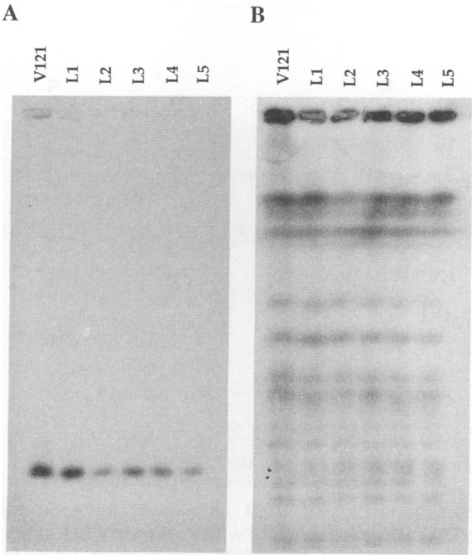


FIG. 3. Comparison of chromosomal localization of gp63 and karyotype of persistent *L. major* isolates with those of V121. Chromosomes of V121 and L1 to L5 were separated by pulsed-field electrophoresis under conditions which separate most of the chromosomes (29). Gels were blotted onto Hybond-N$^+$ membranes and hybridized to probes specific for gp63 (A) or to radiolabeled V121 genomic DNA (B).

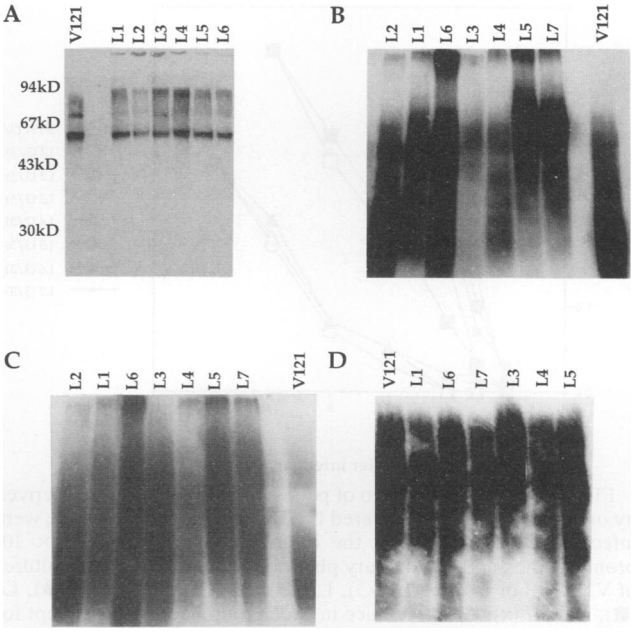


FIG. 4. Western blot analysis of gp63 and LPG expression in persistent *L. major* isolates. Lysates of promastigotes (A, B, and C) or amastigotes (D) prepared from lesions of CBA *nu/nu* mice infected with the isolates were separated on SDS–10% polyacrylamide gels and blotted onto nitrocellulose membranes. Blots were incubated with either rabbit antisera against gp63 (A) or the monoclonal anti-LPG antibodies WIC79.3 (B) and WIC108.3 (C and D).

In vivo infectivity of persistent *L. major* parasites. Persistent parasites could potentially reactivate the disease in immunocompromised hosts, provided that they retained their virulence. It was therefore tested whether injection of promastigotes of the isolates characterized above could produce lesions in susceptible BALB/c or resistant C57BL/6 mice. The progression of disease was monitored by conventional lesion scoring and by monitoring the humoral immune response to the parasites.

In the resistant C57BL/6 mice, all of the isolates induced small, detectable lesions which resolved spontaneously and were indistinguishable from those produced by the cloned line V121 (not shown). In contrast, BALB/c mice infected with the persistent *L. major* isolates developed nonhealing, ulcerating lesions similar to those caused by V121. Figure 5 depicts a representative experiment in which groups of three mice were infected with the persistent isolates. At an infectious dose of 2×10^6 promastigotes, lesions developed in all mice. Animals infected with L2, L5, L6, or L7 developed lesions with kinetics similar to those of mice infected with V121. Lesion development was slower in mice infected with L1, L3, or L4, and one animal infected with L4 did not develop lesions. However, in repeated experiments, only L3 showed retarded lesion development, and all mice infected with L4 developed lesions (data not shown).

As previously shown (32), the *Leishmania*-specific antibody response was low in C57BL/6 mice compared with that in BALB/c mice. Titers of specific antibodies against soluble *Leishmania* antigens in C57BL/6 mice ranged from 1/2,000 for IgM 14 days after infection to 1/25,000 of mainly IgG1 56 days after infection. In contrast, *Leishmania*-specific anti-

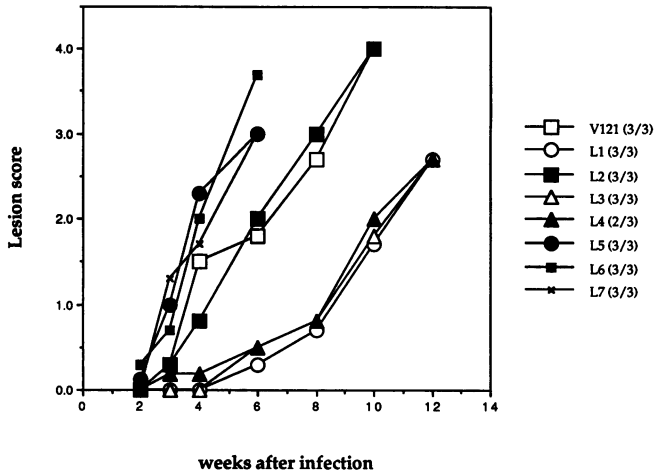


FIG. 5. Infectivity in vivo of persistent *L. major* isolates derived by organ culture from recovered C57BL/6 mice. BALB/c mice were infected subcutaneously at the base of their tails with 2×10^6 promastigotes in the stationary phase of growth from NNN cultures of V121 (□) or isolates L1 (○), L2 (■), L3 (△), L4 (▲), L5 (●), L6 (■), and L7 (x). All three mice in each group had lesions except for those infected with L4 (two out of three mice had lesions). Developing lesions were scored every week (lesion scores: 0, no swelling; 1, swelling or lesion size of <1 mm; 2, lesion size of 2 to 5 mm; 3, lesion size of 5 to 10 mm; 4, lesion size of >10 mm). Mice with lesion scores of more than 3 or 4 were euthanized.

body titers in sera of BALB/c mice infected with V121, L2, L3, L5, L6, or L7 rose to more than 1/400,000 for IgG1 56 days postinfection.

Immunogenicity of persistent *L. major* parasites. *L. major* parasites persisting in an immunocompetent host may be expected to have lost some immunogenic determinants present in the original inoculum, V121, as a result of selective pressure by the host.

To test this hypothesis, we investigated whether sera from BALB/c mice infected with the persistent isolates recognized the same antigens as sera from mice infected with the parent, V121. Sera from three BALB/c mice infected with each isolate were titrated by ELISA, pooled, and diluted to identical titers (1/3,200). These normalized sera were used to detect antigens expressed by V121 amastigotes in a Western blot analysis. As anticipated from the results mentioned above, the sera contained mainly *Leishmania*-specific antibodies of the IgG1 subclass, since detection of bound antibodies with radiolabeled protein G (Fig. 6) produced essentially the same pattern as detection with anti-mouse IgG1 (data not shown). Antigens recognized by all sera from infected mice included molecules with apparent molecular sizes of 145, 92, 63, and 41 kDa (arrows in Fig. 6). With the exception of sera from mice infected with L3 and L4, antigens in the 71-kDa region were also commonly recognized. However, virtually identical patterns were detected with sera from mice infected with the parent *L. major* clone V121 or the persistent isolates L1, L2, and L5 to L7. Note the minor variations in the pattern and intensity of some antigen bands detected with sera from mice infected with two different batches of V121 (Fig. 6).

DISCUSSION

The results reported here show that mice which were experimentally infected with *L. major* and which recovered from that infection still harbored parasites with significant virulence many months later. These organisms persisted in 100% of the individuals, even with highly resistant strains of mice. Moreover, the persistent *L. major* parasites did not lose their virulence and were capable of inducing progressive disease in susceptible hosts. Therefore, persistent parasites are a potential hazard for the host.

Different inbred mouse strains exhibit various degrees of resistance to *L. major* infection (16). In some strains tested, a chronic, ongoing infection may be responsible for parasite persistence, since several mice in these groups had enlarged lymph nodes. However, these mice showed no splenomegaly, a feature associated with progressive disease in susceptible mice. Nevertheless, *L. major* has been shown to visceralize and could be cultured from spleens of resistant C57BL/6 or CBA mice after their recovery (23). In our hands, the lymph nodes draining the initial site of infection were by far the most consistent source of persistent parasites. Whether this is due to temperature preferences of *L. major* (41), the agent of cutaneous leishmaniasis, or to an as-yet-uncharacterized population of long-term host cells resident in lymph nodes needs to be clarified.

Different strategies have been described previously to explain how parasites evade immune attacks in the host (for a review, see reference 2). Blocking host effector mechanisms, e.g., evasion of complement mediated lysis, antigenic variation by mutational mechanisms, or hiding in immunologically privileged compartments have been suggested. Depending on the stage of infection, *Leishmania* spp. may use a combination of these. Resistance to complement lysis seems to be important in the very first moments of infection (21). Antigenic variation on the basis of different glycosylation patterns has been suggested to occur when *L. mexicana* parasites were injected into immune hosts (12). The data reported here allow no conclusive answer on whether antigenic variation occurs. However, persistent *L. major* isolates retained the characteristics of the parent clone, V121. The isolates were genetically stable and exhibited karyo-

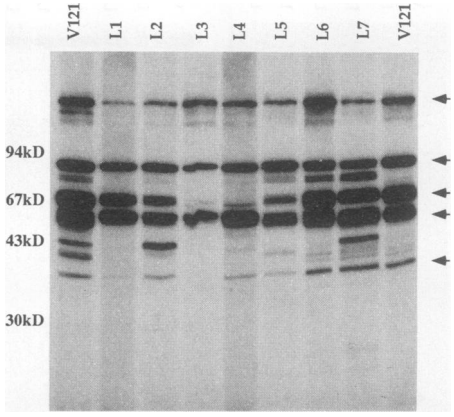


FIG. 6. Comparison of V121 amastigote antigens detected by sera from BALB/c mice infected with *L. major* V121 or persistent *L. major* isolates. Triton X-100-soluble antigens of V121 amastigotes were separated on an SDS–10% polyacrylamide gel and blotted onto nitrocellulose membranes. Blots were incubated with pooled sera from three BALB/c mice infected with V121 (lanes V121) or L1 to L7 (lanes L1 to L7) at equal, *Leishmania*-specific Ig titers. Bound antibodies were detected by ^{125}I-labeled protein G. Sera from uninfected BALB/c mice did not detect any antigens (not shown). Arrows indicate antigens recognized by all sera.

types identical to those of the parent, V121. Yet, the karyotype of *Leishmania* spp. has been shown to be very heterogeneous even within a particular strain (1). In addition, the gene loci investigated by Southern analysis were essentially unaltered, whether expressed in the persisting amastigote (PSA-2 [30], gp63 [3], and Hsp70 [42]) or not (P100.11E [40]). Moreover, analysis of the expression of the major surface antigens, gp63 and LPG, on the relevant amastigote form of the parasite showed no detectable differences between the isolates and the parent clone, V121. Also, the humoral immune response to infection with the persistent *L. major* isolates or the parent, V121, was directed against the same main antigens. This approach may only allow the detection of gross differences, and variation in the patterns of antigens recognized might indeed reflect antigenic variation. However, no correlation between persistence and lack of recognition of one or more antigens was obvious. Therefore, hiding in a safe target cell (27) or downregulation of major histocompatibility complex molecules to prevent the major histocompatibility complex-restricted recognition of parasite antigens on the surface of the infected cells, as suggested for *L. donovani* (36), will probably be more fruitful hypotheses to investigate in order to understand the mechanism of persistence.

It has been reported previously that *L. mexicana* isolated from lesions of previously vaccinated mice were less virulent than parasites from lesions of naive, infected mice (11). This phenomenon may be due to the strong selective pressure on the initial inoculum used to infect the vaccinated mice. In this study, persistent *L. major* isolates were still infective, and a general loss of virulence was not detected in these parasites isolated from recovered, immune mice. The differences between experiments were most probably a consequence of variability in the promastigote cultures used to infect mice. The expression of LPG recognized by WIC79.3 varied among the isolates, and it could explain the variation in the infectivity of the inocula, since this monoclonal antibody can be used to monitor the transition of promastigotes from the noninfective (low-molecular-weight LPG) to the infective (high-molecular-weight LPG) stage (38, 39).

The important finding of this study was that virulent *L. major* parasites persisted in immune mice in all individuals analyzed. Consequently, at least in experimental murine cutaneous leishmaniasis, persistence seems to be the rule rather than the exception. Evidence for persistence of virulent parasites has also been reported recently for mice infected with *L. mexicana* (9). The significance of the persistent parasites has been discussed in the context of immunological memory (23), i.e., to maintain a memory population of protective T cells. This interpretation has been strengthened by the recent observation that T-cell memory is short-lived in the absence of antigen (13). However, it remains to be shown whether live parasites are required to sustain immunological memory and whether this might be relevant for the design of a future vaccine. Thus, parasite persistence might be beneficial to the host under normal circumstances. However, and perhaps more importantly, persistent parasites were able to induce fatal disease in susceptible BALB/c mice. If persistence of *Leishmania* parasites is also frequent in natural human infections, these parasites might indeed represent a source for reactivated leishmaniasis in immunocompromised individuals, for example, AIDS patients.

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