Fibroblasts as Host Cells in Latent Leishmaniosis

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Abstract

Intracellular parasites are known to persist lifelong in mammalian hosts after the clinical cure of the disease, but the mechanisms of persistence are poorly understood. Here, we show by confocal laser microscopy that in the draining lymph nodes of mice that had healed a cutaneous infection with Leishmania major, 40% of the persisting parasites were associated with fibroblasts forming the reticular meshwork of the lymph nodes. In vitro, both promastigotes and amastigotes of L. major infected primary skin or lymph node fibroblasts. Compared with macrophages, cytokine-activated fibroblasts had a reduced ability to express type 2 nitric oxide synthase and to kill intracellular L. major. These data identify fibroblasts as an important host cell for Leishmania during the chronic phase of infection and suggest that they might serve as safe targets for the parasites in clinically latent disease.

Key words: Leishmania major • fibroblasts • persistent infection • nitric oxide • macrophages

Introduction

A hallmark of infections with certain viruses (e.g., herpesviruses), intracellular bacteria (e.g., Mycobacteria, Coxiella, Chlamydiae), or protozoa (e.g., Trypanosoma cruzi, Leishmania) is the long-term persistence of the pathogen after clinical cure of the disease. Based on in vitro results, modulation of host cell antimicrobial activities, synthesis of inhibitory cytokines, impairment of T cell activation, or retreat of the pathogen into cells that do not elicit an immune response have been proposed as viral or microbial survival strategies, but the mechanisms of persistence in vivo remain ill defined (1, 2). One example are infections with Leishmania parasites. Leishmania promastigotes are transmitted by sand flies to mammalian hosts, where they infect macrophages, granulocytes, and dendritic cells, transform into amastigotes, and cause cutaneous, mucocutaneous, or progressive visceral disease. In most strains of mice as well as in humans, infections with Leishmania major usually elicit skin swellings or single ulcers that are ultimately controlled by a CD4+ T cell response involving the production of IFN-γ and the activation of antileishmanial effector mechanisms in macrophages (3–6). In mice, IFN-α/β and IFN-γ induced the production of nitric oxide (NO)† by the inducible (or type 2) NOS synthase (iNOS or NOS2), which was shown to be indispensable for the healing of acute cutaneous lesions (7–10).

After spontaneous or chemotherapy-mediated healing of the infection, both mice and humans continue to harbor small numbers of alive Leishmania parasites in the lymphoid tissue (11–13). This was demonstrated most convincingly by the recrudescence of the disease after treatment with immunosuppressive drugs, depletion of CD4+ T cells, or inhibition of NOS2 activity (14–17). Although the components of the immune system that are responsible for the resolution of acute Leishmania infection are well defined, little is known about the mechanisms that allow the parasites to survive lifelong in the host. In genetically resistant mice that had resolved a skin infection with L. major, we found that NOS2 activity was indispensable for the long-term control of the remaining parasites. 30–40% of the parasites persisting in the draining lymph node colocalized with NOS2-positive macrophages or dendritic cells, whereas 60–70% of the parasites were located in NOS2-negative areas that could not be stained with known markers for mac-
rophones, dendritic cells, granulocytes, or endothelial cells and thus remained undefined (17). As fibroblasts had been reported to be susceptible to infection with various Leishmania species in vitro (18–20), and lymph nodes from chronically infected mice contained strongly increased amounts of fibrous tissue, we considered the possibility that Leishmania might also reside in fibroblasts in vivo. In this study, we identify reticular fibroblasts in lymph nodes as major host cells for L. major during latent disease and provide evidence that these cells might function as “safe targets” (21) for the parasites.

Materials and Methods

Parasites. The L. major strain MHOM/IL/81/FE/BNI (17) was propagated in vitro in RPMI 1640 plus 10% FCS on N-ocular blood agar slants for a maximum of six passages. Fresh L. major promastigotes were derived from amastigotes that were isolated from the ulcerated skin lesions or the spleens of BALB/c mice as described (22)

Mice. Female C57BL/6 mice, weighing 16–18 g, were purchased from Charles River, housed in our own facilities, and used at 8–12 wk of age. C57BL/6 mice were inoculated into the right hind footpad with 3 × 10^6 stationary phase L. major promastigotes. In some experiments, mice were infected bilaterally into both hind footpads. The footpad swelling was measured with a metric caliper. The mice received their skin lesions usually within 60–70 d after infection. For immunohistological analyses, the popliteal lymph nodes draining the site of infection were removed from mice that had been infected for at least 100 d.

Macrophages. Thiglycollate-elicited peritoneal exudate macrophages (PEMs) were prepared from the peritoneal cavity of C57BL/6 mice as described (23). Resident peritoneal macrophages (RPMs) were obtained from C57BL/6 mice by flushing the peritoneal cavity twice with 10 ml ice-cold PBS. The cells were resuspended in RPMI 1640 culture medium (supplemented as described [23] plus 1 or 2.5% fetal bovine serum [Sigma-Aldrich]), seeded into 24-well plates (10 6 cells/well in 500 μl) or 8-well LabTek® chamber slides (Permanox®; N-agle N unc International), and cultured at 37°C in 5% CO_2/95% humidified air. After 90–120 min, nonadherent cells were removed and the macrophage monolayers were further incubated as indicated.

Isolation of Fibroblasts. Mouse skin fibroblasts were established from ear skin explants of C57BL/6 mice as described (24). In brief, after removal of the epidermis, the dermis was cut into 1 × 2 mm pieces, which were then cultured to attach to tissue culture petri dishes before addition of complete RPMI 1640 medium (see above) supplemented with 10–20% FCS. Nonadherent cells were removed by weekly medium exchanges. After 2–4 wk, fibroblasts grew out from the explants that were passaged after treatment with 0.25% trypsin–EDTA solution and further propagated in RPMI 1640 supplemented with 5% FCS by weekly 1:3 splitting.

Reticular fibroblasts were obtained from popliteal lymph nodes (~30–90 mg) of chronically infected mice (>200 d after infection with L. major). Collagenase D (100 U/ml in PBS; Boehringer) was injected into the lymph nodes that had been carefully prepared and freed of extracapsular tissue. The lymph nodes were opened and the cell suspension was harvested in PBS with 100 U/ml collagenase D. Finally, the lymph nodes were teased into small fragments that were further digested in PBS plus 400 U/ml collagenase D at 37°C for 60–90 min. Undigested (capsular) fragments were removed and the cells were pooled with the primary suspension, centrifuged, washed, and seeded in small tissue culture flasks (N-angle N unc International) in RPMI 1640 medium plus 10–20% FCS. Nonadherent cells were removed by weekly medium exchanges. After 2–4 wk, fibroblasts were growing in the cultures.

For infection or phenotypic characterization, skin or lymph node fibroblasts were harvested with a rubber policeman, seeded, and used as confluent monolayers. To minimize growth, the serum concentration was reduced to 1–2.5%.

Infection of Macrophages and Fibroblasts with L. major Parasites. Monolayers of macrophages or fibroblasts in 24-well plates were incubated with a 3–10-fold excess of L. major promastigotes or amastigotes, either continuously (permanent infection) or for 4–14 h after which nonphagocytosed parasites were washed off (pulse infection). The cells were activated with recombinant murine (rm)IFN-γ (provided by Dr. G. Adolf, Ernst Boehringer Institut, Vienna, Austria) with or without rmTNF-α (R&D Systems) or LPS (Escherichia coli O111:B; Sigma-Aldrich). Culture medium was replaced every 24 h.

The infection rate and the number of intracellular parasites per infected cell were determined by immunoenzymatic or immunofluorescence staining of the monolayers. At least 300 cells were evaluated. The total number of intracellular parasites per culture was determined by two different methods that were previously shown to yield comparable results (22). In brief, infected macrophage or fibroblast monolayers were lysed in SDS (0.01% in serum-free RPMI) to release intracellular parasites. After addition of a twofold volume of modified Schneider’s Drosophila medium (mSDM) (25), the cell lysates were spun and the pellets containing the amastigotes were resuspended in 500 μl modified mSDM. The parasite suspensions were seeded in triplicates into 96-well flat-bottomed plates for further incubation (24–48 h) and pulsed with 1 μCi (37 kBq)/well of [3H]thymidine (25 Ci/mmol; Amersham Pharmacia Biotech) for the last 12–18 h. Alternatively, the parasite suspensions were subjected to limiting dilution analyses using serial 2-fold dilutions and 12–24 individual wells per dilution step. After 7 d of culture, the number of wells negative for parasites was determined for each dilution and the number of viable L. major parasites per culture condition was calculated by applying Poisson statistics and the χ² minimization method (8, 22).

Cultures of Macrophages and Fibroblasts. PEMs were allowed to adhere (2 h) to the outer side of the membrane (pore size 0.45 μm) of cell culture inserts (Costar). The inserts were then immersed in culture wells containing complete RPMI 1640 medium with 2.5% FCS. Reticular fibroblasts were added and, after adherence to the inner side of the membrane (6 h), were pulse-infected with L. major amastigotes (ratio 4–10:1) for 12 h. Thereafter, the inserts were transferred to new wells containing culture medium with or without stimuli (see also Fig. 3 D).

Determiniation of Nitrates. As an indirect measurement for the production of NO, culture supernatants were analyzed for their content of nitrite (NO_2⁻) using the Greiss reaction (23). Transient Electrion Microscopy. Incubation of fibroblasts with L. major parasites was stopped by adding an excess amount of cold HTO’s fixative to the cells (26). After overnight fixation at 4°C, the samples were further processed according to established protocols (27). In brief, washes with 0.1 M cacodylate buffer, pH 7.4, were followed by postfixation in ferricyanide-reduced 1% osmium tetroxide, washes with 0.9% saline solution, encapsulation in 2% agar, en bloc staining with an alcoholic mixture of 0.5% phospho-
tungstic acid and 0.25% uranyl acetate, physical dehy-
dration with a graded series of ethanolic solutions ending
with pure acetone, and embedding in Epon 812 resin. U b r a t h i n
sections were placed onto 200-mesh standard square copper
grids, stained with a mixture
of 10% uranyl acetate and 2.8% lead citrate, and viewed with
a Zeiss type 906 transmission electron microscope.

A n t i b o t y  R e a g e n t s .  R a b b i t  a n t i s e r a  a g a i n s t  h u m a n  f i b r o n e c t i n ,
human laminin-1, human collagen VI (recombinant N 9 - N 2
domain), mouse fibulin-2, or mouse perlecan (recombi-
nant III-3 domain) were as described (28-31). The human or rabbit
antisera against L. major, the rabbit antiserum against mouse NOS2
peptide, the rat mAbs against macrophages (M ac-1, F4/80, BM-8,
M O M A - 2 , and ER - M P - 2 3 ) , granulocytes (G R - 1 ) , dendritic cells
(N L D C - 1 4 5 ) , or endothelial cells (M E C A - 3 2 ) were the same as
used previously (17). The rat mAbs M 5 / 1 1 4 . 1 5 . 2 (32) and ER-
TR7 (33) were used for the detection of M H C class II antigens
and mouse reticular fibroblasts, respectively. All rat mAbs as well
as all of the secondary antibodies (affinity-purified biotin-conju-
gated donkey anti-rabbit IgG, mouse anti-rat IgG, or goat anti-
human IgG F(ab')2; fragments affinity-purified Cy5- or lissamine
rhodamine sulfochloride (LRSC)-conjugated donkey anti-rabbit
IgG, Cy5- or dichlorotriazinyl aminofluorescein (DTAF)-conju-
gated donkey anti-rat IgG, or DTAF-conjugated goat anti-human
IgG F(ab')2; fragments) were purchased from Dianova.

I m m u n o m e n o c y t o l o g i c  S t a i n i n g  o f  F r o z e n  T i s s u e  S e c t i o n s  a n d  o f  F i b r o-
b l a s t s and M ac r o p h a g e s .  5-6-μM tissue sections from embedded
lymph nodes were prepared with a cryostat microtome (model
HM 500 OM; Fa. Microm International GmbH), thawed onto
slides coated with Fro-Marker® (Science Services), surrounded
with PAP PEN® (Science Services), air-dried, fixed in acetone
(for 10 min, at −20°C), and briefly washed in PBS/0.05% T w e n
20. Monolayers of macrophages or fibroblasts on Permanox®
chamber slides (N alge M unic International) were washed with
PBS and fixed in acetone without prior air-drying. Nonspecific
binding sites were blocked for 30 min with PBS/0.1% saponin/
1% BSA/20% FCS. Immunoperoxidase staining (with 3-amin-9-
ethyl-carbazole as a substrate), alkaline phosphatase immunoen-
zymatic labeling (with Fast Blue BB salt as a substrate), and hema-
toxylin counterstaining were performed as described previously
(17, 23).

I m m u n o f l u o r e s c e n c e  a n d  L a s e r  S a m m i n g  o n  C o n f o c a l  M i c r o s c o p e .  F o r
double and triple immunofluorescence, acetone-fixed fibroblasts
or macrophage monolayers or cryostat sections were blocked (see
above) and simultaneously incubated (45 min, room temperature)
with two or three different primary antibodies diluted in PBS/
0.1% BSA/0.1% saponin. After washing with PBS/0.05% T w e n
20, the fluorochromes (Cy5, DTAF, or LRSC)-conjugated sec-
ondary antibody reagents (diluted in PBS/0.1% BSA/0.1% sapo-
nin) were added sequentially for 30 min each, followed by exten-
sive washing steps in between. In cases where two of the primary
antibodies were derived from the same species, the complexes of
the first and secondary antibody were formed and free binding sites
of the secondary antibody were saturated with the respective non-
immune serum (10%, 30 min on ice) before adding the com-
plexes to the sections. The slides were finally mounted with
Mowiol® (Hoechst) containing 1,4-diazabicyclo-2,2,2-octane
(DBACO; Sigma-Aldrich) as an antifading reagent. DTAF
(green) was excited at 488 nm and collected using a 515-545-nm
band pass filter. LR SC (red) was excited at 574 nm and collected
with a 590-nm long pass filter. Cy5 (red) was excited at 651 nm
and collected with a 665-nm long pass filter. Nuclei were visual-
ized with the DNA stain TOTO-3 (red; Molecular Probes),
which was excited at 642 nm and collected with a 665-nm long
pass filter. The slides were examined with a Leica laser confocal
microscope equipped with an argon/krypton laser (laser lines of
488, 568, and 647 nm) using the Leica TCS NT software
(v1.6.551). For the three-color presentation of the images, the far
red emission of Cy5 or of T O T O - 3 was turned into blue.

R e s u l t s

I so l a t i o n  a n d  P h e n o t y p e  C h a r a c t e r i z a t i o n  o f  F i b r o b l a s t s .  F i b r o b l a s t s
were isolated from the skin of naive mice and from the draining
(popliteal) lymph nodes of mice that had healed a cutaneous infection with L. major (>day 200 after infection). Three skin fibroblast lines (CHF-1, CHF-2, and
N O S S - 1 ) and two lymph node reticular fibroblast lines
(N O B O - 1 and N O B O - 3 ) were established and used for in vitro experiments at passage numbers <25. The cells were
determined to be fibroblasts on the following grounds: (a)
characteristic morphology, i.e., formation of monolayers
consisting of cells that assumed a pavement-like and spindle-
shaped appearance when confluent (see Fig. 2); (b) se-
rum-dependent growth (data not shown); (c) absence of
markers characteristic for macrophages (F4/80, M ac-1,
BM-8, ER-MP-23), dendritic cells (N L D C - 1 4 5 , M H C
class II), granulocytes (G R - 1 ) , T cells (T h y 1 . 2 ) , B cells
(B220), or endothelial cells (M E C A - 3 2 ) (data not shown);
v) production of a variety of known matrix proteins (fi-
bronectin, laminin-1, fibulin-2, perlecan, and collagen VI)
during in vitro culture for 1-6 d (Fig. 1, A and B, and data
not shown); (e) positive staining with the rat mAb ER-TR7
(Fig. 1 C, and data not shown), which detects an intracellu-
lar component of mouse reticular fibroblasts but also reacts
with an extracellular connective tissue product that is dif-
ferent from the matrix proteins laminin, fibronectin, types
I-V collagen, heparan sulfate proteoglycan, entactin, and
nidogen (33). R P M s or P E M s, in contrast, were negative
for perlecan, fibulin-2, collagen VI, and ER-TR7 through-
out a culture period of 6 d (Fig. 1, E-M) and showed only
a very weak and transient staining with antilaminin or anti-
fibronectin antiserum (not shown). T u n g s t i c  a n d  h e m a-
toxylin counterstaining were performed as described previously
(17, 23).

R eticular F i b r o b l a s t s  o f  L y m p h  N o d e s  o r  S k i n  F i b r o b l a s t s  P h a g o c y t o-
se L. major Parasites. W h e n skin fibroblast monolay-
ergs were continuously incubated with L. major promastigotes
at a parasite/cell ratio of 3-5:1, the average infection
rate at 48 h was 17.8 (± 9) and 24.7 (± 12.4%) in unstim-
ulated or cytokine-stimulated cells, respectively (mean
± SD of three experiments; Fig. 1, A and B). Comparable re-
sults were obtained with lymph node fibroblasts and when
L. major amastigotes were used for infection (Fig. 1, and
data not shown). Most of the infected cells contained one
or two parasites; rarely, three parasites were found within
one cell. Phagocytosis and intracellular localization of pro-
amastigote L. major was confirmed by transmission elec-
tron microscopy (Fig. 2, D and E). T h e s e  r e s u l t s  d e m o-
strate that both dermal and reticular fibroblasts are capable of
taking up L. major pro- and amastigotes.

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Figure 1. Expression of fibroblast markers by NOBO-1 cells (reticular fibroblasts isolated from the lymph node of a C57BL/6 mouse at day 239 of infection with L. major; A–D), RPMs (E–H), or thioglycollate-elicited macrophages (I–M). The cells were kept in cultures for 4–6 d and then analyzed for the expression of fibulin-2 (A, E, and J), perlecan (B, F, and K), or ER-TR7 (C, G, and L) by immunoperoxidase staining and hematoxylin counterstaining. Results similar to those shown for NOBO-1 cells were also obtained with primary skin fibroblasts of C57BL/6 mice (not shown). For control purposes, the primary antibodies were omitted and the cells were incubated with the biotinylated secondary reagent alone followed by streptavidin-peroxidase (D, H, and M). Original magnifications: ×1,000.

Figure 2. Uptake of L. major pro- or amastigotes by skin fibroblasts (NOSS-1, CHF-1) or reticular lymph node fibroblasts (NOBO-1) of C57BL/6 mice. (A–C) Immunoperoxidase staining of intracellular parasites, original magnifications ×1,000: (A) NOSS-1 fibroblasts, 24 h after infection with amastigotes; (B) CHF-1 cells, 48 h after infection with amastigotes; (C) NOBO-1 cells, 24 h after infection with promastigotes. (D and E) Detection of intracellular L. major by transmission electron microscopy: (D) NOBO-1 cells, 20 h after infection with amastigotes. Bar, 2.1 μM. (E) NOBO-1 cells, 24 h after infection with promastigotes. Bar, 2.1 μM.
Fibroblasts Have a Reduced Capacity to Produce NO and to Kill Leishmania Parasites. As NOS2-derived NO has been shown to be indispensable for the control of Leishmania in phagocytes and in mice (8, 9, 34, 35), we analyzed the production of NO by fibroblasts in response to cytokines or microbial products. IFN-γ alone or IFN-γ plus TNF were sufficient to activate resting or inflammatory macrophages for high production of NO. In contrast, skin or lymph node fibroblasts required a microbial costimulus such as LPS (Fig. 3 A) or infection with *L. major* pro- or amastigotes (Fig. 3 B; see also Fig. 3 D). However, whereas >90% of infected macrophages stimulated with IFN-γ/LPS were positive for NOS2 protein by immunofluorescence analysis, the expression of NOS2 in *L. major*-infected and IFN-γ/LPS-stimulated CHF-1 or NOBO-1 fibroblasts was restricted to 33 (±13) or 55 (±14)% of the cells, respectively (mean ± SD of five culture wells from two experiments). Furthermore, in the same experiments, only 34 (±14) or 62 (±9)% of the intracellular parasites were localized in NOS2-positive fibroblasts.

In accordance with previous reports (22, 34), stimulation of amastigote-infected macrophages with IFN-γ or IFN-γ/TNF for 48-72 h caused a reduction of the total number of intracellular parasites by 20- to several hundredfold. In contrast, the average reduction of intracellular amastigotes was 1.2 (±0.2) or 4.0 (±1.8)-fold in reticular fibroblasts (NOBO-1) and 2.6 (±1.1) or 5.65 (±2.2)-fold in dermal fibroblasts (CHF-1) after stimulation with IFN-γ or IFN-γ/TNF, respectively (mean ± SEM of three to seven experiments; Fig. 3 C). However, coculture of infected fibroblasts with uninfected macrophages that were separated by a membrane significantly increased the killing of the intracellular amastigotes compared with cultures of fibroblasts alone (Fig. 3 D).

Reticular Fibroblasts as Host Cells for Persisting Leishmania In Vivo. Next, we addressed the question whether fibroblasts represent (part of) the hitherto undefined NOS2-negative host cell population that harbors 60-70% of all parasites persisting in the lymph nodes of clinically cured mice (17). Initial experiments using immunoperoxidase/al-

![Figure 3](https://example.com/figure3.png)

**Figure 3.** NO production and killing of intracellular *L. major* by RPMs, PEMs, skin fibroblasts (CHF-1), and reticular lymph node fibroblasts (NOBO-1). (A) Nitrite accumulation at 24 or 48 h of stimulation with IFN-γ, IFN-γ (20 ng/ml) plus TNF (10 ng/ml), or IFN-γ (20 ng/ml) plus LPS (20 ng/ml) by equal numbers of uninfected macrophages (RPMs, black bars; PEMs, gray bars) or fibroblasts (CHF-1, hatched bars; NOBO-1, white bars) (mean ± SEM of three to six experiments). (B) Nitrite accumulation in CHF-1 (hatched bars) and NOBO-1 (white bars) fibroblast cultures infected with *L. major* parasites and stimulated with IFN-γ or IFN-γ plus TNF (20 ng/ml each) (mean ± SD of 7-16 experiments). (C) Killing of intracellular *Leishmania* by PEMs and CHF-1 infected with a fourfold excess of amastigotes (6-h pulse infection) and cultured with or without IFN-γ (20 ng/ml) for 54 h. The total numbers of cell-associated parasites were determined by limiting dilution analysis (one of four experiments). (D) Killing of L. major amastigotes by NOBO-1 fibroblasts cultured in the absence or presence of PEMs and stimulated with IFN-γ/LPS (20 ng/ml each, 0 or 72 h), for 72 h as determined by [3H]thymidine incorporation into Leishmania recovered from host cells after SDS lysis (one of two experiments).
Kaline phosphatase double labeling revealed parasites closely associated with the reticular fibroblast marker ER-TR7 (Fig. 4A) or various matrix proteins (not shown). Subsequently, >200 sections of 5 lymph nodes derived from 5 independent time course experiments (day 145–530 after infection) were analyzed by confocal laser microscopy. In a first series, 332 L. major amastigotes were analyzed for their colocalization with NOS2 and extracellular matrix proteins (fibulin-2, perlecan, laminin, or collagen VI). 256 (77%) of all parasites were NOS2-negative, confirming our previous findings (17). 102 of the NOS2-negative Leishmania (i.e., 30% of the total parasites) were tightly embedded in or closely surrounded by matrix. Only 8% of the total parasites colocalized with matrix and NOS2. Similar results were obtained in a second series of sections, in which 824 L. major amastigotes were assessed for their association with the ER-TR7 marker, NOS2, and host cell nuclei. 549 (66%) of all parasites were found within NOS2-negative areas, of which 358 (i.e., 43% of all parasites and 65% of the NOS2-negative parasites) colocalized with ER-TR7 (Fig. 4C). Only 20% of all parasites colocalized with ER-TR7 and NOS2 (Fig. 4D). At least 50% of the ER-TR7-positive amastigotes were located in the close vicinity of host cell nuclei, which further demonstrates that fibroblasts harbor Leishmania in vivo (Fig. 4B). By similar triple immunofluorescence labeling, we found parasites not only within macrophages (Fig. 4E) and dendritic cells (not shown), but also in areas devoid of host cell nuclei and fibroblasts (Fig. 4F). Thus, fibroblasts are host cells for persisting L. major along with other cell types, but Leishmania can also be detected in necrotic areas of the chronically infected lymph nodes.
Discussion

Fibroblasts as Host Cells for Leishmania. Although macrophages are considered to be the most important host cell for Leishmania parasites, several other cell types have been shown to endocytose Leishmania in vitro or in vivo. These include neutrophils (36–38), eosinophils (39–41), dendritic cells (42, 43), and epithelial cells (44). Throughout the 20th century, a possible infection of fibroblasts or fibroblast-like cells (42, 43), and epithelial cells (44). Throughout the 20th century, a possible infection of fibroblasts or fibroblast-like cells (42, 43), and epithelial cells (44). In vitro, several authors demonstrated the uptake of pro- or amastigotes of L. donovani, L. mexicana amazónica, and an L. braziliensis-like species by human skin fibroblasts (18–20). However, none of the above-mentioned studies provided any evidence that the infection of fibroblasts with Leishmania might be relevant for the survival of the parasites in vivo; investigated the expression of NOS2 by fibroblasts and analyzed the antileishmanial activity of fibroblasts after cytokine stimulation; or determined the actual frequency of Leishmania residing in professional phagocytes versus fibroblasts in vivo. Our present study shows that the majority of the parasites that are found in NOS2-negative areas of latently infected lymph nodes and do not colocalize with known macrophage and dendritic cell markers (~60–70% of the total parasites[17]) are tightly associated with reticular fibroblasts. Thus, fibroblasts represent a hitherto unrecognized important host cell for Leishmania persisting in vivo.

Fibroblasts as Safe Targets for Leishmania. Previous reports on the interaction of unstimulated human skin fibroblasts with Leishmania species other than L. major provided evidence that the parasites can persist within these cells in vitro for prolonged periods of time (3−7 d), although replication of the parasites did not occur (18−20). Our present analysis shows that cytokine-stimulated fibroblasts have a limited capacity to kill intracellular L. major parasites in vitro. This is not due to a principal inability to produce NO. However, adherent fibroblasts stimulated with IFN-γ (with or without TNF-α) released much less NO than macrophage monolayers, and even after stimulation with IFN-γ plus LPS the expression of NOS2 protein was restricted to 30−50% of the fibroblasts (depending on the cell line). Similar observations were previously made with embryonic fibroblasts (50). After infection, production of NO was enhanced but still considerably lower than in macrophages. Furthermore, only 35−60% of the parasites were localized in NOS2-positive fibroblasts. Both factors might support the survival of L. major parasites within fibroblasts. Importantly, parasites residing in fibroblasts remained susceptible to the NO produced by neighboring macrophages (Fig. 3 D). Thus, macrophage-derived NO is able to control the Leishmania in nearby NOS2-negative fibroblasts, which might help to maintain a stable balance of parasite killing and evasion in the chronically infected lymph nodes.

We considered the possibility that the localization of Leishmania in fibroblasts might also protect the parasites against the activity of standard antileishmanial drugs and thereby account for the persistence of Leishmania after chemotherapy. When we treated long-term-infected, clinically cured C57BL/6 mice with a combination of pentostam and amphotericin B (Ambisome®), there was a strong (~900-fold) reduction of the parasite burden. However, we did not observe an increase in the number of parasites associated with fibroblast markers. Thus, to date we do not have evidence for a preferential survival of L. major parasites after chemotherapy in fibroblasts.

Extracellular Matrix and Leishmania. Although our confocal laser microscopy analysis strongly suggests that L. major parasites are located within fibroblasts in vivo (Fig. 4 B), we cannot exclude that some of the persisting Leishmania amastigotes are located extracellularly tightly embedded by matrix (Fig. 4, A and C, and data not shown). In addition, in the chronically infected lymph nodes, Leishmania amastigotes were also seen in necrotic areas lacking nucleated cells (Fig. 4 F). In this context, it is important to note that L. mexicana amastigotes express surface molecules that were shown to function as ligands for certain (extracellular) proteoglycans (51). Extracellular amastigotes, surrounded by connective matrix, have also been seen in acute leishmanial skin lesions of humans infected with L. braziliensis guyanensis, but the finding was not discussed (52). Whether a possible extracellular localization of the parasite in a connective tissue matrix contributes to the control (i.e., killing) of the parasite or to its long-term survival in the host is unknown to date.

In conclusion, we have demonstrated that ingestion of Leishmania by fibroblasts is a frequent event during latent disease. Our data suggest that fibroblasts might form a less hostile environment for L. major than macrophages and thereby allow for the persistence of the parasites. Parasite survival and replication, however, are subject to control by neighboring macrophages that are effective against Leishmania residing in fibroblasts and thereby help to maintain a stable host–parasite relationship. Whether such a mechanism also operates in latently infected humans remains to be established.

We are grateful to Dr. Rupert Timpl (Max-Planck-Institut für Biochemie, Martinsried, Germany) for the generous supply of antibodies against matrix components, and to Elke Lorenz for excellent technical assistance.

This study was supported by the Deutsche Forschungsgemeinschaft (SFB263, project A5).

Submitted: 31 March 2000
Acepted: 14 April 2000

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