Parasitism of Epidermal Langerhans Cells in Experimental Cutaneous Leishmaniasis with *Leishmania major*

Christine Blank, Harald Fuchs, Klemens Rappersberger, Martin Röllinghoff, and Heidrun Moll

Institute of Clinical Microbiology, University of Erlangen–Nuremberg, Germany; Department of Dermatology, University of Vienna Medical School, Austria

Murine epidermal Langerhans cells (LC) have been demonstrated to stimulate a vigorous T cell response to *Leishmania major,* a cause of human cutaneous leishmaniasis. It was therefore of interest to analyze whether LC can take up viable parasites. Epidermal cells were obtained from mouse ear skin for incubation with *L. major* and subsequent detection of intracellular parasites by cytochemistry. Freshly isolated LC, but not cultured LC, phagocytosed *L. major* and the uptake was inhibited by antibodies to the complement receptor type 3. Electron microscopic studies revealed the presence of viable amastigotes within LC. Moreover, with double-labeling techniques, *L. major*–containing LC could also be detected in infected skin. The results demonstrate that LC can internalize *L. major.* Since the number of organisms per infected LC remained consistently low, the prime task of LC may not be the promotion of parasite spreading but the presentation of *L. major* antigen to T cells and, thus, the regulation of the cellular immunity during cutaneous leishmaniasis.

Human leishmaniasis comprises a group of infections of the skin, viscera, and mucous membranes caused by protozoa of the genus *Leishmania.* Each form of the disease is initiated by introduction of promastigotes into the skin during a blood meal of an infected phlebotomine sandfly. The promastigotes invade mononuclear phagocytes of the host where they transform into amastigotes and replicate. Depending on the genetic background of the host and on the species of parasite, the infection may be restricted to the primary cutaneous lesion, it may involve metastasis of organisms to mucosal sites, or it may localize to the viscera and result in widespread parasitization of cells of the reticuloendothelial system [1].

Invasion of host cells is mediated by attachment of parasites to a variety of cell-surface receptors, including the receptor for complement component C3bi, CR3 [2, 3], and the mannose-fucose receptor [4] on macrophages. This receptor-ligand interaction is followed by endocytosis of the organisms. In addition to dermal macrophages, however, epidermal Langerhans cells (LC) qualify as host cells for *Leishmania* amastigotes. LC have been reported to express CR3 [5, 6], and their microanatomic location favors the encounter with parasites soon after inoculation of the latter into the skin. It is therefore of interest to analyze their capac-

ity to take up *Leishmania* parasites. This is an intriguing possibility because LC express high levels of major histocompatibility complex (MHC) class II molecules and are potent antigen-presenting cells [6–8]. Thus, they may be involved in induction and regulation of the T cell immune response in lesional skin. In the present study, we used *Leishmania major* parasites, a cause of human cutaneous leishmaniasis, to analyze their interaction with murine epidermal cells.

Materials and Methods

Mice. Female inbred BALB/c mice were 6–10 weeks old at the onset of experiments. All mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany) and, during experimentation, were maintained under conventional conditions in an isolation facility.

Parasites and infection of mice. The origin and propagation of the *L. major* isolate have been described elsewhere [9]. The cloned virulent line used for this study was confirmed to be *L. major* by isoenzyme analysis (D. Evans, London School of Hygiene and Tropical Medicine) and was maintained by passage in BALB/c mice. Promastigotes were grown in vitro in blood agar cultures. Stationary-phase promastigotes were washed in PBS; for infection of mice, 2×10^7 organisms were injected in a volume of 50 μL intradermally on the dorsum.

Amastigote suspensions were prepared from skin lesions 2–3 weeks later as described [10]. Briefly, infected tissue was disrupted and homogenized by passage through a stainless steel mesh into culture medium. Amastigotes were released from host cells by two vigorous passages of the cell suspension through a 27-gauge needle, and cell debris was removed by two steps of centrifugation at 120 g (10 min) before recovery of amastigotes by centrifugation at 1800 g (10 min) and collection of the pellet.

Culture medium. Click's RPMI 1640 medium (GIBCO Laboratories, Eggenstein, Germany) was supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES

buffer, 100 µg/mL penicillin, 160 µg/mL gentamycin (all purchased from Seromed-Biochrom, Berlin), 7.5% NaHCO₃, and 5 × 10⁻⁵ *M* 2-mercaptoethanol.

Antibodies. Rat monoclonal antibodies (MAbs) directed against MHC class II antigens I-A (b, d, q haplotypes) and I-E (d, k haplotypes) from hybridoma M5/114.15.2 [11], against nonlymphoid dendritic cells from hybridoma NLDC-145 [12], and against Mac-1 (CR3) from hybridoma M1/70 [13] were used as culture supernatant. Mouse MAb WIC79.3 [14, 15], recognizing a polymeric epitope specific for the surface lipophosphoglycan of *L. major*, and rat MAb F4/80 [16], directed against mouse macrophages, were used as purified protein. Polyclonal antibodies to *L. major* were raised in rabbits by subcutaneous and intramuscular injections of promastigotes in complete Freund's adjuvant, followed by several boosters of intact promastigotes in PBS and collection of the serum. A fluorescein isothiocyanate (FITC)–conjugated goat anti-rat immunoglobulin (Medac, Hamburg, Germany) served as second-stage reagent for fluorescence staining. Biotin-conjugated mouse anti-rat immunoglobulin or donkey anti-rabbit immunoglobulin (both Dianova, Hamburg, Germany) and gold-conjugated goat anti-rabbit immunoglobulin (Dako, Hamburg, Germany) were used as second-stage reagents for immunocytochemical staining.

Preparation of macrophages and epidermal cells. For the preparation of peritoneal macrophages, thioglycolate-elicited peritoneal exudate cells were washed, resuspended in culture medium, and allowed to adhere for 4 h at 37°C, 5% CO₂, and 95% air humidity. Nonadherent cells were removed by extensive washing with culture medium. Single-cell suspensions of epidermal cells were prepared from mouse ear skin by trypsinization procedures as described [17]. A concentration of 1% trypsin (40 min) was used for processing the ventral, thick ear halves, and 0.6% trypsin (20 min) for the dorsal, thin ear halves. These preparations contained 3%–5% MHC class II–bearing LC and were absolutely devoid of macrophages [17], as documented by the lack of staining with MAb F4/80 after 24 h of culture.

Infection of cell suspensions. Peritoneal macrophages or epidermal cells (3 × 10⁶) were incubated with *L. major* amastigotes at a ratio of 2 parasites per cell in 2 mL. The cultures were harvested after 1–7 days. For depletion of extracellular parasites, the cultures of parasites and adherent macrophages were washed before collection of the cells, and the suspensions of epidermal cells and parasites were incubated with WIC79.3 MAb for 45 min on ice, followed by removal of MAb-coated parasites with goat anti-mouse IgG coupled to magnetic beads (Dynabeads M-450; Dynal, Hamburg, Germany) using a magnet. Interaction of LC with *L. major* was examined in the absence or presence of anti–Mac-1 MAb (0.2 mg/mL) or mannan (1 mg/mL; Sigma, Deisenhofen, Germany). The presence of intracellular amastigotes was assessed by staining with acridine orange and ethidium bromide or by a combination of immunocytochemical staining and FITC labeling using specific antibodies (see below).

Staining with acridine orange and ethidium bromide. Viable intracellular parasites were detected by a modification of the method of Channon et al. [18]. Macrophages or epidermal cells were incubated with a mixture of acridine orange (5 µg/mL) and ethidium bromide (50 µg/mL) for 10 min at room temperature

(RT). After being washed with PBS, the cells were fixed with paraformaldehyde (1%, 15 min) and were analyzed by fluorescence microscopy.

Immunocytochemical and immunofluorescence staining. Epidermal cells at a concentration of 10⁶/mL were mounted onto adhesion slides (Bio-Rad, Munich) according to the manufacturer's instructions and air-dried. Nonspecific binding was blocked by incubating the slides in a solution containing 5% skim milk powder and 0.1% Tween 20 in PBS, pH 7.4 (Blotto), supplemented with 20% FCS.

For immunoenzymatic staining, a three-step avidin-biotin-peroxidase method was used as previously described [19]. Briefly, the cells were overlaid with first-step antibodies (rat anti-CR3 MAb or polyclonal rabbit anti–*L. major* antibodies) in PBS containing 5% bovine serum albumin (10–14 h at 4°C), followed by biotin-conjugated anti-rat or anti-rabbit immunoglobulin (1 h, RT). The slides were rinsed between each incubation step with Blotto and, in the final washing step, with PBS containing 0.1% Tween 20. Subsequently, the cells were incubated with preformed streptavidin-biotin-peroxidase complex (Dako). After cells were thoroughly washed with PBS/Tween 20, labeling was visualized with the substrate 3-amino-9-ethylcarbazole (AEC; Sigma). The AEC solution contained 0.2 mg/mL AEC previously dissolved in *N,N*-dimethylformamide (final concentration, 5%) and 0.015% H₂O₂ in acetate buffer (50 m*M*, pH 5).

For double labeling, the treatment was extended by incubating the slides in Blotto containing 20% rabbit serum, to block free binding sites of the second-stage antibodies of the immunoenzymatic staining, and by consecutively exposing the cells to rat anti-Ia and FITC-conjugated anti-rat antibodies. For control purposes, the first-step MAbs were either omitted or replaced by an irrelevant isotype-matched reagent. These stainings consistently yielded negative results.

Double labeling of skin sections by a combination of gold-silver immunostaining and immunoenzymatic labeling. For immunohistologic analysis of *L. major*–infected skin, tissue samples were snap-frozen in optimal cutting temperature (OCT) compound (Miles, Naperville, IL). Cryostat sections (4 µm) were thawed onto HCl-rinsed slides, air-dried, and fixed in acetone (−20°C, 10 min). OCT compound was washed from slides with Blotto, and nonspecific binding sites were blocked by incubation in Blotto containing 20% FCS (30 min, RT). Tissue sections were labeled as previously described [19]. The following sequence of treatments was used: rabbit anti–*L. major* antibodies (overnight, 4°C); gold-conjugated anti-rabbit immunoglobulin (1 h, RT); blocking with Blotto containing 20% rabbit serum (30 min, RT); MAb NLDC-145 for the second labeling (2 h, RT); biotinylated mouse anti-rat immunoglobulin (1 h, RT); streptavidin–biotin–alkaline phosphatase complex (1 h, RT); Fast Blue solution containing 0.2 mg/mL naphtol AS-MX phosphate (Sigma), 1 m*M* levamisole, and 1 mg/mL Fast Blue BB salt (Serva, Heidelberg, Germany) in TRIS buffer (0.1 *M*, pH 8.2) (5–20 min); gold conjugate–silver enhancement mixture (Amersham, Braunschweig, Germany; 9–14 min).

Counterstaining of sections and microscopic analysis. The skin sections were counterstained with hematoxylin or nuclear fast red and mounted (Aquatex; Merck, Darmstadt, Germany).
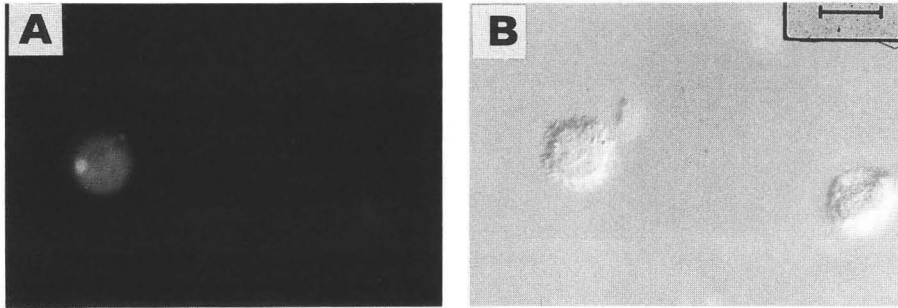
Figure 1. Detection of intracellular amastigotes by staining with acridine orange and ethidium bromide. **A,** Fluorescence micrograph: Epidermal cell contains amastigote. **B,** Same area viewed by interference-contrast microscopy: *L. major*–infected cell (left) displays slender dendrites typical for Langerhans cells. Bar = 10 μm.

For evaluation, a microscope equipped for polarized incident light (epipolarization) and interference-contrast microscopy was used (Axiophot; Zeiss, Oberkochen, Germany).

Electron microscopy. Epidermal cells were fixed in 2% glutaraldehyde for 1 h (RT), washed twice in PBS and then in 0.1 M cacodylate buffer, and postfixed in a 4% aqueous solution of osmium tetroxide (1 h, RT). Then the cells were dehydrated in a graded series of ethanols and embedded in Epon 812. Thin sections of the specimen were stained with uranyl acetate and lead citrate for examination by transmission electron microscopy.

Results

Epidermal LC can be infected by L. major. Freshly isolated epidermal cells were incubated with *L. major* amastigotes for 24 h. Then extracellular parasites were removed, and the presence of viable intracellular parasites was assessed by staining the cell suspension with acridine orange and ethidium bromide. It was evident that some epidermal cells were infected with *L. major* amastigotes, and their dendritic shape, as visualized by interference-contrast microscopy, suggested that they were LC (figure 1). Peritoneal macrophages treated in a comparable manner were used as positive control (data not shown). It should be noted that epidermal cells, in contrast to macrophages, contained intracellular organisms only after incubation with amastigotes but not with promastigotes of *L. major* (data not shown).

In the normal epidermis, LC are the only cells expressing MHC class II (Ia) antigen. Therefore, we used this marker for unambiguous identification of the parasite-containing epidermal cells. Double labeling showed that Ia$^+$ epidermal cells detected by fluorescence staining, but not Ia$^-$ cells, contained *L. major* antigen as visualized by immunoenzymatic labeling (figure 2). This finding demonstrated that epidermal LC can be parasitized by *L. major*.

To exclude the possibility that the parasites had not been internalized by LC but were only attached to their surface membrane, we did ultrastructural studies. Epidermal cells containing intact *L. major* amastigotes were readily detectable and could be identified as LC because they displayed the typical Birbeck granules (figure 3A, B). The amastigotes were secluded within cytoplasmic vacuoles that were surrounded by a unit membrane and thus morphologically

represented phagosomes. Occasionally 2 amastigotes were found within 1 phagosome, indicating mitosis of intracellular *L. major* or, alternatively, concurrent uptake of several parasites. However, the suggestion that amastigotes may replicate within LC was further supported by the demonstration of duplicated kinetoplasts (figure 3C) and flagella (figure 3D). Duplication of these organelles is known to represent the initial event of the parasite's mitosis.

Only freshly isolated LC were able to internalize *L. major* organisms. When LC were cultured for 9 h before addition of parasites, the rate of infection was reduced by >90%. After overnight preincubation of LC (12–16 h), no parasite uptake could be detected (data not shown).

Rate of infection and parasite load of epidermal LC. By using acridine orange and ethidium bromide staining of epidermal cells treated with *L. major*, it was possible to quantitate the proportion of infected LC and the number of parasites per cell. Considering that only freshly isolated LC could take up parasites, it was not surprising that the maximal rate of LC infection was already observed after 24 h of culture
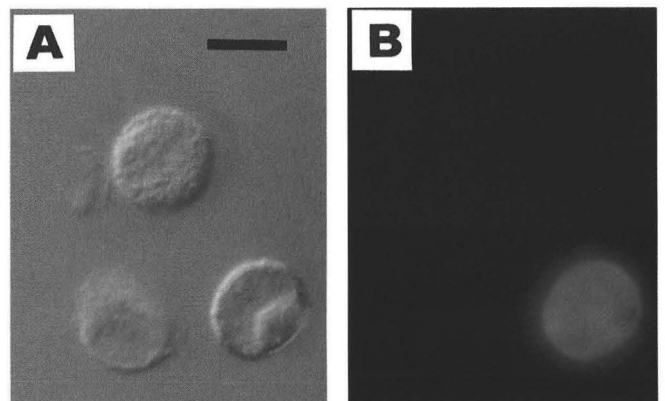
Figure 2. Detection of infected Langerhans cells (LC) by double labeling of epidermal cells with antiserum to *L. major* and anti–major histocompatibility complex (MHC) class II (Ia) monoclonal antibody. Epidermal cells had been incubated with *L. major* amastigotes for 24 h. **A,** Immunoenzymatic labeling: One epidermal cell contains *L. major* antigen (interference-contrast microscopy, peroxidase with substrate aminoethylcarbazole). **B,** *L. major*–infected cell can be identified as LC, as judged by expression of MHC Ia molecules (fluorescence microscopy). Bar = 10 μm.
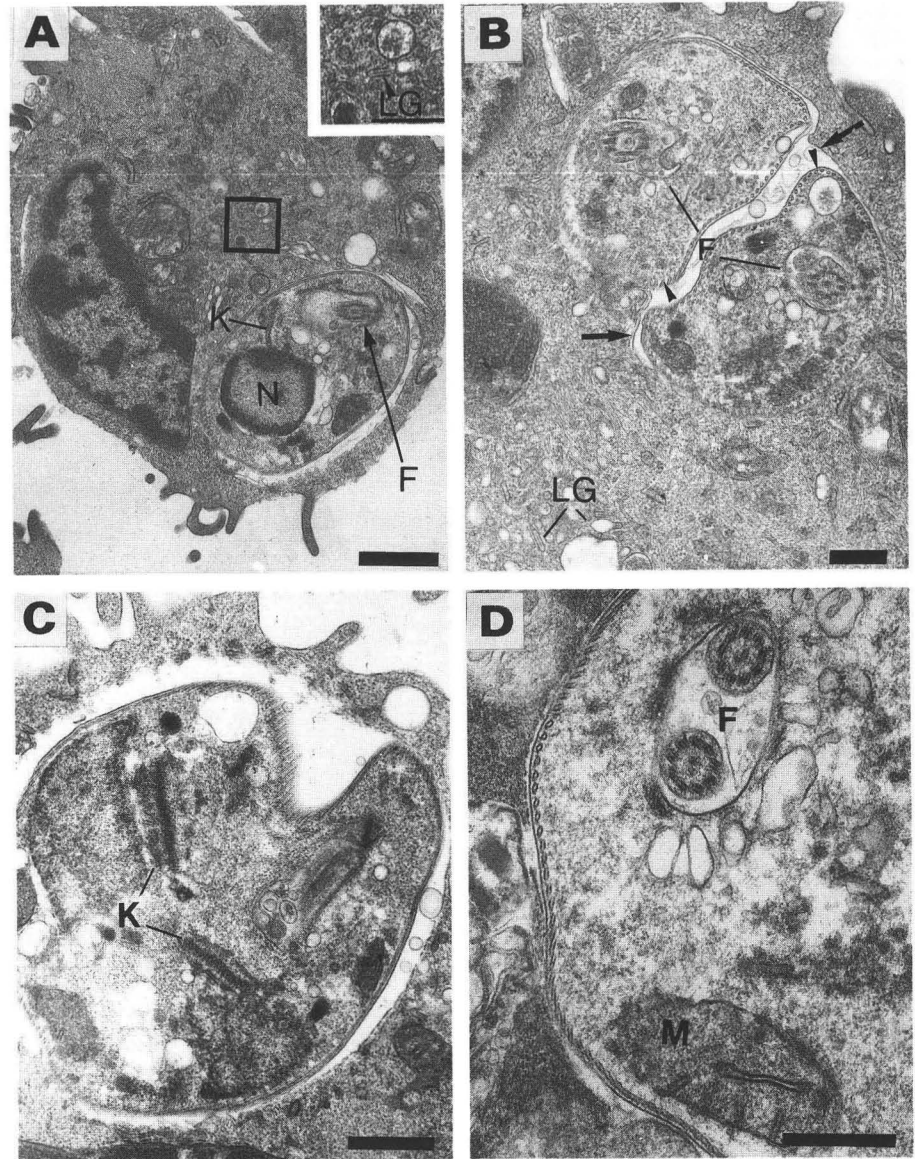
Figure 3. Electron microscopic documentation of *L. major*–infected Langerhans cells (LC). Epidermal cells had been incubated with *L. major* amastigotes for 24 h. **A**, LC with intracellular amastigote exhibiting its nucleus (N), kinetoplast (K), and flagellum (F, arrow). Bar = 1 μm. Area in center of cell (□), at higher magnification in inset, shows LC granule (LG, arrowhead). Bar = 500 nm. **B**, Enlargement of LC, as identified by its granules (LG), containing 2 amastigotes within 1 phagosome. Note phagosomal membrane (arrows), clearly visible subpellicular microtubules beneath parasites' plasma membrane (arrowheads), and flagella (F). Bar = 500 nm. **C** and **D**, Intracellular amastigotes in mitosis. Demonstration of reduplication of kinetoplast (K) in **C** and of flagellum (F) in **D**. Duplication of these organelles is first morphologic evidence of *L. major* cell division. M = mitochondrium. Bars = 500 nm.

with parasites (table 1); 20.4% ± 4.8% of LC contained 1–3 parasites (mean, 1.43 ± 0.02). Thereafter, the percentage of *L. major*–infected LC gradually decreased (table 1), whereas the total number of LC remained constant (data not shown). Most interestingly, there was no evidence for significant intracellular replication of amastigotes in LC because the number of organisms per infected cell did not increase between days 1 and 6 of culture (table 1). The maximal level of infection could not be increased by adding a higher number of parasites to the epidermal cell cultures (data not shown).

Ingestion of L. major by epidermal LC is mediated by CR3. The macrophage plasma membrane receptor for C3bi, CR3, has been identified as an important site for binding and internalization of *L. major*. Expression of CR3 has also been demonstrated for human LC [5] and LC in preparations of murine epidermal sheets [6], and we confirmed this

for LC in the single epidermal cell suspensions used for the present study (figure 4). Therefore, we examined the role of CR3 for the uptake of *L. major* by LC. After culture of epidermal cells and parasites in the presence of anti-CR3 (anti–Mac-1) MAb, the proportion of infected LC was dramatically reduced, whereas the unrelated MAb NLDC-145 had no effect (figure 5). It is unlikely, therefore, that the inhibition caused by anti–Mac-1 MAb was simply due to steric hindrance. On the other hand, the presence of mannan as an inhibitor of mannose-fucose receptor–mediated binding did not cause a reduction in parasite uptake by LC and did not enhance the inhibition observed with anti-CR3 MAb (figure 5). These data provide strong evidence that the CR3 on LC mediates attachment and internalization of *L. major*.
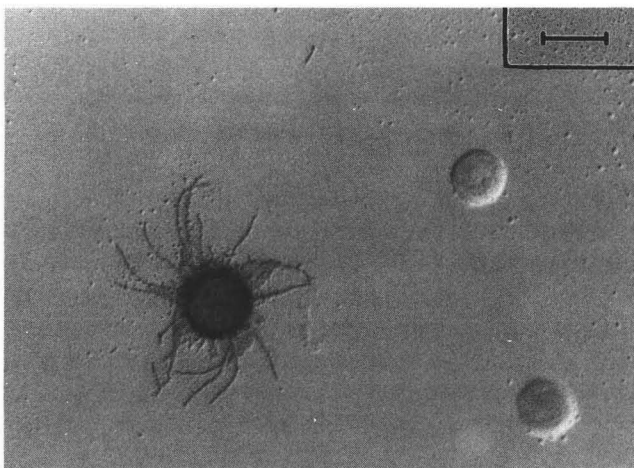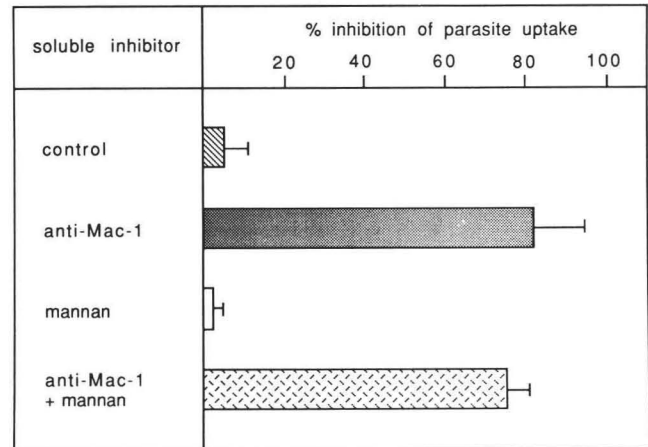
LC expressing L. major antigen can be identified in infected skin. Having demonstrated that epidermal LC can be in-

Table 1. Parasitism of epidermal Langerhans cells (LC) with *L. major* in vitro.

Time after infection (days)	% infected LC	Average no. of organisms/ infected LC
1	20.43 ± 4.82	1.43 ± 0.02
2	14.60 ± 3.53	1.65 ± 0.27
3	9.63 ± 1.61	1.49 ± 0.25
4	6.86 ± 1.39	1.60 ± 0.42
5	4.50 ± 0.50	1.50 ± 0.07
6	1.93 ± 1.15	1.29 ± 0.04
7	0	—

NOTE. Data are mean ± SD of four experiments; at least 1000 epidermal cells (i.e., 100–250 LC) were counted for each experiment and time point.

Figure 5. Effect of inhibitors of CR3 and mannose-fucose receptor on uptake of *L. major* by Langerhans cells. Epidermal cells and *L. major* amastigotes were cultured for 24 h in absence or presence of anti-CR3 (anti–Mac-1) monoclonal antibody (MAb), mannan, or NLDC-145 MAb as a negative control. Numbers of *L. major*–containing cells were determined by fluorescence microscopic analysis after staining with acridine orange and ethidium bromide. Graph shows % inhibition of parasite ingestion from three independent experiments (means + SD).

fected with *L. major* in vitro, we next examined whether parasitized LC are detectable in situ within the lesions of intradermally infected mice. Immunohistologic staining of skin sections with MAb NLDC-145 revealed significant numbers of LC in the epidermis adjacent to the lesion, a distribution pattern resembling that in normal skin (not shown). In the epidermis overlying the parasite-containing dermal infiltrate, however, LC were virtually absent. On the other hand, distinct clusters of NLDC-145+ cells could be detected in this area of the dermis (figure 6A). These cells constituted <1% of the nonlymphoid mononuclear cells in the infiltrate. Double labeling demonstrated that some of the NLDC-145+ LC in the dermis expressed *L. major* antigen (figure 6B, C), and the dense accumulations of parasite antigen strongly suggested the presence of intact organisms. This supposition was confirmed by the finding that intradermal infection of mice with promastigotes and with amastigotes yielded the

same pattern of antigen expression, whereas treatment with lysed parasites resulted in evenly distributed surface staining of LC (data not shown). No LC of the epidermis were ever seen parasitized with *L. major*.

Discussion

In the mammalian skin, two cell populations have been identified that qualify as a sanctuary for *Leishmania* parasites: dermal macrophages and epidermal LC. Whereas the critical role of macrophages has been documented comprehensively (reviewed in [20, 21]), the function of LC in leishmaniasis has remained largely unknown. The present study provides the first direct evidence that LC can be infected with *L. major*, both in vitro and in vivo, and suggests that these cells contribute to the induction and the expression of cell-mediated immunity during cutaneous leishmaniasis.

Although the endocytic potential of LC is far less than that of macrophages, they are capable of phagocytosis [22, 23]. Our observation that LC internalize intact *L. major* amastigotes shows that they can even take up organisms of considerable size (2–5 μm). Endocytosis of *L. major* parasites was inhibited by anti-CR3 MAb, strongly suggesting that the CR3, which is known to be important for the uptake of *Leishmania* organisms by macrophages [2, 3], also mediates binding and ingestion of *L. major* by LC. On the other hand, infection of LC could not be blocked by a soluble inhibitor of the mannose-fucose receptor, another macrophage receptor
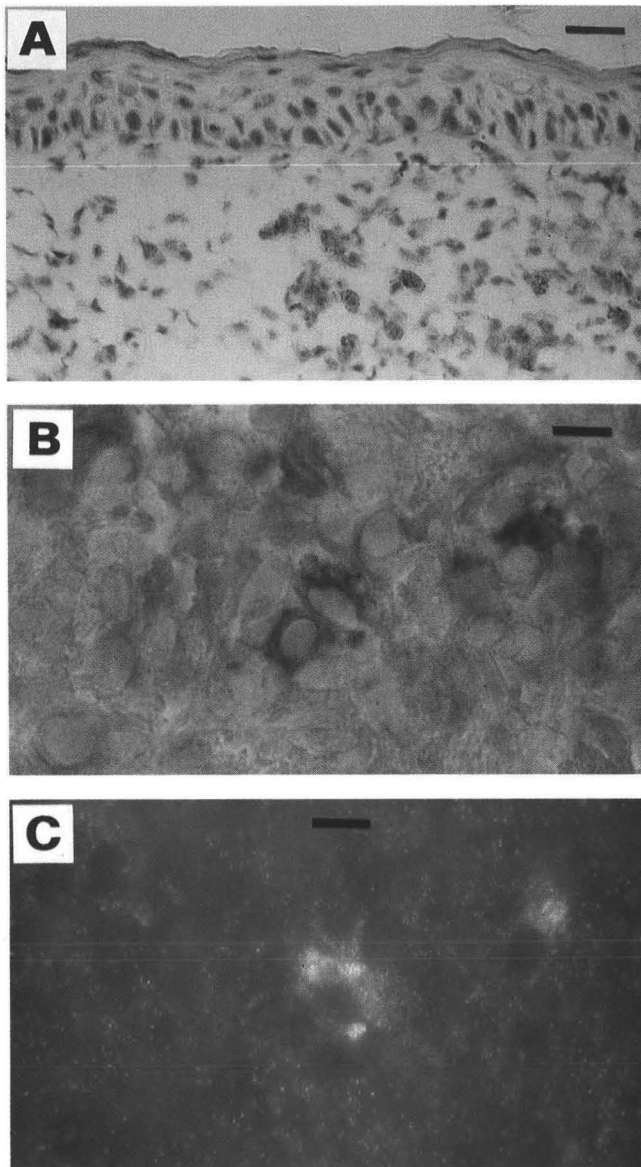

Figure 4. Expression of CR3 by epidermal Langerhans cells (LC). CR3 was visualized by immunoenzymatic labeling with peroxidase and substrate aminoethylcarbazole. Dendritic protrusions identify CR3-positive cell as LC. Bar = 10 μm.

Figure 6. Detection of parasitized Langerhans cells (LC) in ear skin infected with *L. major* promastigotes. **A**, Section of parasite-containing lesion stained for NLDC-145 with peroxidase and substrate aminoethylcarbazole. In infected area, NLDC-145+ LC can be detected only in dermis (hematoxylin counterstaining). **B** and **C**, Double staining for NLDC-145+ cells (immunoenzymatic labeling with alkaline phosphatase and substrate Fast Blue, nuclear fast red counterstaining) and *L. major* (immunogold-silver staining) in dermal infiltrate. Some NLDC+ cells seen under brightfield illumination (**B**) also display *L. major* antigen, which appears light blue under epipolarization illumination (**C**). Bars = 25 μm (**A**); 8 μm (**B**, **C**).

that contributes to phagocytosis of *Leishmania* parasites [2]. This is in line with a report that dendritic cells lack mannose-fucose receptors [24]. Our findings thus indicate that in contrast to the uptake of *Leishmania* organisms by macrophages, which requires the combined action of CR3 and the man-

nose-fucose receptor [2], the ingestion by LC is mediated primarily by CR3.

The maximal level of infection observed in culture was 25% of total LC, and most infected LC had ingested only 1 parasite. This supports the idea that endocytosis of *L. major* by LC is not primarily aimed at antigen scavenging, the characteristic of macrophages, but at processing and presentation of parasite antigen to T cells. Such a concept is in congruence with our recent documentation that epidermal LC are highly active in presenting *L. major* antigen in vitro to parasite-specific T cells [25]. The efficiency of LC in inducing T cell proliferation and lymphokine production was much greater than that of macrophages, suggesting that LC are important antigen-presenting cells in cutaneous leishmaniasis.

The present finding that only freshly isolated LC could be infected with *L. major* corresponds with our previous observation that only fresh LC, not cultured LC, can present *L. major* antigen to T cells. This is reminiscent of earlier reports showing that LC, when freshly explanted from epidermis, can process native antigen for generation of MHC class II–peptide complexes and presentation to primed T cells [8, 26]. After a period in culture, LC lose the ability to present exogenous protein but are the most active antigen-presenting cells for stimuli that do not require processing [8, 27–29]. Recent evidence suggests a causal relationship between the ability to process antigen and the presence of numerous acidic organelles such as endosomes in freshly isolated LC [30]. Furthermore, the synthesis of MHC class II molecules is high in fresh LC but switched off in cultured LC [26, 31, 32].

The phenotypic and functional properties of cultured LC closely resemble those of dendritic cells in lymphoid tissues. It has therefore been suggested that freshly isolated LC reflect the functional repertoire of LC normally found within the epidermis, whereas cultured LC acquire a functional program equivalent to that of LC that have migrated from the skin to the draining lymph node [33, 34]. Such a translocation of LC in vivo provides an efficient means of transporting antigen encountered in the skin to the T cell–dependent areas of lymph nodes [35, 36].

In the course of cutaneous infection with *L. major*, we noticed a significant decrease of the number of LC in the epidermis overlying the lesion. These changes could already be observed at 48 h after infection (unpublished data); at later stages of chronic inflammation, the epidermis became almost devoid of LC. The loss of LC in the epidermal compartment was concomitant with the appearance of NLDC-145+ cells in the dermis, some of which contained *L. major*. These findings indicate that in cutaneous leishmaniasis, LC migrate out of the epidermis to the site of infection in the dermis and that migratory LC in the dermal infiltrate have the capacity to take up parasites. In this context, we also have experimental evidence for further migration of *L. major*–ex-

pressing LC to the draining lymph node and for the ability of those LC to present parasite antigen to T cells (unpublished data).

The ultrastructural studies suggested occasional intracellular mitosis of amastigotes in the early phase after invasion of LC. Over the subsequent days, however, no further reduplication could be seen, and by 1 week of culture, the infection had been resolved. The capacity of cultured LC to restrain parasite replication may be related to the concomitant functional differentiation discussed above.

The interaction of LC with microorganisms has been infrequently investigated. Parasitization of LC has so far been reported only for skin biopsies of patients with leprosy [37] and with granulomatous syphilis [38]. Our findings also extend those of Locksley et al. [39], who reported that isolated LC cannot be infected with the promastigote stage of *L. major*. In the present study, parasitization of isolated LC could be detected only after incubation with amastigotes. However, because it is well known that in vitro cultured promastigotes are usually less infective than amastigotes or promastigotes inoculated by the insect vector [40, 41], this finding does not necessarily reflect the in vivo situation after deposition of promastigotes by a sandfly. Alternatively, the invasion of LC in situ may be launched by amastigotes that had been released from previously infected macrophages in the skin. Although at present we cannot distinguish between the two possibilities, our results demonstrate that parasitization of LC does occur in vivo after cutaneous infection with promastigotes.

In conclusion, our data support the concept that on inoculation of *L. major* parasites into the skin, viable organisms are ingested not only by macrophages but also by LC. Because *L. major*–infected LC constitute a small proportion of parasitized cells in the lesional infiltrate and contain a low number of amastigotes, it seems unlikely that LC account for the massive expansion of parasites usually observed in the infected skin. We propose that the primary function of *L. major*–containing LC, which have been documented to be highly potent accessory cells [25], is the presentation of *L. major* antigen to T cells. They may express this activity after migration to the draining lymph node and initiate the *L. major*–specific T cell response by stimulating quiescent T cells. Moreover, they may be crucial for regulation of the local immune response by presenting parasite antigen to T effector cells infiltrating the cutaneous lesion.

Acknowledgments

References

1. Howard JG. Immunological regulation and control of experimental leishmaniasis. Int Rev Exp Pathol 1986;28:79–116.
2. Blackwell JM, Ezekowitz AB, Roberts MB, Channon JY, Sim RB, Gordon S. Macrophage complement and lectin-like receptors bind *Leishmania* in the absence of serum. J Exp Med 1985;162:324–31.
3. Mosser DM, Edelson PJ. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. J Immunol 1985;135:2785–9.
4. Wilson EM, Pearson RD. Evidence that *Leishmania donovani* utilizes a mannose receptor on human mononuclear phagocytes to establish intracellular parasitism. J Immunol 1986;136:4681–8.
5. Stingl G, Wolff-Schreiner EC, Pichler WJ, Gschnait F, Knapp W, Wolff K. Epidermal Langerhans cells bear Fc and C3 receptors. Nature 1977;268:245–6.
6. Romani N, Stingl G, Tschachler E, et al. The Thy-1 bearing cell of murine epidermis: a leukocyte distinct from Langerhans cells and perhaps related to NK cells. J Exp Med 1985;161:1368–83.
7. Stingl G, Katz SI, Clement L, Green I, Shevach EM. Immunologic function of Ia-bearing epidermal Langerhans cells. J Immunol 1978;121:2005–13.
8. Romani N, Koide S, Crowley M, et al. Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. J Exp Med 1989;169:1169–78.
9. Solbach W, Forberg K, Kammerer E, Bogdan C, Röllinghoff M. Suppressive effect of cyclosporin A on the development of *Leishmania tropica*–induced lesions in genetically susceptible BALB/c mice. J Immunol 1986;137:702–7.
10. Bogdan C, Moll H, Solbach W, Röllinghoff M. Tumor necrosis factor-α in combination with interferon-γ but not with interleukin 4 activates murine macrophages for elimination of *Leishmania major* amastigotes. Eur J Immunol 1990;20:1131–5.
11. Battacharya A, Dorf ME, Springer TA. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. J Immunol 1981;127:2488–95.
12. Kraal G, Breel M, Janse M, Bruin G. Langerhans cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. J Exp Med 1986;163:981–97.
13. Springer T, Galfre G, Secher DS, Milstein C. Mac-1: a macrophage differentiation antigen identified by a monoclonal antibody. Eur J Immunol 1979;9:301–6.
14. delbarra A, Howard JG, Snary D. Monoclonal antibodies to *Leishmania tropica major:* specificities and antigen location. Parasitology 1982;85:523–31.
15. Handman E, Greenblatt CL, Goding JW. An amphipathic sulphated glycoconjugate of *Leishmania:* characterization with monoclonal antibodies. EMBO J 1984;3:2301–6.
16. Austyn JM, Gordon S. F4/80: a monoclonal antibody directed specifically against the mouse macrophage. Eur J Immunol 1981;11:805–15.
17. Schuler G, Koch F. Enrichment of epidermal Langerhans cells. In: Schuler G, ed. Epidermal Langerhans cells. Boca Raton, FL: CRC Press, 1990:139–57.
18. Channon JY, Roberts MB, Blackwell JM. A study of the differential respiratory burst activity elicited by promastigotes and amastigotes of *Leishmania donovani* in murine resident peritoneal macrophages. Immunology 1984;53:345–55.
19. Gillitzer R, Berger R, Moll H. A reliable method for simultaneous demonstration of two antigens using a novel combination of immunogold-silver staining and immunoenzymatic labeling. J Histochem Cytochem 1990;38:307–13.
20. Chang KP, Bray RS, ed. Leishmaniasis. New York: Elsevier Science Publishers, 1985.

21. Bogdan C, Röllinghoff M, Solbach W. Evasion strategies of *Leishmania* parasites. Parasitol Today **1990**;6:183–7.

22. Wolff K, Schreiner E. Uptake, intracellular transport and degradation of exogenous protein by Langerhans cells. An electron microscopic cytochemical study using peroxidase as tracer substance. J Invest Dermatol **1970**;54:37–47.

23. Katz DR, Sunshine GH. Comparative accessory cell function of Langerhans cells isolated from mouse skin. Br J Exp Pathol **1986**;67:157–66.

24. Pugh CW, MacPherson GG, Steer HW. Characterization of nonlymphoid cells derived from rat peripheral lymph. J Exp Med **1983**;157:1758–79.

25. Will A, Blank C, Röllinghoff M, Moll H. Murine epidermal Langerhans cells are potent stimulators of an antigen-specific T cell response to *Leishmania major*, the cause of cutaneous leishmaniasis. Eur J Immunol **1992**;22:1341–7.

26. Puré E, Inaba K, Crowley MT, et al. Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. J Exp Med **1990**;172:1459–69.

27. Schuler G, Steinman RM. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. J Exp Med **1985**;161:526–46.

28. Shimada S, Caughman SW, Sharrow SO, Stephany D, Katz SI. Enhanced antigen-presenting capacity of cultured Langerhans cells is associated with markedly increased expression of Ia antigen. J Immunol **1987**;139:2551–5.

29. Streilein JW, Grammer S. In vitro evidence that Langerhans cells can adopt two functionally distinct forms capable of antigen presentation to T lymphocytes. J Immunol **1989**;143:3925–33.

30. Stössel H, Koch F, Kämpgen E, et al. Disappearance of certain acidic organelles (endosomes and Langerhans cell granules) accompanies loss of antigen processing capacity upon culture of epidermal Langerhans cells. J Exp Med **1990**;172:1471–82.

31. Kämpgen E, Koch N, Koch F, et al. Class II major histocompatibility complex molecules of murine dendritic cells: synthesis, sialylation of invariant chain, and antigen processing capacity are down-regulated upon culture. Proc Natl Acad Sci USA **1991**;88:3014–8.

32. Becker D, Reske-Kunz AB, Knop J, Reske K. Biochemical properties of MHC class II molecules endogenously synthesized and expressed by mouse Langerhans cells. Eur J Immunol **1991**;21:1213–20.

33. Streilein JW, Grammer SF, Yoshikawa T, Demidem A, Vermeer M. Functional dichotomy between Langerhans cells that present antigen to naive and to memory/effector T lymphocytes. Immunol Rev **1990**;117:159–83.

34. Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol **1991**;9:271–96.

35. Larsen CP, Steinman RM, Witmer-Pack M, Hankins DF, Morris PJ, Austyn JM. Migration and maturation of Langerhans cells in skin transplants and explants. J Exp Med **1990**;172:1483–93.

36. Macatonia SE, Knight SC, Edwards AJ, Griffiths S, Fryer P. Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocynate. J Exp Med **1987**;166:1654–67.

37. Poulter LW, Collings LA, Tung KS, Waters MFR. Parasitism of antigen presenting cells in hyperbacillary leprosy. Clin Exp Med **1984**;55:611–7.

38. Mittag H, Klingmüller G. Langerhans cells in granulomatous syphilis. Arch Dermatol Res **1983**;275:190–6.

39. Locksley RM, Heinzel FP, Fankhauser JE, Nelson CS, Sadick MD. Cutaneous host defence in leishmaniasis: interaction of isolated dermal macrophages and epidermal Langerhans cells with the insect-stage promastigote. Infect Immun **1988**;56:336–42.

40. Chang KP, Chaudhuri G. Molecular determinants of *Leishmania* virulence. Annu Rev Microbiol **1990**;499–529.

41. Theodos CM, Ribeiro JMC, Titus RG. Analysis of enhancing effect of sand fly saliva on *Leishmania* infection in mice. Infect Immun **1991**;59:1592–8.