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A method for the isolation and analysis of leucocytic cells from Leishmanial ear lesions in mice

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Abstract

The standard model of experimental cutaneous leishmaniasis involves infection of mice with *Leishmania major* in a single footpad or the rump, and analysis of the subsequent immune response in draining lymph nodes. Relatively few studies have examined the lesion directly. Here, we describe a method for the isolation of cells from established leishmanial lesions in mouse ears. After physical disruption of lesion tissue and isolation of cells on density gradients, a variety of leucocytic cell phenotypes were identified by flow cytometry and cytology. The phenotypes of the viable cells obtained were similar, in proportion, to those observed in histologic sections of ear lesions. This technique may be useful for studying lesion-specific cell function within the first weeks after infection with *Leishmania* parasites. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Leishmania*; Flow cytometry; Ears; Lesions

1. Introduction

Leishmaniasis is a disease affecting millions of people in tropical and subtropical regions of the world. *Leishmania* are obligate intracellular parasites of mammals, borne by sand flies, and delivered during blood feeding at a bite wound into the skin of a mammalian host (Muller and Baker, 1990).

The study of cutaneous leishmaniasis, most commonly utilizing *Leishmania major* (*L. major*), has been greatly assisted by the development of experimental mouse models. In a standard mouse model, an infection is established by injecting 10^5–10^7 *L. major* subcutaneously into one rear footpad. In most mouse strains, the lesion that develops is resolved after several weeks following the onset of a vigorous and protective anti-*L. major* immune response. This immune response is dependent on $CD4^+$ T helper type 1 (Th-1) cells in lesion-draining lymph nodes and the production of high levels of interferon (IFN)-γ (see reviews by Liew and O'Donnell, 1993; Reiner

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Abbreviations: *L. major*, *Leishmania major*; Th-1, T helper type-1; Th-2, T helper type-2; IL-4, interleukin-4; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; NO, nitric oxide; EMA, ethidium monoazide bromide

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and Locksley, 1995; Bogdan et al., 1996; Lohoff et al., 1998). IFN-γ is an important activation factor for macrophages which, when properly activated, can upregulate their levels of inducible nitric oxide synthase (iNOS) and kill *L. major* by the production of nitric oxide (NO) (Green et al., 1990; Liew et al., 1990; Mauel et al., 1991; Stenger et al., 1994).

Unlike most mice, BALB/c mice are not able to control infections with *L. major*. Rather than a Th-1 response, BALB/c mice develop Th-2 responses characterized by the outgrowth of interleukin (IL)-4-producing $CD4^+$ T cells in lesion-draining lymph nodes (Liew and O'Donnell, 1993; Reiner and Locksley, 1995; Bogdan et al., 1996; Lohoff et al., 1998). IL-4 can inhibit the activation of macrophages and the production of NO (Lehn et al., 1989; Sher et al., 1992; Liew and O'Donnell, 1993; Liew et al., 1989). The levels of iNOS in lesion-draining lymph nodes are lower in BALB/c mice than in *L. major*-resistant mice (Stenger et al., 1994), and the footpad lesions of BALB/c mice never heal.

Although the study of anti-*L. major* immune responses in lesion-draining lymph nodes has led to dramatic advances in our understanding of experimental cutaneous leishmaniasis, cellular events in the lesion itself have been less well studied (McElrath et al., 1987; Heinzel et al., 1988; Beil et al., 1992; Sunderkötter et al., 1993; Stenger et al., 1994). This can be attributed, in part, to the structural complexity of the footpad and, thus, greater difficulty manipulating and processing lesion tissue in comparison with lymph nodes. It is currently unknown if results from the study of lymph nodes are truly reflective of anti-leishmanial processes in lesions. Immunological events within the first week following leishmanial infection are pivotal for the development of resistant Th-1 or susceptible Th-2 responses (Titus et al., 1985; Heinzel et al., 1993; Sypek et al., 1993; Launois et al., 1997). Since a lesion forms at the site of first contact with *Leishmania*, early events in the lesion may have a strong bearing on the subsequent anti-*Leishmania* response in mice.

Here, we describe a method for utilizing the mouse ear as a model to study leishmanial lesions. We provide detailed procedures for isolation of cellular infiltrates and compare the composition of those isolates with those of whole lesions and isolates obtained using other methods.

2. Materials and methods

2.1. Media

ACK lysis buffer: 0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2–7.4, filter-sterilized (Kruisbeek, 1994). Blocking medium: staining medium supplemented with 10% normal mouse serum (catalog #005501, Harlan Bioproducts for Science, Indianapolis, IN), 10% fetal bovine serum (FBS, catalog #SH30071.03, Hyclone Laboratories, Logan, UT), and 2 μg/ml of anti-mouse CD16/CD32 (clone 2.4G2, Pharmingen, San Diego, CA). Fixative: 10.7 g/l cacodylate, 7.6 g/l NaCl, 11.8 g/l paraformaldehyde, pH 7.3 (Babcock and Dawes, 1994). 100% Percoll: 1 part 10 × PBS plus 10 parts Percoll (Sigma-Aldrich, St. Louis, MO), adjusted to pH 7.2 with 1N HCl; 40% and 70% Percoll were prepared by diluting 100% Percoll with staining medium. Phosphate-buffered saline (PBS): 49 mg/l NaH_2PO_4, 1.51 g/l Na_2HPO_4, 9 g/l NaCl. SB medium: RPMI 1640 medium (catalog #31800-022, Gibco-BRL/Life Technologies, Baltimore, MD) supplemented with 25 mM Hepes, 10% FBS, and gentamicin at 50 μg/ml. Staining medium: PBS supplemented with 1% bovine serum albumin (catalog #A-7030, Sigma), 0.01% sodium azide, and 2 mM EDTA.

2.2. Materials

2.2.1. Antibodies

R-phycoerythrine (PE)-anti-mouse CD4 (clone H129.19, Pharmingen); fluorescence isothiocyanate (FITC)-anti-mouse CD11b (clone M1/70.15, Caltag, Burlingame, CA); FITC-anti-mouse CD45 (leucocyte common antigen, clone 30F11.1, Pharmingen); biotin-anti-mouse CD45R (B220, clone RA3-6B2, Pharmingen); FITC-anti-CD90.2 (Thy-1.2, clone 53-2.1, Pharmingen); hamster-anti-mouse MHC class II (clone N22 was obtained from American Type Culture Collection, Manassas, VA, and antibody was prepared as culture supernatant in the authors' laboratory); appropriate isotype-matched control antibodies were obtained from the same manufacturers; FITC-anti-hamster IgG (catalog #12104D, Pharmingen); rat IgG (Accurate Chemical and Scientific, Westbury, NY).

2.2.2. Miscellaneous reagents, supplies, and equipment

Diff-Quik® Stain Set (catalog #B4132-1, Baxter Healthcare, McGaw Park, IL); ethidium monoazide bromide (EMA, Molecular Probes, Eugene, OR); nylon mesh (40 μm pore size, Small Parts, Miami Lakes, FL); Poly-Prep™ glass microscope slides (Sigma); stainless steel screen (30 mesh/cm); streptavidin-PE (Pharmingen); strepavidin-Spectral Red™ (Southern Biotechnology Associates, Birmingham, AL); polypropylene 96-well tissue culture plates (Corning, Corning, NY); Cytospin centrifuge (Shandon, Pittsburg, PA); flow cytometer (model XL, Coulter, Hialeah, FL).

2.3. Animals

Female BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME) or the National Cancer Institute (Bethesda, MD) and cared for in accordance with institutional guidelines. A maximum of five animals was housed per polycarbonate shoebox-type cage with aspen chip bedding. Autoclaved tap water and food (Harlan Sprague Dawley, Rodent Maintenance 8640) were provided ad libitum. The temperature in animal rooms was maintained at 69–74°C with approximately 42% humidity, and fluorescent lighting was provided for 12 h each day.

2.4. Parasites and infection of animals

L. major, strain LV39 (RHO/SU/59/P, Neal, or P strain), were maintained as previously described (Sacks and Perkins, 1984). Stationary phase parasites were suspended in Hanks Balanced Salts Solution (HBSS), without phenol red. Mice were sedated by intraperitoneal injection of 1.5 mg Ketamine plus 0.3 mg Xylazine. To prevent drying of eye membranes, Lacrilube (Allergan, Irvine, CA) was applied to each eye. Using a Hamilton microliter syringe and a 30-gauge needle, 15 μl of a parasite suspension (10^6–10^7 parasites) were injected intradermally into the pinna of each ear through the inner face.

2.5. Measurement of ear thickness

After infection, ear thickness was measured as an index of lesion development. Ear thickness was determined using a modified spring-loaded, dial-type thickness gage (model 7326, Mitutoyo Asia Pacific). The gage's readout was in inches; conversion to millimeters has been made here. The thickness gage was modified by reducing the spring tension (the length of the spring's span was reduced). This modification prevented compression of the lesion when measured. The shape of the parasite-filled pocket formed upon injection was irregular and inconsistent between animals; thus, lesion diameter did not provide a sound measure of lesion progression.

2.6. Processing ear tissue for histology

Whole external ears were fixed in 10% neutral buffered formalin and processed normally. Tissue sections (6 μm) were mounted and stained with hematoxylin and eosin.

2.7. Isolation of cells from Leishmania-infected ears

2.7.1. Cell isolation: the lesion disruption method

Mice (3–15 per experimental group) were killed with an overdose of CO_2 and then dipped in 70% ethanol. The pinna of each infected ear was removed with scissors and rinsed with staining medium. Ear tissue and cells were kept on ice or at 4°C in staining medium at all times unless otherwise indicated. The inner and outer cuticular aspects of each ear pinna were separated with forceps in a polystyrene petri dish, and the subcuticular faces were rubbed on a stainless steel screen, using plungers from disposable tuberculin syringes, to liberate lesion-infiltrating cells. The crude cell suspension was transferred from petri dishes into polypropylene tubes. Screens, ear tissues, and petri dishes were rinsed, and this rinse medium was combined with cell suspensions. Cell suspensions were then centrifuged for 7 min at 390 $\times g$. The resultant cell pellets were resuspended in 5 ml ACK lysis buffer and incubated at room temperature for 5 min. Five milliliters of staining medium was then added to each tube followed by centrifugation as above. The resultant cell pellets were resuspended in 5 ml staining medium, passed through nylon mesh, and sedimented at $2560 \times g$ for 11 min on two-layer Percoll density gradients: 40% Percoll over 70% Percoll. Cells at the 40%/70% Percoll interface were collected, washed, and enumerated

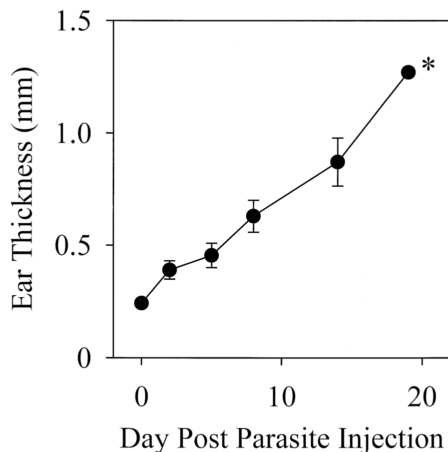


Fig. 1. Development of ear lesions. To follow ear lesion development after infection with 10^7 *L. major*, the thickness of ears was measured with a spring-loaded dial-type thickness gage (see Section 2). Ear thickness is presented as mean ± standard deviation for three mice (six ears) and is representative of several experiments. The first measurement (day 0) was made just prior to injection of parasites. *Indicates that ear thickness did not increase beyond this point due to ulceration and scabbing. All animals were killed after 20 days.

using a hemocytometer. A 40%/70% Percoll gradient was used because live cells sedimented at the interface.

2.7.2. Staining for cytology

Cells were mounted onto slides using a Cytospin centrifuge. Cells (1 to 2×10^5 in 100 μl staining medium) were centrifuged for 2 min at 300 RPM, air dried for 10 min, fixed in methanol, and stained with Diff-Quik® using the protocol provided by the manufacturer. Cell differentials were determined by light microscopy for 100 cells per Cytospin preparation under oil immersion.

2.7.3. Alternate cell isolation: the ear explant culture (EEC) method

For comparative purposes, ear-lesion cells were also isolated using a method adapted from Belkaid et al. (1996). Briefly, ear pinnas were rinsed three times in 70% ethanol, placed in a sterile petri dish at room temperature, and allowed to dry in a sterile laminar-flow hood for 15 min; the inner and outer cuticular aspects of each ear pinna were separated with forceps and floated upon SB medium with the subcuticular faces down, in canted 50 ml polypropylene centrifuge tubes. After 13–18 h of incubation at 37°C in air plus 5% CO_2, the floating ear tissue was discarded and cells in suspension were recovered by centrifugation as above. These cells were passed through nylon mesh and washed twice in ice-cold staining medium prior to staining for flow cytometry.

2.8. Flow cytometry

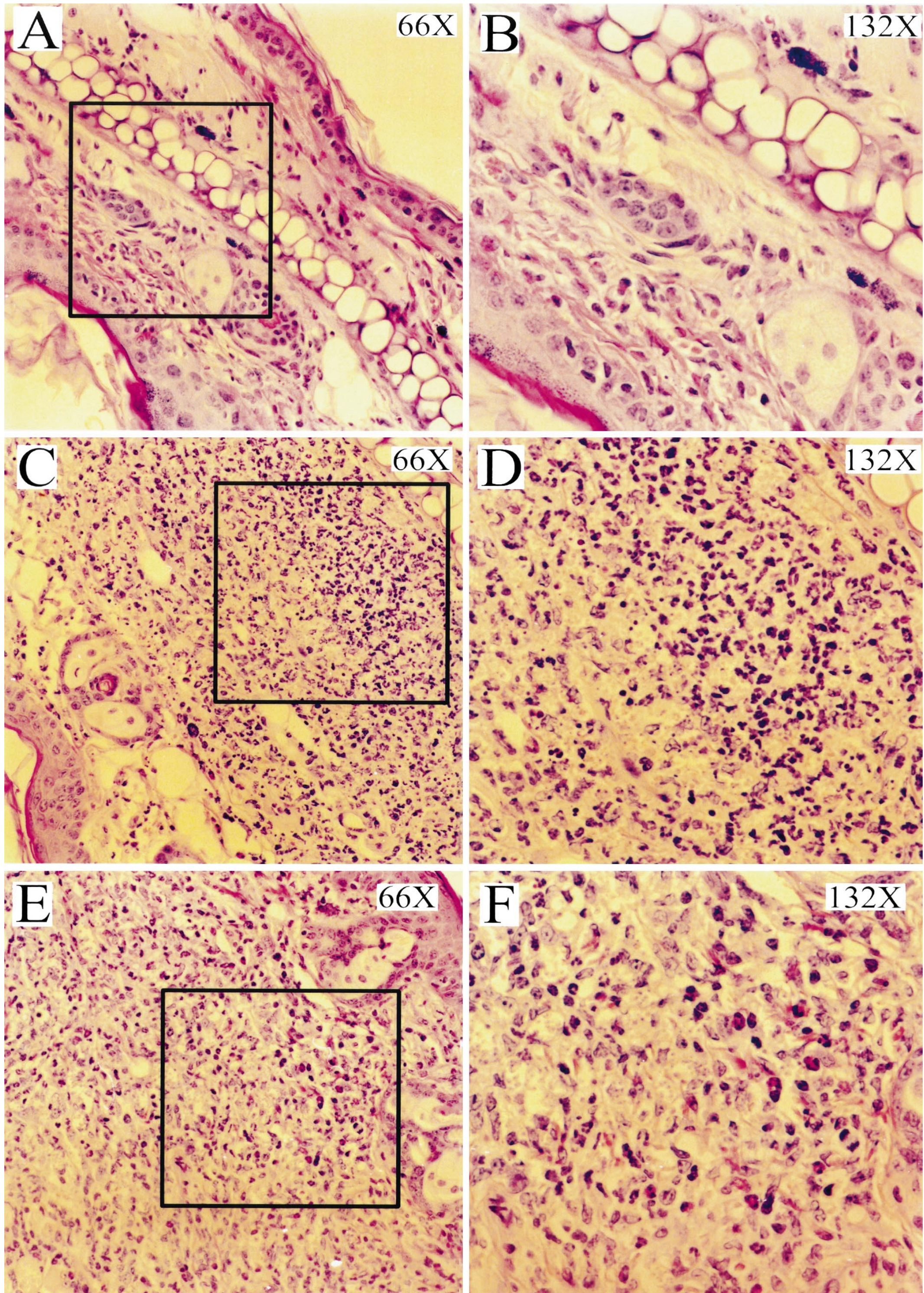
Cells (10^5 per well) were placed in polypropylene 96-well plates and washed with 200 μl staining medium by centrifugation at $210 \times g$ for 2 min. The resultant cell pellets were resuspended in 50 μl blocking medium. After 15 min of incubation, 50 μl of an antibody cocktail was added to each well of cells and incubation was continued for an additional 15 min. Antibody cocktails contained a mixture of antibodies specific for cell surface markers and/or isotype-matched control antibodies at a concentration of 1 μg of each antibody per 10^6 cells. After staining with antibodies, cells were washed as above. Some samples were resuspended in staining medium containing EMA at 5 μg/ml for a minimum of 10 min prior to analysis; this allowed for identification of dead (EMA^+) cells. Alternatively, cells were incubated in 100 μl fixative for 20 min, washed, and stored at 4°C for analysis within 5 days. Cells (20,000–40,000 events) were analyzed by flow cytometry. Off-line analysis of listmode files was performed using XL II (Coulter) and WinMDI (Joseph Trotter) software.

3. Results

3.1. Development of ear lesions

As shown in Fig. 1, the thickness of BALB/c ears increased 5-fold after injection of 10^7 *L. major*,

Fig. 2. Sections of leishmanial ear lesions. Photographs of formalin-fixed ear sections (stained with hematoxylin and eosin) are shown for uninfected ears (A and B), or ears infected with 10^7 *L. major* 4 days (C and D) or 9 days (E and F) prior. Low-power magnifications (66 ×) are shown in the left column; high-power magnifications (132 ×) are shown in the right column. Squares in low-power images delineate, approximately, the areas depicted in high-power images for each day.


A
66X
B
132X
C
66X
D
132X
E
66X
F
132X

from approximately 0.25 mm in uninfected ears (day 0) to approximately 1.27 mm 19 days post-infection. Ear thickness did not increase after day 19 because of ulceration and scabbing. The development of ear lesions in mice infected with 10^6 *L. major* per ear was the same (data not shown).

Ear lesions were examined histologically to identify the nature of the inflammation induced by *L. major* infection. Normal (uninfected) ear architecture is shown in Fig. 2 (panels A and B). The full transverse thickness of an ear pinna could be viewed easily within a low-power ($66\times$) field. Occasional mast cells could be appreciated, but no infiltration of inflammatory cells was evident. Four days after infection with 10^7 *L. major* (Fig. 2, panels C and D), a mixed cellular infiltrate was apparent. This infiltrate consisted predominantly of neutrophils (some degenerate) with fewer monocytes/macrophages and occasional lymphocytes and eosinophils. An increase in the thickness of the dermal layer was evident: at its maximum thickness, only half of a transverse section could be viewed in a low-power field. The cartilage and epidermal layers appeared unchanged. Nine days after infection (Fig. 2, panels E and F), the degree of inflammation was increased when compared with that observed on day 4: at its maximum thickness, only a portion of the dermal layer (transverse section) could be viewed in a low-power field. On this day, the inflammation was transdermal with an intense cellular infiltrate and little or no edema. The infiltrate consisted mainly of neutrophils (some toxic, some degenerate) with fewer monocytes/macrophages and occasional lymphocytes and eosinophils. Amastigote stage *L. major* were apparent diffusely as lightly staining round bodies (center of field, Fig. 2E).

3.2. Analysis of cells isolated using the lesion disruption method

A purification process was required to separate live cells from debris. Prior to filtration and sedimentation on Percoll density gradients, cell suspensions obtained from *Leishmania*-infected ears contained individual cells, cellular debris, hair, and collagen fibers (data not shown). After purification, cell preparations were comprised of various leucocytic cell types as well as some parasites and cell debris. Cell differentials of Diff-Quik®-stained cytospin preparations varied somewhat over multiple experiments, as expected. For example, on day 9 post-infection, the percentages of different cell types obtained were as follows: 3–16% monocytes/macrophages, 48–66% neutrophils, 5–16% eosinophils, and 18–26% lymphocytes.

The yield of cells recovered from ear lesions was dependent on the day after *L. major* infection (see Fig. 3). Relatively few cells were recovered on days 3 and 6 (approximately 10^5 cells per mouse), whereas the largest number of cells recovered was on day 10 ($> 7\times10^5$ cells per mouse), and somewhat fewer cells were recovered on day 14. This pattern of cell recovery was typical of that found in other experiments. Therefore, to achieve the number of cells necessary for phenotypic analysis by flow cytometry, pools of cells from multiple animals were required.

Flow cytometry of lesion-cell isolates revealed four to five major populations, by light scatter, on day 9 post-infection (Fig. 4A). Live leucocytes were identified (68%, Fig. 4B) as EMA negative (EMA^-), $CD45^+$ populations (also displayed in Fig. 4C), clearly separated from low light scatter events (parasites and debris). The percentage of live leucocytes among the isolated cells varied across experi-

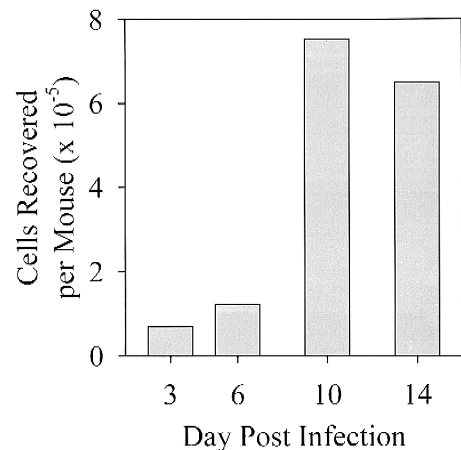


Fig. 3. Recovery of lesion-infiltrating cells. The number of lesion-infiltrating cells recovered from ears is shown for various days after infection with 10^7 *L. major* per ear. Cells were obtained by processing infected ears as described in Section 2. Data are presented as means of cells recovered per mouse for pools of three to five mice per day and are representative of several experiments.

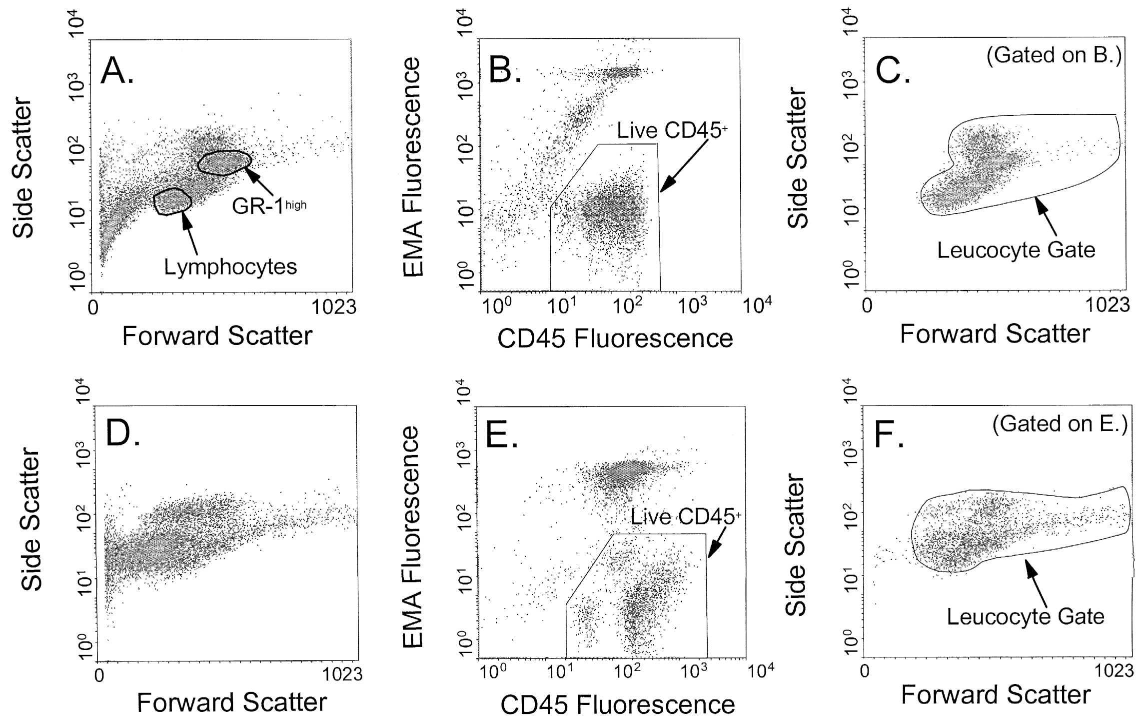


Fig. 4. Light scatter properties and live leucocyte ($EMA^-/CD45^+$) populations of cells isolated from *Leishmania*-infected ears. Cells from *Leishmania*-infected ears were isolated on day 9 post-infection, stained, and analyzed by flow cytometry as described in Section 2. Cells were isolated using either the lesion disruption method (A–C) or the EEC method (D–F). In panels A and D, light scatter data are shown for ungated cells. In panels B and E, FITC fluorescence (anti-CD45) vs. EMA fluorescence (dead cells) data are shown with a gate enclosing live $CD45^+$ cells. In panels C and F, light scatter data are shown for live $CD45^+$ cells gated from panels B and E. Live leucocyte gates, used in further analysis, are indicated. The data presented are representative of two to three experiments.

ments and days of isolation (53%–99%) with the highest yield generally obtained during the second week of infection. Live leucocyte gates were constructed in light scatter histograms (see Fig. 4C) and used to identify subpopulations of cells in subsequent samples. In this way, a variety of cell phenotypes could be identified (see Table 1): Thy-1.2$^+$, CD4$^+$, B220$^+$, and MHC class II$^+$. The largest population identified (66%) expressed CD11b, a general marker found on a variety of cell types including macrophages, granulocytes, and NK cells (Springer et al., 1979; Holmberg et al., 1981; Kishimoto et al., 1989). GR-1^{high}, expressed on differentiated/mature granulocytes (Hestdal et al., 1991; Fleming et al., 1993), was coexpressed on approximately one-third of the CD11b$^+$ cells, and could be identified as a distinct population of large cells (high forward light scatter) with medium-high side (90°) light scatter (Fig. 4A). MHC class II was also coexpressed on CD11b$^+$ cells, but not on those cells expressing GR-1^{high} (data not shown). Lymphocytes (18% of the total population: Thy-1.2 + B220) were identified as

Table 1
Phenotypes of cells isolated from *Leishmania*-infected ears and analyzed by flow cytometry

Percentage of positive cells on day 9 post-infection[a]		
Marker	Lesion disruption	EEC method
Thy-1.2	11	35
CD4	6	22
B220	6	8
CD11b	66	50
GR-1^{high}	23	2
MHC Class II	10	12

[a] Percentages are based on events within leucocyte gates as defined in Fig. 4 and are representative of two to three experiments.

a population with medium light scatter, somewhat overlapped by a $CD11b^+$ population (Fig. 4A).

3.3. Comparison of isolation methods

An alternative method for isolating cells from inflamed or pathogen-infected ears has been previously described (Belkaid et al., 1996), and subsequently used to study ear lesions in mice infected with *L. major* (Belkaid et al., 1998). This method [the ear explant culture (EEC) method] takes advantage of cell motility in inflamed tissue: cells are allowed to ex-migrate out of the dermal face of ears floated on medium for 13–18 h. We used this method to isolate cells from *Leishmania*-infected ears on day 9 post-infection.

Microscopic examination of Diff-Quik®-stained cytospin preparations of EEC isolated cells revealed approximately 40% granulocytes, 30% monocytes/macrophages, and 25% lymphocytes over several experiments. Comparison of cells obtained using the EEC method with those obtained with the lesion disruption method in a single experiment is shown in Table 2. Not quite half as many granulocytes were recovered with the EEC method as when the lesion disruption method was used. Roughly half of the granulocytes obtained with the EEC method were neutrophils, whereas 6-fold more neutrophils than eosinophils were obtained with the lesion disruption method. Approximately equal numbers of lymphocytes were identified in preparations from each method, but approximately 10-fold more monocytes/macrophages were obtained with the EEC method compared with the lesion disruption method.

Table 2
Differentials of cells isolated from *Leishmania*-infected ears

Percentage of cells as determined on day 9 post-infection[a]		
Cell type	Lesion disruption method	EEC method
Neutrophils	61	19
Eosinophils	10	25
Monocytes/macrophages	3	32
Lymphocytes	26	24

[a]Cytospin preparations were stained with Diff-Quik® and analysed by light microscopy as described in Section 2. The data are representative of two to three experiments.

Flow cytometry also revealed differences between the isolates obtained with the two methods. As shown in Fig. 4D, measurement of light scatter revealed three to four major populations of cells isolated by the EEC method on day 9 post-infection. Live leucocytes were identified as EMA^-, $CD45^+$ populations (51%, Fig. 4E), clearly separated from low light scatter events (see Fig. 4F). Forty percent of the $CD45^+$ cells were dead when using the EEC method, whereas 16% of the $CD45^+$ cells were dead (EMA^+) when using the lesion disruption method (Fig. 4). In a representative experiment, cells within a leucocyte light-scatter gate (Fig. 4F) were found to express $Thy\text{-}1.2^+$, $CD4^+$, $B220^+$, $CD11b^+$, and MHC class II^+ as indicated in Table 1.

The percentages of B220 (B cells), CD11b, and MHC class II expressing cells were approximately the same using the two methods, but the percentage of Thy-1.2 and CD4 expressing cells were approximately three- and four-times greater, respectively, when using the EEC method. The percentage of $GR\text{-}1^{high}$ expressing cells obtained with the EEC method was approximately 11-fold less than obtained when using the lesion disruption method.

4. Discussion

The impetus for developing the method described above came from the need for further study of immunological events and cells at the site of first contact with *Leishmania* parasites. Immunological responses of mice to *Leishmania* infection have been most widely studied at the level of the lesion-draining lymph node. It is clear that early immunological events (within the first week following infection) are pivotal for the development of resistant or susceptible immune responses (Titus et al., 1985; Heinzel et al., 1993; Sypek et al., 1993; Launois et al., 1997). However, it is not clear that the results obtained from the study of lesion-draining lymph nodes are wholly reflective of anti-leishmanial processes in leishmanial lesions over the same time course. In addition, early interactions between parasites and cells at the site of infection may have a profound influence on the nature of the subsequent immune response (von Stebut et al., 1998). To study immunological events at the site of infection within the first days–weeks of

infection, we chose to develop a model of *L. major* infection in the ears of mice.

To test the methods under development with the greatest efficiency, we induced rapid lesion progression by injecting BALB/c mice with 10^7 stationary phase *L. major* promastigotes per ear. Analysis of ear lesions was possible through the second week post-infection, but analysis beyond this point was precluded by the development of tissue necrosis as lesions progressed to involve the majority of the external ear. Similarly, rapid lesion development was also observed following injection of 10^6 parasites (data not shown). Thus, because a relatively small cell recovery was obtained at early time points, and because cell recovery was reduced by lesion necrosis, the efficient application of this method may be limited to grossly visible, non-necrotic lesions.

Injection of fewer parasites can lead to less rapid lesion development. Belkaid et al. (1998) has shown that injection of 1000 metacyclic *L. major* per BALB/c mouse ear also leads to the development of nonhealing lesions; however, detection of ear lesions was delayed until the fourth week post-infection with ulceration and tissue necrosis appearing after 2–3 months. It is reasonable to expect that the methods described here for analysis of leishmanial lesions are effective independent of the inoculating dose.

The cellular infiltrates of leishmanial ear lesions (shown here) and those at other sites induced by similar numbers of parasites (shown by others) are similar in composition. Histologic examination of footpad lesions of BALB/c mice infected with 2×10^7 *L. major* (Beil et al., 1992) indicated a predominance of polymorphonuclear leucocytes and mononuclear cells with smaller numbers of eosinophils ($\leq 13\%$) and lymphocytes ($\leq 8\%$), and the presence of extracellular parasites after the fourth day of infection. As shown here, *L. major* lesions in the ears of BALB/c mice also contained a predominance of polymorphonuclear leucocytes (neutrophils) with smaller numbers of monocytes/macrophages, eosinophils, and lymphocytes, as well as (on day 9 post-infection) extracellular parasites (see Fig. 2). Thus, leishmanial lesions in ears are similar in composition to those described at other sites of leishmanial infection.

Cytologic examination of the cells isolated from leishmanial lesions with the lesion disruption method revealed a slightly variant differential when compared to the cell populations evident in sections of *Leishmania*-infected ear lesions. This is most likely due to the removal of some cells during the purification process. Indeed, the $< 40\%$ Percoll fraction of lesion cell isolates contained large numbers of dead cells, predominately macrophages, and the $> 70\%$ fraction contained neutrophils (data not shown). The removal of dead cells is also probably responsible for the larger than expected percentage of lymphocytes obtained in lesion-cell isolates given the frequency of lymphocytes appreciated in lesion sections on day 9 post-infection.

Comparison between cytologic and flow cytometric results showed little variance for cells isolated with the lesion disruption method. For example, CD11b (a common marker for macrophages, granulocytes, and some natural killer cells) was expressed on 66% of the cells isolated by the lesion disruption method on day 9 post-infection (Table 1); this number correlated reasonably well with the 74% of monocytes/macrophages + granulocytes evident by cytology on the same day. GR-1^{high}, expressed on differentiated/mature granulocytes, was expressed on a smaller population than was expected based on the percentage of granulocytes evident by cytology. Because identification of cells with antibodies is dependent on the presence of intact epitopes, this result may reflect a sensitivity of the GR-1 epitope to degradation in an inflammatory and focally necrotic environment. Lymphocytes were identified as 26% of the cell isolate by cytology on day 9 post-infection; this number correlates reasonably well with the 17% of lymphocytes identified by flow cytometry as expressing either Thy-1.2 or B220.

The lesion disruption method described here provides an alternative approach to the study of leishmanial lesions. Some investigators have previously described infiltrating cells and cytokine production in leishmanial lesions (foot pad, rump) within the first weeks post-infection using immunohistochemical and RT-PCR techniques (McElrath et al., 1987; Beil et al., 1992; Sunderkötter et al., 1993; Stenger et al., 1994; Diefenbach et al., 1998), and others have examined cells from *Leishmania*-infected mouse ears within the first 18 h post-infection (Belkaid et al., 1998) using the EEC method. The method described here was developed to permit direct analysis of

viable cells isolated from within an active leishmanial lesion. The most directly comparable method previously described for isolating cells from infected ear tissue is the EEC method of Belkaid et al. (1996). As shown here, the use of these two methods provides, in some respects, markedly different results.

The most striking differences found between the two methods were in the percentages of T cells and granulocytes obtained as determined by either cytologic or flow cytometric techniques. By flow cytometry, the percentage of T cells was markedly higher (3- and 4-fold for Thy-1.2$^+$ and CD4$^+$ cells, respectively) when using the EEC method. Granulocytes, in contrast, composed 44% of the cells obtained with the EEC method (determined by cytology), as compared to 71% granulocytes when using the lesion disruption method. The most likely explanation for these differences lies in the migratory behaviors of these different cell types. Lymphocytes, activated and memory T cells in particular, preferentially recirculate through inflamed tissue (e.g., skin) (Picker and Butcher, 1992). Indeed, the bulk of cells in afferent lymph, downstream of inflamed skin, are T cells (Mackay et al., 1992). It is not expected that most T cells would express receptors specific for antigen presented at an inflammatory site. Therefore, these results suggest that the bulk of the T cells obtained by the EEC method are actively transmigrating through the *L. major* infected ear tissue. Granulocyte migration, on the other hand, tends to terminate once an inflammatory site is reached. The fact that a relatively high percentage (44%) of granulocytes was obtained with the EEC method suggests that cells were falling into the supporting medium from the surface of necrotic sites in ear lesions (offered by Belkaid et al., 1996), rather than actively migrating out of ear tissue. This is supported by the high number of dead cells obtained with the EEC method.

The method described here provides an alternative approach to the study of leishmanial lesions. The ear is a relatively simple structure, when compared to the foot, and permits a straightforward approach to lesion-cell isolation. No enzymatic digestion is required to isolate lesion cells from the ear, thereby eliminating added enzyme-induced loss of epitopes as is known to occur for some cellular markers. Finally, inoculation of *Leishmania* into mouse ears may more closely mimic the route of parasite transmission found in more natural settings (i.e., by sand fly bite). We have demonstrated that cells can be readily isolated from leishmanial ear lesions using the lesion disruption method, and that this technique can provide a cell population that is representative of live cells found in ear lesions. We offer this technique to compliment the EEC method, since no technique can be appropriate in all experimental settings, and so that a broader array of hypotheses can be addressed in the study of experimental cutaneous leishmaniasis.

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