MIXED INFECTION OF HUMAN U-937 CELLS BY TWO DIFFERENT SPECIES OF LEISHMANIA

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Abstract. Mixed infections by different Leishmania species could explain differences in the clinical course of these infections. Moreover, mixed infections of the same macrophage could be the basis for parasite recombination. We stained three strains of Leishmania (L. mexicana amazonensis, L. donovani DD8, and L. infantum D2, respectively) with different fluorescent dyes and analyzed them using a fluorescence-activated cell scanner. The simultaneous infection of one cell by Leishmania belonging to two different species was demonstrated. In additional experiments, cells with mixed infections were separated by a fluorescence-activated cell sorter and monitored for 24 hr. Preinfecting human monocytic U-937 cells with one Leishmania species did not exclude a second species added after 3 hr.

Infections by Leishmania species may cause a broad spectrum of diseases with different clinical pictures known as leishmaniasis. These parasites are widespread in tropical and subtropical countries of the world. Leishmaniasis is considered to be endemic at least in 82 countries (21 in the New World and 61 in the Old World). It presently accounts for more than 75,000 deaths per year. The fact that Leishmania may infect not only residents of endemic areas, but also tourists and military personnel or may be imported by refugees, has attracted increasing interest among scientists and physicians.

The various clinical syndromes associated with this disease, ranging from benign self-healing sores to fulminant, visceral, fatal disease, are attributed to a number of species of the dimorphic protozoa Leishmania of the order Kinetoplastida. Although the parasites live as flagellated, extracellular promastigotes in the gut of blood-sucking female sand flies, they survive and multiply in vertebrate hosts, including humans, in their amastigote form within phagolysosomes (similar to parasitophorous vacuoles of mammalian macrophages). The conversion from promastigotes to amastigotes in macrophages is completed in a matter of hours. The amastigote stage has been used to investigate various aspects of the interaction of the parasites with the host to explain pathogenesis.

Several attempts and various techniques in vitro have been used to study the process of infection of phagocytizing cells by promastigotes. Most of these experiments are complicated and involve time-consuming cell-staining techniques that include laborious microscopic evaluation procedures. The use of multiparameter flow cytometry with multicolor analysis is an important tool in the quantitative examination of the phagocytic capacity of individual phagocytes within a cell population.

There is considerable interest in the genetics of Leishmania, and genetic recombination in this organism is a controversial issue. One way to address these issues would be to cultivate different Leishmania species simultaneously in macrophages. The goal of the present work was to establish a procedure that would produce cells with mixed infections and detect them by flow cytometry. The idea was to label different Leishmania species with various vital fluorescent dyes, which can be readily distinguished by flow cytometry, to infect human U-937 cells simultaneously with two of these differently labeled Leishmania species, and to separate and analyze cells with mixed infections by fluorescence-activated cell sorting (FACS).

MATERIALS AND METHODS

Cell line, media, and culture conditions. The U-937 cells were obtained from Dr. A. F. Kiderlen (Robert-Koch-Institute, Berlin, Germany). This human monocytic cell line was maintained in the suspension culture in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Seromed), 2 mM L-glutamine, penicillin (100 μg/ml) (Jenapharm, Jena, Germany), and streptomycin (100 μg/ml) (Jenapharm) by serial passage. This will be referred to as complete culture medium.

Prior to use in the experiments, the U-937 cells were grown in plastic tissue culture flasks at 37°C in humidified atmosphere containing 5% CO₂. Cells were harvested after 5–6 days of incubation by washing the culture 1–2 times with warm (37°C) phosphate-buffered saline (PBS) or RPMI 1640 medium. The cells were then resuspended at a concentration of 2 × 10⁶ cells/ml in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) without L-glutamine and phenol red in plastic tissue culture flasks. Cell density and viability were ascertained by counting with a hemocytometer using trypan blue dye exclusion. The cells were incubated under identical conditions before subsequent infection with Leishmania.

Parasites. Three strains of Leishmania were used in this study: L. mexicana amazonensis (Lma), which was kindly provided by the Innerklinik (Abteilung Tropenmedizin, Rostock, Germany); L. donovani DD8 (Ld); and L. infantum D2 (Li), which both were kindly provided by the Royal Tropical Institute (Amsterdam, The Netherlands). Promastigotes of Lma, Ld, and Li were maintained in a biphasic medium consisting of a blood agar slant layered with RPMI 1640 medium supplemented with 2 mM L-glutamine (Ferak Laborat, Berlin, Germany), 100 μg/ml of penicillin, 100 μg/ml of streptomycin, and 10% heat-inactivated FCS at 20–25°C.

Vital fluorescent stains. Three vital fluorescent stains were used in this study. The first was BCECF-Am, 2′,7′-bis(2-carboxyethyl)-5-(and-6-) carboxyfluorescein acetoxymethyl ester (lot no. 25317; Molecular Probes, Inc., Leiden,
**Leishmania mixed infection of human U-937 cells**

Table 1

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<tr>
<th>Single-labeling infection</th>
<th>Double-labeling infection</th>
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<td>U-937 + Lma/BCECF-Am</td>
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<td>U-937 + Lma/SYTO-17</td>
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<td>U-937 + Li/BCECF-Am + Ld/SYTO-17</td>
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*U-937 = human cell line; Lma = Leishmania mexicana amazonesi; BCECF-Am = green fluorescent stain; SYTO-17 = red fluorescent stain; Ld = L. donovani; Li = L. infantum.*

The dye stock solution was divided into small aliquots that were stored in the dark at -20°C. The final concentration used was 2.3 μg/ml in 0.9% physiologic saline, pH 7.4. The BCECF-Am working solution was always freshly prepared. The second dye was Cell-Tracker™ Orange CMTMR (5-(and-6)-(4-chloromethyl benzoyl) amino) tetramethylrhodamine), which was obtained from Molecular Probes Europe BV in 1 mg portions (molecular weight = 554.04). The lyophilized product was dissolved in dried DMSO to yield a concentration of 10 mM as a stock solution. It was stored frozen at -20°C. A freshly prepared 5 μM solution was used in the experiments. The third dye used was SYTO-17 red fluorescent universal nucleic acid stain (molecular weight = 534.04). The lyophilized product was dissolved in dried DMSO in 1 mg portions (molecular weight = 554.04), which was obtained from Molecular Probes Europe BV. This dye was supplied as 250 μl of a 5 mM solution. It was stored frozen at -20°C in the dark. A concentration of 5 μM was used in the experiments.

**Leishmania staining procedures.** Staining with BCECF-Am. Promastigotes were washed with PBS by centrifugation at 125 × g for 10 min, at room temperature and resuspended with 1 ml of physiologic saline. They were stained with BCECF-Am for 60–90 min at 20–25°C in the dark. The promastigotes were then washed 2–3 times with PBS by centrifugation. Density and viability were determined using a hemacytometer. Promastigotes were suspended in PBS at a concentration of 2 × 10^6/promastigotes/ml. The probe was kept in the dark.22, 23

Staining with CMTMR. Promastigotes were washed with RPMI 1640 medium without phenol red, L-glutamine, and FCS (this medium was used in all steps of this staining) by centrifugation at 125 × g for 10 min at 20°C. The supernatant was aspirated and the pellet was gently resuspended in prewarmed (37°C) RPMI 1640 medium containing a dye concentration of 5 μM. The promastigotes were stained for 15–45 min under appropriate growth conditions in the dark, centrifuged, resuspended in fresh prewarmed RPMI 1640 medium, and incubated for an additional 30 min. The extracellular dye was removed by centrifuging the promastigotes twice in PBS and resuspending them in RPMI 1640 medium or PBS at a concentration of 2 × 10^6/ml. The probe was kept in the dark as described by the supplier.24, 25

Staining with SYTO-17. Promastigotes were washed with 20 mM HEPES buffer in physiologic NaCl saline solution, pH 7.04, by centrifugation at 125 × g for 10 min at room temperature and resuspended in the same buffer. They were stained with SYTO-17 (5 μM final concentration) for 10–60 min at 20°C in the dark. After staining, the promastigotes were washed 2–3 times in HEPES buffer and resuspended at a concentration of 2 × 10^6/ml in the same buffer. The probe was kept in the dark as described by the supplier.

**Microscopy.** After staining, the labeled parasites should remain fully viable as judged by morphology, motility, and growth rate. Observations were made with a fluorescent microscope. Fluorescent microscopy was performed with a Nikon (Tokyo, Japan) Optiphot-2 microscope using two filter sets (B-2E with excitation [EX] 470–490 nm, a dichroic mirror [DM] 510 nm and emission barrier filter [BA] 520–560 nm) for green fluorescence (BCECF-Am), and G-2A (EX 510–560 nm, DM 580 nm, and BA 590 nm) for orange fluorescence (CMTMR); with an Olympus (Tokyo, Japan) IX-70 microscope with a double exposure unit using WIBA filters (EX 460–490 nm, DM 510 nm, and BA 515–550 nm) for green fluorescence and WIF filters (EX 545/10 nm, DM 580 nm, and BA 610) for red fluorescence; and a Carl Zeiss (Göttingen, Germany) Axioskop MC 80 microscope with a double exposure unit equipped with a 09 filter set (EX 450–490 nm, DM 510 nm, and BA 520 nm) for green fluorescence and a 15 filter set (EX 546/12 nm, DM 580 nm, and BA 590 nm) for red fluorescence.22, 26, 27

**Analysis of stained Leishmania promastigotes.** After staining, the samples were prepared for flow cytometry by mixing the labeled promastigotes Lma/BCECF-Am, Ld/CMTMR, and Li/SYTO-17 as Lma + Ld; Lma + Li, and Ld + Li, respectively. Each mixture was prepared at the ratios of 1:1, 1:10, and 10:1 and incubated at the dark at 20°C. Aliquots were taken immediately after mixing (zero time) and after 1, 2, and 3 hr. The aliquots were washed 1–2 times with RPMI 1640 medium without phenol red, L-glutamine, and FCS, fixed for 30–60 min with 1% paraformaldehyde in PBS, and analyzed.

**Infection of cells.** Before infection, U-937 cells were prepared according to the method described in the culture conditions. The BCECF-Am and SYTO-17 stains were chosen for labeling the three types of Leishmania promastigotes (Lma, Ld, and Li) as described in the Leishmania staining procedures. Each species of Leishmania was divided into two parts. The first part was labeled with BCECF-Am and the second part was labeled with SYTO-17. Labeled promastigotes were used to infect U-937 cells at a total parasite to macrophage ratio of 10:1 and then mixed thoroughly (using one type of Leishmania for a single infection and two types for a double infection) (Table 1). Infection was allowed to proceed at 37°C in a 5% CO2 atmosphere in the presence and absence of 10% FCS. Aliquots were taken at 3, 5, 6, and 24 hr. All nonattached or extracellular promastigotes
were removed by centrifugation 1–2 times with prewarmed (37°C) PBS at 275 × g for 5 min after 3 hr. Cells were transferred to 2 ml of fresh RPMI 1640 medium without phenol red, L-glutamine and FCS, and kept in the dark. In the second series of experiments, mixed infections were not done simultaneously to see if there was exclusion of superinfecting parasites. At the zero time point, cells were infected with five promastigotes of one species per cell and nonattached parasites were removed after 3 hr. The cells were then infected with a second *Leishmania* species (five parasites per cell) labeled with a different dye. Three hours after this second infection, free promastigotes were removed. Three hours after the second infection, aliquots were taken (corresponds to 3 hr of simultaneously infection) and analyzed. Additional aliquots were taken at 5, 6, and 24 hr.

**Flow cytometry.** For flow cytometry and FACS, we used an FACSort flow cytometer (Becton-Dickinson, Heidelberg, Germany) equipped with an argon laser with excitation at 488 nm. Forward angle and orthogonal light scattering, fluorescence 1 (FL1), fluorescence 2 (FL2), and fluorescence 3 (FL3) were recorded on a logarithmic scale. The BCECF-Am fluorescence was detected in channel FL1 (band pass filter 530/30), CMTMR fluorescence was detected in channel FL2 (band pass filter 585/42 nm) and SYTO-17 fluorescence was detected in channel FL3 (long pass filter > 650 nm). Ten thousand events were registered in polygonal light scatter gates. Data analysis were performed using LYSIS II-software (Becton-Dickinson).

**Analysis of of infected cells.** Phagocytosis was evaluated by measuring the fluorescence of the labeled parasites inside the macrophages by flow cytometry. Only macrophages were analyzed, which were separated from free parasites by their different light scattering properties using polygonal light scatter gates.

Since BCECF-Am has a broad emission spectrum with fluorescence overflow to FL2 and FL3, proper compensation of FL3 was essential for detection of mixed infections. This was done by compensating FL3 against FL2. Essential for the analysis of fluorescence of cells is their separation from free *Leishmania* by their light scattering properties. Since light scattering of *Leishmania* is largely different from that of U-937 cells, a logarithmic scale was more appropriate for analysis than a linear scale used commonly in the analysis of blood samples. Since the fluorescence dyes used in this study exhibited a broad emission spectrum, proper compensation of fluorescence overflow is very important to achieve clear separation of singly and doubly (mixed) infected cells. In the flow cytometer we have used, direct compensation of FL1 against FL3 and vice versa was not possible. Therefore, FL3 was compensated against FL2 because the spectral overlap from FL1 runs through FL2. Using this modification, good compensation, and in turn, good resolution of singly and doubly infected cells was possible. After the analysis of the single and double infections, three samples that appeared to have high infection rate percentage of the double labeling were chosen for FACS: U-937 + *Lm*/*BCECF-Am + *Ld*/SYTO-17, U-937 + *Lm*/BCECF-Am + Li/SYTO-17, and U-937 + *Ld*/BCECF-Am + Li/SYTO-17.

Fluorescence gates, in which double-labeled cells appeared, combined with light scatter gates, were used as sort gates. The exclusion mode, which is appropriate for the purity and recovery to be detected, was used. After sorting the target cells, the pellets of the infected cells were collected by centrifugation at 275 × g for 20 min at room temperature, resuspended in RPMI 1640 medium without phenol red, L-glutamine, and FCS, and incubated using the growth conditions of the U-937 cells. After 24 hr, the infected cells were washed with PBS and resuspended in RPMI 1640 medium. Measurement and analysis were done by FACS. Observations and photographs were obtained using fluorescent microscopy.

**Films.** Two color film types were used in this study (for both color photographs and slides): Kodak 36 Ektachrome Elite 400, daylight (Eastman Kodak, Rochester, NY) and Fujichrome 36 process CR-56-E6 RH 135 Dx, daylight (Fuji, Tokyo, Japan).

**RESULTS**

Flow cytometry of fluorescence-stained single strains of *Leishmania* species and experiments with mixtures of two differently stained *Leishmania* spp. at different ratios indicates that the best discrimination of a species can be achieved with BCECF-Am and SYTO-17 as fluorescence labels. Among the samples analyzed after three, five, six, and 24 hr of incubation after infection of U-937 cells, the best results were obtained 6 hr after infection.

Flow cytometry of infected U-937 cells was performed using gates for selection of these cells by their forward and orthogonal light scatter, which differs markedly from that of *free Leishmania*. Ten thousand events of cells, uninfected and infected as well, were analyzed for their specific fluorescence, which indicated their state of infection. All data presented here were obtained with the fluorescence-activated cell sorter (Becton-Dickinson).

Figure 1a shows the distribution of uninfected cells under the experimental conditions selected. Eighty-five percent of the cells are located in the lower left area where cells without or with low autofluorescence are expected. The cells (10.82%) in the upper right area indicate a significant proportion of substantial autofluorescence, which is typical for phagocytizing cells. Less than 10% were detected in the upper left and lower right areas, where one would expect specific red or green fluorescence. Cells infected with unstained parasites resulted in the same pattern.

Figure 1b shows U-937 cells infected with *L. mexicana amazonensis* stained with BCECF-Am (*Lm*/BCECF-Am). In this case, almost 85.69% of all cells were in the lower right area, indicating a significant green fluorescence of these cells, which could only be caused by infection of fluorescent-labeled *Lm*/BCECF-Am. The fact that only 3.64% persist in the lower left area indicates that almost all cells are infected.

Infection with SYTO-17-stained *L. mexicana amazonensis* (*Lm*/SYTO-17) resulted in a totally different picture (Figure 1c). A total of 84.10% of the cells correspond to the upper left area, indicating a high percentage of infection with SYTO-17-stained *Leishmania*, resulting in a red fluorescence of cells.

Figure 1d shows the results of the analysis of *Lm*/BCECF-Am and Li/SYTO-17 mixed-infected cells. The ratio of *Li to Lm* was 1:1, and the ratio of U-937 cells to total
Leishmania was 1:10. A total of 89.69% of the cells are located in the upper right region, where only successfully mixed-infected cells could be expected. Less than 10% of the uninfected cells had enough autofluorescence to appear in this region (compare with Figure 1a). These results could easily be repeated with the same Leishmania species and in principal with other combinations of stained Leishmania. Figure 1e shows the results with Lma/BCECF-Am and Ld/SYTO-17 mixed infected cells. The picture is almost identical.

To obtain more data, we did experiments with reciprocally stained promastigotes. In the case of Ld/BCECF-Am and Lma/SYTO-17 (Figure 1f and compare with Figure 1d), one can see that there is a high proportion of mixed infected cells, but that the maximum of fluorescence is at a different position.

All experiments were evaluated in parallel by microscopic examination. Photographs were taken to demonstrate the infections and mixed infections and to understand what are the causes of the different fluorescence. Figures 2a–2d show cells infected only with one Leishmania species. The BCECF-Am dye is more easily transferred to the host cell.
than SYTO-17. Figures 2e and 2f show two examples of cells with mixed infections.

The cells with mixed infections were then sorted and incubated for 24 hr in RPMI 1640 medium without phenol red, L-glutamine and FCS. After 24 hr, the original cells with mixed infections were scanned again. The results are shown in Figure 1g. More than 60% of the cells appear to be doubly infected (upper right area), but 16.78% of the cells were uninfected (lower left area) and a substantial proportion appeared to be singly infected (lower right area).

In the case of superinfection of previously infected macrophages, 3 hr after infection with a different strain, a slight prevalence of the first infecting strain could be seen only in the first hour. Three hours after infection with the second strain, the result was identical with that after simultaneous infection at zero time.

**DISCUSSION**

There are some data concerning the outcome of cocultivation of promastigotes of different species. When the strains cultivated in suspension were compared, we observed considerable differences in growth rates; therefore, one can find after a certain period only one survivor strain. Our experiments could help to establish an **in vitro** system that enables one to study the coexistence of two species within one host cell.

We attempted to ensure a ratio of 1:1 between the two coinfesting **Leishmania** species and a ratio of 10:1 of **Leishmania** to cells to be infected. The fact that a reciprocal staining procedure resulted in different fluorescence maximums indicates that the infection by different species results in different efficiencies and kinetics of parasite internalization (Figures 1f and 1d). In single infection experiments, the infectivity of **L. mexicana amazonensis** was higher than that of **L. infantum**. Therefore, the dominance of red fluorescence in Figure 1f indicates the higher prevalence of **L. mexicana amazonensis** in the U-937 cells. This fact could also explain the dominance of **L. mexicana amazonensis** in singly infected cells after 24 hr (Figure 1g). The relatively high percentage (16.24%) of cells in the lower right area indicates a single infection with **L. mexicana amazonensis**.

It is apparent that the dyes can influence the course of infection by altering the parasites and eventually the viability of the stained parasites. We stained each strain with both dyes and showed that the dye itself has only minor effects on the outcome of mixed infection. The dyes did not seem to inhibit the growth of promastigotes in culture.

Microscopic examination revealed that even in the sorted cell population with mixed infections, there was a considerable number of uninfected cells. This was consistent with the results of earlier experiments in which the percentage of infected cells reached a maximum at 65%, but contained a high proportion of cells with single infections. The discrepancy could be explained by the fact that the cell sorter also separates cells that have promastigotes only on their surface. Despite the fact that this attachment is reversible in some cases, we did not count these cells as infected during microscopic examination.

The main advantage of using the fluorescence-activated cell scanner is that it can analyze large number of cells or promastigotes; therefore one can obtain statistically significant results. Thus, despite the fact that only a small portion of the cells contain more than four **Leishmania** (and even more rarely two of each species), one can statistically analyze mixed infections with this system. One can enrich doubly infected cells populations by using a fluorescence-activated cell sorter, specifically, by optimizing the procedure: using a smaller gate specific for a given combination of **Leishmania** species and dyes, one can get a cell suspension with a relatively high percentage of doubly infected cells. Our experiments also show that this system could be used to study the outcome of mixed infections over a period of at least 24 hr.

The SYTO-17 dye was used because its strongly stains **Leishmania**. It also appeared that **Leishmania** stained with this dye did not show an alteration in their motility. A disadvantage of this dye was that the emission maximum is at 622 nm without RNA and DNA and at 634 nm with DNA or RNA, which is outside the channel FL2. In addition, it also showed a relative high fluorescence at lower wavelengths. This deficiency was overcome by compensating FL3 against FL2.

There are data indicating that mixed infections occur in humans, which has implications for diagnosis and therapy. However, there are no data indicating that mixed infections occur in one cell. Mixed infections have also been observed in sand flies. However, it has not been shown that genetic recombination occurs in **Leishmania**. We will attempt to show that genetic recombination occurs in this organism by sorting populations of cells with mixed infections. The present work is a prerequisite to do this. In this study, we have shown that mixed infections with different genetically characterized strains occur and cells with mixed infections could be a subject for further studies.

It has been demonstrated that the exchange of resistance to hypomycin (G-418) B introduced into **Leishmania** by plasmids can occur (Laban A, unpublished data). Although actual genetic recombination was not proven, the exchange of genetic material in a mixed promastigote culture was demonstrated. Since we were able to detect cells with mixed infections after 24 hr, it is possible that genetic recombination occurs during this phase of the common presence of the parasites in one cell.

In nature, a simultaneous mixed infection of one cell is probably rare because the multiplicity of infection (number

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**FIGURE 2.** Micrographs taken in parallel to the scanning and sorting procedures. **a**, **Leishmania mexicana amazonensis** (Lma)/BCECF-Am infected U-937 cells showing typical green fluorescence; **b**, Lma/SYTO-17 infected U-937 cells showing typical red fluorescence; **c**, **L. donovani** DD8 (Ld/BCECF-Am infected U-937 cells; **d**, Ld/SYTO-17 infected U-937 cells; **e**, Lma/BCECF-Am and Ld/SYTO-17 doubly infected cells (see Figure 1e); **f**, Ld/BCECF-Am and **L. infantum** D2 (Li/SYTO-17 doubly infected cells; **g**, Lma/BCECF-Am and Li/SYTO-17 doubly infected cells. All micrographs were observed with an oil-immersion objective (100×). (Original magnification × 1,000.)
of parasites per cell) immediately after a sandfly bite is too low. To get a better understanding of the actual events involved in this process, we are attempting to demonstrate whether there is a difference between simultaneous mixed infection and superinfection to show if one preinfecting parasite can exclude (or enhance) superinfection by another one. Initial results indicate that there is no mutual exclusion if a second infection with a different strain occurs after 3 hr. We are also testing other combinations of strains, especially those more epidemiologically relevant, to see if prolongation of the single-infection period up to one day will change the influence of the pre-existing strain.

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