Leishmania donovani infects lymphocyte cell lines *in vitro*

Partha Pratim Manna, Anirban Basu, Asim Saha, Md Quamarul Hassan, Sandeep Mukherjee, Sabita Majumdar, Samit Adhya and Santu Bandyopadhyay

Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Calcutta 700 032, India

Cellular interactions between *Leishmania donovani* parasites and human B (Daudi) and T (HUT78) cell lines were studied *in vitro*. Both promastigotes and amastigotes of *L. donovani* gained entry in approximately 60% of these cells. Transmission electron microscopy revealed the existence of intracellular amastigotes in these cell lines after infection with *L. donovani*. Immunophenotyping by flow cytometry confirmed the lymphocytic lineages of these cell lines. There was no evidence for intracellular replication of *L. donovani* in T and B cell lines. The number of intracellular amastigotes peaked on second day post-infection then gradually declined in a time-dependent manner in both the cell lines. Intracellular parasites remained more viable and transformation-competent when infection was performed with amastigotes. Attachment to the cell surface but no internalization of parasites was observed when infection was attempted on human amnion cell line WISH. Amastigotes, not the promastigotes of *L. donovani* induced IL-12 production by both monocyte and B cell lines.

VISCERAL leishmaniasis (VL) is caused by the kinetoplastid protozoon *Leishmania donovani*, an intracellular parasite which survives and multiplies within mammalian macrophages. Patients usually have many immunological dysfunctions of T cells[1,2] and B cells[3]. For years, the macrophage has been thought to be the predominant cellular target for *Leishmania*, and the immunopathology of VL has been explained on this basis. Previously, a number of reports suggested infection of fibroblasts[4], dog sarcoma cells[5] and dendritic cells[6] by other species of *Leishmania in vitro*. However, the *in vivo* relevance of these observations is unclear. In the present report, we demonstrate, for the first time, the infection of human T and B cell lines by *L. donovani in vitro*.

Human B cell line Daudi[7], T cell line HUT78 (ref. 8) and monocyte cell line U937 (ref. 9) were incubated with *L. donovani* (pathogenic strain AG83 (ref. 10)) amastigotes (freshly isolated from golden hamster spleen) at a multiplicity of infection of 20 for 1 day for 37°C. Free amastigotes were removed by differential centrifugation at 700 rpm for 5 min, the cells were resuspended in RPMI-1640 containing 10% fetal bovine serum and were incubated at 37°C, 5% CO_2. At different time intervals, cells were withdrawn from culture for Giemsa staining. Intracellular amastigotes were quantitated by two parameters by taking into account the number amastigotes/100 randomly selected cells (which incl both uninfected and infected cells) and by the num of amastigotes/100 infected cells only (Figure 1). shown in Figure 1, almost 60% of Daudi and HU cells have intracellular parasites by day 2. On the o hand, over 75% of U937 cells became infected by time. After an early rise, the number of intracell parasites (per infected cell) gradually declined with t

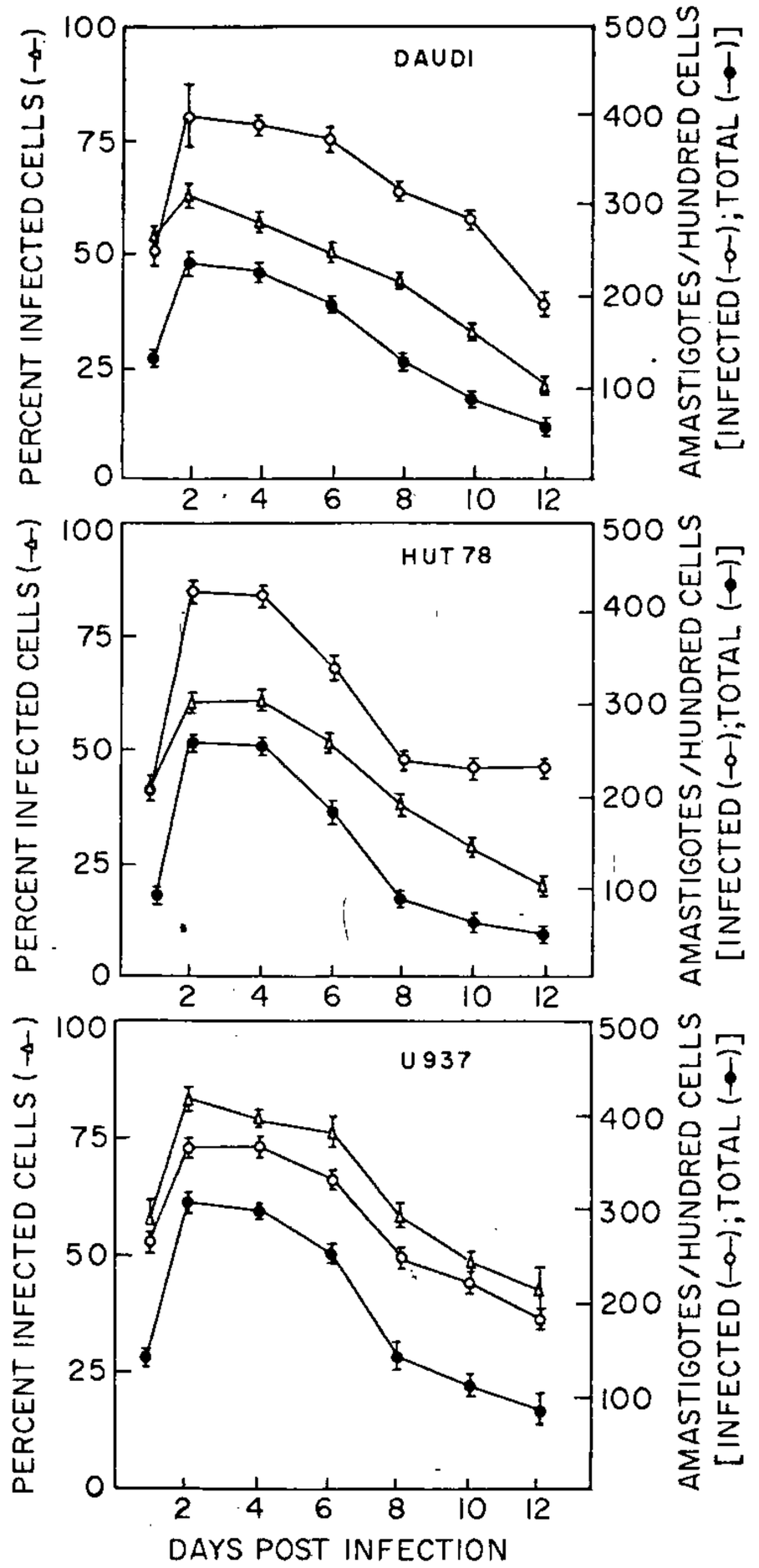

Figure 1. *In vitro* infection of Daudi (B cell line). HUT78 (T line), and U937 (monocyte cell line) cells with *L. donovani* amastigo *L. donovani* (strain AG83) amastigotes were freshly isolated f spleens of 2 month post-infected golden hamsters. Amastigotes v incubated with cell lines at a multiplicity of infection (MOI) of for 1 day at 37°C. Free amastigotes were removed by low sp centrifugation, and the cells were cultured at 37°C, 5% CO_2 in well plates. Medium was changed every other day and cells v withdrawn at different time points for Giemsa staining. At least hundred cells for each time point were examined.

in all the cell lines tested. Parasite clearance was slightly slower in U937 cells. Apparently, there seems to be no replication of parasites even in the monocyte cell line U937. This is expected since U937 cells only after treatment with phorbol myristate acetate (PMA) differentiate into nondividing adherent macrophages and support replication of *Leishmania*[11]. Our results with PMA-treated U937 cells (not shown) are in agreement with the previous report. However, both HUT78 and Daudi cell lines being dividing nonadherent suspension cultures, U937 cells (which are also dividing and nonadherent) rather than PMA-treated U937 cells (which are adherent and nondividing) were used as the positive control. Giemsa-stained micrographs of *L. donovani* infected Daudi, HUT78 and U937 cells are shown in Figure 2. Human amnion cell line WISH[12] was used as a control in *in vitro* infection experiments. Attachment to the cell surface but no internalization of parasites (amastigotes or promastigotes) was observed in this cell line.

To confirm that parasites are truely intracellular rather than merely cell-associated, transmission electron microscopy was performed with *L. donovani*-infected Daudi, HUT78 and U937 cell lines on day 2 post-infection as described earlier[13]. Briefly, infected cells were fixed with 6% glutaraldehyde in 0.125 M phosphate buffer (pH 7.2) for 14–16 h and then with 1% osmium tetroxide for 16–20 h at room temperature. The fixed cells were washed for 2 h in 0.5% uranyl acetate, dehydrated with increasing concentrations of ethanol, embedded in Spurr medium[13] at 70°C for 48 h. Sections were cut with a Du Pont diamond knife in an LKB ultra microtome, stained with uranyl acetate and lead citrate, and examined under a JEOL 100CX transmission electron microscope at 60 kV. All these three cell lines were found to contain intracellular amastigotes. One tramsmission electron micrograph of *L. donovani*-infected Daudi cell is shown in Figure 3, which shows the presence of six intracellular amastigotes.

The early rise in parasite numbers per cell (on day 2 post-infection) in both T and B cell lines may possibly be accounted for by a continuous invasion of cultured cells by residual parasites rather than their intracellular replication in majority of cells; the subsequent decline in parasite numbers could be due to death of infected cells and/or outgrowth of uninfected cells.

In vitro infection of these cell lines was also attempted with the flagellated promastigotes of *L. donovani*. Promastigotes were equally effective as amastigotes in infecting these cells. All promastigotes which entered into Daudi or HUT78 cells transformed into amastigotes, as observed with the monocyte cell line U937. After an early rise on day 2, the number of intracellular parasites declined with time as observed with the amastigotes (not shown). We also examined the interaction of normal human lymphocytes with *L. donovani*. Both resting lymphocytes and T cell blasts were used. Resting

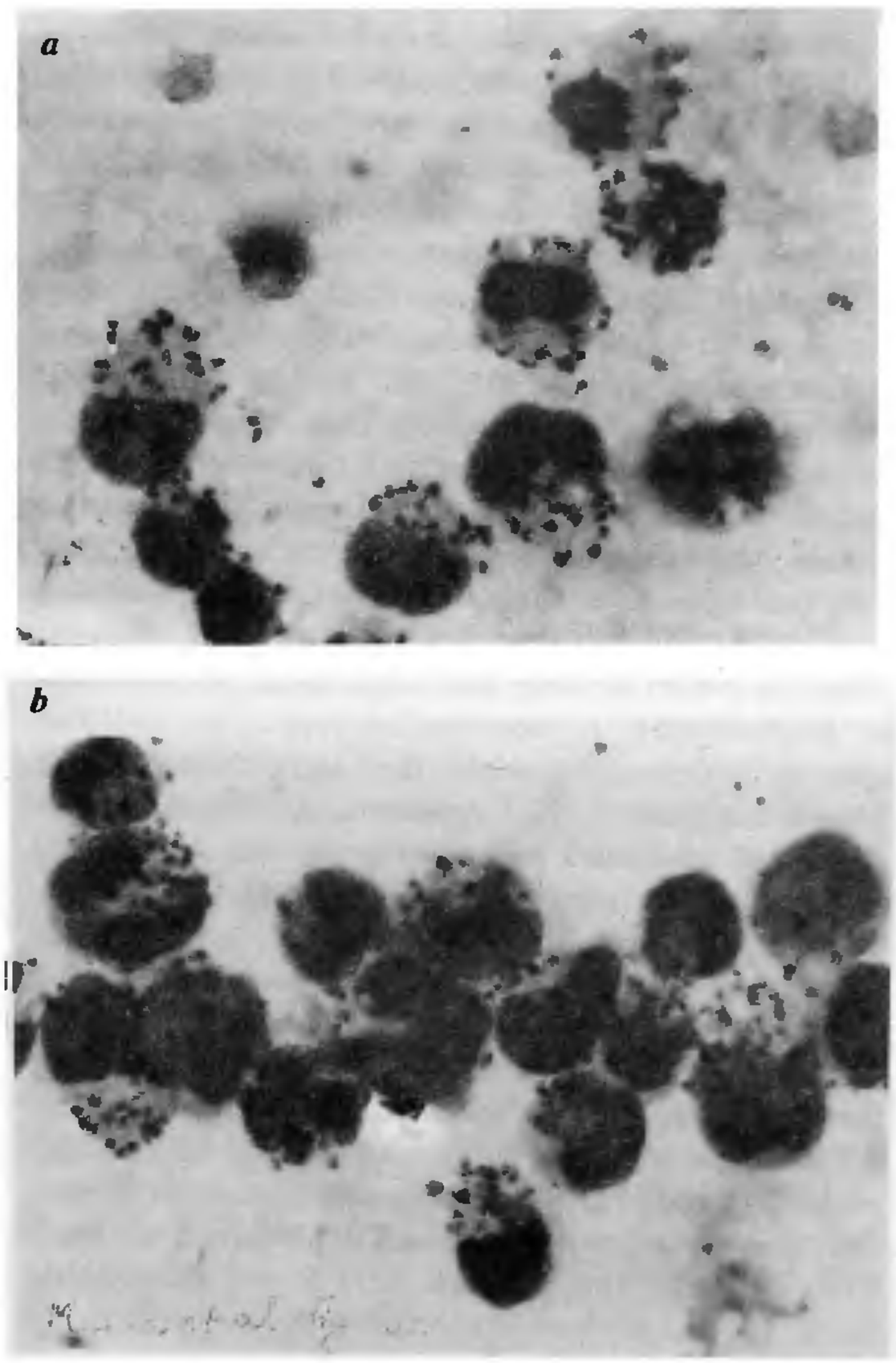

Figure 2. Giemsa staining of *L. donovani* infected Daudi (*a*), HUT78 (*b*) and U937 (*c*) cells. Amastigote/cell ratio, 20 : 1; magnification, × 1000. Two days post-infected cells are shown.

lymphocytes were prepared by exhaustively depleting monocytes from human peripheral blood mononuclear cells (PBMC) by plastic adherence (X2) followed by removal of residual monocytes by treatment with anti-CD14 monoclonal antibody B52.1 (ref. 14) plus baby rabbit complement. Repeated attempts to infect these cells *in vitro* with amastigotes of *L. donovani* failed. However, preliminary data suggest that proliferating T cell blasts prepared by treating human PBMC with PHA (2.5 µg/ml) followed by culture in IL-2 (30 U/ml) containing media for three days were infected by amastigotes of *L. donovani*.

Intracellular parasites in Daudi cells remained viable for at least two weeks when infection was performed with amastigotes (Table 1). Promastigote-infected cells yielded very few, if at all, live promastigotes after transformation. *L. donovani* amastigotes alone cultured in medium (without these cell lines) at 37°C even for a shorter period of time (10 days) failed to transform to promastigotes. *L. donovani* promastigotes cultured alone at 37°C for 10 days also died and did not replicate when transferred to 22°C. As additional control, amastigotes of *L. donovani* were cocultured with WISH cells at 37°C over a period of 14 days and checked for amastigote survival by transforming them at 22°C. All amastigotes died at 37°C when cocultured for 7 days or more even in the presence of WISH cells.

Immunophenotyping of HUT78 and Daudi cells using flow cytometry[15] confirmed their lymphocyte lineages. As shown in Figure 4, 93.4% of HUT78 cells are CD3 positive. Similarly, Daudi cells are positively stained for cytoplasmic immunoglobulins (97.8% cells are positively stained). Thus, studied HUT78 and Daudi cells are T and B cell lines respectively.

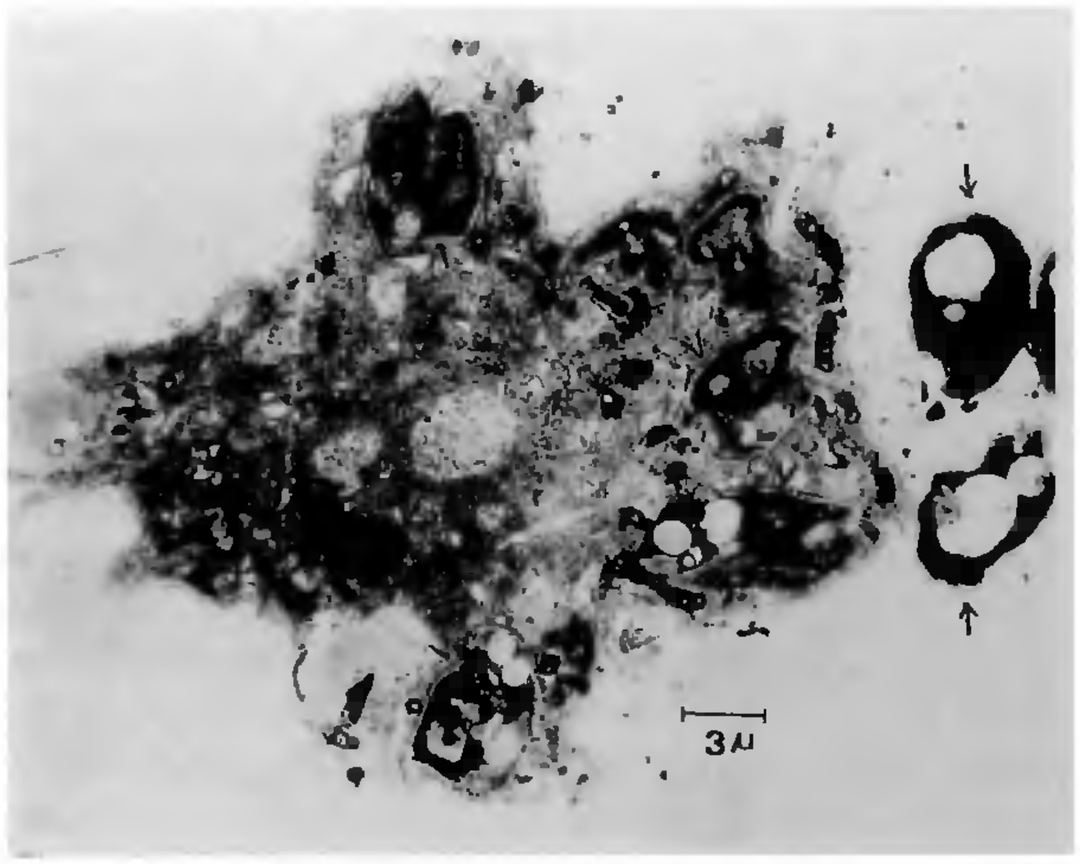

Figure 3. Transmission electron micrograph of a representative Daudi cell 2 days post-infected with *L. donovani* amastigotes (amastigote/cell ratio, 20 : 1). Note the presence of six healthy intracellular amastigotes (^). Two extracellular amastigotes, possibly residual, are also seen in the field (↑).

The specificity of uptake of *L. donovani* by Daudi, HUT78 and U937 cell lines was tested by measuring phagocytic activity of these cells using sheep red blood cells (SRBC) as targets. Effector cells were incubated with target cells at an effector to target ratio of 1 : 10 for 18 h at 37°C in 5% CO_2. Effectors were separated from targets by Ficoll-Hypaque density gradient centrifugation, washed, stained with Giemsa and examined microscopically for quantitation of phagocytosed SRBC. U937 cells phagocytosed SRBC (with 140 ± 13.4 SRBC/100 U937 cells) as expected. However, Daudi or HUT78 cells had no intracellular SRBC. Thus, uptake of *L. donovani* by B or T cell line was not attributable to merely nonspecific phagocytosis, rather specific receptor–ligand interactions may be occurring. Nonphagocytic nature of HUT78 and Daudi cell lines confirmed that these cell lines have not acquired strange characteristics because of immortalization that are not typical of non-transformed lymphocytes.

Induction of IL-12 by monocyte and B cell lines in response to *L. donovani* was also examined. Indicated cell lines were cocultured with *L. donovani* (both promastigotes and amastigotes; multiplicity of infection, 20) at 37°C in 5% CO_2 for 48 h. Cell-free culture supernatants were collected, filtered through 0.2 µm filters and assayed for IL-12 by anti-human IL-12 neutralizable human lymphoblast proliferation assay[16]. As a positive control, cell lines were also incubated with heat killed *Staphylo-*

Table 1. Intracellular survival of *L. donovani* parasites in Daudi, HUT78 and U937 cells

Cells[a]	Number of transformed promastigotes recovered[b]
Promastigote-infected Daudi	3750 ± 1767[c]
Amastigote-infected Daudi	12500 ± 3535
Promastigote-infected HUT78	2500[d]
Amastigote-infected HUT78	3750 ± 1767
Promastigote-infected U937	2500[d]
Amastigote-infected U937	11250 ± 1767
Promastigotes alone	ND
Amastigotes alone	ND
Amastigotes cocultured with WISH	ND

a: Cells were infected *in vitro* with promastigotes or amastigotes of *L. donovani* with parasite/cell ratio of 20 : 1. After removing free parasites by low speed centrifugation, cells were cultured at 37°C in 5% CO_2 in 96 well plates (with medium change every other day).

b: Intracellular amastigotes were released by disrupting 14 days post-infected cells (3×10^5) with one cycle of freeze-thaw. Amastigotes were then allowed to transform into promastigotes by culturing at 22°C in 1 ml of Schneider's *Drosophila* medium for 3 days. Transformed motile promastigotes were counted in a haemocytometer microscopically in 10 µl aliquots, then numbers/ml were calculated.

c: Mean ± SD of duplicate cultures.

d: Representative of duplicate cultures with identical value.

ND: Not detectable.

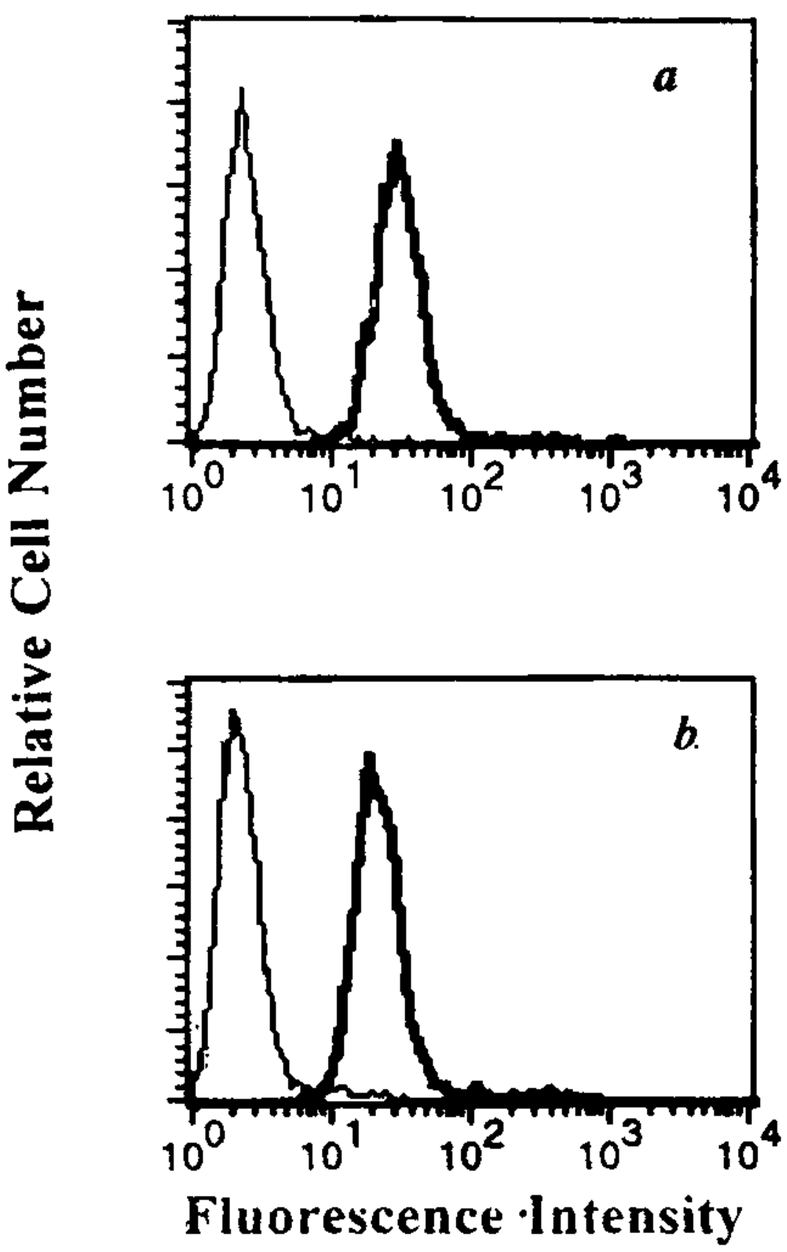

Figure 4. Immunophenotyping of HUT78 (*a*) and Daudi (*b*) cells by flow cytometry. One-parameter histograms showing surface staining of HUT78 cells with FITC-labeled control mAb (light line) and FITC-labeled anti-CD3 mAb (bold line); intracellular staining of Daudi cells with FITC-labeled anti-mouse immunoglobulins (light line) and FITC-labeled anti-human immunoglobulins (bold line). Surface staining of HUT78 cells was performed as described earlier[15]. For cytoplasmic staining, Daudi cells were fixed with 1% paraformaldehyde, permeabilized by treatment with 50% methanol, then stained with FITC-labeled antibodies. After staining, cells were analysed in FACS calibur (Becton Dickinson, USA) using Cell Quest Programme.

coccus aureus (SAC) Cowan strain I (1 : 1000 final dilution, purchased from Pansorbin, Calbiochem-Behring Corp., CA, USA). Each supernatant was also pre-incubated with anti-human IL-12 monoclonal antibody C8.6.2 (ref. 17) (1 : 200 dilution of ascitic fluid, kindly provided by Prof. Giorgio Trinchieri, Wistar Institute, Philadelphia, USA) before addition to lymphoblast cultures. Our data indicate for the first time that *L. donovani* amastigotes not promastigotes induced IL-12 production by both monocyte and B cell lines (Table 2). Promastigotes of *L. donovani* suppressed the induction of IL-12 by secondary stimuli (SAC) in monocyte cell line but not in the B cell line. These data are in agreement with that of Carrera *et al.*[18] who demonstrated that *L. major* promastigotes can actively suppress the induction of IL-12 in murine bone marrow macrophages by secondary stimuli. In contrast to promastigotes, amastigotes of *L. donovani* were not only able to induce IL-12 by both the cell lines, this form of the parasite also augmented IL-12 induction by SAC.

The mechanism of uptake of *L. donovani in vitro* by lymphocytes remains unknown. The interaction between *Leishmania* and the macrophage surface is complex[19] involving as ligand, the parasite surface components gp63 (a glycoprotein), lipophosphoglycan (LPG) as well as opsonized complement factor C3b, C3bi and macrophage receptors including complement receptors CR1, CR3 and the integrins LFA-1 and P150.95. Integrins of the LFA-1 family recognize the carbohydrate moiety of *Leishmania* LPG[19]. Of note, some of these receptors are shared by lymphocytes and one or more of these receptors could be utilized by the parasite to gain entry into lymphocytes.

Table 2. Production of IL-12 by U937 and Daudi cells in response to *L. donovani* and heat-inactivated *Staphylococcus aureus* (SAC)

Inducers	Anti-human IL-12 antibody (C8.6.2)	IL-12 titer in the supernatant (pg/ml)[a]	
		U937	Daudi
Medium	–	< 3.2	< 3.2
SAC	–	60.48 ± 6.79	12.03 ± 0.47
	+	< 3.2	< 3.2
Promastigotes	–	< 3.2	< 3.2
	+	< 3.2	< 3.2
Amastigotes	–	9.6 ± 3.3	12.81 ± 2.72
	+	< 3.2	< 3.2
Promastigotes + SAC	–	< 3.2	15.40 ± 2.72
	+	< 3.2	< 3.2
Amastigotes + SAC	–	133.12 ± 23.70	21.87 ± 6.27
	+	< 3.2	< 3.2

a: IL-12 was assayed in the supernatants as described earlier[16]. Results presented are mean ± SD of triplicate determinations.

In conclusion, our data indicate that *L. donovani* infects and survives in lymphocyte cell lines *in vitro*. Many of the immunopathological responses in visceral leishmaniasis have traditionally been attributed to infection of macrophages. Our present findings raise the possibility that lymphocytes may be infected *in vivo* contributing to their observed functional impairment in visceral leishmaniasis. This possibility merits careful study with patients. Production of IL-12 by B cell line in response to *L. donovani* suggests that B cells may play a key role in the early phase of *Leishmania* infection.

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Estimation of sublethal toxicity of zinc chloride by histopathological analysis of fish (*Heteropneustes fossilis*, Bloch) epidermis

S. Hemalatha and T. K. Banerjee

Histochemistry and Histopathology Laboratory, Centre of Advanced Study, Department of Zoology, Banaras Hindu University, Varanasi 221 005, India

The effect of zinc chloride on the outer (OE) and inner (IE) opercular epidermis of *Heteropneustes fossilis* has been investigated. The main toxicopathological alterations of the OE include extensive intercellular as well as intracellular vacuolization and hyperplasia of the epithelial cells (ECs) with regular exfoliation of round or globular ECs from the skin surface. Splitting of the epidermis from the junction of the outermost (OL) and middle layers (ML) leading to the lifting of the OL is sometimes also noticed. The mucous cells (MCs) show periodic fluctuations in their density and staining properties. Extensive vacuolization of the epidermis along with hyperplasia of ECs along with periodic fluctuations in the density and staining behaviour of the MCs are the main alterations observed in the IE. The mucogenic activity of the IE throughout the exposure period remains mostly above the control level. Hence the damage is comparatively less severe. All these histo-pathological manifestations may be considered for their use for testing the quality of variously contaminated water samples.

HEAVY metal pollution represents a threat to the aquatic biota. The occurrence of metal contaminants especially zinc in excess of natural loads has become a problem of increasing concern. This situation has arisen as a result of the rapid growth in population, increased urbanization, expansion of industrial activities, exploration and exploitation of natural resources as well as lack of environmental regulations. The gills have extensively been used as a potential indicator for disturbed aquatic environment. The other important organ system that also gets similar flooding is the skin. However, data dealing with the impact of various heavy metals including zinc salts are very scanty[1–6]. While studying the acute toxic impact of heavy metal salts on melanophore morphology, Banerjee and his associates[5,7] have demonstrated the importance of melanophore indexing in testing water qualities contaminated with lethal concentrations of mercury and zinc chloride. Application of similar melanophore indexing bio-assay technique, however, failed to evaluate the water samples polluted with sublethal concentration of heavy metal salts including zinc chloride. Hence in this paper efforts have been made to evaluate the effect of zinc chloride on