Distribution of MHC class I and of MHC class II molecules in macrophages infected with *Leishmania amazonensis*

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SUMMARY

Macrophages, being apparently the only cells that in vivo allow the growth of the intracellular pathogen *Leishmania*, are likely candidates to present antigens to *Leishmania*-specific CD4+ and CD8+ T lymphocytes, known to be involved in the resolution or in the development of lesions induced by these parasites, and recognizing processed antigens bound to MHC class I and MHC class II molecules, respectively. In the present study, we analysed by confocal microscopy and by immunoelectron microscopy the subcellular distribution of both MHC class I and class II molecules in mouse (Balb/c and C57BL/6 strains) bone marrow-derived macrophages infected for 12 to 48 hours with *Leishmania amazonensis* amastigotes and activated with gamma interferon to determine the intracellular sites where *Leishmania* antigens and MHC molecules meet and can possibly interact. Double labellings with anti-MHC molecule antibodies and with either propidium iodide or an anti-amastigote antibody allowed localization of MHC molecules with regard to the endocytic compartments housing *Leishmania* amastigotes, organelles known as the parasitophorous vacuoles (PV) and which most likely contain the highest concentration of parasite antigens in the host cell. Both uninfected and infected macrophages from Balb/c mice expressed the MHC class I molecules H-2K^d and H-2D^d on their cell surface but no significant amount of these molecules could be detected in the PV, which indicates that, if infected macrophages play a role in the induction of *Leishmania*-specific CD8+ T lymphocytes, PV are probably not loading compartments for MHC class I molecules.

In contrast, MHC class II molecules were found to be associated with the PV membranes as shown previously with microscopic techniques at lower resolution (Antoine et al. *Infect. Immun.* 59, 764-775, 1991). In addition, we show here that, 48 hours after infection of Balb/c macrophages, in about 90% of PV containing MHC class II molecules, the latter were mainly or solely localized at the attachment zone of amastigotes to PV membranes. This peculiar distribution, especially well demonstrated using confocal microscopy, was confirmed by subcellular fluorescence cytometry of infected macrophages stained for the MHC class II molecules. The following data agree with the idea that PV-associated MHC class II molecules establish specific interactions with plasma membrane components of amastigotes. First, the polarized localization of class II appeared specific to these molecules, since the distribution of the lysosomal glycoproteins lgp110 and lgp120, of the macrosialin (a macrophage-specific marker of endocytic compartments) and of the GTP-binding protein rab7p, shown here as being PV membrane components, was homogeneous. Second, after killing of *Leishmania* with the leishmanicidal drug L-leucine methyl ester, MHC class II molecules remained associated for several hours with remnants of the parasites still bound to the PV membrane. Finally, polarized PV-associated MHC class II molecules of infected Balb/c and C57BL/6 macrophages could be stained with the 14-4-4S and Y-3P monoclonal antibodies, respectively; antibodies that have been described as being much more reactive with the compact conformers of the MHC class II molecules carrying tightly associated peptides.

Key words: macrophage, *Leishmania amazonensis*, phagolysosome, lysosome, prelysosome, MHC class I molecule, MHC class II molecule, confocal microscopy, immunoelectron microscopy

INTRODUCTION

Leishmania are protozoan parasites belonging to the Family Trypanosomatidae. They cycle between diptera vectors and mammalian hosts including man and are at the origin of various human parasitic diseases that are endemic in many parts of the world. In their amastigote stage, they are obligate intracellular parasites that seem to multiply exclusively in

macrophages, within membrane-bound parasitophorous vacuoles (PV). In mouse and rat macrophages infected for 24 to 72 hours, these organelles exhibit at least certain characteristics of the host cell lysosomal compartment. They maintain a strongly acidic pH (Chang, 1980a; Antoine et al., 1990) and contain several if not a full set of active lysosomal enzymes (Antoine et al., 1987; Prina et al., 1990; Russell et al., 1992). Their membrane is positive for lysosomal glycoproteins and at this stage is devoid of the cation-independent mannose 6-phosphate receptor (CI-MPR), a membrane protein of the endocytic pathway generally excluded from the lysosomes (Antoine et al., 1991; Russell et al., 1992). Furthermore, the PV membrane of infected macrophages activated with gamma interferon (IFN-γ) contain MHC class II molecules (Antoine et al., 1991).

Whether infected murine macrophages are good candidates to present *Leishmania* antigens (Ags) to specific CD4^{+} T lymphocytes known to be involved either in the resolution or in the aggravation of some *Leishmania* infections (for a review see Liew, 1989; Locksley et al., 1991; Locksley and Louis, 1992) remains to be determined precisely. The properties of the PV described above suggest however that these compartments are potential sites where limited proteolytic degradation (processing) of *Leishmania* protein Ags could occur as well as the loading of MHC class II molecules with parasite peptides, both events being required for the presentation of exogenous Ags to CD4^{+} T lymphocytes (for a review see Brodsky and Guagliardi, 1991). This hypothesis is sustained by recent findings indicating that phagolysosomes containing killed or degradable bacteria, or PV containing *Leishmania* promastigotes, with properties very similar to those of PV housing *Leishmania* amastigotes, are very likely involved in the processing and presentation of bacterial or parasite antigens (Lang and Kaye, 1991; Pfeifer et al., 1992; Harding and Geuze, 1992). It must be stressed however that a fundamental difference distinguishes these studies from ours, since the microorganisms used were heat-killed before being in contact with the macrophages (Harding and Geuze, 1992) or were used live but rapidly destroyed by macrophages (Lang and Kaye, 1991; Pfeifer et al., 1992).

Recent data also emphasize the importance of *Leishmania*-specific CD8^{+} T lymphocytes in the resolution of murine leishmaniasis due to *L. major* or *L. donovani* (Titus et al., 1987; Stern et al., 1988; Hill et al., 1989; Müller et al., 1991). Triggering of these lymphocytes implies that certain processed parasite Ags bind to MHC class I molecules. How *Leishmania* Ag meet class I molecules is intriguing, since, in general, peptides bound to MHC class I molecules are derived from Ags processed in the cytosol or in the secretory pathway of antigen-presenting cells (APC) (for a review, see Brodsky and Guagliardi, 1991; Monaco, 1992). Recent findings showing that exogenous Ags phagocytized by macrophages can be presented in association with MHC class I molecules are also difficult to reconcile with the classical class I-presenting pathway (Debrick et al., 1991; Rock et al., 1993; Pfeifer et al., 1993). One possible explanation for these unexpected results would be that MHC class I molecules reach phagocytic compartments in macrophages.

One of the purposes of this investigation was thus to determine if MHC class I molecules reach PV of mouse macrophages infected with *L. amazonensis* amastigotes. No

significant amount of MHC class I molecules can be detected in these compartments by confocal microscopy and immuno-electron microscopy, suggesting that *Leishmania* Ag and MHC class I molecules do not meet in PV. The present work also extends our previous results demonstrating the presence of MHC class II molecules in PV of macrophages activated by IFN-γ. Here we show that most MHC class II molecules located in PV membranes are polarized, tightly associated with amastigotes and possibly involved in interactions with parasite components.

MATERIALS AND METHODS

Mice

Female Balb/c and C57BL/6 mice aged 2 to 4 months were obtained from the Pasteur Institute animal facilities. They were used for the preparation of bone marrow cells and for the propagation of amastigotes.

Parasites

Leishmania amazonensis strain LV79 (WHO reference number MPRO/BR/72/M1841) was maintained virulent by passage in Balb/c mice. Amastigotes were inoculated into the hind footpads as described earlier (Antoine et al., 1988). Two to 4 months later, amastigotes were purified from disrupted lesions by differential centrifugations followed by a centrifugation on a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) (Chang, 1980b). Parasites were used within 24 hours after disruption of the lesions.

Culture of macrophages

Bone marrow cells prepared from femurs and tibias of mice were cultured as described previously in RPMI 1640 medium (Seromed, Berlin, Germany) containing 10% heat-inactivated foetal calf serum (FCS) (Dutscher, Brumath, France), 50 μg/ml gentamycin (Seromed), and macrophage colony-stimulating factor (M-CSF) (Antoine et al., 1991). For light-microscopic studies, cells were distributed in 24-well plates (Corning Glass Works, Corning, NY) containing 12 mm round glass coverslips (1×10^5 to 2×10^5 cells per well). For electron-microscopic studies, 35 mm tissue culture dishes (Corning) were seeded with 2×10^6 cells. After 5 days at 37°C, adherent macrophages were washed with Dulbecco's phosphate-buffered saline (PBS) to remove M-CSF. Macrophages on glass coverslips or in tissue culture dishes were then cultured at 37°C in medium devoid of M-CSF.

Infection of macrophage cultures and activation by IFN-γ

Twenty-four hours after the removal of M-CSF, purified amastigotes were added to macrophage cultures to give a parasite-to-host cell count ratio of 4:1. Before infection, macrophage numbers were determined as described earlier (Antoine et al., 1991). Infected cultures and uninfected cultures run in parallel were then incubated at 34°C. Treatment of macrophages with 2.5 ng/ml murine IFN-γ was done before or after infection as described by Antoine et al. (1991). Two batches of IFN-γ were used in these experiments: (1) rIFN-γ, expressed in *Escherichia coli* and containing 3.2 fg endotoxin/μg, was kindly given by G.R. Adolf (Ernst-Boehringer Institut für Arzneimittelforschung, Vienna, Austria); (2) rIFN-γ, produced by transfected CHO fibroblasts, was purchased from Holland Biotechnology (Leiden, The Netherlands). Its contamination by endotoxin did not exceed 12 pg/μg. After 12 to 48 hours of culture at 34°C, macrophages were processed for light- and electron-microscopic studies.

Treatment of infected macrophages with L-leucine methyl ester

The protocol used was adapted from Rabinovitch et al. (1986). In

these experiments, IFN-γ was given to macrophages 24 hours before parasites were added. Twenty-four hours after infection, macrophages were incubated for 1 hour at 34°C in an air atmosphere with Hanks' solution, 10 mM Hepes-3% FCS alone or containing 2 mM of L-leucine methyl ester (Leu-OMe, Sigma Chemical Co., St Louis, MO). After two washes with PBS, cells were fixed immediately with paraformaldehyde (see below) or reincubated in fresh culture medium for various periods of time at 34°C before fixation and processing for light-microscopic studies.

Immunological reagents

A list of monoclonal (mAbs) and polyclonal antibodies (Abs), of immune sera and of control reagents used in this study is shown in Table 1. The binding of these various Abs on cell preparations was detected using the following conjugates: for light microscopy, peroxidase-labelled sheep anti-rat Ig F(ab′)₂ fragments prepared as described previously (Antoine et al., 1991), fluorescein isothiocyanate (FITC)-conjugated goat anti-rat Ig F(ab′)₂ fragments (mouse Ig-adsorbed) (Caltag Laboratories, San Francisco, CA), FITC-conjugated goat anti-hamster F(ab′)₂ fragments (adsorbed on mouse and rat Ig) (Caltag), goat anti-mouse Ig F(ab′)₂ fragments labelled with FITC (Jackson Laboratories, West Grove, PA), goat anti-mouse Ig Ab labelled with Texas Red (TR) (rat Ig-adsorbed) (Jackson) and donkey anti-rabbit Ig Ab conjugated with FITC (Amersham, UK) and for electron microscopy, goat anti-rat Ig Ab and goat anti-mouse Ig Ab, both conjugated to 10 nm gold particles (BioCell, Cardiff, UK).

Immunodetection at the light microscope level

Twelve to 48 hours after infection, macrophages on glass coverslips were fixed for 1 hour at room temperature with 4% paraformaldehyde (Merck-Schuchardt, Darmstadt, Germany) in 0.1 M sodium cacodylate-HCl buffer, pH 7.4. Cells were then quenched with 50 mM NH₄Cl in PBS and permeabilized for 30 minutes with 0.1 mg/ml saponin (Sigma) in PBS containing 10% serum of the same species as peroxidase- or fluorochrome-conjugated Ab. Coverslips were then sequentially incubated for 1 hour at room temperature with primary Ab and with peroxidase or fluorochrome conjugates. Abs and conjugates were

diluted in PBS containing 0.25% gelatin (Bio-Rad Laboratories, Richmond, CA) and 0.1 mg/ml saponin. Each incubation was followed by three washes of 10 minutes with PBS containing 0.1 mg/ml saponin. For double-immunofluorescence labellings, cell preparations were first incubated with both primary Abs belonging to different species and then with both species-specific secondary Abs. In some experiments, nuclei of host cells and of parasites were stained by a 5 minute incubation with 5 μg/ml propidium iodide (Aldrich, Milwaukee, Wisconsin) in PBS. Peroxidase activity associated with macrophages incubated with peroxidase conjugate was detected according to Graham et al. (1965). Cell preparations were mounted in Mowiol (Calbiochem, San Diego, CA) and either examined immediately or stored at −20°C until observation. Fluorescence and conventional transmission microscopy were done using a Carl Zeiss Photomicroscope.

Confocal microscopy of samples labelled with two fluorophores was performed with a Confocal Laser Scanning Microscope (Wild Leitz Instruments, Heidelberg, Germany), which uses an argon-krypton laser operating in multi-line mode. Most of the cell preparations labelled with both fluorescein and Texas Red conjugates were sequentially analysed at 488 nm and 567 nm wavelengths with filters that transmit light very selectively and optimally. Some cell preparations stained with a fluorescein conjugate and propidium iodide were also analysed with the less selective simultaneous mode of excitation and of acquisition. However, under our conditions, no overlap from one channel into the other was detected using this last approach. Emission filters centered at 535 nm, 630 nm and 610 nm were used for fluorescein, Texas Red and propidium iodide, respectively. For each selected cell, 10 to 20 optical sections taken at intervals of 0.2 to 0.6 μm were generally recorded.

Quantitative analysis of MHC class II molecule expression in uninfected and infected macrophages was performed on fixed and permeabilized cell preparations double stained with M5/114 Ab and a fluorescein conjugate and with propidium iodide. An interactive laser cytometer ACAS 570 (Meridian Instruments, Okemos, MI) equipped with an argon ion laser adjusted at 488 nm was utilized in these experiments. Emission filters centered at 530 nm and 605 nm were used for

Table 1. List of Abs and of control reagents used

Reagent	Specificity	Reference
Monoclonals		
34.4.20S (mouse IgG2a)[a]	H-2D[d], H-2K[b]	Ozato et al. (1982)
K9.18 (mouse IgG2a)[a]	H-2K[d]	Hammerling, U.[b] (unpublished)
M5/114 (rat IgG2b)[c]	I-A[b,d], I-E[d,k]	Bhattacharya et al. (1981)
14-4-4S (mouse IgG2a)[a]	I-Eα[d,]	Ozato et al. (1980)
Y-3P (mouse IgG2a)[a]	I-Aα[b]	Janeway et al. (1984)
In-1 (rat IgG2b)[a]	Mouse Ii invariant chain	Koch et al. (1982)
P4H5 (hamster IgG)[d]	Mouse Ii invariant chain	Mehringer et al. (1991)
FA/11 (rat IgG2a)[a]	Mouse macrosialin	Smith and Koch (1987)
2A3-26 (mouse IgG1)[e]	Plasma membrane antigen of *L. amazonensis* amastigotes	Antoine, J.-C. and C. Jouanne (unpublished)
A10.3.2 (mouse IgG2a)[f]	Serotonin	Guesdon, J.-L.[g] (unpublished)
GK1.5 (rat IgG2b)[a]	Mouse CD4	Dialynas et al. (1983)
H35-17.2 (rat IgG2b)[c]	Mouse CD8	Goldstein et al. (1982)
Polyclonals		
Anti-lgp110 (rabbit)[h]	Mouse lysosomal glycoprotein 110	Green et al. (1987)
Anti-lgp120 (rabbit)[h]	Rat lysosomal gycoprotein 120	Lewis et al. (1985)
Anti-rab7p (rabbit)[i]	Small GTP-binding protein rab7p	Chavrier et al. (1990)
Normal rabbit serum	−	−
Anti-ovalbumin[i] (rabbit)	Hen egg ovalbumin	Prina et al. (1990)
Normal rat IgG[j]	−	Levy and Sober (1960)
Normal mouse IgG[i]	−	−

[a]Hybridoma supernatant; [b]Sloan-Kettering Institute, New York; [c]purified from ascitic fluids by adsorption chromatography; [d]purified from hybridoma supernatant by ammonium sulfate precipitation; [e]purified from ascitic fluid by ammonium sulfate precipitation; [f]purified from hybridoma supernatant by HPLC; [g]Institut Pasteur, Paris; [h]immune serum; [i]affinity-purified Ab or IgG; [j]purified from serum by DEAE-cellulose chromatography.
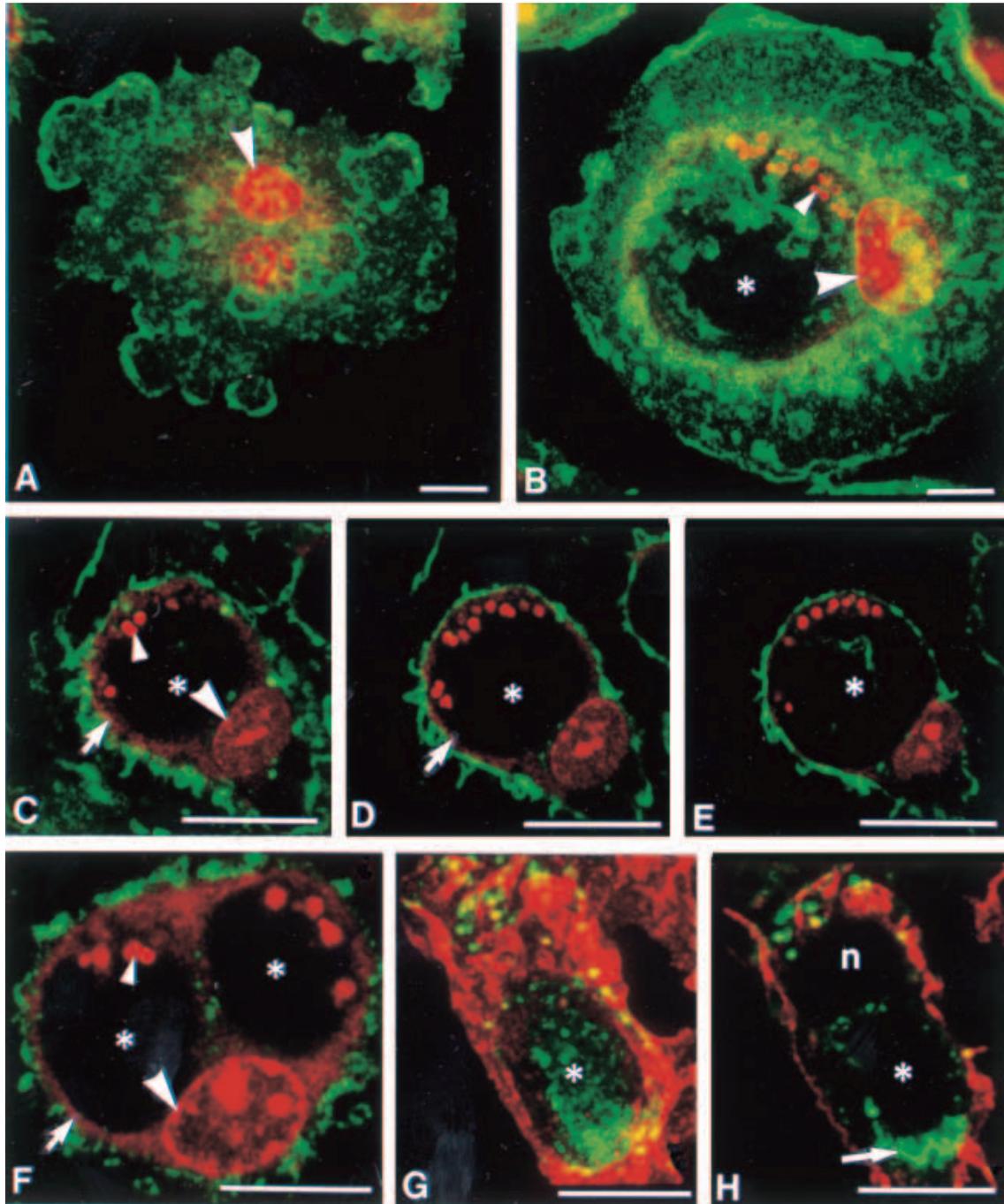
Fig. 1. Analysis by confocal microscopy of the distribution of MHC class I molecules in uninfected (A) and 48-hour-infected (B-H) macrophages from Balb/c mice. Both cell preparations were treated with IFN-γ. Cells were fixed, permeabilized and stained by indirect immunofluorescence before analysis. In some cell samples (A to F), DNA and RNA were stained with propidium iodide (red) (large arrowheads, nuclei of the macrophages; small arrowheads, nuclei of the parasites). A ring of propidium iodide surrounding PV (arrows in C,D,F) allows us to distinguish, in infected macrophages, the PV membrane (located on the inside of the red ring) from the plasma membrane (located on the outside of the red ring). (A,B) Three-dimensional reconstructions of macrophages stained with the anti-H-2D^d mAb 34.4.20S and FITC-labelled goat anti-mouse Ig F(ab′)$_2$ fragments (green). For each cell, 15 optical sections of 0.5 μm thickness were superposed. Plasma membrane is strongly stained, especially at the level of extensions located above the nucleus (A,B) and the PV (B). (C to E) Three serial optical sections (0.5 μm thickness) passing through the PV of a macrophage stained as in A and B. (F) Optical section (0.24 μm thickness) passing through the PV of a macrophage stained with the anti-H-2K^d mAb K9.18 and FITC-labelled goat anti-mouse Ig F(ab′)$_2$ fragments (green). In (C to F) FITC-labelling is only detected on the plasma membrane localized on the outside of the red ring. (G and H) Macrophage, double-stained with the anti-MHC class II molecule M5/114 mAb (a rat Ab) and with the anti-H2D^d mAb 34.4.20S (a mouse Ab). FITC-labelled goat anti-rat Ig F(ab′)$_2$ fragments and TR-labelled goat anti-mouse Ig Ab were used to detect the M5/114 (green) and the 34.4.20S mAb (red), respectively. (G) Three-dimensional reconstruction of 20 optical sections of the cell (0.24 μm thickness). One of them passing through the PV is shown in (H). MHC class I molecules and MHC class II molecules are mainly localized on the plasma membrane and at the level of amastigote-binding sites (arrow in H), respectively. The yellow color of some vesicles is not due to the co-localization of MHC class I and MHC class II molecules but to artefacts occurring during the superposition of the green and red images. In (C-H) only the perinuclear areas of the cells, where PV reside in infected macrophages, are shown. In (B-H) * indicates the centre of the PV. n, macrophage nucleus. Bars, 10 μm.
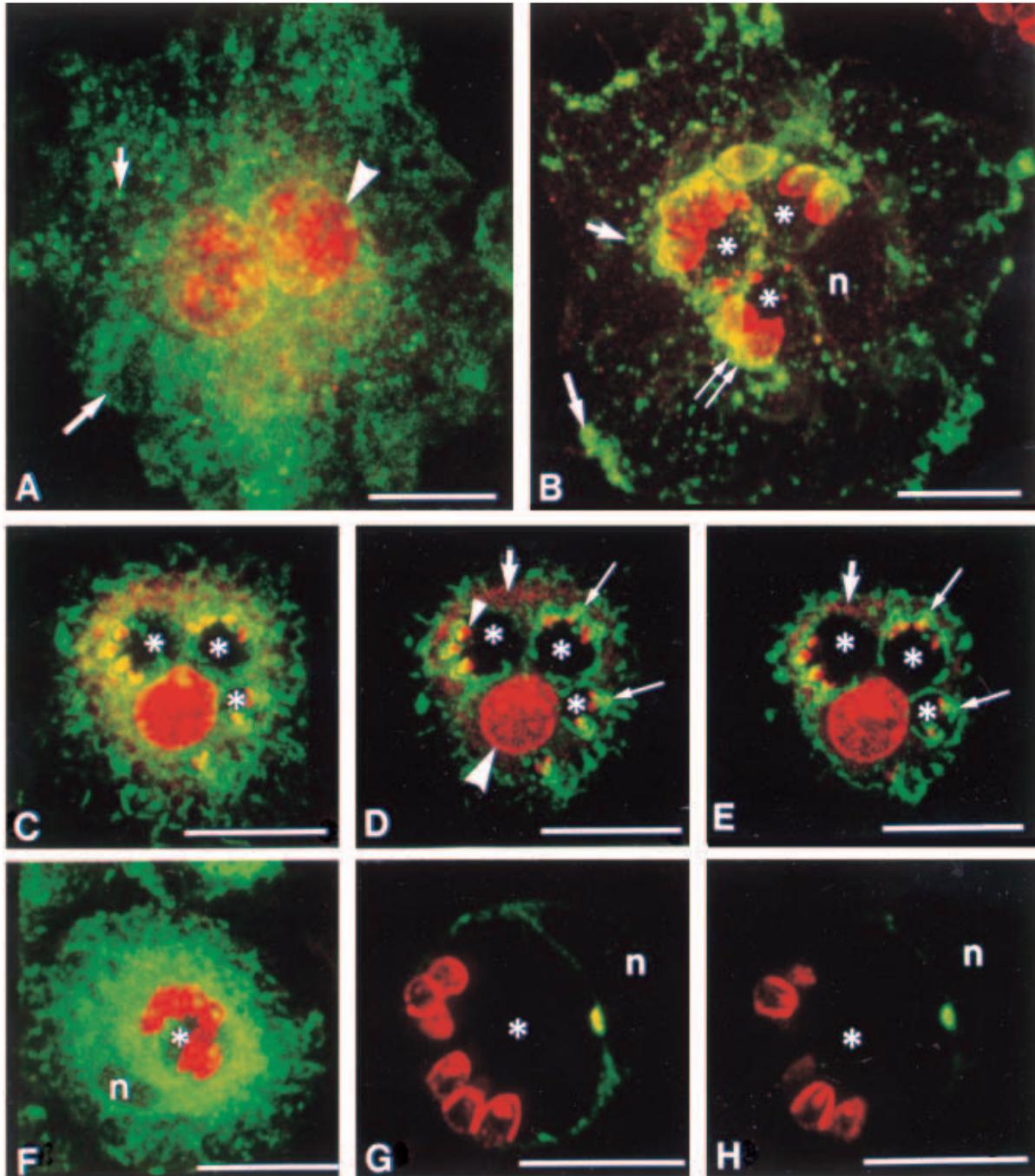
Fig. 2. Analysis by confocal microscopy of the distribution of MHC class II molecules and of the invariant chain Ii in uninfected (A) and 48-hour-infected (B-H) macrophages from Balb/c mice. Before analysis, cell cultures were processed as described in the legend to Fig. 1. In (A,C,D,E) DNA and RNA were stained with propidium iodide (red) (large arrowheads, nuclei of the macrophages; small arrowheads, nuclei of the parasites). A ring of propidium iodide surrounding the PV, visible in D and E (thick arrows) allows us to distinguish, in infected macrophages, the PV membrane (located on the inside of the red ring) from the plasma membrane (located on the outside of the red ring). (A and B) Three-dimensional reconstructions of macrophages stained with the anti-I-A[b,d,q] and anti-I-E[d,k] M5/114 mAb and FITC-labelled goat anti-rat Ig F(ab')$_2$ fragments (green). For each cell, 15 optical sections of 0.5 µm thickness were superposed. Staining of the MHC class II molecules is associated with the plasma membrane (large arrows), with vesicles (small arrows) and in infected macrophages with PV (double arrows). (C to E) Optical sections of a macrophage stained with M5/114 as in (A) and (B). In (C) 5 sections (0.5 µm thickness) were superposed. (D,E) Two serial sections (0.5 µm thickness) passing through the PV are shown. The polarization of the MHC class II molecule staining associated with the PV is indicated by thin arrows. (F to H) Macrophages stained for Ii chains with the In-1 mAb and FITC-labelled goat anti-rat Ig F(ab')$_2$ fragments (green). (F) Three-dimensional reconstruction of a macrophage. Fifteen optical sections 0.6 µm thickness were superposed. (G,H) Two serial optical sections (0.52 µm thickness) passing through the PV are shown. No Ii staining can be detected at the level of the PV. In B,F,G,H, the plasma membrane of amastigotes was stained with the 2A3-26 mAb and TR-labelled goat anti-mouse Ig Ab (red). In (C-E) and (G,H) only the perinuclear regions, which contain PV in infected macrophages, are shown. In (B-H) * indicates the centre of the PV. n, macrophage nucleus. Bars, 10 µm.

Table 2. Expression of MHC class II molecules in infected cell populations and their distribution within the PV

Exp.	Infected macrophages (%)	MHC class II⁺ cells (%)	PV containing MHC class II molecules (% of total PV)	PV with polarized distribution of MHC class II molecules (% of class II⁺ PV)
1	75.0	59.9	57.9	88.3
2	71.0	24.3	30.8	85.1
3	41.9	49.1	72.5	93.7

Balb/c macrophages infected for 48 hours and treated with IFN-γ were fixed and permeabilized. MHC class II⁺ cells were visualized using the M5/114 mAb and peroxidase-labelled F(ab′)$_2$ fragments of sheep anti-rat Ig Ab. Peroxidase activity was detected using 3-amino-9-ethyl carbazole and H$_2$O$_2$ as substrates. For each experiment, about 500 cells were counted.

the measurement of fluorescein and propidium iodide fluorescence, respectively. The microscope of this apparatus was equipped with a motorized stage moving with a linear speed of 20 mm/second and allowing the fluorescence recording of large microscopic fields. Raw data that appeared on the monitor screen as pseudo-color images were smoothed and thresholded. Furthermore, before the final analysis, images of contiguous cells were separated using the mouse of the computer.

Immunodetection at the electron microscope level

Immunoelectron microscopy was performed on Lowicryl K4M sections. Before Lowicryl embedding, adherent macrophages were fixed for 1 hour at 4°C with 2.5% paraformaldehyde, 0.2% glutaraldehyde (grade I, Sigma) in sodium cacodylate-HCl buffer, pH 7.4, containing 0.1 M sucrose, 5 mM MgCl$_2$ and 5 mM CaCl$_2$. Cells were then quenched with 50 mM NH$_4$Cl in the same buffer for 30 minutes at room temperature. Dehydration, embedding, incubation of sections with specific or irrelevant mAbs and with gold conjugates as well as staining with uranyl acetate and lead citrate were as previously described (Whitehouse et al., 1984; Antoine et al., 1988).

Controls of fluorescent and gold-labelled preparations

As controls of cell preparations stained with murine mAb, uninfected and infected macrophages were incubated with the isotype-matched A10.3.2 mAb or with normal mouse IgG. As controls of cell preparations stained with rat mAb, macrophages were incubated with the isotype-matched GK1.5 or H35-17.2 mAb or with normal rat IgG. Finally, cells incubated with rabbit anti-ovalbumin Ab or with normal rabbit serum served as controls of cells stained with rabbit immune sera or Ab directed against organelle markers. All these control preparations were generally unlabelled except for those incubated with normal rabbit serum, which showed a slight diffuse staining.

RESULTS

The PV membrane does not contain significant amounts of MHC class I molecules

When incubated with the anti-H-2D^d mAb 34.4.20S, uninfected Balb/c macrophages analysed by conventional fluorescence microscopy showed very strong plasma membrane staining, and faint or no intracellular staining. Twelve to 48 hours after infection, the plasma membrane staining appeared as strong as in uninfected cells and most if not all PV seemed unstained. However, this intense plasma membrane staining impeded the careful examination of many PV. Confocal microscopy was therefore undertaken to analyse intracellular compartments including PV selectively. In these experiments, infected cells stained for D^d molecules were further incubated with propidium iodide, which labels amastigote and macrophage nuclear DNA. This allowed a more accurate local-

ization of parasites and thus of the PV. A ring of propidium iodide staining surrounding the PV and due to the binding of this fluorochrome to mitochondrial DNA or messenger RNA of the host cells also helped to distinguish the plasma membrane from the PV membrane, which are often very close together. Using this methodology, we noted that D^d molecules were evenly distributed over the plasma membrane of uninfected and infected macrophages (Fig. 1A,B), but no labelling could be detected over intracellular compartments including the PV (Fig. 1C-E). Very similar findings were obtained when using the anti-H-2K^d mAb K9-18 instead of 34.4.20S except that the staining intensity was weaker in this case (Fig. 1F). With both antibodies, staining was stronger on cells treated with IFN-γ than on untreated cells but the pattern of MHC class I distribution was similar whatever the state of the macrophages. These data indicate that, 12 to 48 hours after infection, PV lack significant amounts of both H-2D^d and H-2K^d molecules. In further experiments, we double-stained IFN-γ-treated macrophages with 34.4.20S and with M5/114, a mAb that recognizes I-A and I-E MHC class II molecules. Plasma membrane of these cells expressed both class I and class II molecules; however, only MHC class II molecules could be significantly detected in PV (Fig. 1G,H).

Immunogold labelling of ultra-thin macrophage cryosections and Lowicryl sections confirmed results obtained by confocal microscopy in that plasma membrane and rare small vesicles of infected Balb/c macrophages were labelled with 34-4-20 S but not the PV membrane (data not shown).

MHC class II molecules associated with the PV membrane are mainly localized at the level of amastigote-binding sites

We demonstrated previously that, after infection of IFN-γ-treated macrophages with *L. amazonensis* amastigotes, MHC class II molecules reached PV in less than 2 hours and that in some PV these molecules were preferentially localized at the binding sites of amastigotes to the PV membrane (Antoine et al., 1991). This latter phenomenon was examined in more detail in the present study and its frequency estimated in 48-hour-infected macrophages. Table 2 shows that after treatment of heavily infected populations with IFN-γ, many PV exhibited a class II-specific staining. In 90% of these positive PV, the label was restricted to the site of attachment of amastigotes (Table 2), indicating that this peculiar distribution of MHC class II molecules is a general phenomenon at this stage of infection.

Confocal microscopy allowed a clear analysis of the distribution of MHC class II molecules in macrophages and espe-

cially of those associated with PV. In uninfected macrophages, MHC class II molecules were localized on the plasma membrane and in vesicular structures (Fig. 2A) corresponding to early endosomes and to late endosomes/ prelysosomes as defined previously (Lang and Antoine, 1991). After infection, MHC class II molecules were always present on the plasma membrane of infected cells, as well as in vesicles and PV (Fig. 2B-E). In these cells, double labelling of MHC class II molecules and of amastigotes with the M5/114 mAb and with either propidium iodide or the 2A3-26 mAb, respectively (2A3-26 recognizes an epitope on the plasma membrane of *L. amazonensis* amastigotes), showed that the PV-associated class II staining followed the exact shape of the posterior poles of amastigotes, which were tightly bound to the PV membrane and often protruded deeply within the host cell cytoplasm, as frequently observed by electron microscopy (data not shown). At this level, class II staining thus had a festoon appearance (Fig. 2B-E; see also Fig. 1G,H). This peculiar staining pattern was confirmed by measuring the intensity of fluorescence associated with the PV using the ACAS 570 laser cytometer, which allows quantitative fluorescence microscopy on adherent cells. Pseudo-color images of fluorescent cells showed that the fluorescence intensity was strongest at the attachment zone of the parasites (Fig. 3).

The restricted localization of PV-associated MHC class II molecules described above was also demonstrated by electron microscopy. Fig. 4 shows that the immuno-gold staining of these molecules decorated the junction between amastigotes and PV membrane but was absent from the remainder of the PV membrane not involved in the binding of parasites.

PV-associated MHC class II molecules are apparently not associated with invariant chains

Nonpolymorphic polypeptides termed the invariant chains (Ii) are associated with MHC class II molecules during their intracellular transport from the rough endoplasmic reticulum to endocytic compartments. In the latter, Ii chains are degraded and this process correlates with the ability of MHC class II molecules to bind peptides (for a review see Teyton and Peterson, 1992). To determine whether PV-associated MHC class II molecules are potentially able to bind peptides, Balb/c macrophages stained with the In-1 mAb, which recognizes a cytoplasmic epitope of Ii, were observed by confocal microscopy. In uninfected and infected macrophages, staining was seen in a reticular network, most likely corresponding to the rough endoplasmic reticulum, and in a few vesicles (Fig. 2F). No significant staining could be detected in PV (Fig. 2G,H). Unfortunately, the P4H5 mAb, which recognizes a luminal epitope of Ii chains, was unreactive in our experimental conditions. Nevertheless, since class II-associated Ii chains are progressively degraded starting from the COOH terminus, results obtained with In-1 strongly suggest that MHC class II molecules located in the PV are mature molecules completely devoid of Ii chains and able to bind peptides.

The lysosomal glycoproteins lgp110, lgp120, macrosialin and rab7p are distributed in or on the PV membrane in an unpolarized fashion

The following experiments were designed to determine whether the staining pattern described for the MHC class II molecules located in the PV is a specific phenomenon or if it is a common distribution pattern of the proteins associated with the membrane of these compartments. After fixation and permeabilization, uninfected and 48-hour-infected macrophages from Balb/c mice were stained with immune sera or Ab directed against the membrane proteins lgp110, lgp120, macrosialin or rab7p, which were found to be associated with the PV membrane.

In uninfected macrophages labelled with anti-lgp120 immune serum, the plasma membrane appeared unstained but numerous small and widely dispersed vesicles as well as large perinuclear vesicles were positive (Fig. 5A). Tubular structures were stained in some cells. After infection, small vesicles containing lgp120 were still detected but they were fewer in number, and the degree of depletion appeared to correlate with the size of the PV. Positive large vesicles and tubules almost completely disappeared and the membrane of all PV was strongly and uniformly stained (Fig. 5B). Anti-lgp110 immune serum gave similar staining patterns (data not shown).

Macrosialin labelled with the FA/11 mAb has been previously described as a macrophage-specific marker mainly localized in prelysosomes/tubular lysosomes (Smith and Koch, 1987; Rabinowitz et al., 1992). In the present experiments, macrosialin staining was found to be very similar to the lgp staining. Uninfected macrophages displayed the following positive structures: small vesicles distributed in the cytoplasm, large vesicles clustered in the perinuclear area (Fig. 5C) and long tubules. Both types of vesicles and tubules were less numerous after infection, and this was particularly obvious in macrophages containing huge PV. PV were strongly stained with the FA/11 mAb and the label was evenly distributed on their membranes (Fig. 5D). However, in some PV, the FA/11 staining appeared to be weaker at the level of amastigote-binding sites.
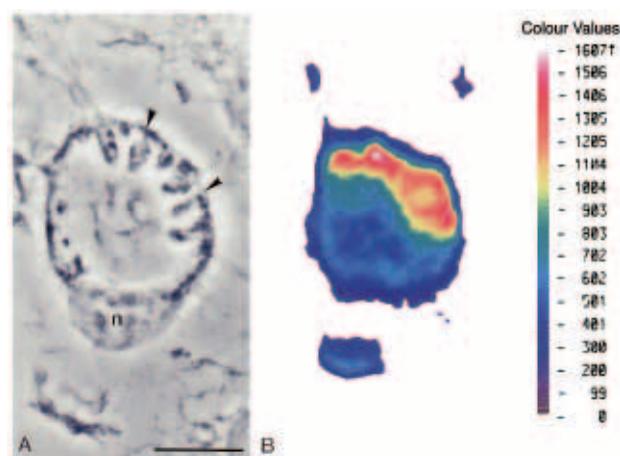
Fig. 3. Quantitative analysis of the MHC class II molecule distribution in PV. Balb/c macrophages were infected, treated with IFN-γ and 48 hours after infection, processed for detection of intracellular MHC class II molecules with M5/114 and FITC-labelled goat anti-rat Ig F(ab′)₂ fragments. The intensity of the fluorescence staining associated with the PV was measured with the ACAS 570 image cytometer. The figure shows the phase-contrast (A) and the MHC class II molecule staining (B) of the same the PV. The fluorescence intensity of the PV is indicated by the scale. Arrowheads, amastigote-binding sites; n, macrophage nucleus. Bar, 10 μm.
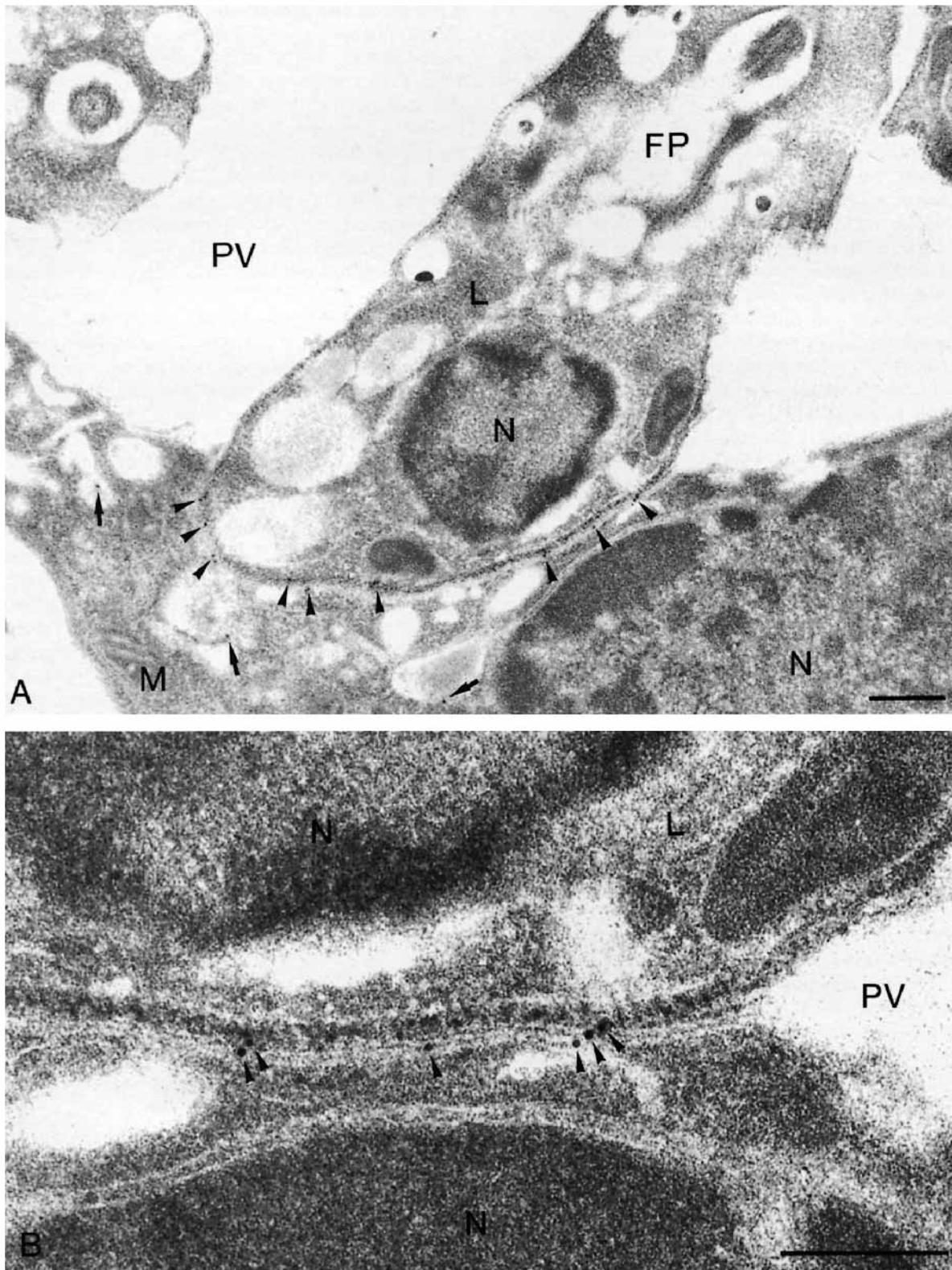
Fig. 4. Localization of MHC class II molecules in PV by immunoelectron microscopy. Balb/c macrophages infected for 48 hours and treated with IFN-γ were fixed and then embedded in Lowicryl K4M. Thin sections were incubated with the anti-MHC class II mAb M5/114 and with gold-labelled goat anti-rat Ig Ab. (A) General view of an infected macrophage. Gold particles can be detected in vesicles (small arrows) and on the PV membrane at the attachment site of the parasite (small arrowheads). (B) Higher magnification of part of the junction between the amastigote and the PV membrane shown in A. MHC class II labelling is indicated by arrowheads. L, amastigote; FP, flagellar pocket; M, mitochondria; N, nucleus; PV, parasitophorous vacuole. Bars: (A) 0.3 μm; (B) 0.2 μm.

Finally, rab7p, which has been previously described as a small GTP-binding protein specifically associated with the cytosolic face of the membrane of late endosomes (Chavrier et al., 1990) and of prelysosomes/tubular lysosomes (Rabinowitz et al., 1992), was detected on small vesicles and on larger perinuclear vesicles of uninfected macrophages (Fig. 5E). The infected macrophages exhibited a lower number of rab7p-positive vesicles and the membrane of most PV was homogeneously stained (Fig. 5F).

MHC class II molecules present in the PV remain associated for a while with parasite remnants after killing of *Leishmania*

From the above results, the restricted distribution pattern of PV-associated MHC class II molecules appears to be specific and in agreement with the idea that these molecules are bound to plasma membrane components of amastigotes, such as peptides or unfolded proteins.

In the next experiments, we took advantage of the fact that after killing of amastigotes some parasite components remain bound to the PV membrane for several hours to determine whether, in infected then cured macrophages, PV-associated MHC class II molecules remained associated with these components. Balb/c macrophages were treated with IFN-γ for 24 hours before adding parasites. At 24 hours post-infection, cells were incubated for 1 hour at 34°C with the leishmanicidal drug Leu-OMe (Rabinovitch et al., 1986). Such treatment cured the macrophage populations almost completely, since the percentage of infected cells dropped from more than 80% to 1% or less. Macrophages just cured or chased for 5 hours or 24 hours in Leu-OMe-free medium could still be stained with the 2A3-26 mAb (93%, 86% and 82% of previously infected macrophages, respectively; means of 3 experiments), indicating the presence of long-lived plasma membrane components in these cells. Just after killing of *Leishmania*, most of the PV were labelled with the
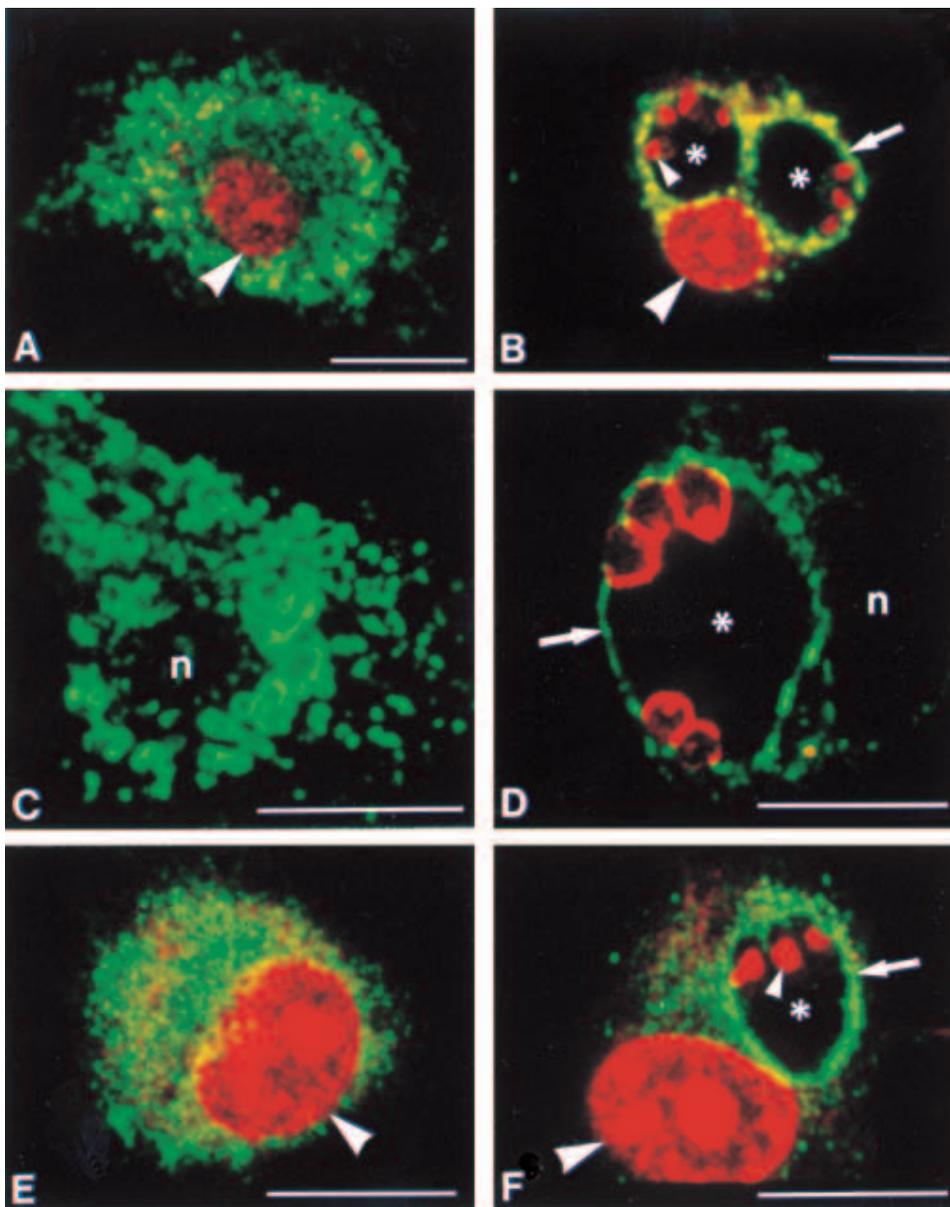
Fig. 5. Distribution of lgp120, macrosialin and rab7p in uninfected (A,C,E) and 48-hour-infected (B,D,F) macrophages from Balb/c mice. Cell preparations were treated (A,B) or untreated (C-F) with IFN-γ. Analysis was performed by confocal microscopy after fixation, permeabilization and staining with the appropriate immunological reagents. (A,B) Optical sections (0.6 μm thickness) of macrophages stained with a rabbit anti-lgp120 immune serum and FITC-labelled donkey anti-rabbit Ig Ab (green). (C,D) Optical sections (0.24 μm thickness) of macrophages stained with FA/11, a rat anti-macrosialin mAb, and FITC-labelled goat anti-rat Ig F(ab′)$_2$ fragments (green). (E,F) Optical sections (0.6 μm thickness) of macrophages stained with rabbit anti-rab7p Ab and FITC-labelled donkey anti-rabbit Ig Ab (green). Except in C, cells were also stained with propidium iodide (A,B,E,F, red) (large arrowheads, nuclei of the macrophages; small arrowheads, nuclei of the parasites), or with the anti-amastigote 2A3-26 mAb and goat anti-mouse Ig Ab labelled with TR (D, red). lgp120, macrosialin and rab7p stainings can be detected all around the PV (arrows). In these micrographs, only the perinuclear areas of the cells are shown. In B,D,F, ∗ indicates the centre of PV; n, macrophage nucleus. Bars, 10 μm.
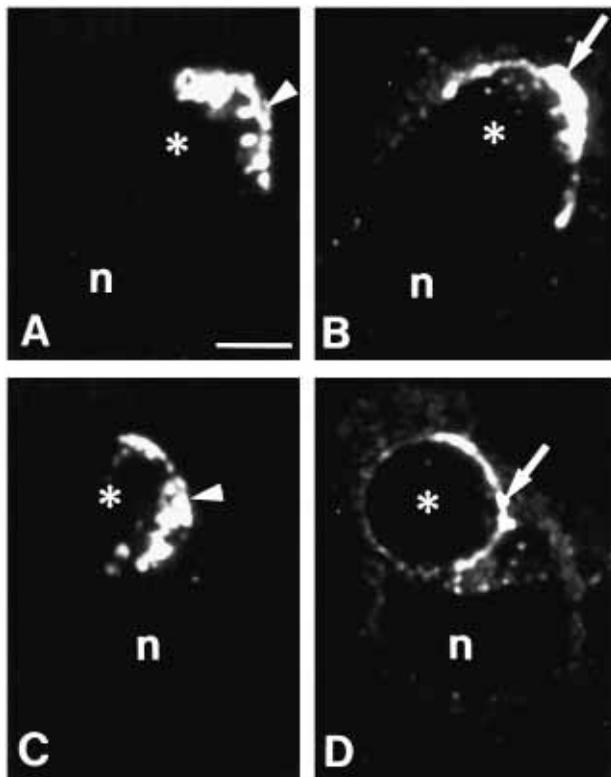
Fig. 6. Distribution of PV-associated MHC class II molecules after killing of amastigotes. Macrophages from Balb/c mice were infected, treated with IFN-γ and 48 hours after infection incubated for 1 hour with Leu-OMe. Cells were then fixed immediately (A,B) or chased for 5 hours in Leu-OMe-free medium before fixation (C,D). After permeabilization, macrophages were double-stained with the anti-amastigote 2A3-26 mAb and with the anti-class II M5/114 mAb. TR-labelled goat anti-mouse IgAb and FITC-labelled goat anti-rat Ig F(ab′)₂ fragments were used to detect 2A3-26 and M5/114, respectively. Cell preparations were analysed by confocal microscopy. 2A3-26 stainings (A,C) and M5/114 stainings (B,D) of the same optical sections (0.4 µm thickness) passing through the PV are shown separately. 2A3-26 parasite components still bound to the PV membranes are indicated by arrowheads (A,C). These remnants are distributed in a polarized fashion. MHC class II molecules remaining in PV are located at the same level as the 2A3-26⁺ components (B,D, arrows). In these micrographs, only the perinuclear areas of the cells are shown. ∗ indicates the centre of the PV. n, macrophage nucleus. Bar, 10 µm.

2A3-26 mAb; the staining was located at one pole of these organelles most likely corresponding to the binding sites of amastigotes. The PV were still stained for class II molecules and the label was very often associated with the parasite remnants detected with 2A3-26 (Fig. 6A,B and Table 3). Similar findings were observed in cured macrophages chased for 5 hours except that, at this time point, PV remnants had already considerably shrunk (Fig. 6C,D and Table 3). Large and small 2A3-26⁺ vesicles were also present at this stage. They were seldom stained with anti-class II Ab. Twenty-four hours after the Leu-OMe treatment, PV had completely disappeared but 2A3-26⁺ vacuoles, most likely derived from PV as well as 2A3-26⁺ large and small vesicles, were still detected in these cells. Among the 2A3-26⁺ vacuoles, some also exhibited an MHC class II staining, which was sometimes polarized in the same area as the 2A3-26 staining (Table 3).

PV-associated MHC class II molecules can be detected with mAb reacting primarily with compact dimers

The above results suggest that PV-associated MHC class II molecules are involved in specific interactions with parasite components. To test this hypothesis further, we examined the reactivity of class II molecules present in PV of infected C57BL/6 and Balb/c macrophages with the anti-Aα^b mAb Y-3P and the anti-Eα^d mAb 14-4-4S, respectively, antibodies that have been described as being much more reactive with the compact conformers of MHC class II molecules carrying tightly associated peptides (Germain and Hendrix, 1991). Forty-eight hours after infection, a large fraction of the class II⁺ PV (detected with the M5/114 mAb, which recognizes all types of class II) could also be stained with 14-4-4S (Balb/c macrophages) or Y-3P (C57BL/6 macrophages) (Table 4). Furthermore, stainings with these conformational Abs were polarized at the level of amastigote-binding sites (Table 4, Fig. 7A,B). Finally, these findings also demonstrated that both I-A and I-E molecules could reach PV and were involved in 'caps'.

Uninfected and infected macrophages express similar levels of MHC class II molecules

As the formation of complexes between peptides and MHC class II molecules has been recently shown to increase the stability and the half-life of the class II (Davidson et al., 1991;

Table 3. Continued association of MHC class II molecules with killed parasite components bound to PV membranes or to membranes of PV-derived vacuoles

| Chase time after Leu-OMe treatment (hours)	Number of 2A3-26⁺ vacuoles observed	2A3-26⁺ vacuoles containing MHC class II molecules (% of total 2A3-26⁺ vacuoles)	Vacuoles with class II molecules polarized towards the 2A3-26 staining (% of 2A3-26⁺, class II⁺ vacuoles)
0	201	66.7	87.3
5	239	53.1	71.6
24	100	31.0	32.3

Macrophages from Balb/c mice were treated with IFN-γ and then infected with amastigotes. Twenty-four hours after infection, cells were cured by a 1 hour incubation at 34°C with 2 mM Leu-OMe (see Materials and Methods). Cells were then fixed immediately or chased for 5 or 24 hours in culture medium devoid of Leu-OMe before fixation. Macrophages were double stained for amastigotes or parasite components and for class II molecules with the 2A3-26 and M5/114 mAb, respectively, and with appropriate conjugates. Data are from a single experiment representative of four similar experiments.

Table 4. Staining of PV-associated MHC class II molecules with antibodies reacting primarily with compact dimers

Origin of macrophages	Antibody used for the staining	Number of PV counted	Labelled PV (% of total PV)	PV with class II staining polarized towards the amastigote-binding sites (% of total labelled PV)
Balb/c	M5/114	1037	42.4	93.4
	14-4-4S	1023	27.9	86.7
C57BL/6	M5/114	535	48.4	74.9
	Y-3P	542	29.9	98.8

Macrophages from Balb/c or C57BL/6 mice were infected and then treated with IFN-γ. Forty-eight hours after infection, cells were processed for the detection of MHC class II molecules by immunofluorescence.

Germain and Hendrix, 1991; Sadegh-Nasseri and Germain, 1991; Neefjes and Ploegh, 1992), we investigated if the expression rate of these molecules was modified in infected macrophages from Balb/c mice. In these experiments, total MHC class II molecules associated with plasma membrane and internal compartments were quantified on cells fixed, permeabilized and stained with M5/114 and a FITC conjugate. Scanning of uninfected and infected cell populations with the ACAS 570 laser cytometer showed very similar distribution of the specific staining (data not shown). Furthermore, the mean fluorescence integrated values of uninfected and infected macrophages were not significantly different ($180,000 \pm 42,000$ and $161,000 \pm 56,000$ arbitrary units, respectively, means ± 1 s.d. of 4 experiments). Thus, 48 hours after infection, the steady-state level of MHC class II molecule expression does not seem to be affected by the presence of the parasites.
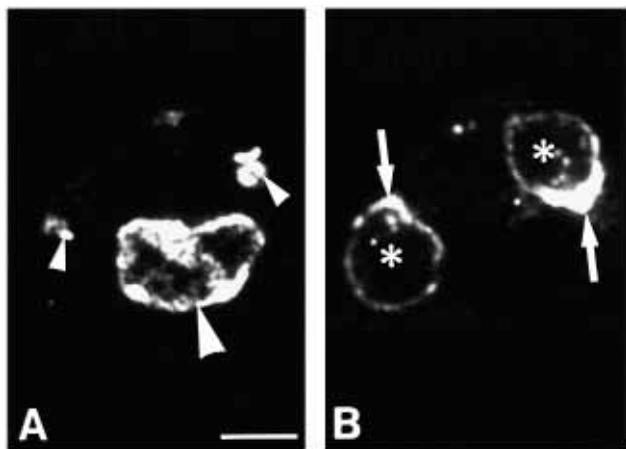
Fig. 7. Staining of PV-associated MHC class II molecules with the conformational mAb Y-3P. Macrophages from C57BL/6 mice were infected and treated with IFN-γ. Forty-eight hours after infection, cells were fixed, permeabilized, stained with Y-3P and FITC-conjugated goat anti-mouse Ig F(ab')$_2$ fragments, and then with propidium iodide before analysis by confocal microscopy. Propidium iodide staining (A) and Y-3P staining (B) of the same optical section (0.4 μm thickness) are shown separately. PV-associated class II molecules stained with Y-3P appear to be concentrated at the level of amastigote-binding sites (B, arrows). Large arrowhead, nucleus of the macrophage; small arrowheads, nuclei and kinetoplasts of the parasites. In these micrographs, only the perinuclear areas of the cells are shown. Bar, 10 μm.

DISCUSSION

PV and MHC class I molecules

How certain *Leishmania* Ags bind to MHC class I molecules is an important question to settle because it has been recently shown that the CD8^{+} T lymphocyte subset is an essential component of the protective immune responses developed in *Leishmania*-infected mice under certain experimental conditions (Titus et al., 1987; Stern et al., 1988; Hill et al., 1989; Müller et al., 1991). This issue is also interesting from a cell biology point of view, since, to our knowledge, *Leishmania* are always located in membrane-bound compartments belonging to the endocytic pathway, which raises the question of the meeting between *Leishmania* Ags and MHC class I molecules. Indeed, until recently, it was generally thought that complexes between processed antigens and MHC class I molecules were formed exclusively in one or several compartments of the biosynthetic pathway of the APC. Several recent findings, however, are also consistent with a peptide loading of MHC class I molecules in endocytic compartments. First, constitutive endocytosis of cell-surface MHC class I molecules has been described in several cell types (Vega and Strominger, 1989; Reid and Watts, 1990). Furthermore, macrophages, and perhaps also dendritic cells, seem to be endowed with a unique capability to present exogenous Ags in association with MHC class I molecules (Debrick et al., 1991; Rock et al., 1993; Pfeifer et al., 1993). The mechanism responsible for this alternative class I processing pathway has not yet been elucidated but the presence of MHC class I molecules in phagosomes or phagolysosomes, which are specific endocytic compartments of macrophages, could be significant.

Leishmania-infected macrophages that contain long-lived phagolysosomes provided an interesting model with which to test the hypothesis presented above. Using sensitive immunocytochemical procedures at the light- and electron-microscopic levels, we were however unable to demonstrate the presence of significant amounts of H-2K[d] and of H-2D[d] molecules in PV, whereas these molecules could be easily detected on the plasma membrane of infected macrophages from Balb/c mice. It can be inferred from these experiments that PV probably do not represent a meeting place for *Leishmania* Ags and MHC class I molecules, with the reservation that we cannot completely exclude the existence of a very small intracellular pool of MHC class I molecules associated with PV, which would be very rapidly transported or recycled towards the cell surface (Reid and Watts, 1990). At this stage, the loading of MHC class I molecules with parasite Ags remains a matter of speculation. However, recent data showing that macrophages

infected with *L. major* promastigotes transfected with the *E. coli* β-galactosidase gene are unable to present β-galactosidase to specific class I-restricted cytotoxic T cell clones (Lopez et al., 1993) suggest that *Leishmania*-infected macrophages are not APC by themselves for class I-restricted *Leishmania*-specific T lymphocytes.

PV and MHC class II molecules

Contrary to MHC class I molecules, MHC class II molecules were present in a large majority of PV when macrophages were activated with IFN-γ. Furthermore, they exhibited a peculiar distribution within these compartments, since 48 hours after infection they were almost entirely located at the site of amastigote anchorage to the PV membrane. This capping phenomenon appears to be specific to the MHC class II molecules, since it was not observed with four other PV-associated proteins lgp110, lgp120, macrosialin and rab7p, and apparently involves only mature molecules devoid of Ii chains and belonging to the I-A or I-E isotypes. Taken together, these results raise the possibility that MHC class II molecules interact with molecules of the amastigote plasma membrane. In favor of this proposal, we also found that if intracellular amastigotes were killed by Leu-OMe, a drug that does not affect the host cells, MHC class II molecules remained associated for several hours with remnants of the parasites still bound to the PV membrane. Alternatively, MHC class II molecules associated with the amastigote-binding sites could interact with soluble products of the parasites preferentially released at their posterior pole. In this case, polarization of class II molecules could be explained by the fact that some ligands like immunogenic peptides decrease the mobility and increase the aggregability of MHC class II molecules (Mecheri et al., 1990). Whatever the correct explanation, the staining of PV-associated class II molecules with the conformational Ab 14-4-4S and Y-3P suggests that, at least for a part of them, the binding site is occupied.

The binding of an immunogenic peptide to MHC class II molecules can increase their stability and their resistance to degradation (Germain and Hendrix, 1991). We therefore asked whether the putative interactions between these molecules and amastigotes up-regulate their expression in infected macrophages. We found that the surface expression of class II (Antoine et al., 1991; Prina et al., 1993) as well as the overall class II level (the present study) were not significantly modified in macrophages infected for 48 hours, suggesting that the half-life of the class II molecules is not grossly affected by the presence of the parasites at this stage of infection. The conclusions drawn from these studies are limited however by the fact that the stabilization of the MHC class II molecules by peptides appears to be strongly dependent on the class II haplotype and on the nature of the peptides.

The presence of complexes between amastigotes/soluble parasite molecules and PV-associated MHC class II molecules could also explain recent findings of our laboratory and of others showing that macrophages infected with *L. amazonensis* amastigotes or with *L. major* promastigotes are partially impaired in their capacity to present several exogenous protein Ags, including *Leishmania* Ags, to specific CD4+ T cell hybridomas (Fruth et al., 1993; Prina et al., 1993). This failure could be due to a direct competition between parasite molecules and exogenous Ags for the same MHC class II molecules or else to the inaccessibility of the class II molecules for exogenous Ags.

Protein composition of the PV membrane

We have shown in previous studies that within 24-72 hours the PV acquires the characteristics of a lysosomal compartment, in terms of contents and membrane. These organelles maintain a very acidic pH (≈4.9), contain high amounts of lysosomal enzymes and accumulate markers of fluid-phase endocytosis (Antoine et al., 1987, 1990; Antoine and Prina, 1992). In addition, the PV membrane housing *L. amazonensis* contains lgp110 and lgp120 but is devoid of CI-MPR, a protein generally excluded from the terminal lysosomal compartment (Antoine et al., 1991). As the PV enlarges during the 72 hours period following infection, a drastic depletion of organelles containing acid phosphatase and arylsulfatase is observed, which is most likely due to fusion of the organelles with the PV (Barbieri et al., 1985). On the basis of all these results, we proposed that the PV corresponds to the morphologically modified (a unique large organelle vs a large number of vesicles and tubules) lysosomal compartment of the host cell. Very similar properties have been described for 'early' PV housing *L. mexicana* (less than 3 days after infection; Russell et al., 1992), and for PV containing *L. donovani* (T. Lang, R. Hellio, P. M. Kaye, and J.-C. Antoine, unpublished data). Although *L. amazonensis* is a pathogenic nondegradable microorganism, the PV in which it multiplies therefore seems to behave like a phagosome containing nonpathogenic and degradable microorganisms such as *Bacillus subtilis* (Lang et al., 1988) and heat-killed *Listeria monocytogenes* (Harding and Geuze, 1992), in that it acquires a membrane of lysosomal composition or at least some of the specific lysosomal membrane constituents such as lgp110 and lgp120.

The question of whether the PV membrane is exclusively of lysosomal composition at this stage of infection remains a matter of debate. We have indeed shown that this membrane is strongly positive for macrosialin, a macrophage-specific protein that seems to be mainly expressed in the tubular lysosomal compartment of these cells, which, according to Rabinowitz et al. (1992), may be the equivalent of the prelysosomal compartment (late endosome) described in other cell types. The same is true for rab7p, which has been reported as being a marker of the late endosomes/prelysosomes (Chavrier et al., 1990; Rabinowitz et al., 1992) and was detected on the PV membrane. A possible explanation to account for these findings could be that the membranes of both prelysosomal and lysosomal compartments participate in the formation of the PV membrane.

The apparent absence of CI-MPR in PV is however difficult to reconcile with this hypothesis. Prelysosomal compartments could be devoid of CI-MPR in macrophages or else this receptor could be limited to certain specialized portions of these organelles not involved in the formation of PV, as observed for the phagolysosomes of inflammatory peritoneal macrophages containing latex beads or bacteria (Rabinowitz et al., 1992).

Alternatively, in bone-marrow-derived macrophages, which exhibit a considerable traffic of lysosomes, markers such as rab7p, considered to be associated with late endosomes/prelysosomes in other cell types, could be also present in terminal lysosomes. In this latter case, the PV

membrane of macrophages infected for 48 hours could still be considered of lysosomal composition.

In conclusion, we show here that *L. amazonensis* amastigotes live in prelysosomal/lysosomal compartments of the macrophages, which contain MHC class II molecules if the host cells are appropriately stimulated. To cope with the harsh conditions they encounter within these organelles, *Leishmania* amastigotes have evolved various strategies ensuring the maintenance of their cytosolic pH near to neutrality, the capture of metabolites and the resistance to lysosomal hydrolases (for a review, see Chang et al., 1990; Alexander and Russell, 1992), but the presence of MHC class II molecules in PV could be also detrimental to the survival of the parasites. The binding of these molecules to the parasite plasma membrane, as is suggested by their distribution, could thus be a means of sequestering them and consequently of avoiding the recognition of infected macrophages by *Leishmania*-specific CD4+ T lymphocytes. To test this issue, we are now attempting to measure the binding of purified MHC class II molecules to isolated amastigotes.

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