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Intracellular *Leishmania amazonensis* amastigotes internalize and degrade MHC class II molecules of their host cells

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SUMMARY

In their amastigote stage, *Leishmania* live in mammalian macrophages within parasitophorous vacuoles (PV), organelles of phagolysosomal origin that, in macrophages activated with IFN-γ, contain major histocompatibility complex (MHC) class II molecules apparently devoid of invariant chains. We have now studied the fate of PV-associated class II molecules in mouse bone marrow-derived macrophages infected with *L. amazonensis* amastigotes using immunocytochemical and biochemical approaches. We have found that at least a part of these class II molecules was internalized by amastigotes and reached structures very often located in their posterior poles. This process was much more obvious if infected macrophages were incubated with protease inhibitors like antipain, chymostatin, Z-Phe-AlaCHN$_2$ and Z-Phe-PheCHN$_2$, or if amastigotes were pre-treated with the irreversible cysteine protease inhibitor Z-Phe-AlaCHN$_2$ before infection, clearly indicating that amastigotes also degraded the internalized class II molecules. Study of infected macrophage cryosections by immuno-electron microscopy allowed the identification of the class II-positive structures in amastigotes as the lysosome-like organelles known as megasomes. Other PV membrane components like the prelysosomal/lysosomal glycoproteins lgp110, lgp120 and macrosialin could not be detected in megasomes of amastigotes even after treatment of macrophages with protease inhibitors, suggesting the involvement of some specific mechanism(s) for the internalization of class II molecules. Interestingly, after treatment of infected macrophages with various protease inhibitors (antipain, leupeptin, E-64, Z-Phe-AlaCHN$_2$, Z-Phe-PheCHN$_2$), PV membrane as well as megasomes of amastigotes become positive for invariant chains. A quantitative analysis of amastigote-associated class II molecules based on enzyme immunoassays showed that: (a) amastigotes extracted from macrophages treated with both IFN-γ and antipain or Z-Phe-AlaCHN$_2$ contained a much greater amount of class II than amastigotes extracted from macrophages treated with IFN-γ alone; (b) class II molecules associated with the former were mainly intracellular and, at least some of them, were complexed with invariant chains or fragments of invariant chains; (c) amastigotes pre-incubated with Z-Phe-AlaCHN$_2$ before infection accumulated a greater amount of intracellular class II than amastigotes pre-incubated without inhibitor, clearly indicating that the blockade of parasite cysteine proteases was sufficient to enhance the pool of these molecules within megasomes. On the whole, these data are consistent with the idea that class II molecules reaching PV are newly synthesized and still complexed with intact invariant chains or with partially degraded invariant chains. The latter are rapidly degraded by proteases, especially cysteine proteases of macrophage origin, whereas at least some class II molecules are internalized by amastigotes and degraded within megasomes by cysteine proteases of parasitic origin. Endocytosis and degradation of MHC class II molecules by *L. amazonensis* amastigotes could be a means of circumventing the host's immune system.

Key words: macrophage, *Leishmania amazonensis*, phagolysosome, megasome, MHC class II molecule, cysteine protease

INTRODUCTION

Leishmania are dimorphic Trypanosomatidae that, in their amastigote forms, parasitize mammals including humans. At this stage, *Leishmania* multiply mainly, if not exclusively, in macrophages of the infected tissues, within endocytic organelles of host cell origin called parasitophorous vacuoles (PV, for a review see Alexander and Russell, 1992). Macrophages are well known to play critical roles in the struggle against some pathogens. These cells are not only involved in the initiation or maintenance of cell-mediated immune responses but they also act as effector cells, functions that are linked to their ability to present endogenous and exogenous antigens (Ag) in the context of MHC class I and class II molecules, and to generate potent microbicidal molecules (Auger and Ross, 1991). On the other hand, persistence of *Leishmania* for years in immune-resistant mice has been reported (Aebischer et al., 1993), which raises the question of the strategies evolved by these parasites to escape or modulate the Ag presentation processes and the leishmanicidal activities of their host cells. Analyses of PV composition and of events occurring in these organelles were undertaken with the intention of resolving some aspects of this question. PV of macrophages infected with *L. amazonensis*, *L. mexicana* or *L. donovani* were found to be very acidic organelles (Chang, 1980a; Antoine et al., 1990; Sturgill-Koszycki et al., 1994) containing various lysosomal enzymes including proteases (Antoine et al., 1987; Prina et al., 1990; Russell et al., 1992; Lang et al., 1994b). They are limited by a membrane of macrophage origin, which, 48 to 72 hours after infection, is enriched with proteins generally associated with prelysosomes/lysosomes (Antoine et al., 1991; Russell et al., 1992; Lang et al., 1994a,b). Furthermore, after stimulation with gamma interferon (IFN-γ), the PV membrane also contains major histocompatibility complex (MHC) class II molecules but not MHC class I molecules (Antoine et al., 1991; Lang et al., 1994a,b). Together, these data support a role for the PV in the processing of parasite molecules and loading of MHC class II molecules with parasite peptides. However, the fate of PV-associated class II molecules requires clarification. Are they truly involved in the presentation of parasite Ag or are they sequestered and eventually degraded within these compartments? During the study of the MHC class II distribution in *L. amazonensis*-infected macrophages, small structures specifically stained with anti-class II monoclonal antibodies (mAb) were regularly observed within amastigotes, suggesting the internalization of class II by the parasites (Antoine, J.-C., unpublished results). In this report, we describe the effects of various protease inhibitors on the location of class II molecules and class II-associated invariant chains expressed by *L. amazonensis* amastigote-infected macrophages stimulated with IFN-γ. We show that some of the inhibitors considerably increased the amount of MHC class II molecules associated with intra-parasite structures, which proved to be megasomes. Similar findings were obtained when amastigotes were pretreated with some protease inhibitors before infection of IFN-γ-activated macrophages. These data support the idea that *L. amazonensis* amastigotes internalize MHC class II molecules of their host cells and degrade them within megasomes. This process could be a parasite mechanism for evading the host immune response.

MATERIALS AND METHODS

Mice

Female Balb/c, C57BL/6 and Swiss nude mice, aged 2 to 4 months, were obtained from the Pasteur Institute animal facilities or from Iffa Credo (St Germain-sur-l'Arbresle, France). 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) and *L. major* strain NIH173 (WHO reference number MHOM/IR/-/173) were maintained virulent by passage in Balb/c and Swiss nude mice, respectively. Amastigotes were purified from disrupted lesions as described earlier (LV79, Antoine et al., 1988; NIH173, Channon et al., 1984). In some experiments, purified *L. amazonensis* amastigotes were treated for 30 minutes at 4°C with 0.15 M NaCl adjusted to pH 2.8 with HCl, after which the parasites were washed with culture medium.

Generation of macrophages, infection with amastigotes and treatment with IFN-γ

The methods for preparation of bone marrow cells and for generation of bone marrow-derived macrophages have been previously described in detail (Antoine et al., 1991). Briefly, cells were cultured for 5 days at 37°C in the presence of L-929 cell-conditioned medium as a source of macrophage colony-stimulating factor. They were allowed to differentiate on 12 mm round glass coverslips for light-microscopic studies, in 35 mm tissue culture dishes (Corning Glass Works, Corning, NY) for electron-microscopic studies and in 100 mm tissue culture dishes (Corning) for biochemical assays. After this time, cultures were washed to remove non-adherent cells and incubated for a further 24 hours at 37°C in the absence of colony-stimulating factor. Amastigotes were then added to macrophage cultures to give a parasite-to-host cell count ratio of 4 or 6:1. *L. amazonensis*-infected cultures and uninfected cultures run in parallel were placed at 34°C while *L. major*-infected cultures and uninfected cultures run in parallel were incubated at 37°C. Some cultures were treated with IFN-γ after infection as described (Antoine et al., 1991). In other experiments, macrophages were pre-activated with IFN-γ 24 hours before infection. At 3 to 48 hours post-infection, macrophages were processed for light microscopy, electron microscopy or biochemical assays.

Protease inhibitors

The serine and cysteine protease inhibitors antipain hydrochloride, chymostatin, leupeptin hemisulfate salt and *N*α-*p*-tosyl-L-lysine chloromethyl-ketone hydrochloride (TLCK), the cysteine protease inhibitor *trans*-epoxy-succinyl-L-leucylamido-(4-guanidino) butane (E-64), the aspartic protease inhibitor pepstatin A, the metalloprotease inhibitor 1,10-phenanthroline monohydrate were purchased from Sigma Chemical Co. (St Louis, MO) and the serine protease inhibitor Pefabloc-Sc [4-(2-aminoethyl)-benzenesulfonyl fluoride] from Interchim (Montluçon France). The cysteine protease inhibitor *N*-benzyloxycarbonyl-phenylalanyl-alanyl-diazomethane (Z-Phe-AlaCHN$_2$) prepared by Elliott Shaw (Friedich Miescher Institut, Basel, Switzerland) (Kirschke and Shaw, 1981) was obtained through Michel Rabinovitch (Institut Pasteur, Paris, France). The cysteine protease inhibitor *N*-benzyloxycarbonyl-phenylalanyl-phenylalanyl-diazomethane (Z-Phe-PheCHN$_2$) was obtained from Bachem (Bubendorf, Switzerland). Stock solutions of the inhibitors (5 mg/ml) were prepared in water (antipain, leupeptin, TLCK), ethanol (phenanthroline), dimethylsulfoxide (DMSO) (chymostatin, E-64, pepstatin, Z-Phe-AlaCHN$_2$, Z-Phe-PheCHN$_2$), or phosphate-buffered saline (PBS) (Pefabloc-Sc), and stored at −20°C until use, except for Pefabloc-Sc, which was stored at 4°C.

Uninfected and infected macrophages were incubated for 20 hours with the following concentrations of inhibitors: 2, 10 or 50 μg/ml for antipain, chymostatin, leupeptin, E-64 and pepstatin A; 0.8, 2, 4 or 20 μg/ml for Z-Phe-AlaCHN$_2$; and 1,10-phenanthroline, 0.02, 0.23 or 2.35 μg/ml for Z-Phe-PheCHN$_2$. Macrophages were then fixed for microscopy or disrupted for biochemical assays as described below. As controls, macrophages were incubated with concentrations of solvent (water, ethanol or DMSO) equivalent to those used in cultures treated with inhibitors. In other experiments, amastigotes were pretreated for 15 hours at 34°C with Z-Phe-AlaCHN$_2$ (2 μg/ml in RPMI, 20 mM Hepes, 10% foetal calf serum) or with a concentration of DMSO equivalent to that used in parasite suspensions incubated with the inhibitor.

Immunological reagents

Hybridoma cells producing the mAb M5/114, a rat anti-mouse I-A^{b,d,q} and I-E^{d,k} IgG2b (Bhattacharya et al., 1981) and those producing the mAb Y-3P, a mouse anti-mouse I-Aα^{b} IgG2a (Janeway et al., 1984), were obtained from the American Type Culture Collection (Rockville, MD). Hybridoma cells secreting the mAb 14-4-4S, a mouse anti-I-Eα^d IgG2a (Ozato et al., 1980), and those producing the mAb MK-D6, a mouse anti-I-Aβ^d IgG2a (Kappler et al., 1981), were kindly supplied by Bernard Vray (Université Libre de Bruxelles, Belgium). Hybridoma cells producing the mAb In-1, a rat anti-mouse invariant chain (Ii) IgG2b (Koch et al., 1982), and those synthesizing the Ab FA/11, a rat anti-mouse macrosialin IgG2a (Smith and Koch, 1987), were kindly provided by Norbert Koch (Institut für Immunologie und Genetik, Heidelberg, Germany), and Gordon Koch (MRC Laboratory of Molecular Biology, Cambridge, UK), respectively. Hybridoma cells secreting the Ab H35-17.2, a rat anti-mouse CD8 IgG2b, were given by M. Pierres (Centre d'Immunologie de Marseille Luminy, France; Goldstein et al., 1982). Cells synthesizing the mAb 2A3-26, a mouse IgG1 recognizing a plasma membrane antigen of *L. amazonensis* amastigotes, were derived from lymph node cells of an infected C57BL/6 mouse (C. Jouanne and J.-C. Antoine, unpublished). The Ab M5/114, H35-17.2 and 2A3-26 were purified as described (Antoine et al., 1991; Lang et al., 1994a). The mAb 14-4-4S and MK-D6 were purified by affinity chromatography on Protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) (Andrew and Titus, 1991). Media of hybridoma cultures were used as source of Ab In-1 and FA/11. The specifically purified mAb TüL3-8, a mouse IgG1 directed against the gp63 metallo-protease of *L. mexicana* promastigotes (Medina-Acosta et al., 1989) was a gift from Peter Overath (Max-Planck-Institut für Biologie, Tübingen, Germany). The mouse anti-serotonin mAb A10.3.2 (IgG2a) and G21.10 (IgG1) were obtained from Jean-Luc Guesdon (Institut Pasteur). They were purified by ion-exchange chromatography. Rabbit immune sera directed against mouse lysosomal glycoprotein (lgp)110 (Green et al., 1987) or rat lgp120 (Lewis et al., 1985) were gifts from Ira Mellman (Yale University, New Haven, CT). Two rabbit immune sera made against the *Leishmania* gp63 metalloprotease were also used. One was specific to gp63 of *L. major* promastigotes (a gift from Clément Bordier and Pascal Schneider, Université de Lausanne, Switzerland; Etges et al., 1986). The other was raised against the hydrophilic form of *L. mexicana* promastigotes gp63 (a gift from P. Overath; Bahr et al., 1993). Rat IgG were purified as mentioned (Antoine et al., 1991).

The following fluorochrome-conjugated antibodies and gold-conjugated antibodies were used as secondary antibodies for immunolabelling at the light and electron microscope level, respectively. Mouse Ig-adsorbed goat anti-rat Ig F(ab′)$_2$ fragments labelled with fluorescein isothiocyanate (FITC) or Texas Red (TR) were obtained from Caltag (San Francisco, CA). TR-conjugated goat anti-mouse Ig Ab (rat Ig-adsorbed) and FITC-labelled donkey anti-rabbit Ig F(ab′)$_2$ fragments (mouse Ig- and rat Ig-adsorbed) were purchased from Jackson (West Grove, PA). Goat anti-rat Ig Ab and goat anti-rabbit Ig Ab linked to 10 nm gold particles were from BioCell (Cardiff, UK).

The following enzyme-conjugated antibodies were used in biochemical assays. Rabbit anti-rat Ig F(ab′)$_2$ fragments labelled with *Escherichia coli* β-galactosidase were prepared as described (Antoine et al., 1991). Goat anti-rat Ig Ab (mouse protein-adsorbed) linked to horseradish peroxidase (HRP) and goat anti-rat Ig Ab coupled to alkaline phosphatase were from Caltag and Sigma, respectively.

Immunofluorescence microscopy

Macrophages on coverslips were fixed with 4% paraformaldehyde (Merck-Schuchardt, Darmstadt, Germany) in 0.1 M sodium cacodylate-HCl buffer, pH 7.4, for 1 hour at room temperature. Cells were then permeabilized with 0.1 mg/ml saponin (Sigma) in phosphate-buffered saline (PBS) before processing for simple or double immunolabelling as already described (Lang et al., 1994a). In some cell preparations, nuclei of macrophages and parasites were also stained for 5 minutes with propidium iodide (5 µg/ml in PBS). After mounting in Mowiol (Calbiochem, San Diego, CA), cell preparations were examined under a conventional Zeiss Axiophot fluorescence microscope (Oberkochen, Germany) or under a Leitz confocal laser scanning microscope (Wild Leitz Instruments, Heidelberg, Germany). Conditions of observations by confocal microscopy were detailed previously (Lang et al., 1994a). Cell sections were sequentially analysed using 488 nm and 567 nm excitation wavelengths for the FITC and TR or propidium iodide signals, respectively.

As controls, fixed and permeabilized cells were incubated with irrelevant isotype-matched mAb (H35-17.2, A10.3.2, G21.10), with normal rat IgG or with normal rabbit serum according to the primary mAb or immune sera used in the specific labellings. These controls were generally unstained or exhibited a slight diffuse staining.

Immunocytochemistry on cryosections

Cryosections were prepared as follows. Adherent macrophages were fixed with 4% paraformaldehyde, 1% acrolein (Agar, UK) in 0.1 M sodium cacodylate-HCl buffer, pH 7.4, for 2 hours at room temperature, after which adherent cells were scraped off. Cells were then embedded in 5% gelatin in 0.1 M phosphate buffer, pH 7.2, impregnated overnight with 2.1 M sucrose in phosphate buffer and frozen in liquid nitrogen. Ultrathin cryosections collected on Formvar-coated nickel grids were floated face down for 1 hour at room temperature on the mAb M5/114 or on anti-gp63 immune sera diluted in PBS containing 0.1% bovine serum albumin, and then on gold conjugates diluted in the same medium. As controls, sections were incubated with the irrelevant isotype-matched mAb H35-17-2 or with normal rabbit serum. After incubation with Ab or sera and conjugates, sections were washed 3 to 4 times with PBS, 0.1% bovine serum albumin and stained for 5 minutes with 3% neutralized uranyl acetate before embedding in Methocel, 0.15% uranyl acetate (Fluka, Buchs, Switzerland).

Extraction of *L. amazonensis* amastigotes from infected macrophages and preparation of parasite lysates

Macrophages on 100 mm tissue culture dishes were washed with Dulbecco's PBS and then scraped off using a rubber policeman. Recovered cells were centrifuged at 1,200 ***g***, 20°C for 10 minutes and then resuspended in 45% isotonic Percoll (Pharmacia). Cells were disrupted by passage through a 27-gauge needle. Amastigotes released were laid over 90% isotonic Percoll and centrifuged at 4,200 ***g***, 15°C for 30 minutes. Purified amastigotes located at the interface 45% / 90% Percoll were washed twice with Dulbecco's PBS (Chang, 1980b). At this stage, amastigotes were incubated or not for 30 minutes at 4°C with 0.15 M NaCl, pH 2.8, to remove macrophage components bound to their plasma membrane. After counting, parasites were solubilized at 2×10^8 to 3×10^8 cells/ml in 50 mM Tris-HCl buffer, pH 7.5, containing 25 mM KCl, 5 mM $MgCl_2$, 0.5% (v/v) Nonidet-P40 (Sigma), Pefabloc-Sc, TLCK and phenanthroline, 1 mM of each, 5 µM pepstatin, 15 µM leupeptin and 0.1 mM antipain (lysis buffer). After 10 minutes at room temperature and 20 minutes at 4°C, lysates were immediately tested for the presence of MHC class II molecules or stored at −80°C until titration.

Quantitative assay of amastigote-associated MHC class II molecules

Serial dilutions of parasite lysates were made in lysis buffer and then mixed with an equal volume of the mAb M5/114 (360 ng/ml) diluted in twofold concentrated PBS containing 1% bovine serum albumin (BSA) (Sepracor, Villeneuve-la-Garenne, France), and 0.2% NaN_3. In parallel, serial dilutions of a mixture of purified I-A^d and I-E^d molecules (a kind gift from John Sidney, Cytel Corporation, San Diego, CA) were prepared as above and mixed with M5/114. After 1 hour at 37°C and overnight at 4°C, dilutions were centrifuged for 1 minute at 10,000 ***g***, 4°C before their deposition in the wells (100 µl/well) of a polystyrene flat-bottomed microtiter plate (Dynatech, Denkendorf, Germany) previously coated with I-A^d and I-E^d molecules. Coating was carried out for 2 hours at 37°C and overnight at 4°C with purified MHC class II molecules (1 µg/ml of each isotype in 50 mM potassium phosphate buffer, pH 8, 100 µl/well). The plate was then extensively washed with PBS and non-specific binding sites were blocked by a 30 minute incubation at room temperature with PBS, 0.5% BSA, 0.1% NaN_3. The coated plate with serial dilutions of parasite lysates or of class II molecules was incubated for 3 hours

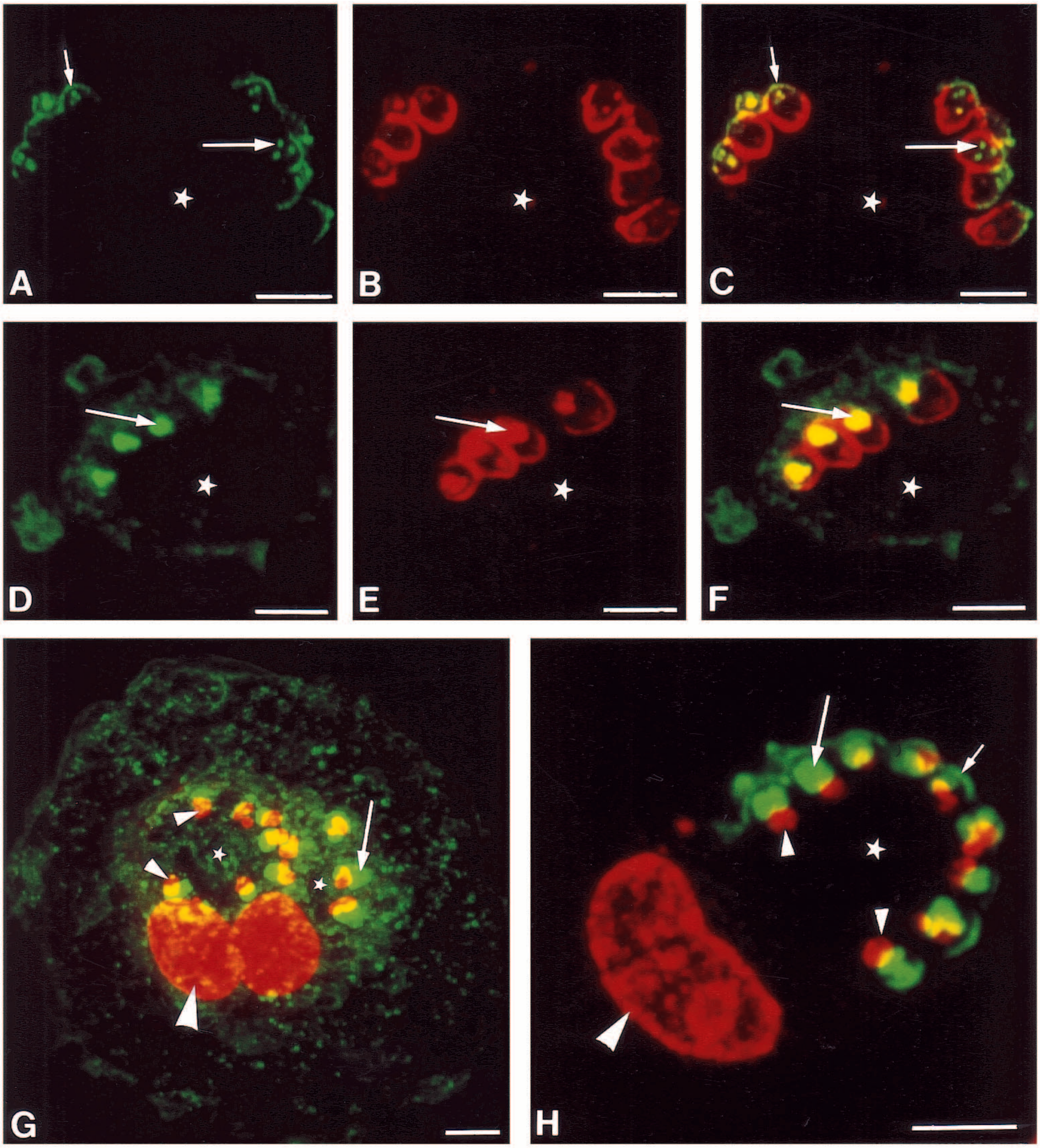

Fig. 1. Immunolabelling of PV-associated MHC class II molecules in 48 hour-infected macrophages from Balb/c mice. After infection, macrophages were activated with IFN-γ and then treated (D-H) with protease inhibitors for 20 hours before fixation or left untreated (A-C). Analysis was performed with a confocal laser scanning microscope. The three-dimensional reconstruction of a macrophage (G) or optical sections of macrophage perinuclear regions, which contain PV in infected cells (A-F,H), are shown. MHC class II molecules were labelled using the mAb M5/114 and goat anti-rat Ig $F(ab')_2$ fragments coupled to FITC (green staining). Amastigotes were detected using the mAb 2A3-26 and TR-conjugated goat anti-mouse Ig Ab or by labelling of the DNA with propidium iodide (red staining: small arrowheads, parasite nuclei and kinetoplasts; large arrowheads, macrophage nuclei). (A,B) PV of a macrophage not treated with protease inhibitor and scanned for the class II signal (A) and for the 2A3-26 epitope signal (B). Superimposed images of A and B are shown in C. PV-associated class II molecules are detected in the membrane of these organelles (small arrows) and within parasites (long arrows). (D-F) PV of a macrophage treated with antipain (50 µg/ml) and double-stained as in A-C. The M5/114, 2A3-26 and superimposed signals are shown in D, E and F, respectively. Under these conditions, internal structures of amastigotes (arrows) are much more reactive with the mAb M5/114. They also become strongly positive for the epitope recognized by 2A3-26. (G-H) Three-dimensional reconstruction (G) and optical section (H) of macrophages treated with Z-Phe-$AlaCHN_2$ (2 µg/ml) and labelled with the mAb M5/114 and propidium iodide. Class II molecules are associated with the plasma membrane and cytoplasmic vesicles of the macrophages (G), with the PV membrane (H, small arrow), and with large intracellular structures of amastigotes (G,H, long arrows). *The centre of the PV. Bars, 5 µm.

at 4°C. After washing with PBS, binding of the Ab M5/114 was assessed with β-galactosidase-linked rabbit anti-rat Ig $F(ab')_2$ fragments diluted in PBS, BSA, NaN_3 (1 µg/ml, 100 µl/well, 2 hours at 37°C). β-Galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside (Sigma) as substrate (Antoine et al., 1988) and after 30 minutes at 37°C the absorbance of the reaction product was read using a test wavelength of 414 nm and a reference wavelength of 690 nm. Amounts of amastigote-associated class II molecules were calculated by referring to the standard curve made with purified class II and the mAb M5/114. Results are expressed in arbitrary units.

Detection of Ii chains or Ii fragments bound to amastigote-associated MHC class II molecules

Microtiter plates were coated for 2 hours at 37°C and overnight at 4°C with the Ab 14-4-4S or with a mixture of the Ab 14-4-4S and MK-D6 diluted in 0.1 M sodium carbonate buffer, pH 9.5 (1 µg/ml of each, 100 µl/well). Plates were then washed with PBS, 0.1% Tween 20 (Merck-Schuchardt, Darmstadt, FRG). Parasite lysates prepared as described above and diluted with PBS, 0.1% Tween, 0.25% gelatin (Bio-Rad Laboratories, Richmond, CA) were incubated in coated wells for 2 hours at 37°C and overnight at 4°C. After washings, wells were incubated successively for 2 hours at 37°C with: (a) the mAb M5/114, In-1 or H35-17.2 (1 µg/ml); and (b) HRP-conjugated species-specific goat anti-rat Ig Ab. Ab and conjugate were diluted in PBS-Tween-gelatin. HRP activity was measured at room temperature, using *o*-phenylenediamine as chromogen. The absorbance of the reaction product was read using a 492 nm test wavelength and a 690 nm reference wavelength.

In other experiments, amastigotes lysates were electrophoresed on 12% SDS-polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose membranes (Burnette, 1981), and Ii chains or Ii fragments were detected by binding of mAb In-1 and of goat anti-rat Ig Ab linked to alkaline phosphatase. The blots were developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma) as substrates. As controls, blots were incubated with the mAb H35-17.2 instead of In-1.

Viability assay

The viability of *L. amazonensis* amastigotes incubated with antipain was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Mosmann, 1983). Amastigotes purified from lesions were suspended at 10^7 cells/ml in RPMI 1640 (without $NaHCO_3$) supplemented with 20 mM Hepes, 10% foetal calf serum, 50 µg/ml gentamycin and they were then treated for 20 hours at 34°C with various concentrations of antipain (from 10 to 100 µg/ml), after which parasites were centrifuged at 1,200 ***g*** (10 minutes, 20°C), resuspended in fresh medium without inhibitor and distributed into the wells of a microtiter plate (TPP, Switzerland; 100 µl/well). A 10 µl sample of MTT (5 mg/ml in PBS) was added. After 24 hours at 34°C, formazan product was dissolved and titrated as described (Mosmann, 1983). In some experiments, amastigotes were allowed to differentiate into promastigotes by a 24 hour incubation at 25°C after removal of antipain. At this stage, MTT was added and the 25°C incubation was continued for a further 24 hours.

RESULTS

Intracellular amastigotes endocytose MHC class II molecules

Earlier studies had shown that intracellular MHC class II molecules of macrophages activated with IFN-γ undergo an important redistribution after infection with *Leishmania* amastigotes and that, at 48 hours post-infection, a large proportion of these molecules is associated with PV membrane (Antoine et al., 1991; Lang et al., 1994a, b). Moreover, the MHC class II molecules associated with PV containing *L. amazonensis* are preferentially localized at the attachment zones of amastigotes to PV membranes (Lang et al., 1994a; Fig. 1A,C), and can also be detected within parasite structures (unpublished results; Fig. 1A,C). These structures were often located at the posterior poles of the parasites, near the site of their attachment to the inner face of the PV membrane. Because this region also contains megasomes, large lysosome-like organelles that are particularly evident in *Leishmania* of the *mexicana* complex (Pupkis et al., 1986; Antoine et al., 1988), we hypothesized that MHC class II molecules present in PV membrane could have been internalized by the parasites and degraded within megasomes. To test this hypothesis, infected macrophages activated with IFN-γ were treated with antipain (50 µg/ml), an inhibitor of cysteine and serine proteases, for 20 hours before fixation and immunolabelling of class II molecules with the mAb M5/114. Under these conditions, class II-specific staining of intra-parasite structures was considerably increased (Fig. 1D,F). Furthermore, these structures also became strongly reactive with the mAb 2A3-26 (Fig. 1E,F), a reagent that in the absence of protease inhibitor binds mainly to the plasma membrane of amastigotes, including the membrane of the flagellar pocket (Fig. 1B). Both findings could be explained by the internalization of class II together with some components of the amastigote plasma membrane and by the degradation of these molecules within parasites. Under these conditions, PV membrane could always be stained with anti-class II Ab but generally with a weaker intensity than

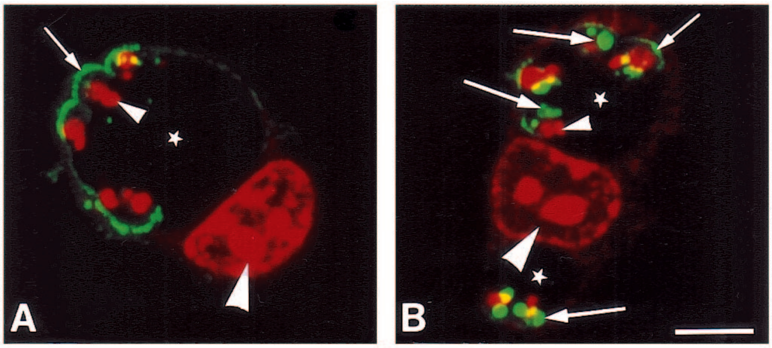

Fig. 2. Localization of MHC class II molecules in PV housing amastigotes treated with DMSO (0.2%) or Z-Phe-AlaCHN_2 (2 µg/ml) before infection. Macrophages from Balb/c mice used as host cells were activated with IFN-γ 24 hours before infection. Immunolabelling with M5/114 and a FITC conjugate (green staining) was performed at 24 hours post-infection. DNA was stained with propidium iodide (red staining: small arrowheads, parasite nuclei and kinetoplasts; large arrowheads, macrophage nuclei). Optical sections of macrophage perinuclear regions obtained by confocal microscopy are shown. (A) Macrophage infected with amastigotes pre-treated with DMSO. PV-associated class II molecules are mainly detected in the membrane of the organelle (arrow). (B) Macrophage infected with amastigotes pre-treated with Z-Phe-AlaCHN_2. PV-associated class II molecules are localized in PV membrane (small arrow) and in strongly stained internal structures of the parasites (long arrows). *The centre of the PV. Bar, 5 µm.

in control cells (data not shown). Antipain had no apparent effect on the viability of amastigotes (data not shown), a result that is in contrast with those reported by Coombs and Baxter (1984) for *L. mexicana* amastigotes. This discrepancy could be related to the short period of treatment used here or to other differences in the two protocols.

We next analysed the effects of other protease inhibitors on intra-amastigote class II staining. Some of the inhibitors tested (antipain, chymostatin, Z-Phe-AlaCHN_2, Z-Phe-PheCHN_2) strongly increased the internal class II staining, whereas others had only a moderate effect (leupeptin, E-64) or no effect (pepstatin A, 1,10-phenanthroline). Most of the inhibitors tested also increased with various degrees the class II-specific staining associated with intracellular vesicles and tubules of the macrophages. However, the effects of the inhibitors on macrophages and on amastigotes did not correlate (data not shown). For instance, E-64 and pepstatin A strongly increased the intracellular class II staining of the macrophages but had a moderate or no effect on that of the amastigotes. This lack of correlation suggested that the increase in intra-amastigote staining was due to a block of class II molecule degradation by parasite proteases. More specifically, amastigote cysteine proteases were probably implicated, since the most potent effect was noted with Z-Phe-AlaCHN_2 and Z-Phe-PheCHN_2, irreversible inhibitors of this type of enzyme (Fig. 1G,H). The absence of toxicity of most of these inhibitors (leupeptin, E-64, Z-Phe-AlaCHN_2, pepstatin A) on intracellular or isolated LV79 *L. amazonensis* amastigotes has been previously reported (Alfieri et al., 1988, 1989).

Internal structures of amastigotes located in IFN-γ-stimulated macrophages from Balb/c or C57BL/6 mice could be also labelled with mAb 14-4-4S or Y-3P, respectively, and the staining associated with these structures considerably increased after treatment of macrophages with antipain or Z-Phe-AlaCHN_2 (data not shown). As these Ab recognize conformational epitopes associated with class II αβ dimers (Germain and Hendrix, 1991), the class II molecules internalized by amastigotes are very likely to be undegraded, assembled molecules.

A similar analysis was performed on macrophages infected with *L. major* amastigotes. After infection, the macrophages were activated with IFN-γ and treated, as described above, with Z-Phe-AlaCHN_2. PV membrane from both treated and non-treated macrophages contained MHC class II molecules. Furthermore, in the presence of the protease inhibitor, class II-positive structures were detected within parasites. These structures were however less numerous and/or less intensively stained than in *L. amazonensis* (data not shown). In the experiments described below, only the latter species was used.

A strong intracellular staining of amastigotes by anti-class II Ab is observed when parasites are treated with Z-Phe-AlaCHN_2 before infection

The preceding experiments demonstrate that intracellular amastigotes are able to internalize host MHC class II molecules and that this process is much more obvious after treatment of infected macrophages with certain protease inhibitors. However, under these conditions, both macrophage and parasite proteases are inhibited. It could thus be argued that the apparent increase in intra-amastigote class II molecules arose from changes in the transport or maturation of these molecules, or else from the accumulation of class II that must occur within host cells, as reported for other class II-synthesizing cells treated with leupeptin (Neefjes and Ploegh, 1992; Zachgo et al., 1992; Loss and Sant, 1993). To assess this possibility, purified amastigotes were incubated in culture medium for 15 hours at 34°C with or without 2 μg/ml Z-Phe-Ala CHN_2. They were then washed and used to infect macrophages activated with IFN-γ for 24 hours. Treatment of amastigotes with Z-Phe-AlaCHN_2 apparently did not detectably interfere with their ability to infect macrophages. The PV they induced were, however, slightly smaller than PV housing amastigotes treated with DMSO alone (data not shown). Immunolabelling of class II molecules was performed at 3, 8 and 24 hours post-infection. At 3 hours, no difference could be detected between the two groups of infected macrophages, but at 8 hours class II staining was stronger within amastigotes treated with Z-Phe-AlaCHN_2 than within the control amastigotes (data not shown). At 24 hours post-infection, amastigotes treated with DMSO contained small class II-positive structures (Fig. 2A), whereas amastigotes treated with Z-Phe-AlaCHN_2 exhibited large and strongly labelled class II-positive internal structures (Fig. 2B). These results were very similar to those observed when infected macrophages were incubated with protease inhibitors.

Intracellular amastigotes treated with protease inhibitors either before or after infection accumulate large amounts of MHC class II molecules

We next quantified the amount of class II molecules present on/in amastigotes extracted from macrophages activated or not with IFN-γ and treated or not with antipain or Z-Phe-AlaCHN_2. The indirect enzyme immunometric assay used allowed determination of the amount of amastigote-associated class II by referring to a standard curve obtained with purified class II molecules. Typical results are shown in Fig. 3. In the absence of IFN-γ, amastigotes contained none or only a very small amount of class II; the amount of parasite-bound class II increased after treatment with IFN-γ and increased even more after treatment with both IFN-γ and protease inhibitors. A 3 to 4- and a 5 to 10-fold increase was noted between parasites from cells treated with IFN-γ alone and those treated with IFN-γ plus antipain, or between simple IFN-γ treatment and treatment with IFN-γ plus Z-Phe-AlaCHN_2, respectively (Fig. 3 and data not shown).

Very likely, the important increase in amastigote-associated class II after treatment of macrophages with antipain or Z-Phe-AlaCHN_2 was due to the apparently large amounts of class II detected by confocal microscopy in internal structures of the parasites. To test this point, amastigotes extracted from macrophages treated with IFN-γ alone or with both IFN-γ and Z-Phe-AlaCHN_2 were incubated for 30 minutes at 4°C with 0.15 M NaCl, pH 2.8, to remove cell surface-bound class II. Fig. 4 shows that about 70% of the class II molecules associated with amastigotes extracted from macrophages treated with IFN-γ was removed by the acid treatment and was thus extracellular. This was an expected result, since we had previously shown that, under these conditions, a large part of the associated class II is bound to the plasma membrane of the parasites (Antoine et al., 1991). In contrast, more than 80% of the class II molecules associated with amastigotes extracted from macrophages treated with both IFN-γ and Z-Phe-AlaCHN_2 were resistant to the acid treatment and thus intracellular.

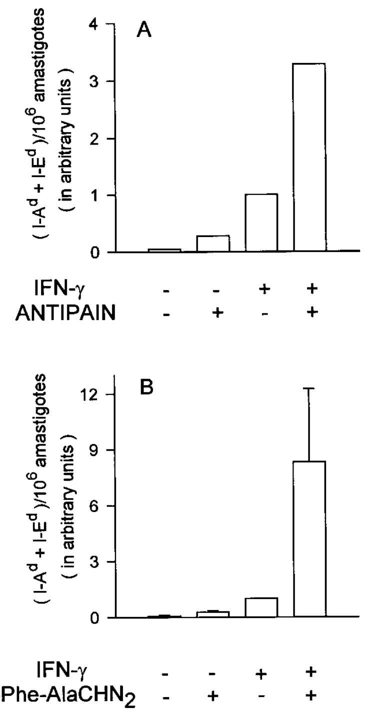

Fig. 3. Quantification of amastigote-associated MHC class II molecules. After infection, macrophages from Balb/c mice were activated or not with IFN-γ and treated or not with antipain (50 µg/ml, A), or Z-Phe-AlaCHN$_2$ (2 µg/ml, B) for 20 hours. At 48 hours post-infection, macrophages were disrupted and the amastigotes they contained were purified and lysed. MHC class II molecules present in parasite lysates were titrated as described in Materials and Methods. Results are expressed in arbitrary units. The amounts of MHC class II molecules associated with 10^6 amastigotes extracted from macrophages treated with IFN-γ, which gave the same inhibition as 1.33 to 4.83 ng of purified class II molecules, were taken as 1 unit. Data presented in A are from a single experiment. Data presented in B are means of two to three experiments. Error bars represent one s.d.

Finally, we found that intracellular amastigotes pre-incubated with Z-Phe-AlaCHN$_2$ before infection accumulated a greater amount of class II molecules in internal structures than amastigotes pre-incubated without inhibitor (Fig. 5). This result clearly indicates that the effect of enzyme inhibitors on the pool of class II molecules detected within parasites is mainly due to the inhibition of their own proteases.

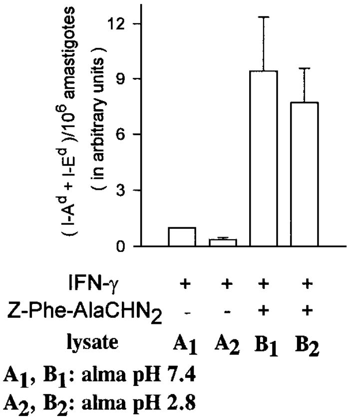

Fig. 4. Quantification of MHC class II molecules associated with internal structures of amastigotes. Infected macrophages from Balb/c mice were activated with IFN-γ and treated or not with Z-Phe-AlaCHN$_2$ (2 µg/ml) for 20 hours. At 48 hours post-infection, intracellular amastigotes (alma) were extracted, purified and incubated for 30 minutes at 4°C with either Dulbecco's PBS, pH 7.4, or 0.15 M NaCl, pH 2.8, before lysis. MHC class II molecules present in lysates were then titrated. Lysates A: amastigotes extracted from macrophages treated with IFN-γ alone and incubated at pH 7.4 (A1), or at pH 2.8 (A2) before lysis. Lysates B: amastigotes extracted from macrophages treated with IFN-γ plus Z-Phe-AlaCHN$_2$ and incubated at pH 7.4 (B1), or at pH 2.8 (B2), before lysis. Data are the means of three experiments. Error bars represent one s.d. Results are expressed in arbitrary units. The amounts of MHC class II molecules associated with 10^6 amastigotes extracted from macrophages treated with IFN-γ alone, which gave the same inhibition as 1.33 to 3.17 ng of purified class II molecules, were taken as 1 unit.

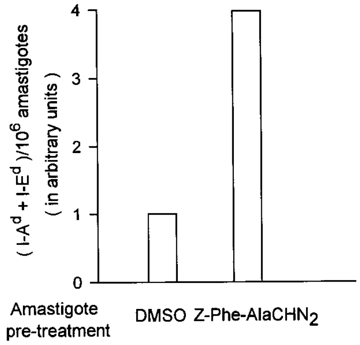

Fig. 5. Quantification of MHC class II molecules associated with internal structures of amastigotes pre-treated with DMSO or Z-Phe-AlaCHN$_2$ before infection. Lesion amastigotes were purified, treated at pH 2.8 and then incubated for 15 hours at 34°C with either 0.2% DMSO or 2 µg/ml Z-Phe-AlaCHN$_2$ in culture medium, after which parasites were washed and used to infect Balb/c macrophages activated with IFN-γ 24 hours before. At 24 hours post-infection, amastigotes were extracted, treated at pH 2.8 and lysed to titrate endocytosed MHC class II molecules. Results are expressed in arbitrary units. The amount of MHC class II molecules associated with 10^6 amastigotes pre-treated with DMSO, which gaves the same inhibition as 2.17 ng of purified class II molecules, was taken as 1 unit.

Invariant chains can be detected in PV membrane and in amastigotes after treatment of infected macrophages with protease inhibitors

Invariant (Ii) chains are non-polymorphic polypeptides that form complexes with newly synthesized class II αβ dimers. They are involved in the folding of class II, in their targeting towards endocytic compartments and impede their premature binding with peptides (Anderson and Miller, 1992; Teyton and Peterson, 1992). As observed in earlier studies, PV are apparently devoid of Ii chains (Antoine et al., 1991; Lang et al., 1994a,b), and are seen as holes in the cytoplasm following immuno-labelling with the mAb In-1, which recognizes the cytoplasmic tail of Ii (Fig. 6A). However, the PV became positive for these polypeptides in the presence of antipain, leupeptin, E-64, Z-Phe-AlaCHN$_2$ or Z-Phe-PheCHN$_2$, Z-Phe-AlaCHN$_2$ and Z-Phe-PheCHN$_2$ having the strongest effect (Figs 6 and 7). PV-associated Ii chains or fragments could be detected in the membrane of the organelles, mainly in the

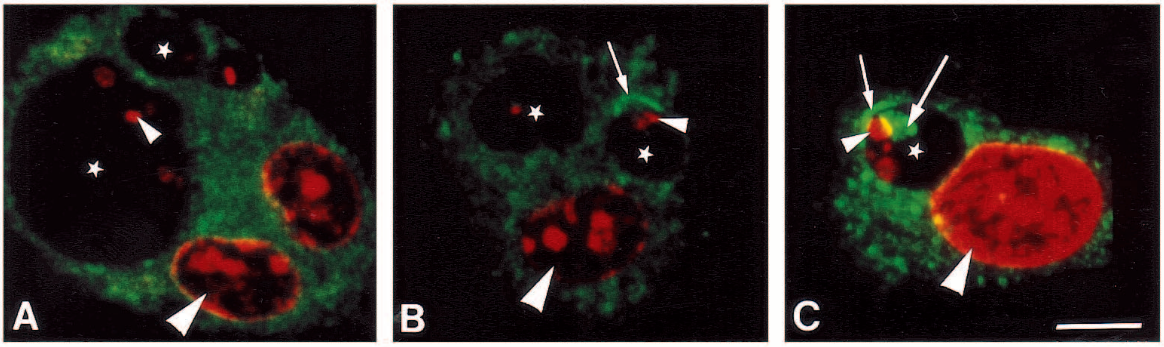

Fig. 6. Localization of Ii chains in PV of macrophages treated with protease inhibitors. Macrophages from Balb/c mice were infected, activated with IFN-γ and then untreated, or treated with protease inhibitors, as described in the legend to Fig. 1, before fixation and labelling for Ii chains using the mAb In-1 and a FITC-conjugate (green staining) at 48 hours post-infection. Amastigotes were detected by propidium iodide staining (red staining: small arrowheads, parasite nuclei and kinetoplasts; large arrowheads, macrophage nuclei). Optical sections of macrophage perinuclear regions obtained by confocal microscopy are shown. (A) Macrophage not treated with protease inhibitors. PV are devoid of Ii chains. (B) Macrophage treated with antipain (50 μg/ml). Note the presence of Ii chains in the membrane of one of the two PV (arrow). (C) Macrophage treated with Z-Phe-AlaCHN$_2$ (2 μg/ml). Ii chains are present in PV membrane (small arrow) and within parasites (long arrow). *The centre of the PV. Bar, 5 μm.

region of amastigote-binding sites, as PV-associated class II molecules (Fig. 6B). Ii chains or fragments were also present within amastigotes (Fig. 6C). Double labelling with the anti-class II mAb 14-4-4S and In-1 showed that class II and Ii chains co-localized in the same internal structures of the parasites (data not shown). In contrast, pepstatin A didn’t induce the appearance of Ii in PV (Fig. 7).

At 24 hours post-infection with amastigotes pre-treated with Z-Phe-AlaCHN$_2$, neither membranes of PV nor internal parasite structures could be stained with the anti-invariant chain mAb In-1 (data not shown).

MHC class II molecules present in PV of macrophages treated with Z-Phe-AlaCHN$_2$ are complexed with Ii chains or Ii fragments

MHC class II molecules reaching the PV were found to be associated with the plasma membrane of amastigotes and they remain bound to the membrane even after extraction of the parasites. A second pool of class II molecules is observed within amastigotes (see above). A distribution very similar to that observed for class II molecules was also found for PV-associated Ii chains in macrophages treated with several protease inhibitors. Using a two-site enzyme immunoassay, we tested whether PV-associated class II molecules remained complexed with Ii chains or Ii fragments. Amastigotes were extracted from IFN-γ-activated macrophages incubated in the presence or absence of Z-Phe-AlaCHN$_2$. Parasite lysates were prepared and deposited in microtiter wells previously coated with the mouse anti-class II mAb 14-4-4S. The wells were washed and incubated successively with either the anti-class II

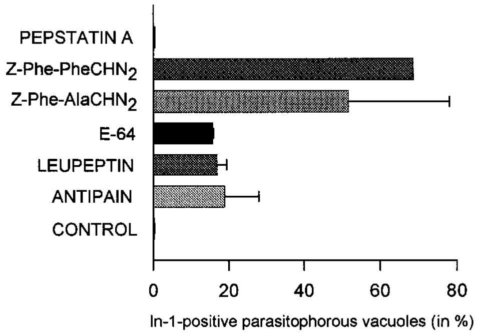

Fig. 7. Percentage of PV expressing Ii chains under various conditions. Macrophages from Balb/c or C57BL/6 mice were infected, activated with IFN-γ and treated or not for 20 hours with protease inhibitors. The final concentrations of inhibitors used were: 50 μg/ml (antipain, leupeptin, pepstatin A), 10 μg/ml (E-64), 2 μg/ml (Z-Phe-AlaCHN$_2$) and 2.35 μg/ml (Z-Phe-PheCHN$_2$). Forty-eight hours post-infection, macrophages were fixed, permeabilized and stained for Ii chains with the mAb In-1 and an appropriate FITC-conjugate. For each determination, about 500 VP were counted. Data are the means of 1 to 8 experiments. Error bars represent one s.d.

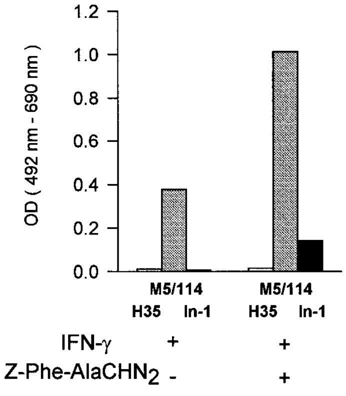

Fig. 8. Detection of amastigote-associated class II-Ii complexes. Amastigotes were extracted from 48 hour-infected macrophages of Balb/c mice activated with IFN-γ and treated or not with Z-Phe-AlaCHN$_2$ (2 μg/ml). Parasite lysates were deposited in wells of a microtiter plate coated with the mouse anti-class II mAb 14-4-4S. Wells were then incubated with the control rat mAb H35, the rat anti-class II mAb M5/114, or the rat anti-Ii mAb In-1, and finally with goat anti-rat Ig Ab coupled to HRP. Enzyme activity expressed in absorbance units was read 30 minutes after adding the substrate. Data are from one of three comparable experiments.

mAb M5/114, the anti-Ii mAb In-1 or H35, a control isotype-matched mAb (all three are rat Ab), and then with goat anti-rat Ig Ab coupled with HRP. As expected, the class II signal was higher with lysates of amastigotes extracted from macrophages treated with IFN-γ plus the protease inhibitor. Furthermore, the In-1 epitope was associated with these class II molecules but not with class II molecules present in lysates of amastigotes extracted from macrophages treated with IFN-γ alone (Fig. 8). To determine the size of the amastigote-associated Ii chains, Western blots of parasite lysates were incubated with In-1 and an alkaline phosphatase conjugate. As shown in Fig. 9, no band could be detected in lysates of amastigotes extracted from macrophages treated with IFN-γ alone but the presence of a 10-12 kDa In-1 reactive band corresponding to the NH_2 terminus of the Ii chains was noted in lysates of amastigotes extracted from macrophages treated with IFN-γ and Z-Phe-Ala CHN_2. Interestingly, a weak signal associated with a 41 kDa band, probably corresponding to the Ii chain p41, was also detected in lysates of parasites treated with the protease inhibitor. These results probably indicate that MHC class II molecules reaching the PV are still assembled with Ii chains or Ii fragments but that the latter are rapidly degraded in these organelles by host cysteine proteases.

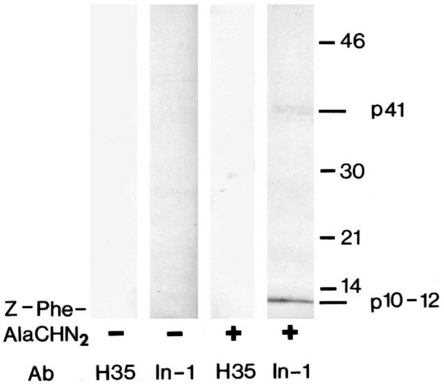

Fig. 9. Size of the Ii chains or Ii fragments associated with amastigotes. Amastigotes were extracted from 48 hour-infected macrophages of Balb/c mice activated with IFN-γ and treated or not with Z-Phe-AlaCHN_2 (2 μg/ml). Parasites were lysed in the presence of a large panel of protease inhibitors as described in Materials and Methods. Lysates were subjected to SDS-PAGE (each lane received the equivalent of 4.5×10^6 parasites). Then, they were blotted onto nitrocellulose sheets and successively incubated with In-1 or the control mAb H35-17.2 and an alkaline phosphatase conjugate. Molecular mass markers in kDa are indicated to the right of the panel.

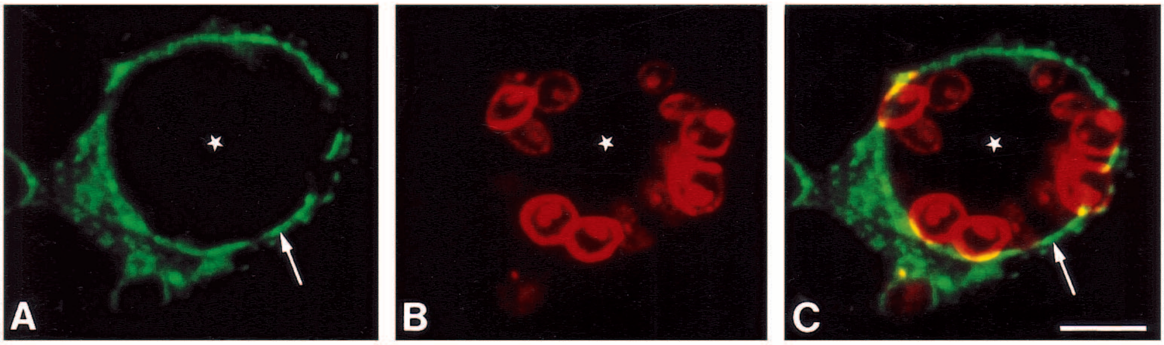

Fig. 10. Localization of macrosialin in PV of macrophages treated with Z-Phe-AlaCHN_2. Macrophages from Balb/c mice were infected, activated with IFN-γ and incubated with the inhibitor (2 μg/ml) before processing for confocal microscopy. The double-labelling of a 48 hour-infected macrophage with the anti-macrosialin mAb FA/11 and the anti-amastigote mAb 2A3-26 is shown. FITC-labelled goat anti-rat Ig F(ab′)$_2$ fragments and TR-conjugated goat anti-mouse Ig Ab were used to detect FA/11 (green staining) and 2A3-26 (red staining), respectively. (A,B) Optical section of a PV scanned for the FA/11 signal (A) and for the 2A3-26 signal (B). Superimposed signals of A and B are shown in C. Macrosialin is clearly detected in the PV membrane (arrows) but not within parasites. *The centre of the PV. Bar, 5 μm.

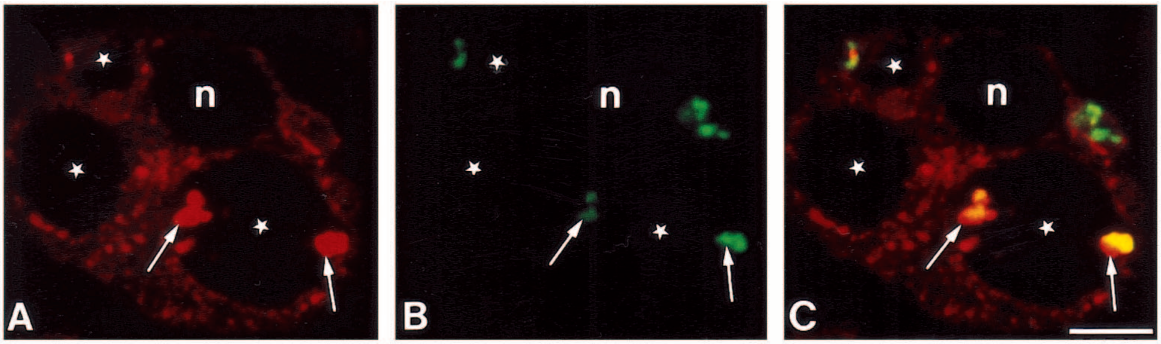

Fig. 11. Localization of MHC class II molecules and of *Leishmania* gp63 in infected macrophages of Balb/c mice that were treated with IFN-γ and antipain (50 μg/ml). At 48 hours post-infection, macrophages were fixed and labelled for both Ag, with M5/114 and a rabbit immune serum raised against the hydrophilic form of *L. mexicana* gp63, respectively. TR-labelled goat anti-rat Ig F(ab′)$_2$ fragments and FITC-conjugated donkey anti-rabbit IgF(ab′)$_2$ fragments were used to detect M5/114 (red staining) and anti-gp63 Ab (green staining). Analysis was performed by confocal microscopy. (A,B) PV scanned for the class II signal (A) and for the gp63 signal (B). Superimposed signals of A and B are shown in C. Some internal structures of amastigotes are positive for both class II and gp63 (arrows). *The centre of the PV. n, macrophage nucleus. Bar, 5μm.

PV membrane proteins other than class II molecules and Ii chains are not detected within parasites

To determine whether the internalization of class II and Ii by amastigotes reflects a general engulfment of PV membrane components, we tested for the presence, within parasites, of the prelysosomal/lysosomal glycoproteins macrosialin, lgp110 and lgp120, which are known to occur in PV membranes (Antoine et al., 1991; Lang et al., 1994a). As illustrated in Fig. 10 for macrosialin, these proteins were undetectable in internal structures of amastigotes even after treatment of macrophages with antipain or Z-Phe-AlaCHN_2. The results of these experiments suggest that the internalization of class II molecules by amastigotes involves a selective process.

Megasomes are involved in the internalization of MHC class II molecules by amastigotes

To determine whether the class II-containing structures of amastigotes could be the megasomes, macrophages treated with IFN-γ and protease inhibitors were double-labelled with anti-class II mAb and various anti-gp63 Ab. This approach was based on the fact that amastigotes of the *mexicana* complex express this enzyme mainly within megasomes (Medina-Acosta et al., 1989; Bahr et al., 1993). Fig. 11 shows that class II molecules internalized by amastigotes and gp63 were co-localized in the same structures, probably the megasomes. Similar findings were obtained using three anti-gp63 reagents: two rabbit immune sera specific to gp63 of *L. major* promastigotes and to the hydrophilic form of *L. mexicana* promastigote gp63, respectively, and the mAb TüL3.8 directed against the gp63 of *L. mexicana* promastigotes.

Direct demonstration of the presence of class II in megasomes was achieved by immunogold labelling of infected macrophage cryosections (Fig. 12). In macrophages treated with IFN-γ alone, PV-associated class II were detected mainly at the level of amastigote-binding sites and weakly in megasomes (Fig. 12A). In contrast, in macrophages treated with IFN-γ and Z-Phe-AlaCHN_2, PV-associated class II molecules were detected mainly in megasomes, which sometimes appeared dilated, but also in the PV membrane (Fig. 12C,D). In both conditions, megasomes were strongly positive for gp63 (Fig. 12B,E).

DISCUSSION

This paper describes the internalization by intracellular *L. amazonensis* amastigotes of MHC class II molecules synthesized by their host macrophages. This process occurs spontaneously and can be detected in infected macrophages that have not been treated with any additional agents. However, the visualization of class II molecules endocytosed by the parasites was much more evident if infected macrophages or amastigotes were treated with certain protease inhibitors. This protection of the class II molecules as well as their presence in parasite lysosomes called megasomes suggests that, after internalization, class II molecules are degraded within these organelles by parasite proteases. The protease inhibitors antipain and chymostatin, which react with both serine and cysteine proteases (Umezawa, 1982), and Z-Phe-AlaCHN_2 and Z-Phe-PheCHN_2, which irreversibly block cysteine proteases and especially cathepsin B and cathepsin L (Kirschke and Shaw, 1981), increased the accumulation of class II molecules within megasomes. The strongest effect was seen with Z-Phe-AlaCHN_2 and Z-Phe-PheCHN_2. Both cathepsin B-like and cathepsin L-like enzymes have been identified in *Leishmania* of the *mexicana* complex and some of these enzymes were localized in megasomes (Coombs et al., 1991; Robertson and Coombs, 1993; Duboise et al., 1994). Our data are thus consistent with the involvement of megasomal cathepsin B-like and/or cathepsin L-like enzymes in the degradation of internalized class II molecules.

The amount of PV-associated Ii chains or Ii fragments detected with the mAb In-1 was also clearly dependent on the presence, nature and subcellular localization of protease inhibitors. At 24 to 48 hours post-infection, in both infected macrophages cultured without protease inhibitor and macrophages infected with Z-Phe-AlaCHN_2-pre-treated amastigotes, the PV were generally devoid of detectable Ii chains. In contrast, in infected macrophages treated with antipain, leupeptin, E-64, Z-Phe-AlaCHN_2 or Z-Phe-PheCHN_2, about 20 to 60% of PV contained Ii chains or Ii fragments localized in the membrane of these organelles and/or in megasomes of the parasites and bound, at least in part, with class II molecules. An interpretation consistent with these results is that MHC class II molecules reaching PV are newly synthesized and still complexed with intact Ii chains or with Ii fragments. The latter would be rapidly degraded by macrophage proteases present in these compartments (Prina et al., 1990; Russell et al., 1992; Lang et al., 1994b), and more specifically by cathepsin B and/or cathepsin L, since the highest percentage of In-1-positive PV was obtained after treatment of macrophages with Z-Phe-AlaCHN_2 or Z-Phe-PheCHN_2. The possible involvement of cysteine proteases in the degradation of Ii chains has been already well documented (Blum and Cresswell, 1988). In contrast, no In-1-positive PV could be detected after treatment of infected macrophages with the inhibitor of aspartic proteases pepstatin A (Umezawa, 1982). This finding agrees with a recent paper showing that pepstatin A does not block the degradation of Ii chains in human B lymphoblastoid cells (Morton et al., 1995), but appears to be at variance with the report of Marić et al. (1994) demonstrating that an aspartic protease initiates Ii chain processing in transformed human B lymphocytes.

The mode and site of entry of MHC class II molecules and, in some experimental conditions, of Ii chains within parasites remain to be identified. In Trypanosomatidae, the main site of endocytosis is the flagellar pocket (Webster and Russell, 1993). It is difficult, however, to imagine that MHC class II molecules, which are integral proteins of the PV membrane, could be taken up by that route. An alternative possibility could be the internalization of class II and associated polypeptides by the parasite posterior pole, located on the opposite of the flagellar pocket. Several observations support this proposal: (1) class II present in the PV membrane are very often associated with the membrane binding site on the amastigote, which is the posterior pole (Antoine et al., 1991; Lang et al., 1994a); (2) in several Trypanosomatidae, including *Leishmania* amastigotes, subpellicular microtubules end at the subterminal region of the posterior pole. Consequently, the tip of the apex, which is often invaginated, is devoid of microtubules and thus represents a potential site for endocytosis (Angelopoulos, 1970; Gardener, 1974; Pan and

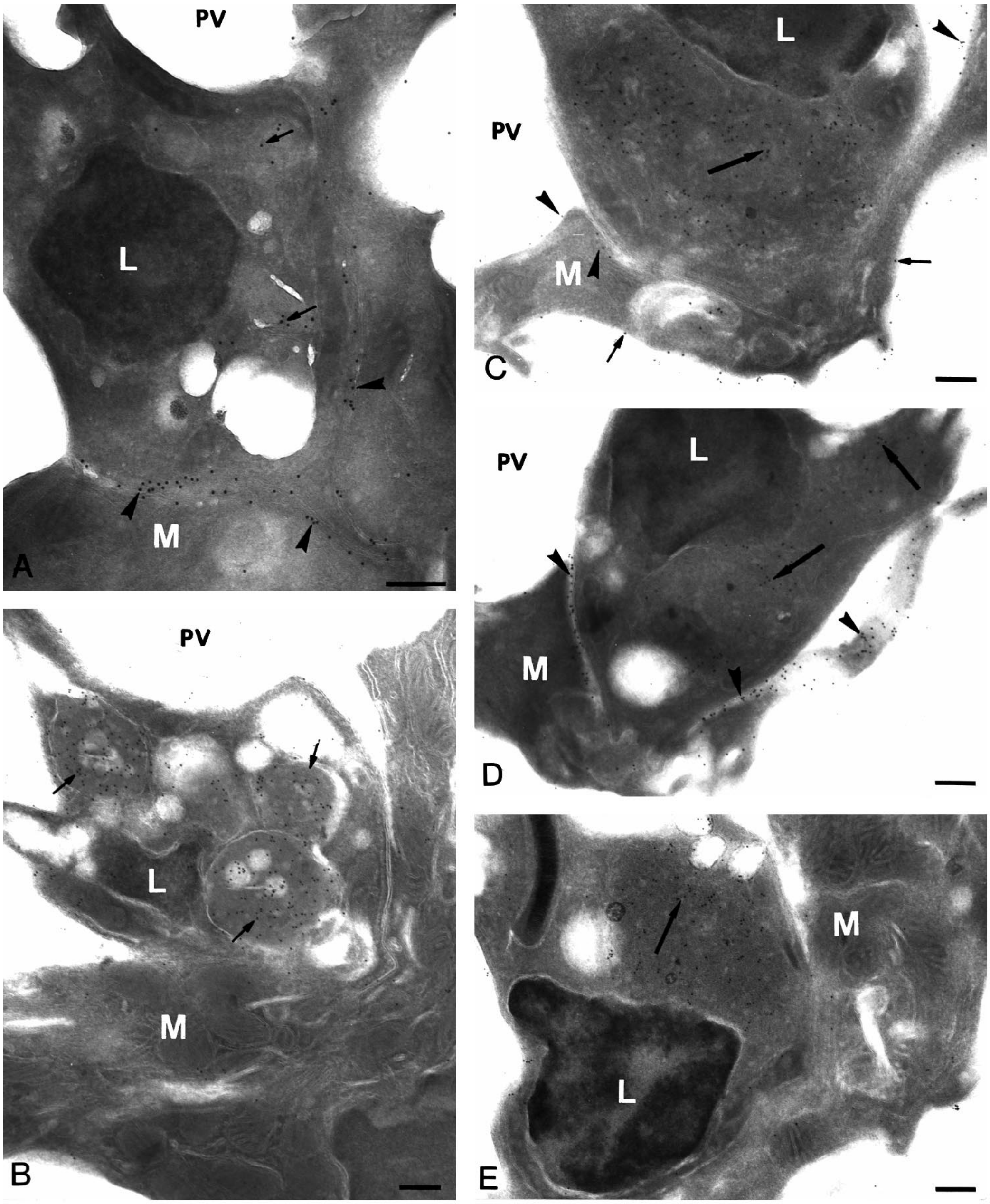

Fig. 12. Immuno-gold labelling of amastigotes located in Balb/c macrophages activated with IFN-γ and untreated (A,B), or treated for 20 hours with Z-Phe-AlaCHN$_2$ (C-E). At 48 hours post-infection, macrophages were fixed and processed for immuno-electron microscopy. Cryosections were labelled for MHC class II molecules with M5/114 and 10 nm gold-conjugate (A,C,D) or for gp63 with rabbit anti-*L. mexicana g*p63 immune serum and 10 nm gold-conjugate (B,E). (A) MHC class II molecules are detected at the level where amastigotes bind to PV membrane (arrowheads). A few gold particles are also present in megasomes (arrows). (B) gp63 is mainly localized in megasomes (arrows). (C,D) Class II molecules are present in PV membrane (C) including the zone of amastigote attachment (D) (arrowheads). After treatment with Z-Phe-AlaCHN$_2$, megasomes are strongly labelled for class II (long arrows, compare with megasomes in A). The plasma membrane of macrophages also contains class II (small arrows). (E) After treatment with Z-Phe-AlaCHN$_2$, gp63 is detected in slightly distended megasomes (arrow, compare with B). L, amastigote; M, macrophage; PV, lumen of parasitophorous vacuoles. Bars, 0.2 µm.

Pan, 1986); (3) electron micrographs show events that could be interpreted as endocytic processes occurring at the posterior poles of *Leishmania* amastigotes (Pham et al., 1970; Gardener, 1974). The observation that both the luminal parts of class II molecules (recognized by M5/114, 14-4-4S, Y-3P) and the cytosolic tail of Ii (recognized by In-1) can be detected in megasomes indicates that the parasites not only internalize molecules of the PV membrane but also portions of the PV membrane. However, the prelysosomal/lysosomal glycoproteins lgp110, lgp120 and macrosialin, which, like class II proteins, are integral proteins of the PV membrane, cannot be detected in megasomes even after treatment of the macrophages with protease inhibitors. This difference suggests that there are some specific mechanisms for the internalization of class II molecules and their associated polypeptides. Of course, plasma membrane components of amastigotes are also endocytosed during the process, as indicated by the strong reactivity of the megasomes with the mAb 2A3-26 noted after treatment of infected macrophages with antipain or Z-Phe-AlaCHN$_2$ (this Ab recognizes an epitope that is normally mainly expressed on the plasma membrane of amastigotes). Parasite proteins analogous to bacterial or viral superantigens (Herman et al., 1991), or partially unfolded (Jensen, 1993), could mediate the endocytosis of class II. But molecules other than proteins, for instance glycolipids that are highly expressed in the plasma membrane of amastigotes, could be also involved in this process. It is interesting to note in this context that the O-chain of *Brucella abortus* lipopolysaccharide appears to bind to mouse class II, based on its ability to induce compact conformers of these molecules (Escola et al., 1994). In any case, whatever the mechanisms brought into play, endocytosis apparently occurs independently of the class II isotype (I-A, I-E) and haplotype (H-2^d, H-2^b).

Both *L. amazonensis* and *L. major* amastigotes internalize host cell MHC class II molecules. The former species appears, however, to internalize much higher amounts of the class II proteins. The origin of this difference is still unknown, but could be linked to the expression of species-specific molecules on the amastigote plasma membrane, or to some peculiarities of the endocytic pathway(s) and/or to the level of protease activity. Several elements shown here to be involved in the internalization and degradation of class II vary greatly in different *Leishmania* species. Thus, the lysosome-like organelles called megasomes have, so far, been clearly identified only in amastigotes of *Leishmania* belonging to the *mexicana* complex. This complex is also distinguished by an abundant protease activity mainly due to numerous cysteine proteases (Coombs et al., 1991). Interestingly, to our knowledge, only *Leishmania* amastigotes endowed with megasomes and a high cysteine protease activity live in huge PV. Whether there is a correlation between the formation of large PV and an elevated capacity of amastigotes to internalize and degrade some PV membrane proteins could be an important topic for future study. For instance, in addition to class II molecules, amastigotes might endocytose and inactivate proteins involved in fission events.

The polarization of PV-associated MHC class II molecules towards amastigote-binding sites (Lang et al., 1994a) and the internalization and degradation of class II by *L. amazonensis* amastigotes, demonstrated here, are very likely two linked phenomena, which need to be considered in evaluating the antigen presentation capacity of infected macrophages. They could explain, at least partially, recent findings by our laboratory showing that macrophages infected with *L. amazonensis* are partially impaired in their capacity to present several exogenous protein Ag (Prina et al., 1993) including *Leishmania* Ag (Prina et al., unpublished data) to specific $CD4^+$ T cell hybridomas.

The present data suggest that class II molecules that reach the PV are newly synthesized molecules, which, consequently, are potentially able to bind peptides and especially parasite peptides. Such complexes, if they reach the plasma membrane of infected macrophages, could be detrimental for the parasite's survival. One way for *Leishmania* species to cope with the antigen presentation process could be the internalization of class II molecules, which are conveyed to PV. However, it is not yet known whether this mechanism is of sufficient magnitude to make infected macrophages invisible to the $CD4^+$ T lymphocytes, but its efficiency should be directly related to the number of amastigotes present in PV. Curiously, at 48 hours post-infection, neither MHC class II molecule expression on the macrophage plasma membrane nor the total amount of class II present on the cell surface and in internal compartments seems to be affected by the presence of the parasites (Prina et al., 1993; Lang et al., 1994a). This constant level of class II molecules suggests that the bulk of the newly synthesized class II molecules that are delivered from the secretory pathway to the plasma membrane bypasses the PV. Alternatively, a large part of newly synthesized class II could transit through the PV, but their sequestration/removal by amastigotes could have a noticeable effect on the steady-state level of class II only at post-infection times later than 48 hours, when 20 or more amastigotes can be located in each PV. A careful study of the rate of biosynthesis and of the turnover of class II molecules in *L. amazonensis*-infected macrophages should help to answer these questions.

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