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Distribution of Endosomal, Lysosomal, and Major Histocompatibility Complex Markers in a Monocytic Cell Line Infected with *Chlamydia psittaci*

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The intracellular fate of *Chlamydia psittaci* during infection of a monocytic cell line, THP1, was characterized. Cytochalasin D inhibited phagocytosis of latex beads but had no effect on infection by *C. psittaci*, and vacuoles expressed the transferrin receptor, suggesting accessibility to the endocytic pathway. Early *Chlamydia*-containing vacuoles expressed major histocompatibility complex (MHC) class I molecules, and most vacuoles fused with host cell lysosomes, since they expressed LAMP-1 and had acidic pHs. In cells prestimulated with gamma interferon, vacuoles also expressed MHC class II molecules, suggesting that the monocytes might effectively process *Chlamydia*-derived antigens for presentation by MHC class I and class II molecules.

Infection with a given *Chlamydia* strain confers short-term protection against subsequent infections by the same strain (23), and much of the protection appears to be T-cell mediated, as B-cell-deficient mice can resolve chlamydial infections as effectively as immunocompetent mice (29), whereas athymic (T-cell-deficient) mice display persistent infections (31). Furthermore, experimental animals have been shown to generate both $CD8^+$ and $CD4^+$ T cells in response to *Chlamydia* infections (reviewed in reference 30). Thus, in adoptive-transfer experiments, $CD4^+$ T cells obtained from mice challenged with *Chlamydia trachomatis* were effective in transferring antichlamydial immunity to naive mice (37), and isolated cytotoxic $CD8^+$ T cells were able to reduce the number of *Chlamydia* organisms in infected mice (36) or resolve the infection in persistently infected athymic mice (20). However, the source of the bacterial antigens presented by major histocompatibility complex (MHC) class I and class II molecules remains to be elucidated. MHC class I molecules generally present peptides derived from cytosolic (endogenous) antigens and are recognized by $CD8^+$ T cells, while class II molecules present peptides derived from antigens that have entered the antigen-presenting cell through endocytosis (exogenous antigens) and are recognized by $CD4^+$ T cells (9, 14). This poses an apparent paradox for pathogens such as *Chlamydia* organisms, which spend their entire infection cycle in epithelial cells within membrane-bound vacuoles that presumably do not intersect the MHC class I molecule-loading pathway (26). Moreover, epithelial cells express MHC class I molecules but, in the absence of previous stimulation by cytokines, normally do not express MHC class II molecules; this implies that other cells first encountered by the bacteria, such as macrophages or dendritic cells, most likely stimulate the *Chlamydia*-specific $CD4^+$ T cells.

Macrophages are thought to play an important role in the immunopathology of chlamydial infections, since they not only attempt to eradicate chlamydiae in the early stages of infection but in some cases are themselves infected and become sites of chlamydial replication. For several *Chlamydia* species, including *Chlamydia psittaci*, it has been shown that the bacteria can be internalized by macrophages, leading to either infection or bacterial lysis (reviewed in reference 22). Antigens degraded within macrophages may therefore be presented by MHC class I and class II molecules, thus providing the immune system with one of the earliest signals of infection.

In order to determine whether chlamydiae internalized by macrophages can provide antigens for presentation by MHC molecules, we investigated the distribution of MHC class I and class II molecules in the monocytic cell line THP1 (38, 40, 41), in which vacuoles harboring *C. psittaci* can be detected for at least 2 days of infection. As antigen processing by both class I and class II molecules is probably favored by an acidic pH (26), the localization of *Chlamydia*-containing vacuoles with respect to acidic compartments and those expressing lysosomal markers was also examined.

Infection of THP1 cells by *C. psittaci*. The guinea pig inclusion conjunctivitis strain of *C. psittaci* (3) was obtained from Roger Rank (University of Arkansas). The chlamydiae were grown as described previously (2, 17), and they were used unpurified. The monocytic leukemia cell line THP1 was maintained at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) and 2 mM L-glutamine.

THP1 cells were infected with *C. psittaci* for a total period of 2, 4, 6, 8, 24, or 48 h; the monocytes were fixed by paraformaldehyde, permeabilized with saponin, incubated with fluorescein isothiocyanate (FITC)-labelled anti-*Chlamydia* antibodies (lipopolysaccharide [LPS]-specific monoclonal antibody [MAb] clone C4; Argene, Varilhes, France), and mounted for confocal microscopy as previously described (25, 27). At all incubation times, bacterium-containing vacuoles were observed within the host cell at a concentration of one vacuole per one to five monocytes (shown for a 24-h infection period in Fig. 1). The vacuoles seen by confocal microscopy were generally less than 1 μm in diameter, similar to the sizes of early *C. psittaci* and *C. trachomatis* vacuoles in epithelial cell lines (12, 19, 24, 32, 42). However, unlike the vacuoles characterized for *C. trachomatis* in Vero cells (18) or for *C. psittaci* in HeLa cells (our unpublished observations), which developed into inclusions with diameters of about 10 μm after a 1-day infection, the *Chlamydia* vacuoles in THP1 cells remained essentially the same size for up to 48 h of infection (shown for 24 h in Fig. 1).

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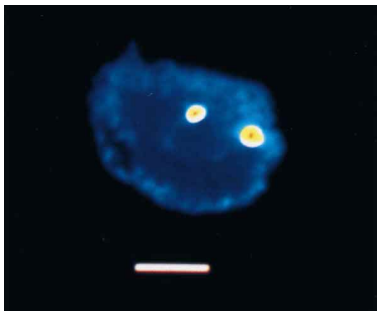

FIG. 1. Confocal micrograph of a representative THP1 cell infected with *C. psittaci* for 24 h, showing a single section of the cell with two intracellular vacuoles revealed by an FITC-labelled anti-LPS MAb. Most of the infected cells had only one *Chlamydia*-containing vacuole, and no difference in the vacuoles was noted in cells infected for 4, 24, or 48 h. The vacuoles are shown in red and yellow; pseudocolors were used to highlight the contours of the cell, which are visible due to background labelling with the anti-LPS MAb and are shown in blue. Bar, 5 μm.

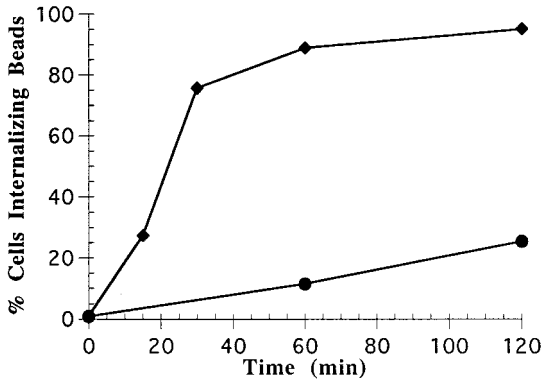

FIG. 2. Phagocytosis of fluorescent latex beads by THP1 cells. Cells were incubated at 37°C with latex beads for the indicated times with (●) and without (◆) CCD. The cells were then fixed with paraformaldehyde to arrest further phagocytosis, and the fluorescence was measured by cytofluorometry. Cells incubated with beads at 4°C for 2 h were used as negative controls for phagocytosis. The kinetics were measured on three separate days, with similar results.

Phagocytosis by THP1 cells and distribution of endosomal markers. The effect of cytochalasin D (CCD) (Sigma, St. Louis, Mo.) or recombinant human gamma interferon (IFN-γ) (Endogen, Cambridge, Mass.) on infection by *C. psittaci* was determined by cytofluorometry. THP1 cells were preincubated for 30 min at 37°C with 10 μg of CCD per ml in RPMI medium or with RPMI alone as a control, and the cells were then incubated for 24 h with bacteria in RPMI-FBS containing 10 μg of CCD per ml or in control buffer. The cells were centrifuged, washed once in phosphate-buffered saline (PBS), fixed with paraformaldehyde, permeabilized with saponin, and incubated with the fluorescein-labelled anti-*Chlamydia* MAb for confocal microscopy, as described above (25, 27). The cells were transferred into Falcon 2052 fluorescence-activated cell sorter tubes (Becton Dickinson, San Jose, Calif.), and the relative infection rate of THP1 cells was measured by cytofluorometry in the FL1 fluorescence range, which measured the fluorescence of the anti-*Chlamydia* MAb. Data from 5,000 THP1 cells were collected on a FACScan flow cytometer (Becton Dickinson), and the mean fluorescence intensity was obtained from the recorded data.

Given that the phagocytosis inhibitor, CCD, did not interfere with infection by *C. psittaci*, which survived in THP1 cells at a rate of 114% ± 13% (mean ± standard deviation) after a 24-h incubation with CCD, we verified whether the monocytes were capable of cytoskeleton-dependent phagocytosis. THP1 cells have been reported to be capable of ingesting opsonized erythrocytes, but not unsensitized erythrocytes, through phagocytosis (40). We measured phagocytosis with 1-μm-diameter yellow-green fluorescent carboxylate-modified polystyrene latex beads (Molecular Probes, Portland, Oreg.). A 2.0% (wt/vol) suspension of fluorescent beads in distilled water was diluted 25 times with RPMI containing 10% FBS, and this suspension was sonicated thoroughly. One million THP1 cells were placed in a tube and centrifuged, and the pellet was resuspended with 0.5 ml of the latex bead-RPMI-FBS solution. This mixture was incubated at 37 or 4°C for up to 2 h, with and without CCD, and phagocytosis was stopped by diluting the mixture with ice-cold PBS, centrifuging, and washing twice more with cold PBS. The cells were fixed with paraformaldehyde for confocal microscopy as described above (25, 27). The relative extent of phagocytosis was determined by cytofluorometry, as described above. As a negative control, THP1 cells were incubated for 2 h at 4°C, a temperature at which phagocytosis does not proceed. The results of a typical cytofluorometry experiment, in which almost all of the cells internalized at least one bead, are shown in Fig. 2. The kinetics of phagocytosis observed, with a half time of about 15 min, were consistent with phagocytosis in general, and essentially all phagocytosis was inhibited by CCD (Fig. 2).

We found that, consistent with the lack of effect of CCD on *Chlamydia* infection, most vacuoles containing bacteria expressed the transferrin receptor (TfR) (Fig. 3), a marker for early endosomes and recycling compartments (10, 16). The TfR was visualized by using ascitic fluid (1:1,000 dilution) containing the anti-TfR MAb 5E9 (American Type Culture Collection), revealed with rhodamine-coupled $F(ab')_2$ fragment goat anti-mouse immunoglobulin G antibodies (Jackson ImmunoResearch) at 10 μg/ml. These results are in agreement with a previous study, which found that cytochalasin B, at a concentration that inhibits phagocytosis of latex beads, has no effect on ingestion of *C. psittaci* by mouse peritoneal macrophages (15). Chlamydiae have also been reported to enter epithelial cells through a CCD-resistant mechanism characterized as endocytosis (19, 33). However, other studies using *C. psittaci* and *C. trachomatis* in both macrophages and epithelial cells gave conflicting results (reviewed in reference 3), raising the possibility that, depending on the *Chlamydia* strain and the host cell type, the bacteria may be capable of exploiting both endocytic and phagocytic entry mechanisms.

Interaction of *Chlamydia*-containing vacuoles with host cell lysosomes. Chlamydiae survive in epithelial cell lines (11, 12, 18) due presumably to their ability to inhibit fusion between *Chlamydia*-containing vacuoles and host cell lysosomes. In order to determine if the vacuoles in THP1 cells remain small because they fuse with lysosomes, cells were infected for 4 or 24 h and lysosomes and/or late endosomes were detected by using the MAb against human LAMP-1, H4A3 (Developmental Studies Hybridoma Bank, Iowa City, Iowa), at a 1:5,000 dilution. At both time points (shown for 4 h in Fig. 4A), most of the *Chlamydia*-containing vacuoles colocalized with the lysosomal marker. However, a small minority of vacuoles appeared to exclude LAMP-1 (data not shown). The degree of colocalization was determined semiquantitatively by taking advantage of the fact that the fluorescence intensities in confocal microscopy are digitized (35). Hence, by normalizing the fluorescence values for fluorescein (chlamydiae) and rhodamine (LAMP-1) so that the highest values were set at 100, 80% of the vacuoles were found to colocalize partially, with values for

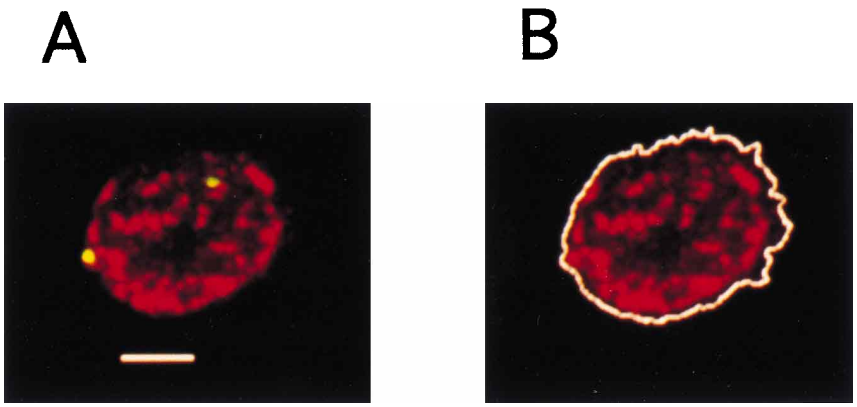

FIG. 3. Double-immunofluorescence labelling of *Chlamydia*-containing vacuoles and the TfR, a marker for early endosomes. (A) Endosomes (red) were revealed with a MAb against the TfR, and *Chlamydia*-containing vacuoles (green) were revealed with the anti-LPS MAb. Regions where both markers colocalized are shown in yellow. (B) The same cell is shown only in red. The contours of the cell (drawn in white) were identified by enhancing the contrast of low-fluorescence-intensity regions. The cell had been infected with *C. psittaci* for 4 h before fixing and incubating with antibodies. Bar, 5 μm.

both fluorescein and rhodamine equal to at least 25, and 50% of the vacuoles were found to colocalize strongly, with values for both colors equal to at least 50.

Similarly, when acidic compartments were revealed with the weakly basic amine DAMP [3-(2,4-dinitroanilino)-3-amino-*N*-methyldipropylamine] (Molecular Probes) and 2.5 μg of anti-2,4-dinitrophenol polyclonal antibodies (1) per ml followed by 10 μg of rhodamine-conjugated $F(ab')_2$ goat anti-rabbit immunoglobulin G antibodies (Immunotech, Marseilles, France) per ml, the majority of *Chlamydia*-containing vacuoles were found to be acidic (Fig. 4B). However, there was exclusion between a small fraction of the *Chlamydia*-containing vacuoles and the acidic vesicles (Fig. 4B). Hence, most of the vacuoles harboring bacteria fused with acidic lysosomes and/or late endosomes, but a small fraction of the vacuoles avoided fusion.

Distribution of MHC molecules in infected cells. No MHC class II molecule expression was observed by immunofluorescence in unstimulated THP1 cells, a result which is consistent with these cells having the characteristics of undifferentiated monocytes (39, 40). THP1 cells were therefore infected with chlamydiae after a 24-h incubation with 100 U of IFN-γ per ml. The effect of IFN-γ treatment on *Chlamydia* infection was tested by incubating THP1 cells with bacteria for 24 h, fixing and permeabilizing the cells, and incubating them with fluorescein-labelled anti-*Chlamydia* antibody. The presence of bacteria was detected by cytofluorometry. IFN-γ treatment de-

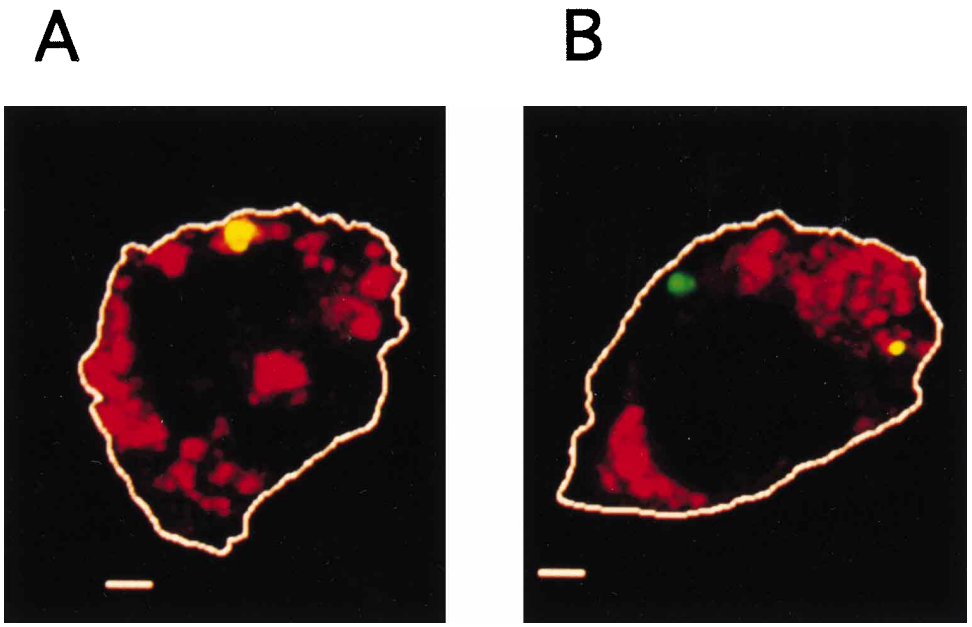

FIG. 4. Double-immunofluorescence labelling of *Chlamydia*-containing vacuoles and markers for lysosomes or acidic compartments. (A) The *Chlamydia*-containing vacuole (green) was revealed with an anti-LPS MAb, and the lysosomes (red) were revealed with an anti-LAMP-1 MAb. (B) The *Chlamydia*-containing vacuole (green) was revealed with an anti-LPS MAb, and the acidic compartments (red) were revealed with DAMP and anti-2,4-dinitrophenol polyclonal antibodies. Regions of colocalization appear in yellow. The infection was carried out for 4 h, and a single medial cross section of each cell is shown by confocal microscopy. Cell contours (drawn in white) were identified as described in the legend to Fig. 3. Bar, 2 μm.

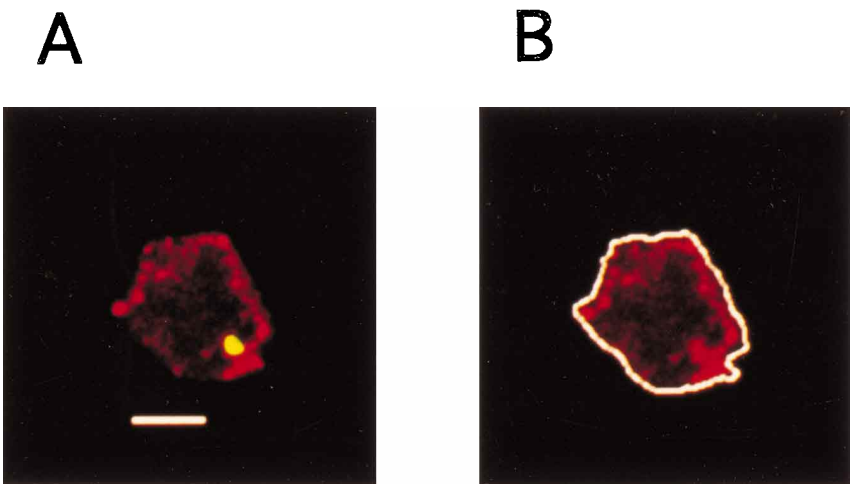

FIG. 5. Double-immunofluorescence labelling of *Chlamydia*-containing vacuoles and MHC class II molecules. (A) *Chlamydia*-containing vacuoles were revealed with FITC (green), and MHC class II molecules were revealed with rhodamine isothiocyanate (red); areas of colocalization appear in yellow. (B) The same cell is shown only in red (MHC class II molecules) with cell contours in white. The confocal micrograph shows a single cross section of an IFN-γ-treated THP1 cell that had been infected with *C. psittaci* for 4 h. Bar, 5 μm.

creased the amount of chlamydiae present after a 24-h incubation to 38% ± 7% (mean ± standard deviation), suggesting that cytokine treatment either caused bacteria to be killed or inhibited proliferation of the surviving bacteria. In previous work, it was shown that the inhibition of *C. psittaci* growth in lymphokine-activated macrophages is a static rather than lethal effect, since chlamydial replication is observed after removal of lymphokines from infected cells (6, 7).

The colocalization of chlamydiae and MHC class II molecules in IFN-γ-stimulated THP1 cells was then evaluated by confocal microscopy with the anti-MHC class II MAb BT2.9, which recognizes HLA-DR, used at a 1:5,000 dilution (8). As was the case for LAMP-1 expression or acidity, most of the vacuoles harboring chlamydiae expressed MHC class II molecules (Fig. 5). However, a small fraction of the vacuoles present after a 4-h infection appeared to exclude the MHC class II markers (not shown). The degree of colocalization was determined semiquantitatively as described above for LAMP-1. Ninety percent of the vacuoles displayed partial colocalization between chlamydiae and MHC class II molecules, while 30% of the vacuoles displayed strong colocalization between the two colors.

MHC class I molecules were detected in both untreated and IFN-γ-stimulated THP1 cells. The MHC molecules were revealed with the FITC-labelled anti-human MHC class I MAb W6/32 (Sigma), and the bacteria were detected with an anti-*Chlamydia* LPS MAb (1:300 ascitic fluid dilution) provided by J. Orfila (Amiens, France). In uninfected cells, the distribution of MHC class I molecules was restricted primarily to the surface, which is consistent with plasma membrane localization (14). After a 4-h incubation with chlamydiae, MHC class I molecules were detected on the host cell surfaces and in intracellular vesicles (Fig. 6). In both untreated and IFN-γ-stimulated THP1 cells, essentially all *Chlamydia*-containing vacuoles colocalized with MHC class I molecules (Fig. 6), and the colocalization was detected within 2 h of infection (data not shown).

In conclusion, we have analyzed the intracellular behavior of *C. psittaci* in THP1 cells at various times of infection up to 2 days. Intracellular bacteria were observed within 2 h by confocal microscopy, and bacteria were present for at least 2 days. It is thus likely that the vacuoles observed at 2 days represented the minority that had avoided fusion with lysosomes at earlier time points. However, the vacuoles never developed into large inclusions, suggesting that THP1 cells may lack as-yet-unknown signals required for homotypic fusion between *Chlamydia*-containing vacuoles. Furthermore, most *Chlamydia*-containing vacuoles expressed endosomal and lysosomal markers and were acidic, and they expressed MHC class II molecules, which was consistent with their ability to interact with the endocytic-lysosomal pathways used for loading MHC class II molecules (43).

Although the intracellular fate of *Chlamydia* organisms within macrophages or monocytes had not been previously characterized with the markers we used, the interaction between *Chlamydia*-containing vacuoles and host cell lysosomes has recently been studied by confocal microscopy in Vero cells (18). After a 24-h infection with *C. trachomatis*, the inclusion bodies excluded the lysosomal markers LAMP-1, LAMP-2, acid phosphatase, cathepsin D, and H^+ ATPases, and the Golgi and late endosomal marker, the cation-independent mannose 6-phosphate receptor. These vacuoles also failed to accumulate the acidotropic base acridine orange (18), and it was shown that the vacuole pH remained above 6 in the epithelial cell line HEC-1B (34). Nonetheless, the pH of the vacuoles decreased to 5.3 when heat-killed *C. trachomatis* was added to HEC-1B cells (34), suggesting that at least a fraction of the acidic vacuoles we observed in THP1 cells may contain damaged bacteria that were present in our preparation.

The MHC class I protein has been observed on essentially all vacuoles containing chlamydiae within 1 h of internalization in THP1 cells, suggesting that the MHC molecule may have been cointernalized with the bacteria. It still remains to be seen whether these MHC molecules can present chlamydial antigens. Antigens from intracellular bacteria such as *Shigella* and *Listeria*, which are phagocytosed by the host cell but then escape into the cytoplasm, are presented efficiently by MHC class I molecules of the infected cells; however, bacteria lacking the hemolysin that lyses the phagosomal membrane remain within the vacuoles, and the infected cells are poorly recognized by $CD8^+$ T cells (5, 13). This does not appear to be a general rule, though, since $CD8^+$ T cells are able to recognize

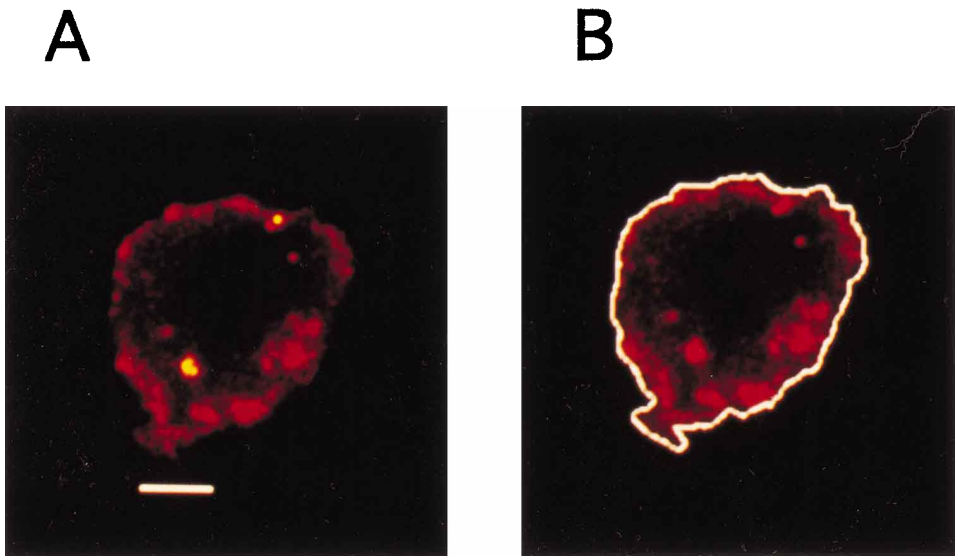

FIG. 6. Double-immunofluorescence labelling of *Chlamydia*-containing vacuoles and MHC class I molecules. (A) MHC class I molecules (red) were revealed with an anti-class I MAb in a THP1 cell that had been infected for 4 h with *C. psittaci* (green). Most of the labelling for MHC class I molecules was at the cell surface except for two brightly stained intracellular vesicles. Colocalization between *Chlamydia* and MHC class I molecules appears in yellow. (B) The same cell is shown in red (MHC class I molecules) with cell contours in white. A single cross section of the cell is shown. Bar, 5 μm.

Chlamydia antigens in infected L cells through the MHC class I molecule-loading pathway normally used by endogenous antigens (4), since recognition was blocked by inhibitors that inhibit conventional class I presentation. Along similar lines, a novel pathway linking phagosomes with the cytosol of antigen-presenting cells has recently been described (21, 28), and it is conceivable that *Chlamydia* could exploit this pathway. The observation that most vacuoles containing bacteria and expressing MHC class I molecules are also acidic favors the possibility that these MHC class I molecules exchange their contents for *Chlamydia*-derived peptides (26). Furthermore, most of the *Chlamydia*-containing vacuoles fuse with lysosomes and compartments expressing MHC class II molecules, suggesting that this monocytic cell line might effectively present antigens to *Chlamydia*-specific $CD4^+$ T cells.

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