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Internalization of Chlamydia by Dendritic Cells and Stimulation of Chlamydia-Specific T Cells

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Chlamydia species are the causative agents of trachoma, various forms of pneumonia, and the most common sexually transmitted diseases. Although the infection cycle has been extensively characterized in epithelial cells, where the Chlamydia entry-vacuoles avoid fusion with host-cell lysosomes, the cellular immune response has received less attention. Moreover, despite the abundant presence of dendritic cells (DC) in the sites of infection, the interaction between Chlamydia and DC has never been studied. We observe that DC kill Chlamydia trachomatis and Chlamydia psittaci. The chlamydiae are internalized by the DC in a nonspecific manner through macropinocytosis, and the macropinosomes fuse subsequently with DC lysosomes expressing MHC class II molecules. The interaction induces maturation of the DC, since presentation of an exogenous Ag is severely inhibited after a 1-day incubation, although chlamydial Ags are still presented and recognized by Chlamydia-specific CD4+ T cells. Thus, DC most likely play a role in initiating the T cell response in vivo and could potentially be used in adoptive transfer therapies to vaccinate against Chlamydia. The Journal of Immunology, 1998, 160: 1297–1303.

The chlamydiae are among the most successful pathogens on earth and have been implicated in a wide spectrum of diseases in humans and other mammals as well as in birds (1, 2). The three species pathogenic to humans are Chlamydia trachomatis, Chlamydia psittaci, and Chlamydia pneumoniae. Members of the C. trachomatis species alone afflict over 500 million people and are the causative agents of trachoma (the leading cause of preventable blindness) and of the most frequently encountered bacterially acquired sexually transmitted diseases.

Chlamydia is an obligate intracellular Gram-negative bacterium whose proliferation in host epithelial cells is characterized by two distinct developmental stages (1). Infection is initiated by adherence to the host cell of the infectious but metabolically inert elementary bodies (EBs, 0.3 μm in diameter). Although the mechanism of internalization remains controversial (2), the bacteria appear to survive within host epithelial cells through their ability to inhibit fusion between the entry vacuoles and host cell lysosomes (3, 4). Several hours after internalization, the EBs differentiate into 1.0-μm reticulate bodies (RBs), which are noninfectious and proliferate within vacuoles that give rise within 24 h to large “inclusion bodies” that may contain up to a thousand bacteria. After approximately 2 days of infection, the RBs differentiate back to EBs, the EBs are released from the infected cell, and a new cycle of infection can begin.

Consistent with the intracellular localization of Chlamydia, cell-mediated immune responses against Chlamydia genital infections have been observed in humans, mice, and guinea pigs (2). Transfer of either CD4+ or CD8+ T lymphocytes into naive mice has been shown to protect the mice against challenge with C. trachomatis, and studies with mice deficient in MHC molecules confirm the importance of the T cell-dependent response. Nonetheless, despite the abundance of dendritic cells (DC) in the cervix and vagina and in other epithelial tissues (5–8), the possible participation of DC in warding off Chlamydia infections has not been investigated.

DC are very efficient APCs and play a pivotal role in activating T cell-dependent immune responses (9, 10). DC present in epithelial tissues are thought to perform a “sentinel” function, due to their ability to internalize pathogens and process their Ags before migration to secondary lymphoid organs where they stimulate CD4+ and CD8+ T cells (10). Accordingly, we have studied the interaction of two Chlamydia species, C. trachomatis and C. psittaci, with a fully functional DC line. Key features of the interaction were confirmed with primary DC. The bacteria were internalized through macropinocytosis in what appeared to be a nonspecific manner. However, unlike the results described for other microbes, the DC killed the chlamydiae, apparently due to early fusion between Chlamydia vacuoles and host-cell lysosomes, a fusion that is inhibited in epithelial cells. The chlamydiae induced secretion of TNF-α and maturation of the DC, since presentation of an exogenous Ag was inhibited after a 24-h incubation with the bacteria. Most importantly, the mature DC presented efficiently chlamydial Ags to Chlamydia-specific T cells. Given the lack of success in the development of effective Chlamydia vaccines (11) and the recent use of DC as adjuvants for inducing antitumor immunity in vivo (12), the use of Chlamydia-fed DC transferred in...
vivo should be considered as part of future immunization strategies against Chlamydia infections.

Materials and Methods

Cells and materials

The two Chlamydia species used here, the guinea pig inclusion conjunctivitis (GPC) serovar of C. psittaci and the C. trachomatis agent of mouse pneumonitis (MoPn), have been described elsewhere (2). The DC line, D25C/1 (13), was generously provided by Dr. P. Ricciardi-Castagnoli (University of Milan, Italy). Primary dendritic cells were isolated from the bone marrow of BALB/c mice as described (14).

The following have been previously described (15): FITC-labeled anti-Chlamydia mAb; the anti-Chlamydia LPS mAb; the mAb against human LAMP-1, H4A3; the mAb against the human transferrin receptor (TIR), 5E5; the mAb against dextran (neutral; 40,000 kDa) and 5-(2,4-dinitroanilino)-3'-amino-N-methylidipropylamine (DAMP); cytochalasin D (CCD); 1,4-diazalicyclo[2.2.2]octane (DABCO); rhodamine-coupled F(ab')2 fragment goat anti-mouse IgGs; and rhodamine-conjugated F(ab')2 goat anti-rabbit IgGs. The mAb against murine LAMP, anti-CD107a, was from Pharmingen (San Diego, CA). The hyridoma producing the anti-murine TIR, R17 217.1.3, was from American Type Culture Collection (ATCC, Rockville, MD), and the biotinylated anti-MHC class II mAb recognizing IAα-d was from Pharmingen. Rhodamine B-labeled dextran (neutral; 40,000 kDa) and 5-(N,N-dimethylamino)rhode from Molecular Probes (Leiden, The Netherlands). The Factor-Test X mouse TNF-α ELISA kit was from Genzyme (Cambridge, MA).

Cell culture conditions and infection with Chlamydia

Chlamydiae were obtained from infected HeLa cells as described (16). A partially purified suspension was aliquoted and stored at −80°C until ready for use. For heat pretreatment experiments, the bacteria were incubated at 55°C for 30 min before incubating with HeLa or DC.

Typically, the DC line was prestimulated for 2 days with 100 U/ml IFN-γ, then incubated with bacteria in RPMI 1640 for the indicated times at 37°C in 5% CO2. Unless indicated otherwise, the cells were incubated in RPMI 1640 for 1 h at 37°C before adding them to an equivalent volume of HeLa cells or DC in culture medium. After an hour, unbound bacteria were removed by washing once with PBS and replacing the supernatant with culture medium. After an additional 3 h, the cells were fixed, permeabilized, and incubated with FITC-labeled anti-Chlamydia mAb. Bacteria were then counted with a 63X lens using a Zeiss Axioskop fluorescence microscope.

Electron microscopy

DC or HeLa cells infected with chlamydiae for 0, 4, or 24 h were fixed with 2.5% glutaraldehyde for at least 2 h at room temperature. The fixed cells were then prepared for electron microscopy as previously described (18). Thin sections were poststained with uranyl acetate and lead citrate for examination on a Zeiss electron microscope at an accelerating voltage of 50 kV.

Measurement of TNF-α secretion

Secretion of TNF-α from DC was measured using the Genzyme mouse TNF-α ELISA kit following the manufacturer’s instructions. DC were stimulated in 96-well plates with 100 IFN-γ for 2 days, and then incubated with chlamydiae (at an m.o.i. of 1:3 for GPC and 1:30 for MoPn) for the indicated times. The supernatant was collected, centrifuged in Eppendorf tubes to remove cellular debris, and kept frozen at −80°C until use. Supernatants were diluted in the ELISA kit wash buffer, and the absolute concentrations of TNF-α were obtained by calibrating the ELISA kit with a known concentration of TNF-α provided by the manufacturer.

HEL Ag presentation assays

DC were plated at a concentration of 3 × 104 cells/ml and stimulated with 100 U/ml murine IFN-γ (Genzyme) and 2 ng/ml murine rGM-CSF (Genzyme) for 2 days. The DC were washed with PBS and trypsinized for 20 min at 37°C. After washing twice with complete medium, infected or uninfected cells were ready for use in Ag presentation assays.

DC were fed GPC (m.o.i. = 1:30) or MoPn (1:300) for 1 day. The DC and the HEL-specific T cell hybridoma B9.1 (19) were plated into 96-well tissue culture plates at a concentration of 103 cells/well of each cell type in 200 μl of complete medium. The DC were used to present HEL (10 mg/ml) or HEL-derived 103–117 peptide (1 mg/ml) to stimulate IL-2 production from the B9.1 hybridoma. Cells were incubated for 24 h at 37°C and plated in triplicate in 96-flat-bottom well microtiter plates (Costar, Cambridge, MA). IL-2 secretion after 24 h of culture was measured by transfecting the supernatants to microcultures wells to stimulate proliferation of the IL-2-dependent cell line CTLL-2. Proliferation was assessed by the addition of 1 μCi of [3H]thymidine per well during the last 12 h of a 36-h culture period. Cells were harvested onto paper with an automatic cell harvester (Skatron, Sterling, VA), and radioactive counts were measured in a beta plate counter (Beckman, Fullerton, CA).

Purification of Chlamydia-specific CD4+ cells and proliferation assay

The ILN were removed from five mice 13 days after vaginal inoculation with the MoPn and pooled (20). Single-cell suspensions were incubated for 30 min on ice with TIB146 (anti-B20) and HB85 (anti-κ light chain) from ATCC and anti-CD11b (clone M1/70) and anti-CD8 (53-6.7) from Pharmingen. The cells were then incubated with microbeads conjugated to goat-anti-rat Ig (Miltenyi Biotech, Auburn, CA) for 30 min on ice. After washing, the cells were applied to MiniMACS columns (Miltenyi Biotech). The resulting population was 96% pure for CD4 cells. The DC line was seeded at 100 cells/ml with rIFN-γ (100 U/ml) and recombinant granulocyte/macrophage-CSF (2 ng/ml) for 2 days at 37°C. The DC were trypsinized and treated with 100 μg/ml of mitomycin C for 20 min at 37°C. ILN CD4 cells (20,000) were incubated for 5 days in 96-well plates with 10,000 DC and 5 μg of UV-inactivated MoPn. Proliferation was measured by [3H]thymidine incorporation (20).
Results

Differential internalization mechanisms of Chlamydia in epithelial and dendritic cells

The internalization rate of chlamydiae in epithelial cells has not been rigorously characterized before, and it was not known whether chlamydiae could infect DC. The bacteria were therefore incubated with HeLa cells or DC for different times, the unbound bacteria were removed, and the host cells with associated bacteria were fixed. Bacteria that had not entered the host cells were then revealed by incubating the cells with an anti-Chlamydia mAb, followed by incubation with a RITC-labeled second Ab. Internalized bacteria were then revealed by permeabilizing the cells and incubating them with a FITC-labeled anti-Chlamydia mAb, which would bind to both internalized bacteria and external bacteria. Thus, internalized bacteria appeared green, while external bacteria were red or yellow. Figure 1A shows the kinetics for bacterial entry into HeLa cells and DC. The epithelial cells, representing the preferential physiologic target for Chlamydia (1), were infected inefficiently, with only 20 to 30% of the associated bacteria having entered within 6 h. The DC not only internalized the bacteria faster, but to a greater extent, with >60% of the bacteria entering within 6 h. Cytoskeleton-dependent phagocytosis or macropinocytosis (21) appeared to be involved in the entry in both cell types, however, since CCD inhibited entry by 76 ± 12% in HeLa cells and 87 ± 13% in DC (not shown). Macropinocytosis was further implicated by the observation that dimethyl amiloride, which inhibits macropinocytosis in primary DC and bone marrow macrophages (22, 23), decreased chlamydial internalization by the DC by 75 ± 7% (not shown).

Heparin has previously been reported to inhibit the adherence of C. trachomatis (24, 25) and C. psittaci (16) to epithelial cells. To consider, additionally, the possible effects of heparin on early stages of entry, we measured the number of bacteria associated with host cells after a 4-h incubation with chlamydiae. Heparin efficiently inhibited bacterial attachment by HeLa cells, with >90% inhibition by 100 μg/ml heparin (Fig. 1B). On the other hand, heparin did not have a significant effect on internalization by DC lines (Fig. 1B) or primary DC (not shown). In addition, heat inactivation, which inhibits C. psittaci attachment to HeLa cells (16), had no effect on the kinetics of internalization by DC (not shown). These data suggest that different receptors and/or entry mechanisms may be used by Chlamydia in DC.

Differential intracellular fate of Chlamydia in epithelial cells and dendritic cells

To investigate the possibility that the Chlamydia vacuoles may be routed differently in epithelial cells and DC, host cells were incubated with bacteria for 4 or 24 h, and the interaction between the entry vacuoles and lysosomes was analyzed by confocal microscopy using LAMP as a marker for lysosomes (26). Most Chlamydia vacuoles in epithelial cells avoided fusion with lysosomes at early stages of entry (Fig. 2A), and within 24 h the vacuoles had developed into the expected inclusions, having diameters of approximately 10 μm, which also excluded the lysosomal marker (Fig. 2B). However, the Chlamydia vacuoles displayed a very different behavior in DC, as essentially all vacuoles fused with lysosomes within a few hours. Figure 2C shows one vacuole that had fused with lysosomes in the DC line; the same results were obtained with a 20-fold increase in the bacterial concentration. Fusion between Chlamydia vacuoles and lysosomes was also observed in primary DC (not shown). No inclusions were ever detected at 24 h. To exclude the possibility that the intracellular fate of Chlamydia in DC may be due to prestimulation of the DC.
line with IFN-γ, the same experiments were repeated in the absence of IFN-γ prestimulation. No differences were observed in vacuole fusion with lysosomes or survival of bacteria in either stimulated or unstimulated DC (not shown).

Interaction with other host cell compartments was analyzed using Abs against the TR, a marker for early and recycling endosomes (27, 28), and DAMP, a weakly basic amine that concentrates in acidic compartments and is revealed with fluorescently labeled Abs (15). Three cell types were studied: HeLa cells, L cells (fibroblasts), and DC. The L cells behave the same as HeLa cells, in the sense that infection gives rise to large inclusions after 24 h and the vacuoles avoid fusion with lysosomes (not shown). The vacuoles in DC display lysosomal markers and are acidic, as expected for phagolysosomes (29). In addition, consistent with the observation that CCD inhibits internalization by HeLa cells and DC, the TR was not detected in most of the Chlamydia vacuoles in either of these two cell types (not shown).

Extracellular material that is phagocytosed and sorted to phagolysosomes is normally destined for degradation, although microbes that survive in phagolysosomes have also been identified (30, 31). The Chlamydia epitope recognized by the mAb LPS was sometimes detected in DC 24 h after infection, but as LPS from both Shigella and Chlamydia can detach from the bacteria and may be present even when the bacteria are degraded (18, 32), we evaluated the state of the chlamydiae in HeLa cells and DC by electron microscopy. Chlamydia EBs in tight-fitting vacuoles were observed in HeLa cells within 4 h of infection (Fig. 3A), and at 24 h most of the bacteria had differentiated into RBs and were proliferating within large inclusions (Fig. 3B). In DC, many EBs were observed in large, transparent vacuoles at 4 h (Fig. 3C), and many bacteria that appeared to be severely damaged were also observed (not shown), although in most cases it was difficult to distinguish them from other cellular debris. No intact bacteria were ever found after a 24-h infection.

**Effect of Chlamydia on presentation of exogenous Ags**

Pathogen-induced maturation of DC implies that, among other changes, the DC lose the capacity to present new exogenous Ag (9, 10). We therefore determined whether a 24-h incubation with chlamydiae had any effect on the ability to subsequently present HEL to a HEL-specific CD4+ T cell hybridoma (19). DC were able to present HEL efficiently to the hybridoma, but the ability to stimulate the hybridoma decreased dramatically following incubation with either C. psittaci (Fig. 4) or C. trachomatis (not shown). The inhibition of HEL presentation was not due to loss of surface expression of MHC class II, since the DC incubated with either of the two Chlamydia species could still present an HEL-derived peptide added exogenously, which can presumably bind to surface MHC class II without being internalized (33).

**Interaction between Chlamydia vacuoles and dendritic cell macropinosomes and MHC class II compartments**

Given the ability of chlamydiae to inhibit presentation of exogenous HEL, we examined the interaction between Chlamydia vacuoles and compartments involved in Ag presentation by MHC class II. The DC line displayed numerous macropinosomes with diameters of 2 μm to over 10 μm, as determined by incubating DC with rhodamine-labeled dextran. At early stages of Chlamydia entry, bacteria also colocalized with the dextran (Fig. 5A), suggesting that many if not all bacteria are internalized through macropinocytosis. Since entry of bacteria into DC is slow, whereas the fluorescence signal from macropinosomes loaded with dextran is intense and saturates within 30 min, the cells were incubated with chlamydiae for 2 h before incubating with dextran for an additional

**FIGURE 3.** Electron micrographs of HeLa cells infected with C. psittaci for 4 h (A) or 24 h (B) and DC incubated for 4 h (C). Vacuoles containing Chlamydia EB were observed in both HeLa cells and DC at 4 h, but only HeLa cells had RB-laden inclusions at 24 h. Scale bars: 0.25 μm (A), 1 μm (B), 0.5 μm (C).

**FIGURE 4.** Effect of Chlamydia infection on presentation of exogenous Ags. Control DC presented either HEL or an HEL-derived peptide recognized by an HEL-specific hybridoma (black left-hand columns). DC-fed C. psittaci for 24 h (gray right-hand columns) were unable to present whole HEL, but could still stimulate the hybridoma after incubation with the HEL-derived peptide.
The general outlines of the development and migration of DC have been previously described (9, 10). Bone marrow-derived DC obtained and incubated with C. trachomatis and DC. When the proliferative response was assessed, DC or CD4+ T cells incubated separately with Ag displayed little if any proliferation, while a large increase in proliferation was observed when CD4+ T cells were incubated in the presence of DC and Chlamydia Ag (Fig. 7). These data demonstrate that the DC not only express Chlamydia Ag-MHC complexes on the cell surface, but that these complexes are capable of providing a stimulatory signal for sensitized CD4+ T cells.

Presentation of Chlamydia-derived Ags by DC

To determine whether the DC were capable of presenting Ag to sensitized T cells, mice were infected intravaginally with C. trachomatis, and ILN were removed 13 days later. CD4+ T cells were
precursors translocate to nonlymphoid tissue, including epithelia of the genital tract, where they develop into "immature DC" capable of internalizing and processing Ag and expressing high levels of MHC molecules. Pathogens and inflammatory cytokines can induce their differentiation into "mature DC" with a decreased capacity for Ag processing but stable presentation of previously acquired Ags by the MHC molecules. The mature DC migrate into secondary lymphoid tissues, where they initiate T cell-dependent immune responses.

We have characterized the interaction between two species of *Chlamydia*, *C. trachomatis* and *C. psittaci*, and DC using a fully functional DC line (13). In parallel, key features of internalization, namely the effects of heparin and fusion of *Chlamydia* vacuoles with lysosomes, were confirmed with primary DC. These results were compared with the intracellular behavior of the chlamydial macropinosomes in an epithelial cell line, HeLa, representing the preferred physiologic target cell of the bacteria. The most striking difference between the epithelial cells and the DC is that the entry vacuoles avoid fusion with lysosomes in the epithelial cells, evolving within a day into large inclusions, while in DC there is rapid fusion between the *Chlamydia* vacuums and host cell lysosomes. By electron microscopy, chlamydial, mostly at the metabolically active RB developmental stage, were found proliferating in large numbers in the epithelial cells after 1 day. In DC, on the other hand, no intact bacteria were detected in DC after a 1-day incubation, suggesting that the bacteria were killed by the DC.

At early time points, chlamydial were detected in large vacuoles in the DC, consistent with macropinosomes, and the internalization was inhibited by dimethyl amiloride. This internalization appears to be nonspecific or at least different from that used in epithelial cells, since heparin, which inhibits invasion of epithelial cells (16, 24, 25), has no effect on DC internalization. Thus, it is conceivable that DC could also internalize other bacteria through macropinocytosis.

Interaction of the DC with *Chlamydia leads to maturation of the DC, as defined by down-regulation of macropinocytosis and loss of the ability to present new exogenous Ag. The decrease in macropinocytosis was observed by fluorescence microscopy, which revealed a large drop in the number of acidic vesicles expressing LAMP and capable of accumulating dextran. The intracellular distribution of MHC class II in the DC line suggests that the DC line behaves as immature primary DC (14), while the disappearance of intracellular class II molecules after incubation with *Chlamydia* is consistent with the phenotype of mature primary DC (14). In addition, after a 1-day incubation with *Chlamydia*, the DC were no longer able to present exogenous HEL to HEL-specific T cells, but they still presented chlamydial Ags efficiently to T cells, in line with the role of mature DC to present Ags from the pathogen that induced the maturation.

As both infectious and inactivated chlamydial were used in our experiments, it is likely that some of the stimulatory activity of *C. trachomatis* and *C. psittaci* may be due to TNF-α secretion induced by simple contact with *Chlamydia* surface epitopes. Assuming that surface epitopes (e.g., LPS) from other bacteria may also induce differentiation of DC and that other bacteria may also be internalized through macropinocytosis, the only requirement for subsequent presentation of bacterial Ags by the DC is that the macropinosomes fuse with lysosomes (39), where the bacteria could be degraded for subsequent presentation by MHC class II. Although this appears to be the case for *Chlamydia*, this argument cannot be generalized to all microbes, as seen in the example of *L. monocytogenes*, which escapes from DC phagolysosomes (40).

The observation that infection with *Chlamydia* gives rise to short-lived CD4+ -dependent immunity against subsequent infections (2) implies that the frequency of T cells recognizing *Chlamydia* Ags is low. The frequency of active, *Chlamydia*-specific T cells may thus be augmented by transferring *Chlamydia*-fed DC into animals, as has recently been done with DC to induce protective immunity to lethal challenge by tumors (12). This strategy could be extended to microbes that normally survive within DC by incubating DC with previously inactivated microbes.

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**References**