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Chlamydial Antigens Colocalize within IncA-Laden Fibers Extending from the Inclusion Membrane into the Host Cytosol†

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Chlamydial IncA localizes to the inclusion membrane and to vesicular fibers extending away from the inclusion. Chlamydial outer membrane components, in the absence of developmental forms, are found within these fibers. This colocalization may explain how chlamydial developmental form antigens are localized outside of the inclusion within infected cells.

Chlamydia are obligate intracellular bacteria that develop inside nonacidified host vacuoles, termed inclusions. Chlamydial inclusion biogenesis remains relatively uncharacterized and, to date, only two host cell macromolecules are known to interact with the inclusion membrane (IM [9, 19]). *Chlamydia*, however, synthesize and export several proteins to the IM (reviewed in reference 17). IncA, the best characterized of the proteins in the IM, is exposed at the cytoplasmic face of the inclusion by ca. 12 h postinfection, where it remains until lysis of the host cell (10, 15, 16). In addition to its localization on the IM, IncA is also found within fibers that appear as chains of vesicles extending away from the inclusion along distinct routes into the host cytoplasm (2, 17). The significance and composition of the IncA-laden fibers is unknown.

In the present study, we examined the abundance, distribution, and composition of the IncA-laden fibers present in HeLa 229 cells infected with *Chlamydia psittaci* guinea pig inclusion conjunctivitis, *C. trachomatis* L2 434/Bu, and *C. pneumoniae* TWAR. Cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum (MEM-10; standard growth conditions), in MEM-10 containing ampicillin, or in MEM-5 lacking tryptophan (5), as indicated. Methanol-fixed *Chlamydia*-infected cells cultured under these different environmental conditions were fluorescently labeled (5) and examined by laser scanning confocal microscopy. Antibodies to HSP60 (24), *C. trachomatis* major outer membrane protein (MOMP) (25), *C. psittaci* (16), *C. trachomatis* (2), *C. pneumoniae* IncA (1), and *C. trachomatis* Cap1 (8) have been described. Monoclonal antibody (MAb) to chlamydial Mip (MAb 147), chlamydial lipopolysaccharide (LPS; MAb EVI-H1), and *C. psittaci* MOMP (MAb 62) were kindly provided by John H. Pearce, Harlan Caldwell, and You-Xun Zhang, respectively. Data from three independent experiments were pooled and subjected to statistical analysis (Table 1) using a commercial data analysis program (Minitab, Inc., State College, Pa.).

Infection of HeLa cells by each tested chlamydial species led to the formation of IncA-laden fibers that were attached to the intracellular inclusions. Approximately 31% (95% confidence interval [CI] = 28 to 35%) of *C. psittaci*, 47% (CI = 41 to 53%) of *C. trachomatis*, and 5% (CI = 3 to 7%) of *C. pneumoniae* inclusions were associated with IncA-laden fibers (Fig. 1A to C).

There are several reports of chlamydial antigens localizing to areas outside the inclusion during growth (6, 11, 14, 22, 23). It is unclear how these chlamydial macromolecules, generally associated with the developmental forms, become distributed within host cells. The presence of antigens normally found in developmental forms was examined within the IncA-laden fibers of the three chlamydial species under different growth conditions. For *C. psittaci*-infected cells, these antigens included three membrane-associated antigens (LPS, MOMP, and Mip) and one cytosolic protein (HSP60). Under standard growth conditions, trace amounts of LPS were found within 27% (CI = 19 to 37%) of the IncA-laden fibers. In contrast, MOMP, Mip, and HSP60 remained associated with the developmental forms. The distribution of these same antigens, and Cap1, were examined in *C. trachomatis*-infected cells. Cap1 is an IM-associated antigen that is a cytotoxic-T-cell target (8).

TABLE 1. Statistical summary of the antigens localizing within IncA-laden fibers

Cell type and antigen	% Normal growth conditions (CI)	% Ampicillin exposure (CI)	% Tryptophan depletion (CI)
C. psittaci-infected cells			
IncA-laden fibers	31 (28–35)	71 (68–73)	26 (22–30)
LPS in fibers	27 (19–37)	94 (92–96)	0
Mip in fibers	0	27 (24–31)	0
HSP60 in fibers	0	0	0
C. trachomatis-infected cells			
IncA-laden fibers	47 (41–53)	70 (64–76)	6 (4–8)
Cap1 in fibers	12 (9–16)	30 (25–35)	NE[a]
LPS in fibers	0	8 (6–10)	0
HSP60 in fibers	0	0	0
C. pneumoniae-infected cells			
IncA-laden fibers	5 (3–7)	6 (4–8)	NE
LPS in fibers	0	27 (20–36)	NE
HSP60 in fibers	0	0	NE

[a] NE, not examined.

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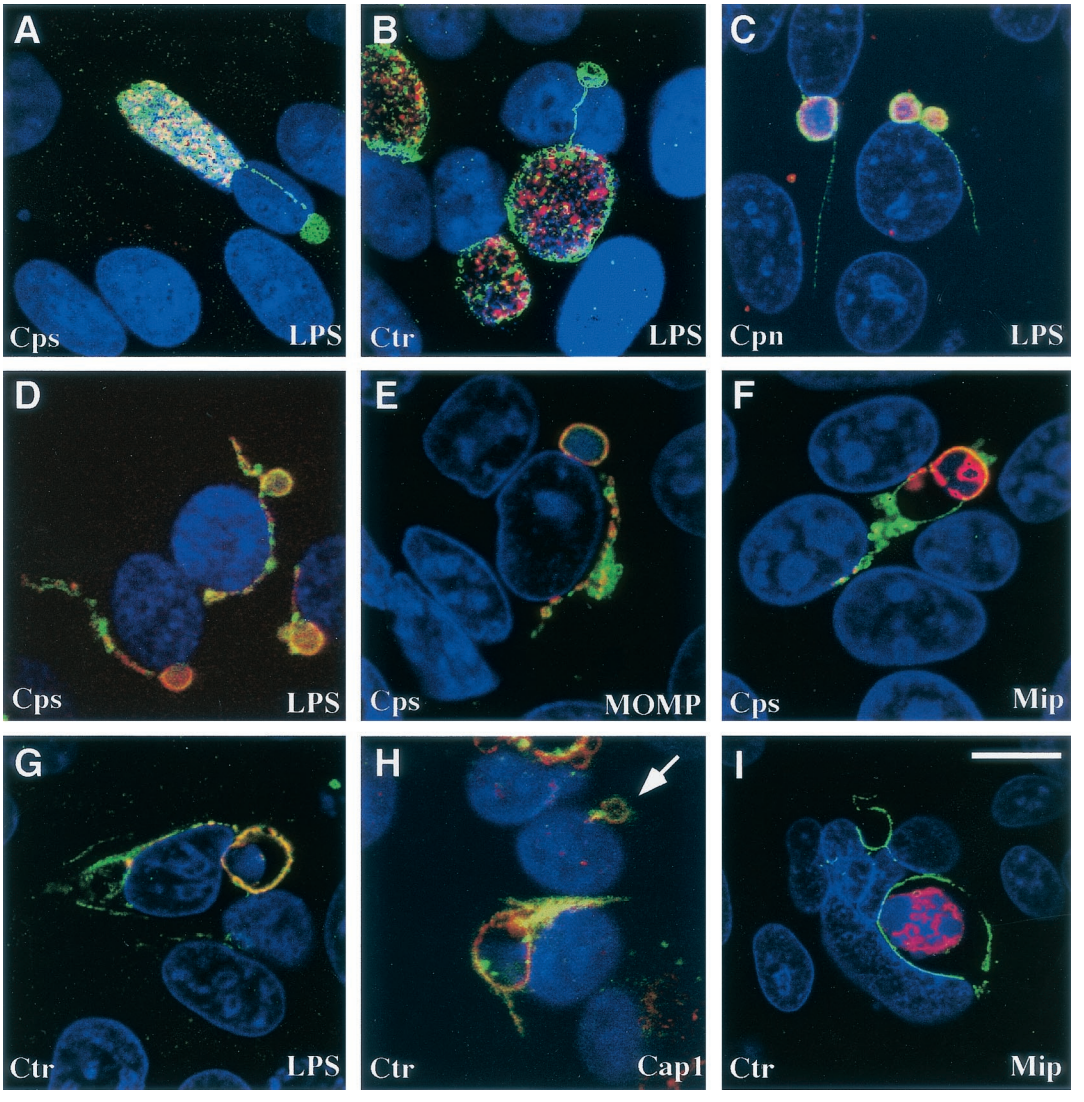

FIG. 1. Antigen distribution within IncA-laden fibers of *Chlamydia*-infected cells cultured during standard growth conditions and in the presence of ampicillin. *C. psittaci* (Cps)-, *C. trachomatis* (Ctr)-, and *C. pneumoniae* (Cpn)-infected cells were cultured in MEM-10 (A to C) or MEM-10 containing ampicillin (D to I), prior to methanol fixation. All cells were labeled with anti-IncA antibodies (i.e., with fluorescein isothiocyanate). The antigens labeled with TRITC are depicted in the bottom right corner of each panel. Antigen colocalization was examined by using a confocal microscope. DAPI was used to label nucleic acids. The arrow in panel H shows the location of a vacuole embedded with chlamydial antigens that lack developmental forms. Bar, 8 μm (for all images).

When cultured in MEM-10, *C. trachomatis* MOMP, LPS, and Mip remained associated with developmental forms, but Cap1 was detected in 12% (CI = 9 to 16%) of the IncA-laden fibers. Within *C. pneumoniae*-infected cells, LPS and HSP60 were the only antigens examined, both of which were not detected in IncA-laden fibers.

Culturing *Chlamydia* in the presence of certain antibiotics or the absence of certain amino acids results in the aberrant development of chlamydiae and can lead to a persistent growth state (reviewed in reference 3). These culture conditions also promoted a change in the appearance and frequency of the IncA-laden fibers. *C. psittaci*-infected cells cultured in the presence of 10 μg of ampicillin/ml showed a significant increase in the size and abundance of the cytosolic fibers ($P < 0.0001$). Enlarged IncA-laden fibers extended off 71% (CI = 68 to 73%) of the inclusions (Fig. 1D to F, fluorescein isothiocyanate). Exposure to ampicillin also led to an increase in the amount and type of other antigens localizing within the enlarged fibers. Ninety-four percent (CI = 92 to 96%) of the IncA-laden fibers contained increased amounts of LPS (Fig. 1D, TRITC [tetramethyl rhodamine isothiocyanate]). A similar percentage of IncA-laden fibers contained large amounts of MOMP (Fig. 1E, TRITC), whereas only 27% (CI = 24 to 31%) of the fibers contained trace amounts of Mip (Fig. 1F, TRITC). HSP60, however, was never observed outside the chlamydial developmental forms (data not shown), which is consistent with the electron microscopic observations of Raulston et al. (13). The extrainclusionary distribution of LPS, MOMP, and Mip was always associated with the IncA-laden fibers.

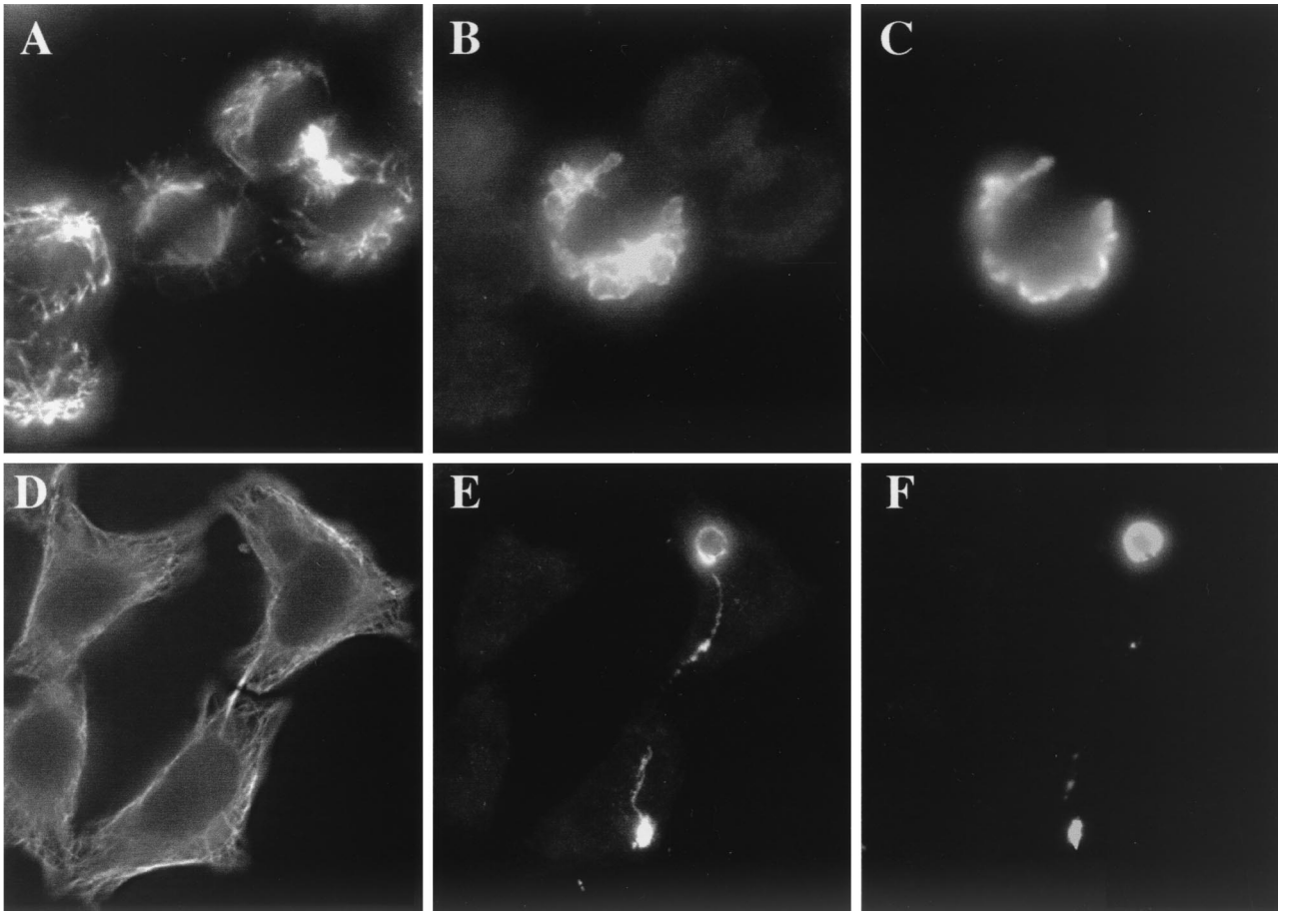

FIG. 2. Distribution of IncA-laden fibers during host cell division. *C. psittaci*-infected cells were cultured in MEM-10 (A to C) or MEM-10 containing ampicillin (D to F) for 30 h prior to methanol fixation. Cells were triple labeled with anti-tubulin (A and D), anti-IncA (B and E), and anti-LPS (C and F) antibodies and then examined by using a standard fluorescence microscope. The image in panel E shows an IncA fiber stretching between two inclusions in different cells.

Similar to the observations made in *C. psittaci*-infected cells, the number of *C. trachomatis* inclusions containing IncA-laden fibers significantly increases to 70% (CI = 64 to 76%) in cells cultured in the presence of 1 μg of ampicillin/ml ($P < 0.0001$; Fig. 1G to I). LPS (Fig. 1G, TRITC) was found within ca. 8% (CI = 6 to 10%) of the IncA-laden fibers, whereas Cap1 was present in 30% (CI = 25 to 35%) of the fibers (Fig. 1H, TRITC). MOMP was also observed within the fibers, and the abundance of MOMP was similar to that seen with LPS (not shown). During culture in ampicillin, MOMP, LPS, and Cap1 were also found within the membrane of IncA-laden vacuoles that lack developmental forms (assessed by the absence of DAPI [4′,6′-diamidino-2-phenylindole] labeling; Fig. 1G and H). *C. trachomatis* Mip (Fig. 1I, TRITC) was occasionally localized to the IM but was not found in these empty vacuoles or IncA-laden fibers. Exposure of *C. pneumoniae*-infected cells to 10 μg of ampicillin/ml had no significant effect on the abundance of the IncA-laden fibers.

Immunofluorescence of dividing HeLa cells showed that, during host cytokinesis, inclusions containing *C. psittaci* also divided, distributing the inclusions between the two daughter cells (Fig. 2). IncA-laden fibers commonly formed between the divided inclusions present in the two different cells (Fig. 2E), linking cells that otherwise may appear unassociated and, in some cases, cells that are devoid of chlamydial developmental forms (Fig. 1H). These cells could routinely be found within infected monolayers and, commonly, the vacuole in the apparently uninfected cell was connected via a fiber to an inclusion in a neighboring cell (data not shown). LPS was often found within the fibers extending between the two inclusions (Fig. 2F). These results suggest host cell division may distribute the IncA-laden fibers, and empty IncA-laden vacuoles, between the daughter cells, providing a mechanism for chlamydial antigens to be present in the cytosol of uninfected host cells.

We showed, in immunofluorescence analyses, that chlamydial fibers appeared to follow distinct routes through the host cell, suggesting that fiber distribution may be directed along the host cytoskeletal network. To examine this theory, we examined fiber formation in cells that lacked intact microfilaments (21), microtubules (7, 12), or intermediate filaments (18). Morphologically typical IncA-laden fibers were found extending off 36% (CI = 32 to 40%) of the inclusions in the absence of microfilaments (Fig. 3B) and 55% of infected cells (CI = 51 to 58%) lacking microtubules (Fig. 3C); both percentage values were significantly less than that found in control cells ($P < 0.0001$). Culture of chlamydiae in a cell line lacking

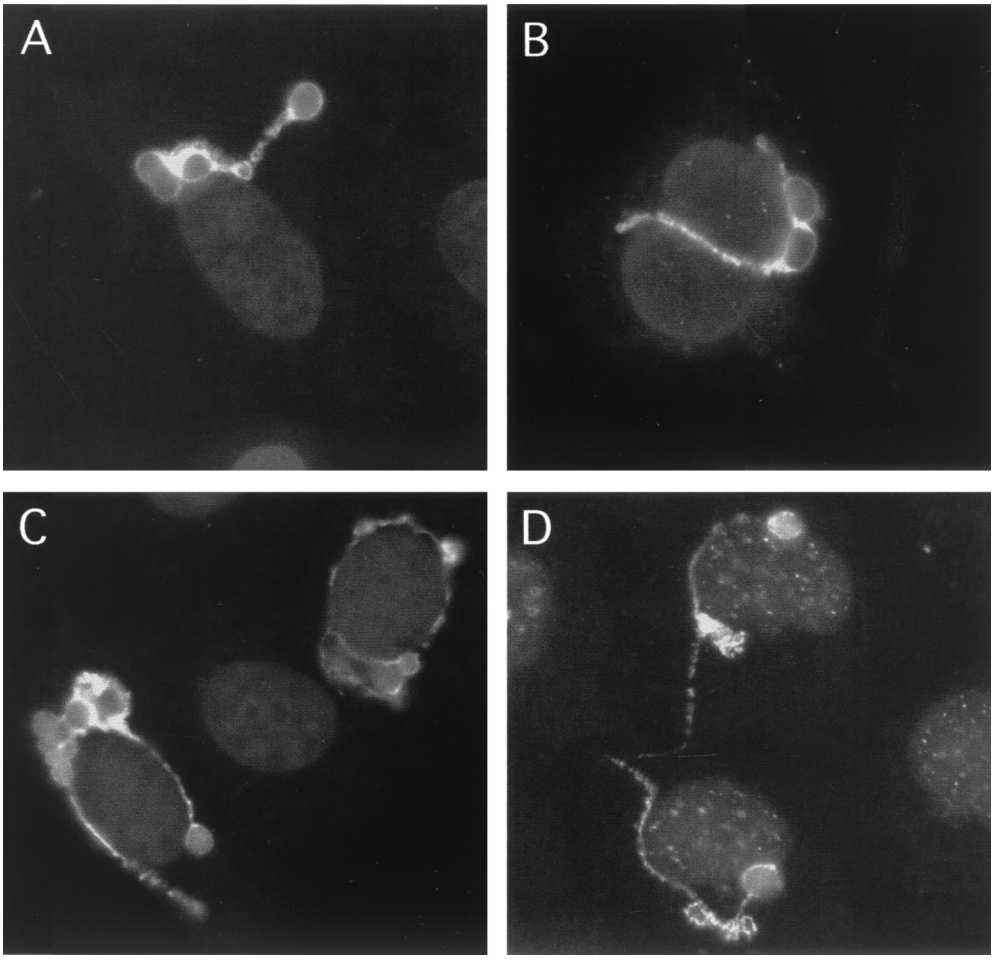

FIG. 3. IncA-laden fibers are present in cells lacking intact cytoskeleton structures. The cytoskeleton of *C. psittaci*-infected HeLa cells cultured in the presence of MEM-10 and ampicillin was disrupted by using colchicine (A), cytochalasin D (B), or nocodazole (C). (D) Additionally, *C. psittaci*-infected SW-13/c1.2 intermediate filament-free cells were cultured in the presence of MEM-10 containing ampicillin. Cells were labeled with anti-IncA antibodies and examined by using a standard fluorescence microscope. IncA-laden fibers were still present after the three different drug treatments and in cells lacking intermediate filaments. Dark gray areas represent nucleic acid staining with DAPI.

intermediate filaments (SW-13/c1.2 [18]) had no effect on the production of IncA-laden fibers. These results suggest that the production and localization of the IncA-laden fibers is not a function of the host cell cytoskeleton.

One possible mechanism of liberating components from the reticulate body surface is through the formation of outer membrane vesicles which have been documented in several bacterial species (reviewed in reference 4) and have been found within the inclusions of *Chlamydia*-infected cells (20, 23). These vesicles would then fuse with the IM and incorporate, with other material in the IM, into growing IncA-laden fibers extending off the inclusion.

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