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Localization of *Chlamydia trachomatis* Heat Shock Proteins 60 and 70 during Infection of a Human Endometrial Epithelial Cell Line In Vitro

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Unlike chlamydial lipopolysaccharide, which is released from the developing inclusion to the surface of infected genital epithelial cells, both *Chlamydia trachomatis* heat shock protein (hsp) 60 and 70 antigens remained confined within the inclusion during the course of the chlamydial developmental cycle. Exposure of the infected cells to penicillin to induce a persistent infection or to a lipophilic microbicide did not potentiate secretion or exocytosis of the chlamydial hsp.

Chlamydia trachomatis (serovars D to K) is one of the most common causes of sexually transmitted diseases worldwide. In the United States, more than 4 million cases of chlamydial genital infection occur annually. Indeed, chlamydial infections were the most frequent of the top 10 diseases reported to the Centers for Disease Control and Prevention in the United States in 1995 (7). In women, genital tract infections with *C. trachomatis* are frequently asymptomatic and, if untreated, lower genital tract infection can ascend to the upper genital tract and result in salpingitis, ectopic pregnancy, or infertility (15).

It is well known that chlamydial hsp60 and hsp70 are highly immunogenic during the course of natural infections (3). Women with *C. trachomatis*-associated pelvic inflammatory disease, tubal infertility, and ectopic pregnancy have high titers of serum antibodies to chlamydial hsp60 (4, 26). In contrast, the presence of serum antibodies to chlamydial hsp70 has been correlated in female patients with protective immunity against tubal disease (4), and antibodies against chlamydial hsp70 were able to neutralize chlamydial infectivity in vitro (6).

Virtually nothing is known about chlamydial antigen trafficking in mucosal epithelial cells, but previous studies have demonstrated that chlamydial lipopolysaccharide (LPS) (9, 20, 28), an exoglycolipid termed GLXA (23), and the major outer membrane protein (MOMP [28]) can escape from the confines of the membrane-bound inclusion midway through the chlamydial developmental cycle and before the release of progeny at the end of the cycle, and the antigens can be detected on the surface of the infected host epithelial cells. Subsequent studies revealed that the early release of chlamydial antigens from the inclusion could be increased by the lipophilic microbicide compound C31G, presumably due to destabilization of the chlamydial inclusion membrane as well as direct removal of LPS from the chlamydial envelope (30). The early release of chlamydial LPS, which fixes complement by both the classical and alternative pathways to generate the potent chemoattractant C5a (10), likely serves as one of the first signals for the marked influx of polymorphonuclear leukocytes, observed in patients with cervicitis and endometritis (14). Further, in the case of LPS, it has been postulated that the altered fluidity of the plasma membrane of the infected epithelial cells may thwart destruction by cytotoxic T cells (27). There is also evidence that, on exposure of infected cells to gamma interferon (IFN-γ), chlamydiae may persist in a noncultivable state within mucosal epithelial cells for an extended period in vitro (2). Under these conditions, production of the MOMP is reduced but production of hsp60 is maintained.

The purpose of this study was to determine if chlamydial hsp60 and hsp70 were among the antigens released prematurely from the developing chlamydial inclusion in (i) normally infected cells, (ii) infected cells exposed to penicillin as another in vitro model of persistent infection, and (iii) infected cells exposed to the microbicide C31G.

The human urogenital isolate *C. trachomatis* E/UW-5/CX was grown in McCoy cells propagated on microcarrier beads, and the progeny were purified, counted, and titrated for infectivity as described previously (21). The human epithelial cell line HEC-1B (HTB-113; American Type Culture Collection, Rockville, Md.) was grown in a polarized manner (29) in Dulbecco's modified Eagle's medium with high glucose (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah) and 10 mM HEPES, pH 7.3. The HEC-1B cells were determined to be free of mycoplasma contamination by staining with the Hoechst 33258 reagent (B2883; Sigma, St. Louis, Mo.).

Polarized HEC-1B cell monolayers were inoculated on their apical surfaces with a concentration of infectious elementary bodies (EB) titered to infect 50% of the host cells and were incubated at 35°C in an atmosphere of 5% CO_2. At 12, 24, 36, 48, 60, and 72 h postinoculation (p.i.), the chlamydia-infected monolayers were harvested and processed in Lowicryl resin for immunoelectron microscopy (28). In some test and control samples at 24 h p.i., the culture medium was replaced with medium containing noncytotoxic concentrations of C31G (0.0005% [30]) or penicillin (20 U/ml [28]) and incubation was continued for an additional 24 h and 14 days, respectively. C31G was obtained from Biosyn Corporation (Philadelphia, Pa.) as a stock containing 4% actives, consisting of equimolar amounts of a C_{14} alkyl amine oxide and a C_{16} alkyl dimethyl glycine. Unstained Lowicryl thin sections were probed with (i) a monoclonal antibody directed against a chlamydia-specific epitope for the chlamydial hsp60, kindly provided by Richard Morrison, or (ii) a polyclonal antibody generated against a peptide spanning the carboxyl terminus of the *C. trachomatis*

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serovar E hsp70 (19) (prepared by Genosys Biotechnologies, Inc., The Woodlands, Tex.); the sections were subsequently exposed to 30-nm-diameter gold-conjugated goat second-affinity antibodies (Auroprobe; Amersham Internationals, Buckinghamshire, United Kingdom). After being stained with uranyl acetate, the sections were examined on a Zeiss EM900 electron microscope operating at 50 kV.

Localization of chlamydial hsp60 in infected HEC-1B cells. In all samples examined, the chlamydial hsp60 protein was restricted to the chlamydial inclusion; the gold particles were predominately associated with EB and reticulate bodies (RB) (Fig. 1A and B). The specificity of the anti-chlamydial hsp60 monoclonal antibody was confirmed with the following controls: (i) infected cells exposed to gold-conjugated second-affinity goat anti-mouse antibody alone and (ii) uninfected HEC-1B cells (Fig. 1C). Each control sample revealed negligible background labeling, thereby confirming that the conserved epitopes of the HEC-1B cell hsp60 were not recognized by the primary monoclonal antibody. While it was expected that the predominate location of the chlamydial hsp60 would be the bacterial cytoplasm, it is important to note that immunolabeling of this protein was also localized to the chlamydial envelope (Fig. 1A, inset, and B). Bavoil et al. (1) have shown an association of chlamydial hsp60 with the outer membrane by using differential detergent extraction methods. As a positive control for chlamydial antigen escaping from the inclusion, duplicate thin sections were exposed to a monoclonal antibody directed against *C. trachomatis* LPS (donated by Shirley Richmond and Steve Campbell) followed by labeling with a gold-conjugated second-affinity antibody. Figure 2A illustrates LPS bound to chlamydial EB and RB, as well as LPS distributed throughout the eukaryotic cell at 48 h p.i. Again, sections of uninfected control HEC-1B cells revealed no nonspecific labeling with anti-LPS primary antibody (Fig. 2B). Since this monoclonal antibody recognizes the unique chlamydial α-3-deoxy-D-manno-octulosonic acid (KDO) linkage αKDO-(2-8)-αKDO-(2-4)-αKDO, the cytoplasmic distribution of the LPS gold label in RB (Fig. 2A, inset) is most likely due to recognition of precursors, because this portion of the molecule is synthesized and linked on the cytoplasmic side of the inner membrane.

Localization of chlamydial hsp60 in persistently infected HEC-1B cells. Other researchers have reported high expression levels of chlamydial hsp60 following penicillin-induced (12) or IFN-γ-induced (2) persistent infection, but only in the case of penicillin-induced persistent infection was there a hint by fluorescence microscopy that chlamydial hsp60 might have been released from the inclusion (12, 13). In our studies, when chlamydia-infected epithelial cells were exposed to penicillin for 14 days, some enlarged, abnormal RB were present, and a greater density of hsp60 was detected in these chlamydiae than in RB grown in the absence of penicillin (compare Fig. 2C with 1A). Once again, hsp60 was rarely found beyond the inclusion boundary whereas LPS was distributed throughout the host cell as well as bound to chlamydiae following penicillin-induced persistence (data not shown).

Localization of chlamydial hsp60 in microbicide-exposed, infected HEC-1B cells. The lipophilic microbicidal agent C31G has been shown to enhance the release of chlamydial LPS and other antigens in RB from the inclusion following exposure of infected cells to noncytotoxic concentrations (30). This surface-active agent penetrates eukaryotic cells and eventually destabilizes both the chlamydial inclusion membrane and the chlamydial envelope by intercalation of alkyl chains into membrane bilayers (5, 25, 30). When chlamydia-infected HEC-1B cells were exposed to noncytotoxic concentrations of C31G, hsp60 remained localized within the inclusion (Fig. 2D). Duplicate thin sections stained with the monoclonal antibody to chlamydial LPS confirmed the enhanced release of LPS (data not shown).

Localization of chlamydial hsp70 in infected HEC-1B cells. A polyclonal peptide antibody generated against the carboxyl terminus of chlamydial hsp70, a region of less sequence homology with other hsp70 proteins, was used to examine both uninfected and *C. trachomatis*-infected HEC-1B cells (Fig. 3). It was not surprising that a small degree of cross-reactivity was observed with mammalian hsp70 in uninfected HEC-1B cells (Fig. 3B) because of the extent of amino acid homology within the hsp family of antigens. Therefore, the number of gold particles per unit area in the host cell cytoplasm from several infected cells was compared with the number of gold particles per unit area in uninfected cells. No increase in the number of gold particles was observed in the cytoplasm of infected cells over the number in the cytoplasm of uninfected cells (Fig. 4), suggesting that, similar to hsp60, there was no escape of chlamydial hsp70 from the inclusion. Immunolabeling of infected HEC-1B cells exposed to C31G also revealed no increase in the amount of hsp70 labeling in the host cell cytoplasm (Fig. 3D and 4). However, the most interesting observation was that unlike hsp60, immunolabeling of the chlamydial hsp70 in the penicillin model of persistent infection showed no increase relative to labeling within normally infected HEC-1B cells (compare Fig. 3C to A).

In summary, these data provide evidence that unlike chlamydial LPS, GLXA, and the MOMP, the chlamydial hsp60 and -70 homologs are not released beyond the confines of the intracellular inclusion, at least during a single cycle of chlamydial development in polarized HEC-1B cells in vitro. These observations do not preclude the possibility that chlamydial hsp antigens are presented by infected epithelial cells to surveillant dendritic cells or released by dying infected epithelial cells (i) later in the infectious process in vivo and (ii) during upper genital tract complications to be processed by other professional antigen-presenting cells.

From one perspective, the differential secretion of chlamydial components by the infected mucosal epithelial cell could reflect a clever strategy by these pathogens to ensure their survival and/or persistence. The accumulation of LPS in the host eukaryotic plasma membrane has been shown to decrease plasma membrane fluidity, as assessed by electron spin resonance spectroscopy, which has been speculated to alter subsequent immune recognition and possibly immune cytolysis (27). Although chlamydial LPS has been shown to elicit secretion of proinflammatory cytokines, such as tumor necrosis factor alpha, Ingalls et al. (8) showed that the potency of chlamydial LPS was 100-fold less than that elicited by phenotypically similar structures, such as the lipooligosaccharide from *Neisseria gonorrhoeae* or the rough ReLPS from *Salmonella minnesota* R595. According to these investigators, this relatively weak induction of an acute immune response may partially explain the asymptomatic nature of initial chlamydial infection and, in turn, allow progression of infection to the upper genital tract. Perhaps, restriction of chlamydial hsp70 to the inclusion may also favor progression of infection by slowing the development of protective humoral immunity, since (i) certain antibodies against chlamydial hsp70 neutralize infectivity in vitro (6) and (ii) the presence of chlamydial hsp70 antibodies in female patients is correlated with protection against ascending infection (4).

It is not clear that chlamydial hsp60 is one of the key mediators in destructive immunopathology. Morrison et al. (12, 13) have shown that isolated hsp60 induces a delayed hypersensitivity (DH) reaction when administered to the conjunctiva of

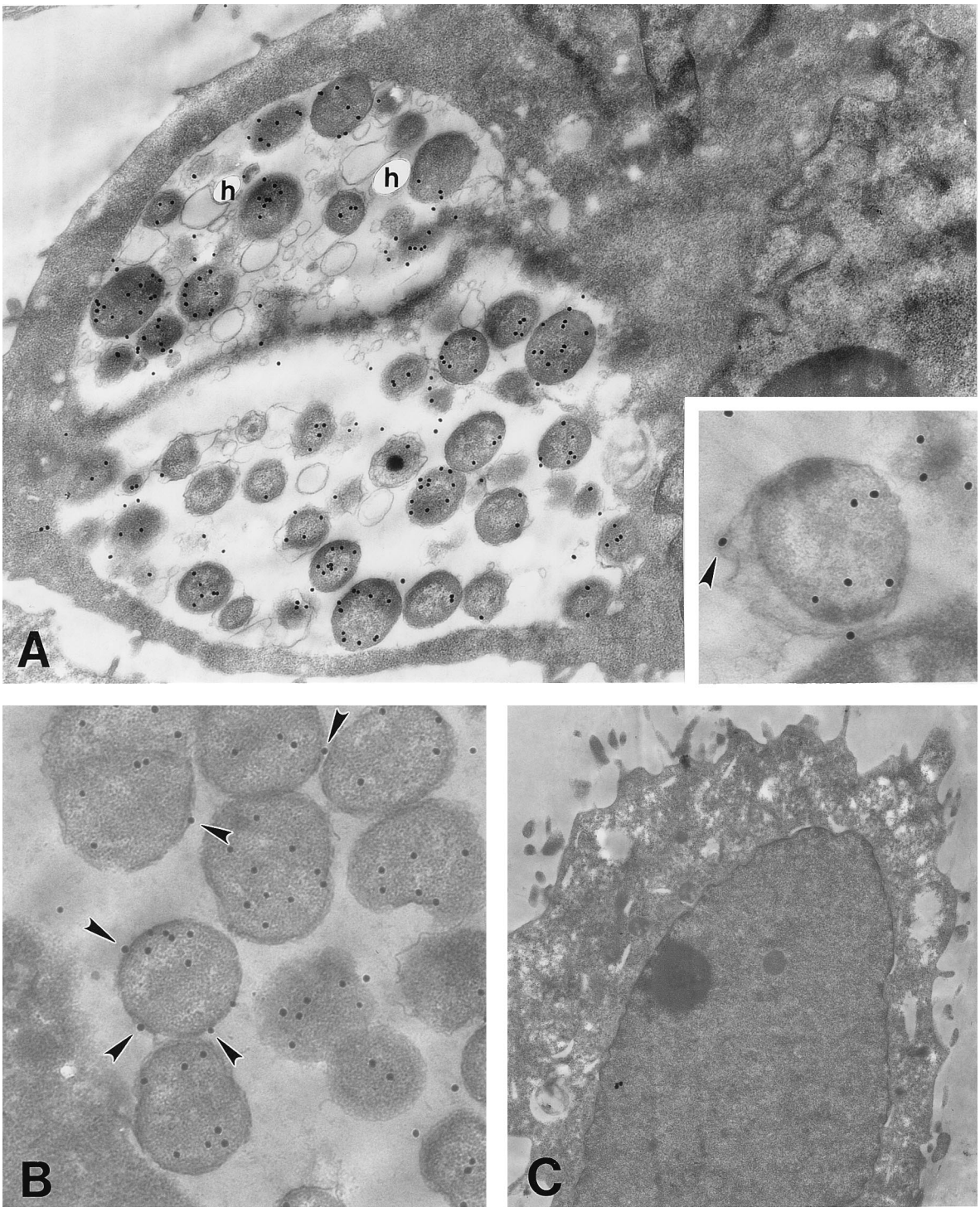

FIG. 1. Localization of chlamydial hsp60 in infected HEC-1B cells. Thin sections were probed with a chlamydia-specific hsp60 monoclonal antibody and labeled with 30-nm-diameter gold-conjugated second-affinity antibodies. Labeling of hsp60 in normally infected cells (A and B) at 48 h p.i. and in uninfected cells (control) (C) is shown. The arrowheads indicate gold particles associated with the chlamydial cell envelope (A, enlarged inset, and B). Translucent holes (h) are often observed in samples embedded in the fragile Lowicryl resin. Magnifications: ×15,750 (A); ×35,777 (inset); ×16,285 (B); and ×7,700 (C).

chlamydia-sensitized guinea pigs; chlamydial MOMP and LPS appeared to serve only minor, supporting roles in the DH reaction. However, in studies by Rank et al. (16), subcutaneous immunization of guinea pigs with recombinant hsp60 (rhsp60) following a primary guinea pig inclusion conjunctivitis (GPIC) infection actually produced a reduction in the pathologic response in the conjunctiva. In the same studies, guinea pigs immunized subcutaneously with rhsp60 and challenged in the conjunctiva with avirulent but viable *Salmonella typhimurium* expressing GPIC rhsp60 did not produce an exacerbated DH

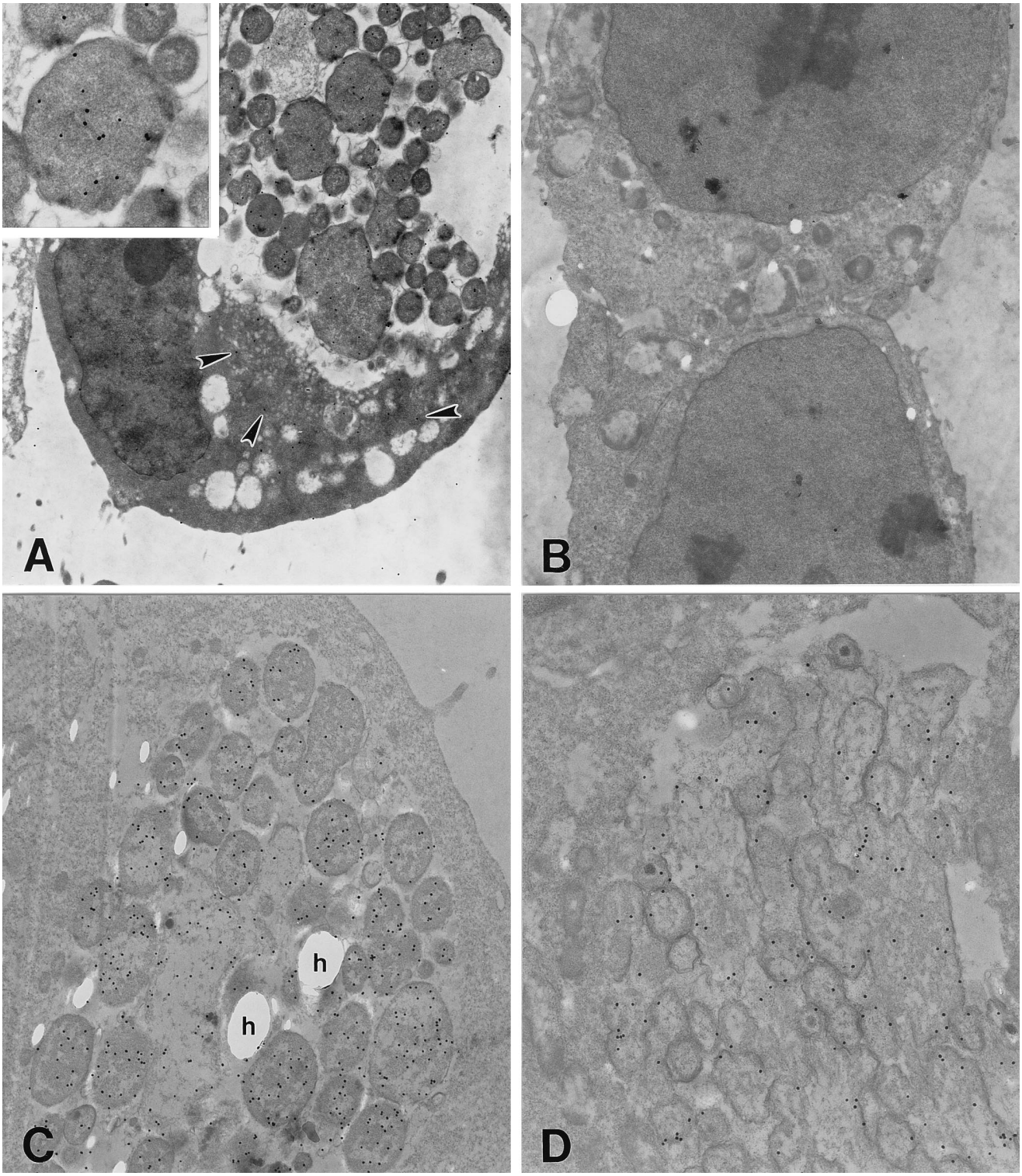

FIG. 2. Localization of chlamydial hsp60 and LPS in normal, penicillin-exposed, and microbicide-exposed infected HEC-1B cells. Lowicryl thin sections were probed with monoclonal antibodies directed against the chlamydial hsp60 or the chlamydial LPS and labeled with 30-nm-diameter gold-conjugated second-affinity antibodies. Labeling of chlamydial LPS in normally infected cells at 48 h p.i. (A), LPS in uninfected cells (control) (B), chlamydial hsp60 in penicillin-induced, persistently infected cells after 14 days (C), and chlamydial hsp60 in infected cells exposed to C31G after 48 h (D) is shown. Magnifications: ×6,500 (inset, ×12,666) (A); ×7,700 (B); ×9,744 (C); and ×8,993 (D). h, translucent holes.

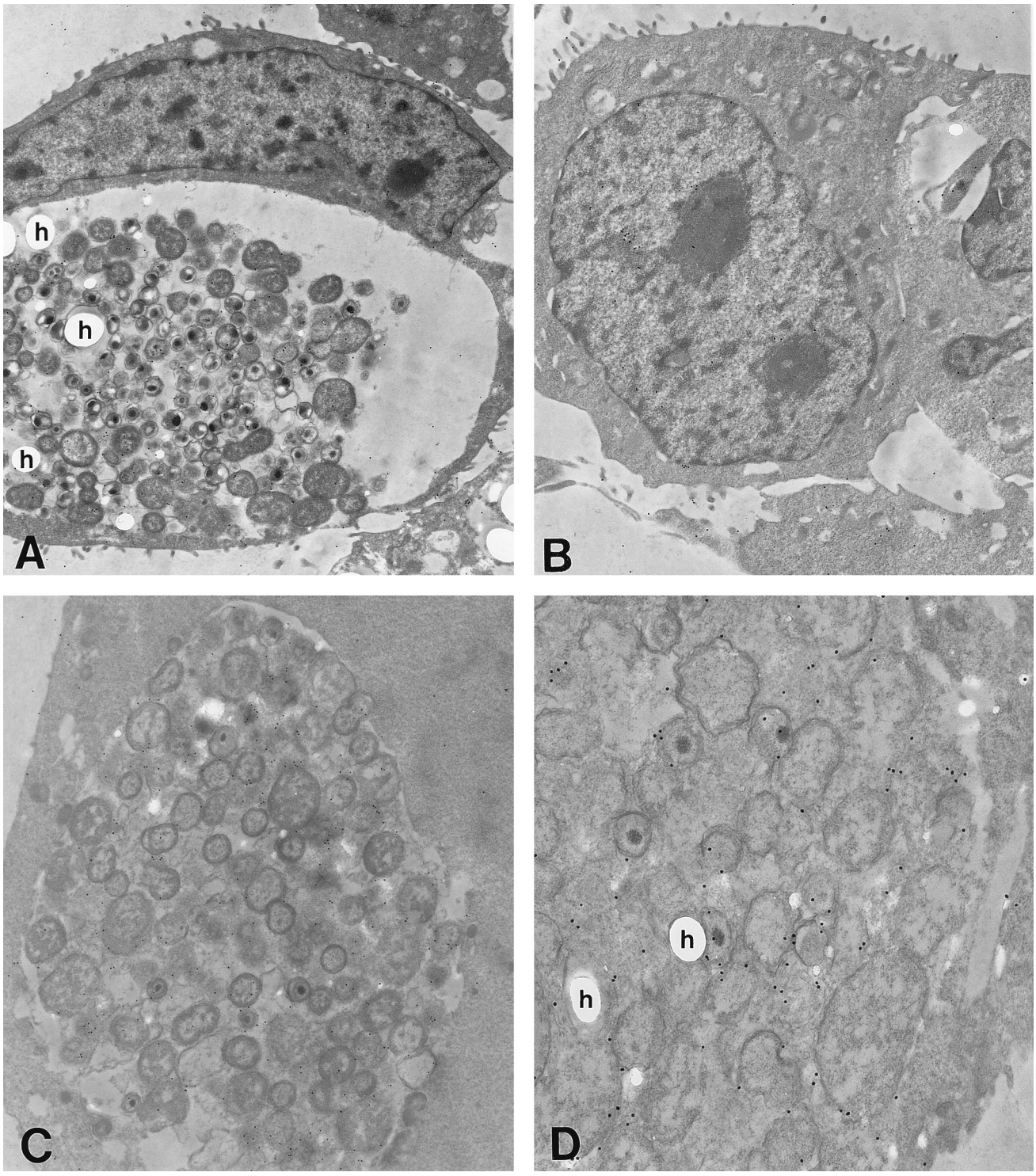

FIG. 3. Localization of chlamydial hsp70 in normal, penicillin-exposed, and microbicide-exposed infected HEC-1B cells. Thin sections were probed with a polyclonal antibody generated against the carboxyl terminus of *C. trachomatis* hsp70 and labeled with a 30-nm-diameter gold-conjugated second-affinity antibody. Labeling of hsp70 in normally infected cells at 48 h p.i. (A), uninfected cells (control) (B), penicillin-induced persistently infected cells after 14 days (C), and hsp70 in infected cells exposed to C31G at 48 h p.i. (D) is shown. Magnifications: ×6,978 (A); ×8,723 (B); ×6,978 (C); and ×13,956 (D). h, translucent holes.

reaction in the conjunctiva. These data suggest that the form of the hsp60 or the route of administration, or both, may affect the development of a pathologic response, at least in the guinea pig model. Thus, the confinement of chlamydial hsp60 to the inclusion early in the course of infection might be important in preventing an overt DH response which could otherwise lead to the destruction or depletion of host mucosal epithelial cell layers.

Another interesting observation was that there was no substantial increase in the amount of chlamydial hsp70 expressed

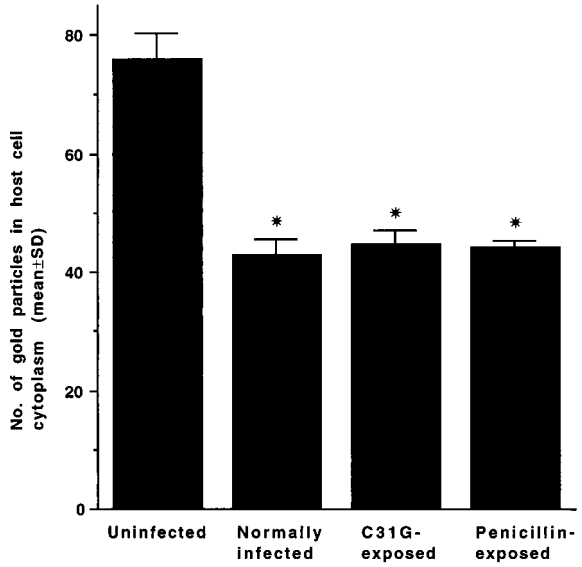

FIG. 4. Quantitation of gold particles in the cytoplasm of uninfected and infected HEC-1B cells. Lowicryl thin sections were exposed to a primary polyclonal peptide antibody generated against the carboxyl terminus of chlamydial hsp70, followed by gold-conjugated second-affinity antibody. Gold particles in several random 2-cm^2 areas per host cell cytoplasm were counted in 10 different photomicrograph images, at the same magnification, of uninfected and infected HEC-1B cells. Utilizing equivalent measurements per unit area accounted for the overall decrease in volume in the cytoplasm of infected host cells attributed to the large space occupied by the chlamydial inclusion, which effectively reduced the surface area exposed to gold labeling. An asterisk indicates that there was a significant decrease ($P < 0.02$) in the number of gold particles in the cytoplasm of infected versus that in uninfected HEC-1B cells. Exposure of infected HEC-1B cells to C31G or penicillin did not increase immunolabeling in the host cell cytoplasm relative to that in normally infected cells. SD, standard deviation.

in the penicillin-induced model of persistence. It is often debated whether the chlamydiae survive within a hostile intracellular environment or exploit an environment to which they are well adapted. The high level of expression of chlamydial hsp60 in IFN-γ-exposed cells (2), penicillin-exposed cells (12), and, more recently, under conditions of iron limitation (18) could suggest that enhanced production of hsp60 is a signal for chlamydial survival when more adverse conditions arise. However, this response may differ among chlamydial serovars or within different host cell systems (17). The lack of such a correlation for hsp70, at least to date, may be obfuscated by the complexities of regulation of these heat shock genes, about which very little is known in chlamydiae. Our observations (22) and those by Tan and colleagues (24) indicate that the chlamydial *dnaK* and *groE* operons may be regulated by a mechanism similar to that described for *Bacillus subtilis*, in which there are at least three differential mechanisms for heat shock regulation. The *groE* and *dnaK* operons in *B. subtilis* are negatively regulated at the level of transcription by a repressor termed HrcA, which interacts with an operator termed CIRCE (class I regulation). However, the GroEL or -ES chaperonin modulates HrcA in a posttranscriptional control mechanism, whereas the DnaK (hsp70) chaperonin does not (11). The result is that when GroEL or -ES is turned off, the *dnaK* operon is activated, and if GroEL (hsp60) is overproduced, there is decreased expression of the *dnaK* operon.

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