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Differences in the Association of *Chlamydia trachomatis* Serovar E and Serovar L2 with Epithelial Cells In Vitro May Reflect Biological Differences In Vivo

CAROLYN H. DAVIS AND PRISCILLA B. WYRICK*

Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina

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Chlamydia trachomatis **serovar E is one of the most common bacterial sexually transmitted pathogens. Since it is an obligate intracellular bacterium, efficient colonization of genital mucosal epithelial cells is crucial to the infectious process. Serovar E elementary bodies (EB) metabolically radiolabeled with ^{35}S-Cys-Met and harvested from microcarrier bead cultures, which significantly improves the infectious EB-to-particle ratio, provided a more accurate picture of the parameters of attachment of EB to human endometrial epithelial cells (HEC-1B) than did less infectious ^{14}C-EB harvested from flask cultures. Binding of serovar E EB was (i) equivalent at 35 and 4°C, (ii) decreased by preexposure of EB to heat or the topical microbicide C31G, (iii) comparable among common eukaryotic cell lines (HeLa, McCoy), and (iv) significantly increased to the apical surfaces of polarized cells versus nonpolarized cells. In parallel experiments with *C. trachomatis* serovar L2, serovar E attachment was not affected by heparin or heparan sulfate whereas these glucosaminoglycans dramatically reduced serovar L2 attachment. These data were confirmed by competitive inhibition of serovar E binding and infectivity by excess unlabeled live and UV-inactivated serovar E EB but not by excess serovar L2 EB. The noninvasive serovar E strains in the lumen of the genital tract enter and exit the apical domains of target columnar epithelial cells to spread canalicularly in an ascending fashion from the lower to the upper genital tract. In contrast, the invasive serovar L2 strains are primarily submucosal pathogens and likely use the glucosaminoglycans concentrated in the extracellular matrix to colonize the basolateral domains of mucosal epithelia to perpetuate the infectious process.**

Chlamydia trachomatis serovars D to K are the leading cause of bacterially acquired sexually transmitted infections and their sequelae (18). The infectious process begins by attachment and entry of chlamydiae in the form of elementary bodies (EB) into epithelial cells of the genital mucosa. Following endocytosis, rapid diversion of the EB-containing vesicles to the exocytic pathway (17), and fusion of individual EB-containing vesicles with one another, the metabolically inactive EB transform into metabolically active reticulate bodies (RB), which grow and divide by binary fission. As the number of progeny increases, the surrounding membrane expands via added sphingomyelin from the Golgi complex (6, 7) to keep the developing microcolony within a membrane-bound protective niche, termed an inclusion. Since the RB are noninfectious, conversion of the RB back into infectious EB in the later stages of this developmental cycle must occur in order for the released EB to infect neighboring epithelial cells and perpetuate the infectious process.

Since chlamydiae are obligate intracellular bacterial pathogens and must get inside host cells in order to grow and survive, adherence of the EB to its target host cell is crucial to a successful infectious process. Understanding the mechanisms involved may also offer opportunities for interfering with the infectious process via microbicides as well as vaccine candidates. Thus, studies of attachment to and entry of chlamydiae into host cells have been an area of intense investigation for many years. As new information has emerged, it is clear that the interactions between the microbe and the host cell are more complex than perhaps previously appreciated and that multiple parameters, including culture condition nuances, which may reflect biological nuances, are important.

One of the leading candidate chlamydial molecules demonstrated to play an important role in the attachment process is the major outer membrane protein (MOMP). In an elegant study by Su et al. (19) using the trachoma-inducing strain *C. trachomatis* serovar B and hamster kidney (HaK) cells, it was shown that MOMP displayed at least two independent but probably cooperative adhesion functions. Negatively charged divergent hydrophilic residues exposed in MOMP variable domains II and IV were involved in initial electrostatic interactions to bring the highly negatively charged EB close to the host cell, after which more specific hydrophobic interactions took over. Following a conformational change in variable domain IV, an invariant nonapeptide sequence—previously located in a cryptic, immunoinaccessible cleft—became exposed and immunoaccessible and promoted binding.

In more recent exciting studies, Zhang and Stephens (31) reported that Lymphogranuloma venereum (LGV) strain L2, often designated as *C. trachomatis* serovar L2, synthesizes a unique heparan sulfate-like molecule that is required for attachment of chlamydiae to HeLa cells. Exogenous addition of heparan sulfate or heparin to the culture system effectively blocked binding of EB to the HeLa cells in a dose-dependent fashion. In addition, removal of the chlamydial heparan sulfate adhesin by exposure of EB to heparitinase also blocked attachment; saturation of the enzyme-exposed EB with heparan sulfate restored binding of EB to HeLa cells. When identical experiments were performed on the trachoma-inducing *C. trachomatis* serovar B, serovar B infectivity was found to be less

* Corresponding author. Mailing address: Department of Microbiology and Immunology, UNC School of Medicine, CB# 7290, 804 Mary Ellen Jones Building, Chapel Hill, NC 27599-7290. Phone: (919) 966-5051. Fax: (919) 962-8103. E-mail: pbwyrick@med.unc.edu.

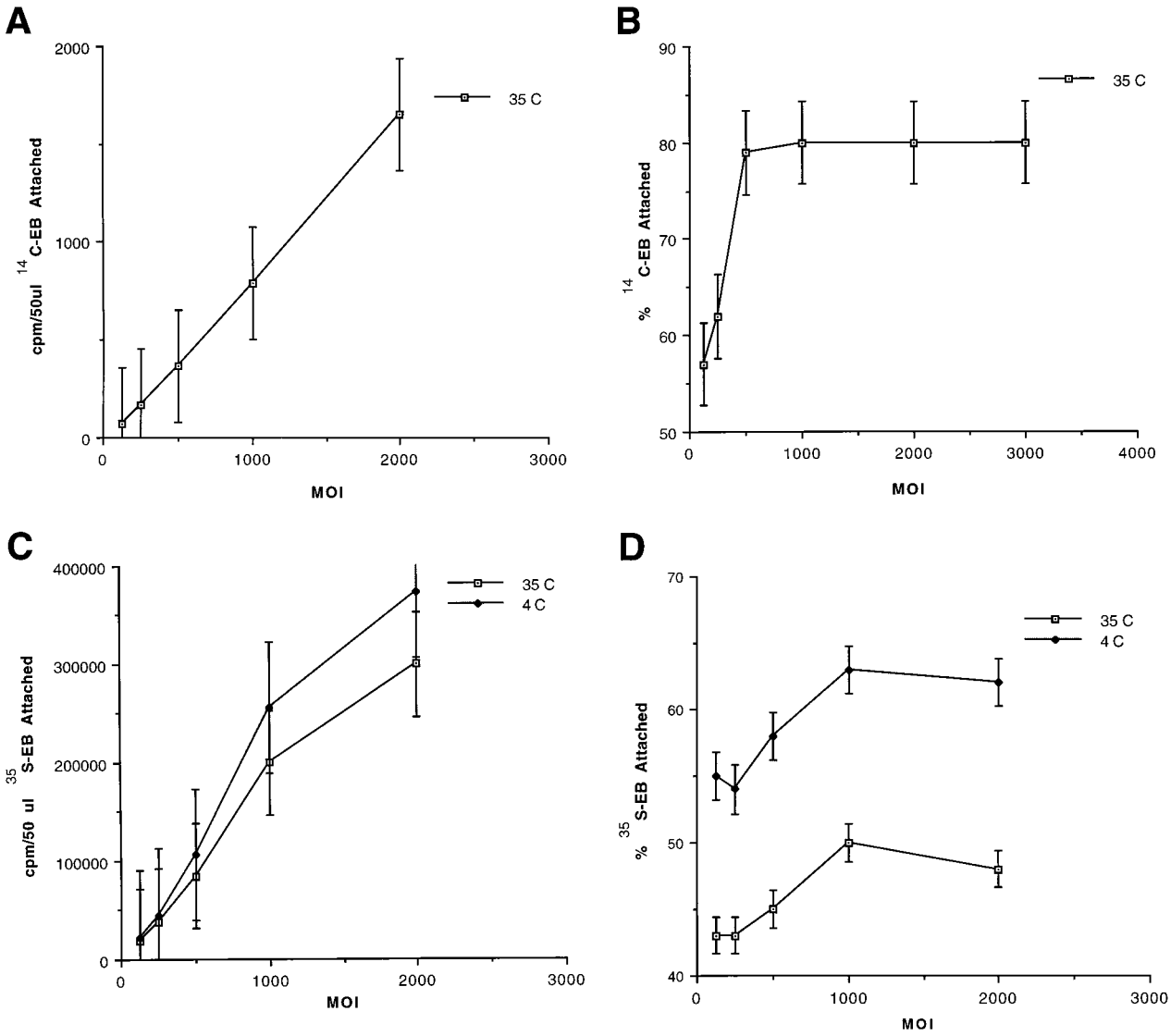


FIG. 1. Comparison of attachment of flask-grown ^{14}C-radiolabeled versus microcarrier-grown ^{35}S-radiolabeled *C. trachomatis* serovar E EB to HEC-1B cells. Counts per minute of ^{14}C-labeled EB per 50 μl attached to HEC-1B cells (A) and the percentage of EB attached after 1 h at 35°C (B) were increased as a function of increasing MOI. Saturation was achieved between 500 and 1,000 EB per host cell. Counts per minute of ^{35}S-labeled EB attached to HEC-1B cells (C) and the percentage of EB attached after 1 h at 35 and 4°C (D) were increased as a function of increasing MOI. Saturation was apparent at 1,000 EB per host cell. Binding of EB to HEC-1B cells was slightly better (~10%) at 4°C compared to binding at 35°C.

sensitive to the effects of heparan sulfate and heparitinase (2, 3).

In light of the compelling evidence for the role of a heparan sulfate-like glucosaminoglycan in chlamydial adherence, Su and colleagues (20) revisited the issue of MOMP as a cytoadhesin for HeLa cells with the murine *C. trachomatis* mouse pneumonitis (MoPn) strain. To more directly assess the role of MOMP as an adhesin, in the absence of other chlamydial envelope components, the mature MOMP polypeptide was expressed as a fusion protein with the *Escherichia coli malE* gene product (MBP-MOMP). Surprisingly, exposure of intact MoPn EB to heparitinase did not reduce chlamydial infectivity for HeLa cells. However, exposure of the HeLa cells to heparitinase or exogenous heparan sulfate markedly reduced the binding of MBP-MOMP to HeLa cells and the infectivity of whole EB for those treated cells. These findings provided stronger support for the role of MOMP as an adhesin and, indeed, pointed to the heparan sulfate proteoglycans as the host cell receptor to which the MOMP binds. The same strategy has been reported for tropism and infection of Chang epithelial cells by *Neisseria gonorrhoeae* MS11—selective binding of heparan sulfate receptors by gonococcal opacity-associated protein adhesin(s) (25).

Host cell surface-anchored glucosaminoglycans have also been shown to be involved in several virus-host cell interactions, including those of human immunodeficiency virus (13), and bacterium-host interactions, including those of *N. gonorrhoeae* (4, 14, 25). With the added success of the chlamydia-host cell studies, investigations into the potential use of sulfated polysaccharides in vaginal formulations for the purpose of inhibiting transmission of sexually transmitted disease agents were prompted (30). Both heparin and heparan sulfate were, again, capable of blocking infectivity of *C. trachomatis* serovar L2 and, in addition, serovar E for nonpolarized ME180

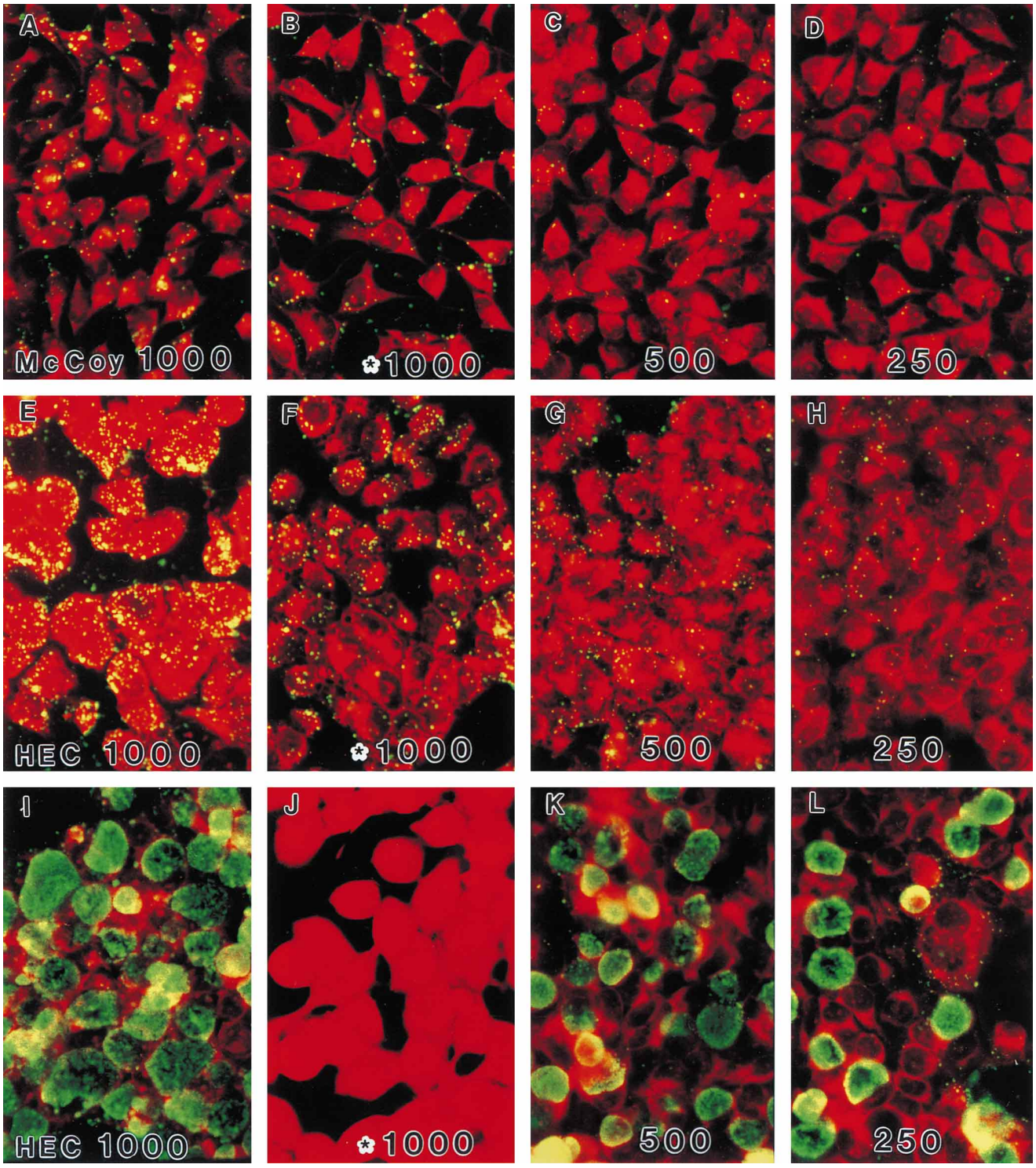


FIG. 2. Morphological assessment of binding of flask-grown ^{14}C-labeled *C. trachomatis* serovar E to McCoy and HEC-1B cells and the infectivity for HEC-1B cells as a function of MOI. Binding of live, infectious EB to McCoy cells (A, C, and D) and HEC-1B cells (E, G, and H) after 1 h at 35°C was decreased as a function of decreasing MOI (1,000, 500, and 250). Binding of heat-exposed EB (60°C for 10 min) to McCoy cells (B) and HEC-1B cells (F) at an MOI of 1,000 was considerably decreased relative to control, unexposed EB (A and E). Infectivity of live EB for HEC-1B cells (I, K, and L), based on the number of inclusions formed at 48 h postinfection, decreased with decreasing MOI. Preexposure of EB to 60°C in a water bath for 10 min resulted in a loss of infectivity for HEC-1B cells (J). The fluorescent photomicrographs also confirmed that the eukaryotic cell monolayers were subconfluent, there was no background binding of EB to glass coverslips, and there was no clumping of EB, especially at the high MOI or following exposure to heat. Magnification, approximately ×300 (A to D and F to L) and approximately ×500 (E).

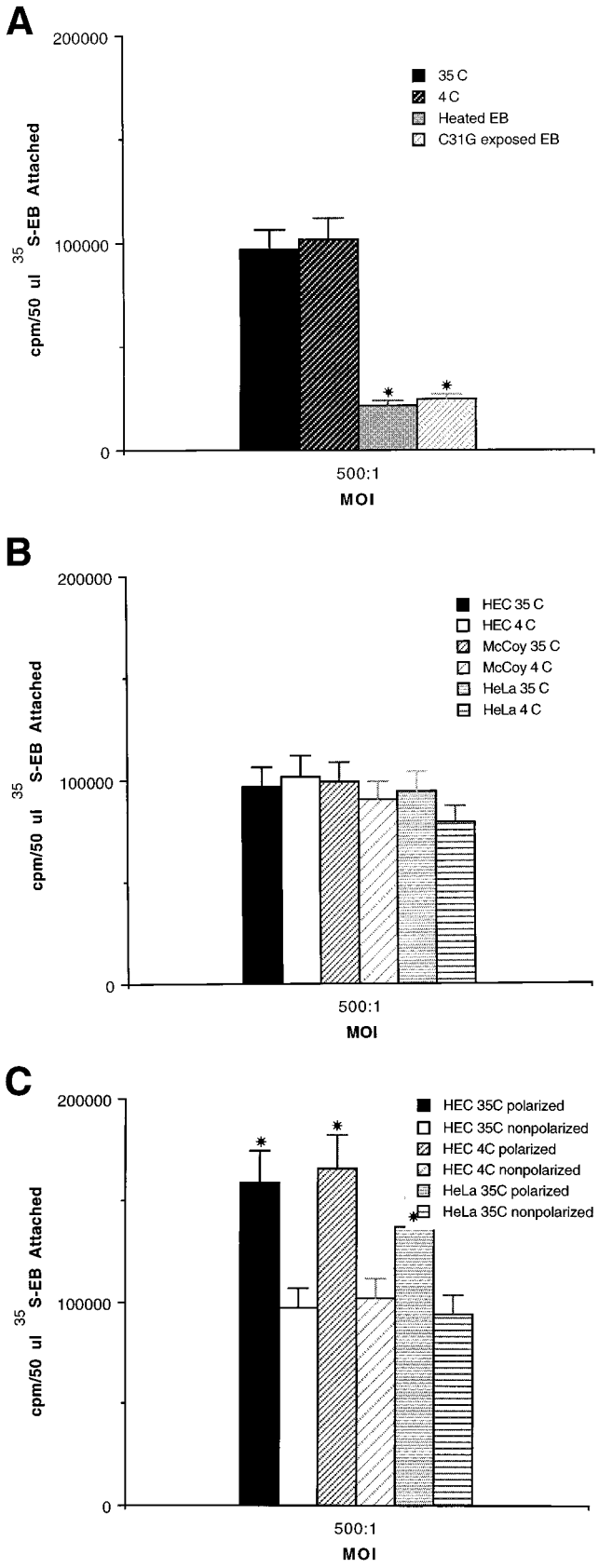


FIG. 3. Parameters influencing attachment of microcarrier-grown ^{35}S-radiolabeled *C. trachomatis* serovar E EB (MOI, 500:1) to various host cells. (A) Preexposure of EB to heat (60°C for 10 min) or topical microbicide (C31G) dramatically reduced attachment of EB to HEC-1B cells after 1 h at 35°C relative to control, unexposed EB. (B) There was no significant difference in the attachment of EB to nonpolarized HEC-1B cells versus McCoy cells versus HeLa cells at either 35 or 4°C. (C) However, there was a statistically significant difference (asterisks signify $P < 0.05$) in attachment of EB to polarized versus nonpolarized host cells.

cells, although serovar E was considerably less susceptible. To further analyze the specificity of the heparan sulfate inhibitory activity, additional sulfated polysaccharides were tested. In essence, the more charged the polyanion, the more it inhibited chlamydial infectivity for ME180 cells. The investigators concluded that the blocking of chlamydial infectivity by sulfated polysaccharides, including heparin, was a nonspecific, charge-mediated event. Further, antigenically distinguishable variants displayed differing sensitivities to heparan sulfate, as reported by Chen, Zhang, and Stephens (3).

The chlamydial developmental cycle is slow and asynchronous, at least when performed in vitro in eukaryotic cell lines grown in plastic tissue culture flasks. For the more rapidly growing chlamydiae, the *Chlamydia psittaci* species and the LGV biovar (*C. trachomatis* serovars L1, L2, and L3), the developmental cycle requires ~48 h to complete, and one or two of every three EB particles is usually infectious. For the more slowly growing chlamydiae, such as the trachoma-inducing strains (*C. trachomatis* serovars A to C) and the genital strains (*C. trachomatis* serovars D to K), the developmental cycle requires ~72 to 96 h to complete and the infectious EB-to-particle ratio is significantly greater. For serovar D, the infectious EB-to-particle ratio has been estimated to range from 1:100 to 1:1,678 (11). Incomplete maturation of RB to infectious EB as well as asynchronous release of infectious progeny into a rapidly deteriorating environment, consisting of acidic conditions and lysed host cell debris, may alter outer membrane components, including adhesins, and compromise infectivity. Such circumstances might influence the sensitivity of the trachomatis biovar types (A to K) to heparan sulfate.

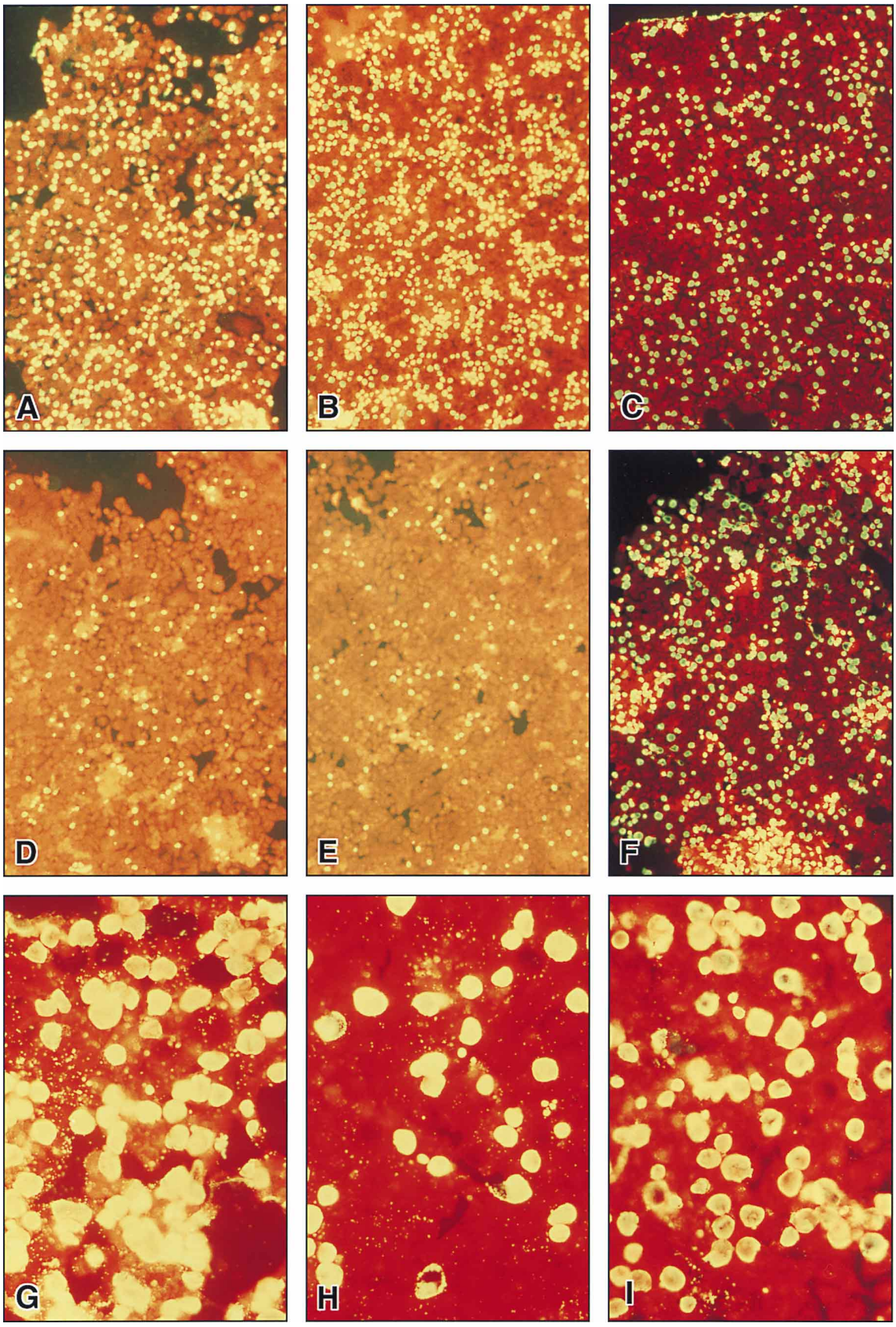
Our laboratory has adapted the microcarrier bead culture method for growing *C. trachomatis* genital serovar E, and the results have been dramatic relative to growth of the same serovar in plastic flasks (21, 29). Two findings are pertinent to this theme: (i) the developmental cycle is markedly accelerated, and (ii) the isolated EB progeny are as much as 100 to 1,000 times more infectious. The purpose of this study was to analyze the adherence of the highly infectious microcarrier-harvested *C. trachomatis* serovar E EB to host cells (i) by using various culture condition parameters known or suspected to influence binding and (ii) by comparing the sensitivity of this serovar E to heparan sulfate and heparin relative to that of the *C. trachomatis* L2 prototype.

MATERIALS AND METHODS

Chlamydiae and host cell lines. *C. trachomatis* serovar E (UW5-CX) and the *C. trachomatis* biovar LGV L2/434/Bu were cultured in McCoy cells grown on Cytodex 3 microcarrier beads (29) (Pharmacia, Uppsala, Sweden) for approximately 60 to 65 and 40 h, respectively. The chlamydiae were harvested as crude stocks and frozen at −70°C in storage buffer (0.02 M phosphate buffer, 0.2 M sucrose, 5 mM glutamine [2SPG]). An aliquot of frozen stock was rapidly thawed in a 37°C water bath, and the titer of infectious units was determined, as previously described (29).

Three eukaryotic cell lines were used for these studies, and all were determined to be free of mycoplasmas by routine, periodic screening: (i) McCoy cells, a fibroblast cell line (CRL 1696; American Type Culture Collection [ATCC], Rockville, Md.); (ii) a transformed human endometrial epithelial cell line, HEC-subclone 1B (HTB-113; ATCC); and (iii) HeLa 229 (CCL 2.1; ATCC), an epithelial cell line originating from an adenosquamous carcinoma of the cervix.

Metabolic radiolabeling. McCoy cells, seeded on 75-cm^2 flasks for ^{14}C labeling or Cytodex beads for ^{35}S labeling, were grown in minimal essential medium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(MEM) with Hanks' salts (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (MEM-10). When the monolayers were slightly subconfluent, they were infected with *C. trachomatis* serovar E and incubated at 35°C in MEM-10 containing 0.5 μg of cycloheximide per ml. At 16 h postinfection, the medium was replaced with fresh medium containing 2 μg of cycloheximide per ml and incubation was continued for an additional 2 h. Prior to addition of radiolabel, MEM-10 was replaced with MEM-Selectamine (Gibco) containing a 1/10 volume of each amino acid with a labeled counterpart, 10% FCS, and 1.5 μg of cycloheximide per ml. For ^{14}C labeling, 0.2 μCi of an L-^{14}C-amino acid mixture (Dupont NEN, Boston, Mass.) per ml was used. ^{35}S Easy Tag EXPRESS protein labeling mix (Dupont NEN) was used at a concentration of 10 μCi/ml for cysteine and methionine labeling. Radiolabeled EB were harvested at 60 to 65 h and purified by a Percoll (Sigma, St. Louis, Mo.) gradient procedure described by Newhall et al. (10). Infectious unit (IFU) titrations and protein determinations (bicinchoninic acid; Pierce, Rockford, Ill.) were performed on Percoll-purified radiolabeled EB.

The efficiency of radiolabeling was as follows. For ^{14}C labeling, there were 5×10^{10} EB particles/ml and 1×10^8 IFU/ml; 1 μl of EB = 1 μg of protein = 5×10^7 particles = 1×10^5 IFU, giving 160 dpm. For ^{35}S-Cys-Met labeling, there were two batches: (i) in the first batch, there were 6×10^9 EB particles/ml and 1.3×10^7 IFU/ml; 1 μl of EB = 1 μg of protein = 6×10^6 particles = 1.3×10^4 IFU, giving 40,000 dpm; (ii) in the second batch, there were 1.65×10^{10} EB particles/ml and 4×10^8 IFU/ml; 1 μl of EB = 1 μg of protein = 1.65×10^7 particles = 4×10^5 IFU, giving 70,000 to 100,000 dpm. The overall results obtained from the two preparations were virtually the same.

Attachment assay. HEC-1B, HeLa 229, or McCoy cells were resuspended in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FCS and 2 mM glutamine, and 0.5 ml of a suspension containing 5×10^4 cells was added per well (ca. 11.3 mm in diameter) of 48-well plates (Costar). The cultures were incubated at 37°C for 48 to 72 h until subconfluent monolayers were obtained, and then they were washed with phosphate-buffered saline (PBS) containing Ca^{2+} (0.7 mM) and Mg^{2+} (0.5 mM) prior to addition of radiolabeled EB. Appropriate dilutions of purified, radiolabeled EB (in sucrose-phosphate-glutamic acid [SPG] storage buffer) were made in 50% SPG–50% DMEM (devoid of serum), each of which was sonicated for 15 s in a water bath to disperse any aggregated EB, and 50-μl aliquots were inoculated onto monolayers in each well. Attachment of EB to host cells was allowed to proceed for 1 h at 35 or 4°C, with gentle tapping of the monolayer every 15 min. For attachment assays performed at 4°C, the monolayers were prechilled to 4°C prior to the addition of the EB inoculum.

At the end of the 1-h adsorption period, supernatants were removed, the monolayers were washed three times in PBS, and the washes were combined with the supernatants and added to vials containing 4 ml of scintillation fluid (Scintisafe Plus 50%; Fisher Scientific, Fair Lawn, N.J.). Host cell monolayers were solubilized in 2% sodium dodecyl sulfate, the wells were washed three times in PBS, and these washes were combined with the host cells for scintillation counting in a Beckman LS 6500 (Beckman, Schaumburg, Ill.). The results are expressed as (i) counts per minute per 50 μl of radiolabeled EB bound to host cells, (ii) the number of radiolabeled EB bound per host cell, or (iii) the percentage of radiolabeled EB attached.

For some analyses, the HEC-1B cells and HeLa cells were grown in a polarized orientation. In these experiments, Falcon 24-well Biocoat cell culture inserts (ca. 9 mm in diameter; Becton-Dickinson, Bedford, Mass.) were seeded with 10^4 eukaryotic cells suspended in DMEM-10. The cultures were incubated at 37°C and monitored until the cells became confluent and reached a polarized state. The attachment assays were then performed as described for the 48-well cultures. The raw data were then adjusted for equivalence with the data from the 48-well cultures for comparison with attachment of EB to nonpolarized cells.

Each experiment was performed in triplicate and was repeated a minimum of five times on separate days. Data were analyzed by Student's *t* test, and a value of $P < 0.05$ was considered significant.

Exposure of EB to heparin and heparan sulfate. In accordance with the basic protocol of Chen, Zhang, and Stephens (3, 31), dilutions of heparin, from porcine intestinal mucosa (Sigma H-3393), were mixed with 10^5 IFU of ^{35}S-labeled serovar E EB, and 50 μl of each suspension was adsorbed to HEC-1B monolayers for 1 h at 4 or 35°C; the samples were then processed for scintillation counting. After the initial titration experiments were analyzed, 500 μg of heparin or heparan sulfate per ml, from bovine intestinal mucosa (Sigma H-7641), was used in all subsequent attachment assays.

For the fluorescence microscopy experiments, heparan sulfate (500 μg/ml)-exposed or unexposed, nonradiolabeled, control LGV L2 EB (10^5 IFU) or *C. trachomatis* serovar E EB (10^5 IFU) were incubated with HEC-1B cells on coverslips for 1 h at 4 or 35°C for morphological assessment of attachment or were incubated at 37°C for 36 h (serovar L2) or at 35°C for 48 h (serovar E) for assessment of infectivity based on inclusion development (16). The monolayer cultures were then fixed with methanol, stained with a fluorescein-conjugated pool of monoclonal antibodies generated against the *C. trachomatis* MOMP, suspended in an Evans blue counterstain (Syva, Palo Alto, Calif.), and examined on a Zeiss Axiovert 10 microscope equipped with 495-nm excitation and 520-nm emission filters.

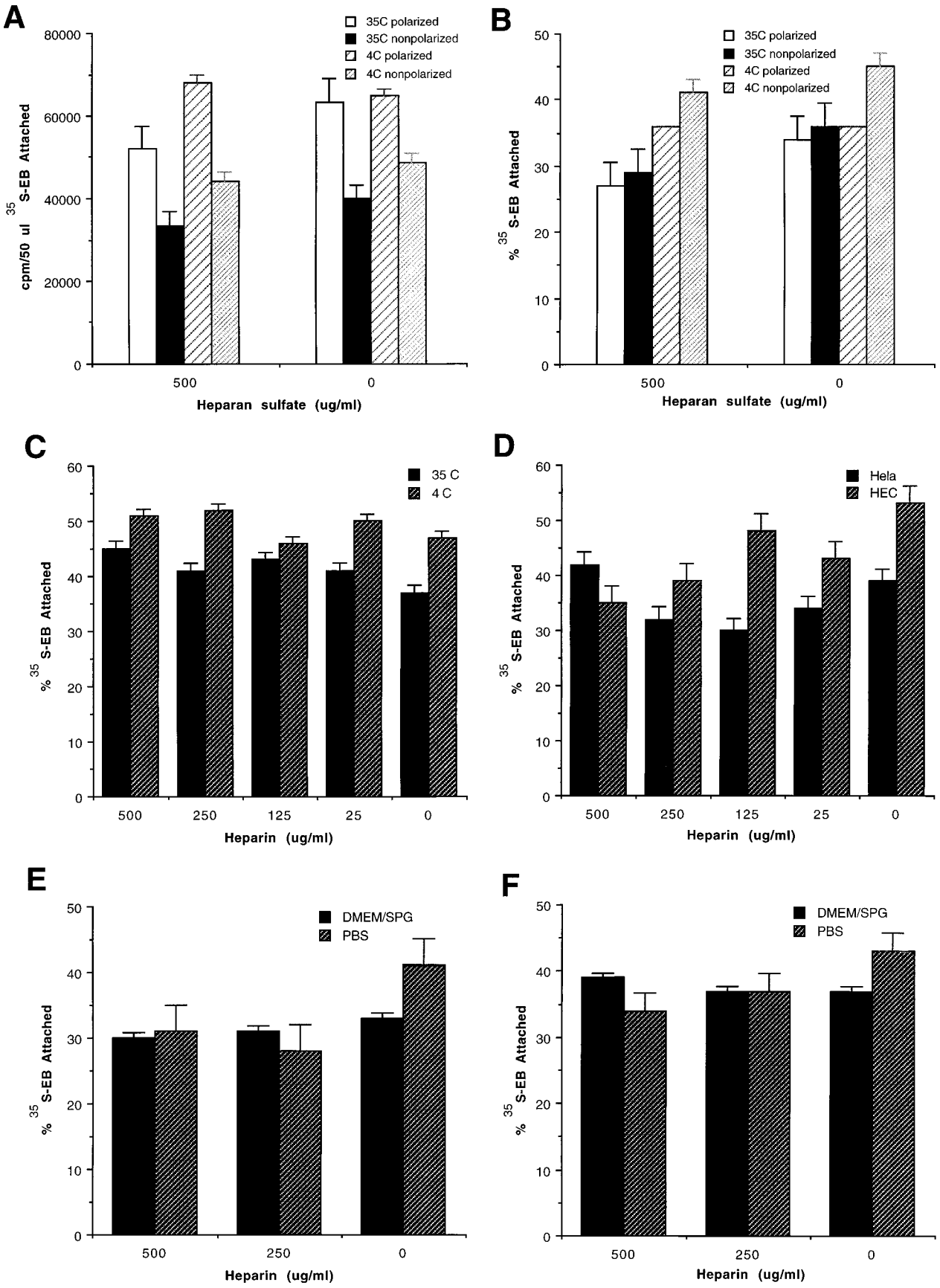
Competitive inhibition. In order to evaluate the specificity of serovar E attachment, competitive inhibition experiments were performed according to the method of Su et al. (20). ^{35}S-labeled serovar E EB (10^5 IFU/ml) were added to increasing concentrations of nonradiolabeled serovar L2, and the mixtures were added to HEC-1B cells for 1 h at 4°C and processed for scintillation counting for analysis of attachment of radiolabeled serovar E EB. For infectivity competition assays, serovar E EB or serovar L2 EB were irradiated by UV light (at a 70-cm distance in an open petri dish for 10 min), added in increasing concentrations to HEC-1B monolayers on coverslips, and incubated for 1 h at 4°C. After three washes of the monolayers with PBS, live serovar E EB (10^4 IFU per coverslip) were inoculated onto the monolayers and the coverslip cultures were incubated for an additional 1 h at 4°C. Following additional washes, the cultures were topped up with fresh DMEM and incubated at 37°C for 36 h (serovar L2) or at 35°C for 48 h (serovar E). The infected monolayers were fixed with methanol and stained with Syva reagent, and the fluorescent chlamydial inclusions in 30 grid fields at an ×40 magnification were counted on each of three coverslips.

RESULTS

Binding parameters of flask-grown ^{14}C-labeled EB. These studies initially used ^{14}C-metabolically labeled serovar E EB harvested from infected cells grown in flasks as a baseline for comparison with previous chlamydial attachment studies (8, 19) and with subsequent attachment studies using microcarrier-harvested EB. The basic protocol involved a 1-h adherence time of radiolabeled EB to subconfluent monolayers of HEC-1B cells in 48-well cluster plates incubated at 35°C in an atmosphere of 5% CO_2.

Increasing the number of EB per cell (multiplicity of infection [MOI]) resulted in a concomitant increase in attachment of EB per HEC-1B cell (Fig. 1A). Saturation was achieved between 500 and 1,000 MOI (Fig. 1B). These quantitative data were confirmed morphologically with both HEC-1B cells and McCoy cells although the number of EB attached to McCoy cells (Fig. 2A, C, and D) appeared slightly less than the number of EB attached to HEC-1B cells (Fig. 2E, G, and H). The dose-dependent increase in EB particle attachment was further confirmed by a corresponding dose-dependent increase in HEC-1B cell infectivity as reflected by an increase in the number of inclusions detected at 48 h (Fig. 2I, K, and L). Preexposure of EB to 60°C in a water bath for 5 to 10 min resulted in a noticeable decrease in attachment of EB to McCoy cells (Fig. 2B versus A) and HEC-1B cells (Fig. 2F versus E) and a loss of infectivity of EB for HEC-1B cells (Fig. 2J versus I). The fluorescent photomicrographs in Fig. 2 provide additional important information for evaluating the quality of the attachment assays: (i) the eukaryotic cell monolayers were subconfluent; (ii) there was no background binding of EB to the plastic wells; and (iii) there was no evidence of aggregation of EB, especially at the high MOI or following exposure of EB to heat. Ting and colleagues (24) had reported spontaneous aggregation of *C. psittaci* EB following heat exposure, which

FIG. 4. Fluorescent microscopy analysis of the effect of heparan sulfate on the infectivity of *C. trachomatis* serovar L2 and serovar E for HEC-1B cells and the specificity of serovar E infectivity. Numerous serovar L2 inclusions were obtained in HEC-1B cells at 36 h postinfection, whether the initial adherence was performed at 4°C (A) or 35°C (B). Exogenous addition of heparan sulfate (500 μg/ml) to the serovar L2 EB inoculum during the 4°C adherence (D) or the 35°C adherence (E) period dramatically reduced subsequent L2 inclusion formation. In contrast, exogenous addition of heparan sulfate to the serovar E inoculum during the 1-h adherence period had no effect on subsequent serovar E inclusion formation (F) compared to control infections in the absence of heparan sulfate (C). The specificity of the association of serovar E EB with HEC-1B cells was demonstrated by competitive inhibition of infectivity of live serovar E EB (G) with excess UV-irradiated serovar E EB (H), whereas excess UV-irradiated serovar L2 EB (I) were not able to competitively inhibit infectivity of serovar E EB. Magnification, ×100 (A to F) and ×400 (G to I).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interfered with an interpretation of decreased binding and infectivity.

Binding parameters of microcarrier-grown ^{35}S-labeled EB. While the fundamental experimental conclusions to this point were in agreement with those of previous chlamydial attachment studies, we were concerned that the low efficiency of ^{14}C labeling of EB (essentially 1 dpm per 10^5 EB) might mask important aspects of chlamydial adherence. Therefore, we switched to metabolic radiolabeling of EB with ^{35}S-cysteine-methionine, which increased the efficiency of serovar E EB labeling. In addition, all ^{35}S-labeled EB were harvested at 60 h postinoculation from microcarrier bead-grown cultures, which increased the infectious titer of these EB progeny.

Again, there was a linear increase in attachment of ^{35}S-EB to HEC-1B cells with an increase in MOI, and this finding was true for incubation temperatures of 35 as well as 4°C (Fig. 1C). There was also a shift in saturation to ~1,000 particles per cell (Fig. 1D), which agrees with the findings of Ting et al. (24) of 750 particles of *C. psittaci* GPIC for HeLa cells.

Quantitatively, there was an 80% decrease in binding of heat-exposed EB to HEC-1B cells (Fig. 3A), which correlates with the well-recognized heat sensitivity of chlamydiae (26) via conformational alterations in outer envelope components (19). For comparison, the radiolabeled EB were preexposed to the microbicide C31G (0.01%) for 1 h at 4°C prior to the binding assay. C31G is a mixture of an alkyl dimethyl glycine (alkyl betaine) and an alkyl dimethyl amine oxide (23). As an amphoteric surface active agent, its mechanism of action is suspected to involve binding to microbial surfaces via the polar head group of the amine oxide-betaine and subsequent intercalation of the alkyl chains into membranes. The result is a general destabilization of the outer membrane of gram-negative bacterial envelopes. Indeed, binding of C31G-exposed EB to HEC-1B cells was decreased by 75%.

There is sometimes concern that use of different eukaryotic cell lines may be responsible for variations in data from laboratory to laboratory. However, in these adherence studies, cell type did not appear to be an issue. There were no significant differences in the binding of ^{35}S-labeled serovar E EB to HEC-1B cells versus McCoy cells versus HeLa cells (Fig. 3B). In fact, the only statistically significant difference found was that binding of ^{35}S-labeled serovar E EB to polarized host cells was significantly greater ($P < 0.05$) than binding to nonpolarized cells (Fig. 3C).

Effect of heparan sulfate and heparin on serovar E EB attachment and infectivity. The parameters involving microcarrier bead-harvested *C. trachomatis* serovar E adherence to HEC-1B cells in vitro having been established, the second goal of these studies was to examine the effects of heparin and heparan sulfate on serovar E adherence. The prototype studies by Zhang and Stephens (31) were performed with *C. trachomatis* serovar L2 and HeLa cells at 4°C. Since the first phase of these studies demonstrated virtually no difference in adherence of serovar E to HEC-1B or HeLa cells and that serovar E was equally adherent to HEC-1B cells at 4 or 35°C, HEC-1B cells were used for the heparin-heparan sulfate analyses.

The infectivity of *C. trachomatis* L2 for HEC-1B cells was demonstrated by the appearance of numerous inclusions at 36 h postinoculation, whether the initial adherence was performed at 4°C (Fig. 4A) or at 35°C (Fig. 4B). The results were comparable to the infectivity of serovar E for HEC-1B cells (Fig. 4C). Addition of heparan sulfate (500 μg/ml) to the inoculating suspension resulted in a marked decrease in serovar L2 infectivity (Fig. 4D and E), similar to that reported previously (31), and proved that the reagents and protocol were working in our in vitro system. In contrast, heparan sulfate had no obvious effect on serovar E infectivity (Fig. 4F versus C). To examine these findings with serovar E in a more quantitative manner, ^{35}S-labeled serovar E EB were adjusted to an MOI sufficient to yield a 30 to 50% infection of HEC-1B cells, suspended in heparan sulfate (500 μg/ml), and analyzed for adherence to HEC-1B cells. Again, in the presence of heparan sulfate, there was no significant decrease in either the number of EB attached (Fig. 5A) or the percentage of EB bound (Fig. 5B) to HEC-1B cells at 4 versus 35°C or to nonpolarized cells versus polarized cells relative to control, radiolabeled EB not exposed to heparan sulfate.

Even though heparin is more negatively charged than heparan sulfate, the two glucosaminoglycans appear to behave in a functionally equivalent manner in reducing the association of serovar L2 EB with HeLa cells (3). Over a range of concentrations of heparin (25 to 500 μg/ml), there was no statistically significant difference in the number or percentage (Fig. 5C) of ^{35}S-serovar E EB bound to HEC-1B cells at either adherence temperature. In one experimental series only, suspension of serovar E EB in 500 μg of heparin per ml resulted in an 18% decrease in the percentage of (nonpolarized) HEC-1B cell-bound EB at 35°C (Fig. 5D). By comparison, the presence of 500 μg of heparin per ml was reported to reduce serovar L2 binding by 90% (31) and serovar B infectivity by 56% (2).

To determine if a longer exposure of EB to heparin or, alternatively, different buffers were necessary to effect reduced association of serovar E with HEC-1B cells, ^{35}S-serovar E EB were diluted in our standard buffer (SPG-DMEM) or PBS and preexposed to heparin for 1 h at 4°C prior to the 1-h adherence time. Extended preexposure of EB to heparin (Fig. 5E) versus immediate exposure (Fig. 5F) did not alter the binding of serovar E EB to HEC-1B cells when SPG-DMEM was the suspension buffer. Preincubation of heparin-EB in PBS resulted in a ~10% reduction in binding.

The specificity of the association of serovar E EB with HEC-1B cells was demonstrated by competitive inhibition of infectivity of live serovar E EB with excess UV-irradiated (20) serovar E EB (Fig. 4G and H and 6A) whereas excess UV-irradiated serovar L2 EB were not able to competitively inhibit infectivity of serovar E EB (Fig. 4I and 6B). Further, excess viable serovar L2 EB were unable to competitively inhibit binding of ^{35}S-labeled serovar E EB to HEC-1B cells (Fig. 6C).

DISCUSSION

Relative to previous studies (8), three important differences in binding of *C. trachomatis* serovar E EB to host cells were noted in this study. First, binding did occur at 4°C. Second, the use of more infectious EB harvested from microcarrier beads did provide for a greater number of EB bound rapidly per host

FIG. 5. Quantitative effect of heparan sulfate or heparin on the association of ^{35}S-radiolabeled *C. trachomatis* serovar E EB with HEC-1B cells. Exogenous addition of heparan sulfate (500 μg/ml) made no difference in the number of labeled EB bound to nonpolarized or polarized HEC-1B cells after 1 h at 35 or 4°C (A) or the percentage of ^{35}S-EB bound (B). Similarly, exogenous addition of increasing concentrations of heparin did not significantly alter the percentage of radiolabeled EB bound to HEC-1B cells at 35 or 4°C (C) or to HEC-1B or HeLa cells at 4°C (D). Alternatively, EB were diluted either in our standard SPG-DMEM buffer or in PBS and were then preexposed to heparin for 1 h at 4°C prior to the 1-h adherence assay (E) or subjected to immediate exposure to heparin during the adherence assay (F). None of these manipulations affected the percentage of EB binding to HEC-1B cells more than 10%.

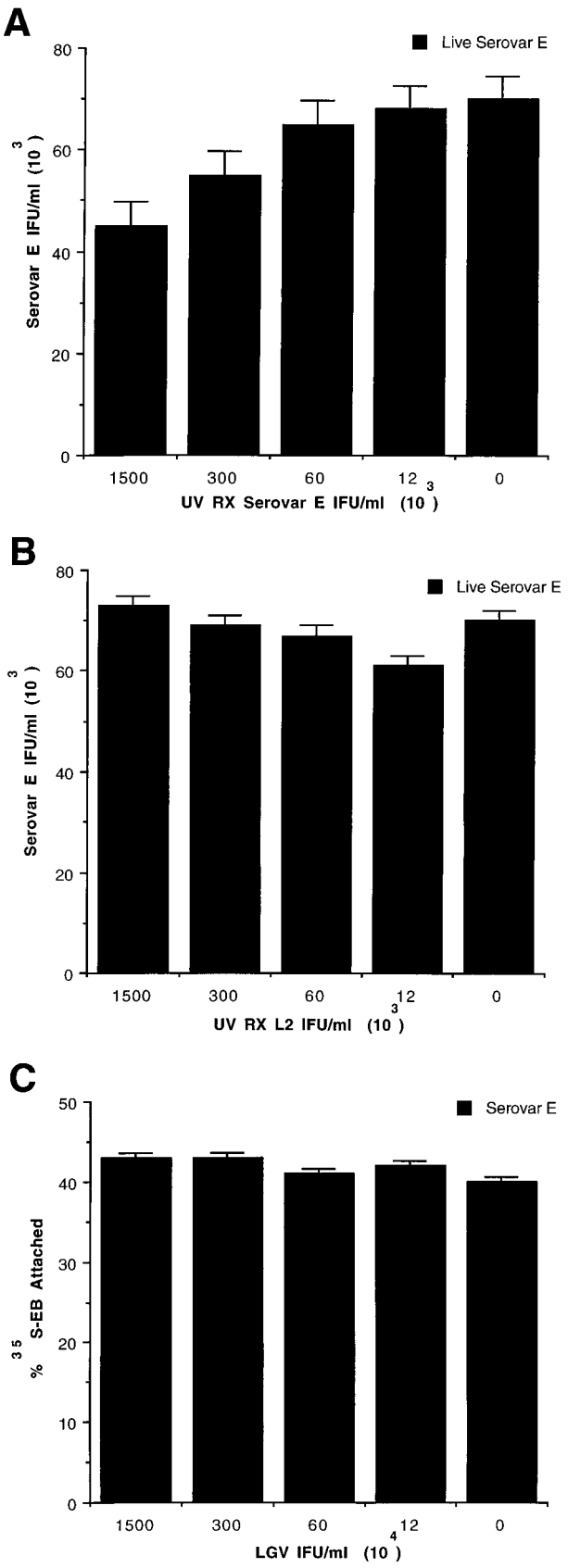

FIG. 6. Competitive inhibition of infectivity and binding of serovar E EB to HEC-1B cells. Infectivity of serovar E EB for HEC-1B cells was competitively inhibited by increasing concentrations of unlabeled, UV-irradiated serovar E EB (A) but not by increasing concentrations of UV-irradiated serovar L2 EB (B). Binding of ^{35}S-serovar E EB to HEC-1B cells at 4°C was not competitively inhibited by increasing concentrations of live serovar L2 EB (C).

cell, which could be better documented by using more efficiently radiolabeled ^{35}S-EB. Previous attempts to increase the efficiency of labeling of EB by using ^{125}I were unsuccessful due to a marked loss of EB infectivity (5). Third, attachment of EB to the apical surfaces of polarized epithelial cells was significantly greater than attachment of EB to nonpolarized cell surfaces.

The number of serovar E EB bound to HEC-1B cells at 4°C was always equal to or even slightly better, by ~10%, than that of EB bound at 35°C. While it would be understandable that the number of organisms bound at 4°C could be slightly greater than the number bound at 35°C due to an increase in the number of receptors available, from lack of receptor recycling, the same phenomenon should be true for HeLa and McCoy cells. One of the difficulties in obtaining an accurate measurement of EB binding at 4°C is that eukaryotic cells tend to round up from cold shock and detach from plastic surfaces. In our hands, HEC-1B cells seemed to be somewhat less susceptible to the 4°C temperature shift than were HeLa or McCoy cells, especially when we used the SPG-DMEM diluent containing 10 mM HEPES buffer; the sucrose provides some cryoprotection, and the buffer maintains the pH of the cultures near neutral. Alternative strategies employed to circumvent the difficulties of a 4°C temperature shift for binding analyses have included prefixation of host cells with glutaraldehyde or sodium azide and then performance of binding analyses at 35°C. HEC-1B cells were more susceptible to these prefixation maneuvers, and significant numbers of cells were lost from the monolayers during the numerous washing steps. We and others (24) have seen a 40% reduction in EB binding to glutaraldehyde-fixed cells. Even though some of the reduction may be explained by glutaraldehyde-mediated receptor cross-linking, we were concerned about the accuracy of such analyses.

Another explanation for the slight discrepancies in binding of *C. trachomatis* EB at 4°C to HeLa and McCoy cells is that comparisons have been made relative to binding analyses at 37°C. The reason that we use the 35°C incubation temperature is that Lee (9) reported a 10 to 30% decrease in serovar A inclusion development with an incubation temperature of 37 versus 35°C. In extensive studies of the kinetics of neutralization of chlamydiae, Peeling and Brunham (11) also documented that the sensitivity of trachoma serovars to heat is greater than that of the LGV serovars. Without an added component for thermal protection in PBS buffer, serovar D infectivity dropped 30 to 50% in 30 min at 37°C. While these culture condition nuances may often explain discrepancies in results from laboratory to laboratory, the emphasis should be on what such nuances tell us about the biology of chlamydiae.

As to the heparin-heparan sulfate experiments, exogenous addition of the glucosaminoglycans did not affect association or infectivity of serovar E EB with host eukaryotic cells at either 4 or 35°C, while in side-by-side experiments with LGV serovar L2, both binding and infectivity were dramatically affected, as reported by Zhang and Stephens (31). The specificity of the attachment was confirmed by competitive inhibition studies.

How might our understanding of the biological differences between the two serovars help us explain the quite different results obtained in our binding analyses involving heparin and

heparan sulfate? The *C. trachomatis* species is subdivided into (i) the *C. trachomatis* MoPn biovar, (ii) the *C. trachomatis* biovar, and (iii) the LGV biovar. The latter two biovars are strictly human pathogens and exhibit nearly 100% homology at the DNA level. The human *C. trachomatis* biovars can be further subdivided into serological variants or serovars: A, B, Ba, and C are responsible for the blinding eye disease trachoma, and D to K are responsible for sexually transmitted diseases. The three serovars (L1, L2, and L3) of the LGV biovar are also responsible for causing sexually transmitted diseases, but the sexually transmitted diseases caused by the two human biovars are markedly different. *C. trachomatis* serovars D to K are luminal pathogens. Following initial infection of the epithelial cells lining the endocervical canal, the chlamydiae may spread canalicularly to the fundus of the uterus to cause endometritis and ascend into the fallopian tubes to cause salpingitis (18). In contrast, the more invasive LGV biovar preferentially infects cells of the lymph nodes. The first manifestation of the disease is a painless, superficial ulcer or vesicle on the genitals. Within 1 to 3 weeks after appearance of the primary lesion, regional lymphadenopathy develops. While the course of the disease is variable, in cases of persistent infection, progressive destruction of the genitalia and genitourinary tract may occur. Due to perirectal drainage of lymph nodes from the vagina, women may experience proctitis and perirectal abscesses. LGV organisms also spread systemically via the bloodstream and can enter the central nervous system (12).

At the in vitro level, several differences between serovar E and serovar L2 have been documented experimentally, besides the more rapid maturation of serovar L2 inclusions at 48 h versus the later maturation of serovar E inclusions at 60 to 72 h. Previous studies using inhibitors of the cytoskeletal network demonstrated that disruption of microfilaments with cytochalasin D markedly reduced entry, early vesicle fusion, and inclusion development of serovar E in HEC-1B cells but not that of serovar L2. Conversely, disruption of microtubules with colchicine or nocodazole had no effect on serovar E inclusion development but resulted in altered serovar L2 early and midinclusion development (16). These results implied that *C. trachomatis* serovar E may utilize a different pathway for uptake and infection from that of LGV serovar L2, a finding which has been suggested in these studies. Chlamydial development in the trans-Golgi network occurs in the supranuclear apical region of polarized mucosal epithelia. Either the aberrant exocytic inclusion (7, 22) containing serovar E then fuses with the apical membrane of the infected cell to release EB progeny into the lumen, or the EB "sneak out" the apical domain gradually (29). In contrast, the L2-containing inclusion or released progeny may exploit the apical-to-basolateral axis of microtubule bundles to position themselves for basal exit into the submucosa (16). Further, in accordance with the directional infectious process in vivo, we have demonstrated that, in polarized epithelial cells in vitro, serovar L2 progeny do exit through the basal domain, as if en route to the lymph nodes (27).

The isoform of heparan sulfate associated with simple epithelia in vivo is localized to basolateral surfaces (1, 28). Thus, it makes sense topologically for the more invasive chlamydial strains, such as LGV, to exploit heparan sulfate, perhaps as an adhesin, and for the heparan sulfate receptors to concentrate at mucosal epithelia for reentry at the basolateral domains from their submucosal location to perpetuate the infectious process. This rationale, if true, could also explain the absence of a heparan sulfate effect on luminal serovar E EB.

Chlamydiae do not possess flagella or appendages such as pili or fimbriae. Thus, the mechanisms used by chlamydiae to facilitate colonization of target mucosal epithelial cells have remained unknown. A plausible explanation could be that invasive chlamydiae exploit numerous low-affinity heparan sulfate receptors to reduce the dimensionality of their diffusion in the extracellular matrix from three to two dimensions (15), thereby increasing their local concentration at the basolateral membrane; this sets up the probability for other domains of the chlamydial adhesin GAG acceptor ligand (3), MOMP (19, 20), the 60-kDa OMP (24), or some other chlamydial ligand then interacting with high-affinity receptors for effecting entry.

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