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The Microbicidal Agent C31G Inhibits *Chlamydia trachomatis* Infectivity In Vitro

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Safe and effective vaginal microbicidal compounds are being sought to offer women an independent method for protection against transmission of sexually acquired pathogens. The purpose of this study was to examine the efficacy of two formulations of one such compound, C31G, against *Chlamydia trachomatis* serovar E alone, its host epithelial cell (HEC-1B) alone, and against chlamydiae-infected HEC-1B cells. Preexposure of isolated, purified infectious chlamydial elementary bodies (EB) to C31G, at pHs 7.2 and 5.7, for 1 h at 4°C resulted in reduced infectivity of EB for HEC-1B cells. Examination of the C31G-exposed ^{35}S-EB on sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiographs and by Western blotting revealed a C31G concentration-dependent and pH-dependent destabilization of the chlamydial envelope, resulting in the release of chlamydial lipopolysaccharide and proteins. Interestingly, when the host human genital columnar epithelial cells were infected with chlamydiae and then exposed to dilute concentrations of C31G which did not alter epithelial cell viability, chlamydial infectivity was also markedly reduced. C31G gained access to the developing chlamydial inclusion causing damage to or destruction of metabolically active reticulate bodies as well as apparent alteration of the inclusion membrane, which resulted in premature escape of chlamydial antigen to the infected epithelial surface. These studies show that the broad-spectrum antiviral and antibacterial microbicide C31G also has antichlamydial activity.

It is becoming abundantly clear that the growing global AIDS epidemic requires new strategies that would allow women to exercise greater control over the prevention of transmission of sexually transmitted diseases in general and human immunodeficiency virus (HIV) transmission in particular. The link between sexually transmitted diseases, particularly genital ulcer diseases, and the sexual transmission of HIV is strong (18, 24). More recently, the recruitment of polymorphonuclear leukocytes by *Chlamydia trachomatis* in females with cervicitis has been shown to be a factor for induction of HIV in mononuclear cells present in semen and for HIV seroconversion (13). The realization that strategies designed around highly efficacious chemical and physical barriers which women can use discretely has led to the concept of vaginal microbicides (7, 10, 25). While some research has been directed towards existing microbicides, including nonoxynol-9 (N-9), octoxynol-9 (15, 35), benzalkonium chloride, menfegol, and mercuric compounds (2, 6), other investigators are exploring new potential microbicides, such as magainins (9) and sulfated polysaccharides (22, 23, 34).

Our studies have focused on C31G, a broad-spectrum antibacterial and antiviral compound (4, 8, 28). C31G is a mixture of an alkyl dimethyl glycine (alkyl betaine) and an alkyl dimethyl amine oxide, buffered with citric acid. C31G has been shown to have potent activity against enveloped viruses, a large number of gram-positive and gram-negative bacteria, and numerous fungi and yeasts, including *Candida* (4, 8). Because both components of C31G are amphoteric surface-active agents, it is suspected that the mechanism of action involves binding to microbial surfaces via the polar head group of the amine oxide-betaine mixture and subsequent disruption of the microbial membrane by the alkyl portion of the molecule. In addition, the compound has potent spermicidal activity and penetrates into cervical mucus (28). Thus, it appears to be well suited as a vaginal spermicide and microbicide.

In the present study, we have examined the action of two preparations of C31G on purified *C. trachomatis* infectious forms, known as elementary bodies (EB), on host genital epithelial cells alone, and on *C. trachomatis* infection of a human endometrial epithelial cell line (HEC-1B). Our data show that C31G appears to be effective in reducing and inhibiting chlamydial infection in vitro.

MATERIALS AND METHODS

Chlamydia and host epithelial cells. A human urogenital isolate of *C. trachomatis* E/UW-5/CX was used throughout these studies. Stock cultures of EB were grown in McCoy cells propagated on microcarrier beads, purified, counted, and titrated as previously described (33). The purified stock EB preparations were resuspended in storage buffer (twice-concentrated 0.2 M phosphate buffer–0.2 M sucrose–5 mM glutamine [2SPG]) and frozen at −80°C until use.

To obtain radiolabeled EB, the Hanks buffered Eagle minimum essential medium (MEM; GIBCO, Grand Island, N.Y.) (pH 7.3), supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah) from 16-h-infected McCoy cells on microcarrier beads, was replaced with fresh MEM containing the eukaryotic protein synthesis inhibitor cycloheximide at a concentration of 2 μg/ml. Following an additional 2 h of incubation, the tissue culture medium was replaced with MEM-Selectamine (GIBCO) containing 10-fold-reduced amounts of cysteine and methionine and supplemented with 1.5 μg of cycloheximide per ml. ^{35}S-labeled Cys-Met (7 mCi/ml; EASYTAG Express Protein Labeling Mix; NEN Research Products, Boston, Mass.) was added to the infected culture to give a final concentration of 10 μCi/ml, and the culture was reincubated at 35°C. Radiolabeled EB were harvested at 60 h postinfection (hpi) and purified. Harvests of radiolabeled chlamydiae, typically consisting of 10^9 to 10^{10} EB/ml, had specific activities ranging between 4×10^5 and 7×10^5 cpm/ml. One microliter

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was equivalent to 1 μg of protein. Infection-forming units ranged between 10^7 and 10^8 per ml.

The human endometrial epithelial cell line HEC-1B (HTB-113; American Type Culture Collection, Rockville, Md.) was used as the host cell for all of these experiments. The epithelial cells were maintained in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (pH 7.2 unless otherwise stated) supplemented with 10% (vol/vol) fetal calf serum (Hyclone) and 10 mM HEPES and devoid of cycloheximide. The cells were routinely checked for mycoplasma contamination during the course of these studies.

HEC-1B cell viability. Epithelial cell viability was assessed by staining the microbicide-exposed monolayers with a mixture of the fluorescent dyes fluorescein diacetate and propidium iodide (0.1 μg of each per ml; Sigma, St. Louis, Mo.) in phosphate-buffered saline. Viable cells cleave and metabolize the diacetate, which in turn activates the fluorescein; thus, viable cells fluoresce green. Propidium iodide rapidly penetrates and diffuses into dead cells, and the entire cell fluoresces red within 3 min (27). Visualization of the HEC-1B monolayers with a fluorescence microscope fitted with dual excitation-emission filters provides easy determination of the status of the eukaryotic cells.

Preparation of microbicides. C31G and N-9 were prepared as stock solutions. N-9 was obtained as a concentrate from Rhone-Poulenc-Ruhr (IGEPAL-CO-630) and diluted with distilled water to a final concentration of 4% actives. C31G was prepared by and obtained from Biosyn Corporation (Philadelphia, Pa.) in two stock solutions, each containing 4% actives: a low-molecular-weight solution, consisting of equimolar amounts of alkyl amine oxide and alkyl dimethyl glycine with alkyl chain lengths of 12 (C12/C12), and a high-molecular-weight solution, consisting of equimolar amounts of a C_{14} alkyl amine oxide and a C_{16} alkyl dimethyl glycine (C14/C16).

Action of C31G on EB. Isolated, purified EB were exposed to 10-fold dilutions of C31G in 100 mM phosphate buffer, adjusted to pH 5.7 or 7.2, and for comparison, to N-9 for 1 h at 4°C. The microbicide-exposed EB were then centrifuged at 15,000 × *g* for 10 min and washed three times by centrifugation to remove excess microbicide. At the higher concentrations, residual microbicide could be cytotoxic to the host cells and, thus, potentially interfere with interpretation of the C31G effect on EB infectivity. Mock-exposed EB were processed identically. The C31G-exposed EB were then assayed for attachment and inclusion-forming capability and were analyzed for extraction of proteins and lipids. We chose pH values of 5.7 and 7.2 to evaluate the effects of the microbicide under acidic and neutral conditions, approximating the in vivo situation before and after the introduction of seminal fluid.

Attachment and infectivity assays. Unexposed EB and microbicide-exposed EB were briefly sonicated to disperse any aggregated EB and then adsorbed in amounts of 50 μl onto subconfluent HEC-1B cell monolayers for 1 to 2 h at 35°C, with gentle agitation of the inoculum every 15 min. For analysis of attachment, two methods were employed. In the first method, the monolayers on coverslips (10^5 HEC-1B cells) were washed to remove excess inoculum, fixed with methanol, labeled with a pool of fluorescein-labeled monoclonal antibodies specific for the chlamydial major outer membrane protein (MOMP; Syva, San Jose, Calif.), and examined by fluorescence microscopy. A minimum of 500 to 1,000 eukaryotic cells per coverslip were analyzed, and the number of fluorescent EB attached per cell was recorded and confirmed by computer-aided morphometry (Metamorph; Universal Imaging Corp., West Chester, Pa.). In the second method, a radioactive binding analysis was performed. ^{35}S-labeled EB (5×10^7 particles suspended in either 100 mM phosphate-buffered saline or SPG storage buffer) were inoculated (50 μl) onto subconfluent monolayers of HEC-1B cells grown in the wells of 48-well microtiter plates. This standardized inoculum was calculated to provide for approximately a 50% attachment ratio. Following a 1-h incubation at 35°C, the supernatant fluid and three washes were combined (supernatant), and the amount of EB bound to the HEC-1B cells (pellet) was determined by scintillation counting.

For analysis of infectivity, the infected cultures were incubated for an additional 46 h, fixed with methanol, stained with the pool of fluorescein-labeled anti-MOMP monoclonal antibodies (Syva), and examined by microscopy for inclusion development.

All experimental parameters were set up in duplicate or triplicate and were performed a minimum of two times on separate occasions. The results from the fluorescence analyses were expressed as means ± absolute deviations from the means, and the results from the radiolabeled analyses were expressed as standard errors of the data.

Protein and LPS analyses. Purified EB were exposed to C31G for 1 h at 4°C in 100 mM sodium phosphate buffer at a pH of 7.2 or a pH of 5.7. Each EB sample contained 25 μg of total protein (BCA microassay; Pierce, Rockford, Ill.) or 9.8×10^8 particles. Following exposure, EB were pelleted by centrifugation (12,000 × *g*, 15 min, 4°C) and solubilized in lysis buffer for resolution by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualization of (i) total protein (16) by autoradiography and/or staining with Coomassie blue or (ii) lipopolysaccharide (LPS) by silver staining as described in detail by Hitchcock and Brown (12). Duplicate samples resolved by SDS-PAGE were transferred to nitrocellulose for immunoblot analysis, essentially as outlined by Caldwell and Hitchcock (3). One exception in the immunoblot procedure was that detection of the primary monoclonal chlamydia-specific LPS antibody was performed with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G secondary antibody (Sigma) as a reporter probe instead of radiolabeled protein A.

Transmission electron microscopy. Fixation and processing of samples for morphological analysis in Epon resin were performed as previously described by Wyrick et al. (31). Fixation and processing of samples for postembedding staining for immunoelectron microscopy in Lowicryl resin were performed as previously reported for identifying exocytosis of chlamydial antigens from inclusions (32).

The primary antibodies selected for use in the postembedding immunolabeling portion of this study were (i) rabbit polyclonal antibodies generated against purified *C. trachomatis* serovar E reticulate bodies (RB), prepared in the authors' laboratory, and (ii) a monoclonal antibody (ascites) directed against a chlamydia-specific epitope for the chlamydial heat shock protein 60 (hsp60), donated by Richard Morrison. Both primary antibodies were used at a dilution of 1:50, and the second-affinity gold-conjugated antibodies (30-nm-diameter gold particles; Auroprobe; Amersham Internationals, Buckinghamshire, United Kingdom) were also used at a dilution of 1:50.

RESULTS

C31G activity on host HEC-1B cells. Subconfluent monolayers of HEC-1B cells on coverslips were exposed to various concentrations of C31G (0.1 to 0.00001% [vol/vol]), prepared in Hank's balanced salt solution or DMEM, and adjusted to pH 5.7 and pH 7.2, respectively. Following 2, 6, 18, 24, 36, 48, and 72 h of incubation at 35°C, the epithelial cells were stained with a mixture of fluorescent dyes and examined by fluorescence microscopy for viability or signs of cytotoxicity. At pH 7.2, both formulations of C31G resulted in loss of viability for the endometrial epithelial cells at concentrations up to 0.001%, whereas dilutions of 0.0001 to 0.00001% were not damaging to the eukaryotic cells, even after a 72-h exposure. In contrast, in vitro-cultured HEC-1B cells underwent immediate cytotoxicity on exposure to pH 5.7 solutions.

C31G activity on *C. trachomatis* EB. The effect of C31G on the infectivity of isolated, purified *C. trachomatis* serovar E EB for HEC-1B cells was examined. Even though we previously reported studies of the action of N-9 on *C. trachomatis* EB and chlamydiae-infected McCoy cells (14), we included for comparison the infectivity of N-9-exposed EB at pH 7.2. The results, recorded in Table 1, can be summarized as follows: (i) at a concentration of 0.1%, C31G was clearly more effective than N-9 at reducing EB infectivity, based on inhibition or reduction of inclusion formation at 48 hpi; (ii) at a concentration of 0.01%, the C14/C16 formulation of C31G was more effective than the C12/C12 formulation at both pH 5.7 and 7.2 for reducing EB infectivity; and (iii) the preliminary breakpoint concentration for C31G activity on EB infectivity was approximately 0.001%.

Duplicate samples on coverslips were stained with a commercially obtained pool of fluorescein-conjugated monoclonal antibodies directed against *C. trachomatis* MOMP suspended in an Evans Blue counterstain (Syva) and examined by fluorescence microscopy. Representative photographs are shown in Fig. 1A to C. The infectivity of chlamydial EB preexposed to 0.1% C12/C12 (Fig. 1B) and C14/C16 (Fig. 1C), at pH 5.7, was markedly reduced for HEC-1B cells compared to control HEC-1B cells infected with EB not exposed to C31G (Fig. 1A).

An additional parameter was also incorporated in this series of experiments. In an attempt to determine if the reduced infectivity of C31G-exposed EB was due to reduced attachment, a duplicate set of samples was fixed and stained at 2 hpi, and the number of EB attached per epithelial cell among a total of 3,000 epithelial cells was determined. The results of one representative experiment are recorded in Table 1. At a concentration of 0.1%, both formulations of C31G profoundly reduced the population of EB binding to the epithelial cell surface. Further, the C14/C16 formulation at pH 5.7 was more

TABLE 1. Effects of C31G and N-9 on *C. trachomatis* infectivity in vitro

Detergent and pH	% Inhibition of chlamydial inclusions at concn (% [vol/vol])[a]					No. of adherent chlamydial EB/field at concn (% [vol/vol])[b]			
	0.1	0.01	0.001	0.0001	0	0.1	0.01	0.001	0
C31G (C12/C12)									
5.7	98.5 ± 2.3	25.0 ± 28.5	0	0	0	3.8 ± 3.5	145.3 ± 2.0	147.9 ± 9.2	139.4 ± 16.8
7.2	98.5 ± 1.8	4.5 ± 4.5	4.3 ± 4.4	10.0 ± 10.0	0	3.2 ± 2.0	28.3 ± 5.2	33.8 ± 1.6	68.6 ± 14.0
C31G (C14/C16)									
5.7	100	93.2 ± 6.2	1.6 ± 2.2	0	0	0.5 ± 0.4	3.6 ± 1.2	75.5 ± 17.6	139.4 ± 16.8
7.2	80.8 ± 19.4	30.4 ± 26.9	2.5 ± 3.2	0	0	0.4 ± 0.4	78.1 ± 33.3	80.7 ± 45.3	68.6 ± 14.0
N-9									
7.2	16.5 ± 13.5	0	0	ND[c]	0				

[a] *C. trachomatis* EB were exposed to each of three detergents for 1 h at 4°C, washed three times, and adsorbed onto HEC-1B monolayers. The labeling of inclusions at 48 hpi was accomplished with fluorescent monoclonal antibody directed against *C. trachomatis* MOMP. Twenty-five microscopic fields from each monolayer were examined for fluorescent inclusions at ×400 magnification for a total of approximately 1,000 HEC-1B cells. The percent inhibition is a reflection of the reduction in the inclusion count in infected cells exposed to C31G compared to the inclusion count in control infected cells not exposed to C31G. Results are expressed as means ± averages of the absolute deviations from the means for triplicate monolayers from four experiments for C12/C12, five experiments for C14/C16, and two experiments for N-9.

[b] Twenty-five microscopic fields from each monolayer were examined for adherent fluorescent EB at ×400 magnification for a total of approximately 1,000 HEC-1B cells. Three monolayers for each concentration of C12/C12 and six monolayers for each concentration of C14/C16 were evaluated. Results are expressed as means ± absolute deviations from the means.

[c] ND, not done.

effective at reducing EB binding to HEC-1B cells than the C12/C12 formulation. Thus, the data confirm that both formulations of C31G were effective in reducing the infectivity of chlamydiae for genital epithelial cells and that part of the reason for the reduced infectivity of C31G-exposed EB was due to reduced binding of the EB to the epithelial cell surface.

To further analyze the binding of C31G-exposed chlamydial EB to the human genital epithelial cells, ^{35}S-labeled EB were

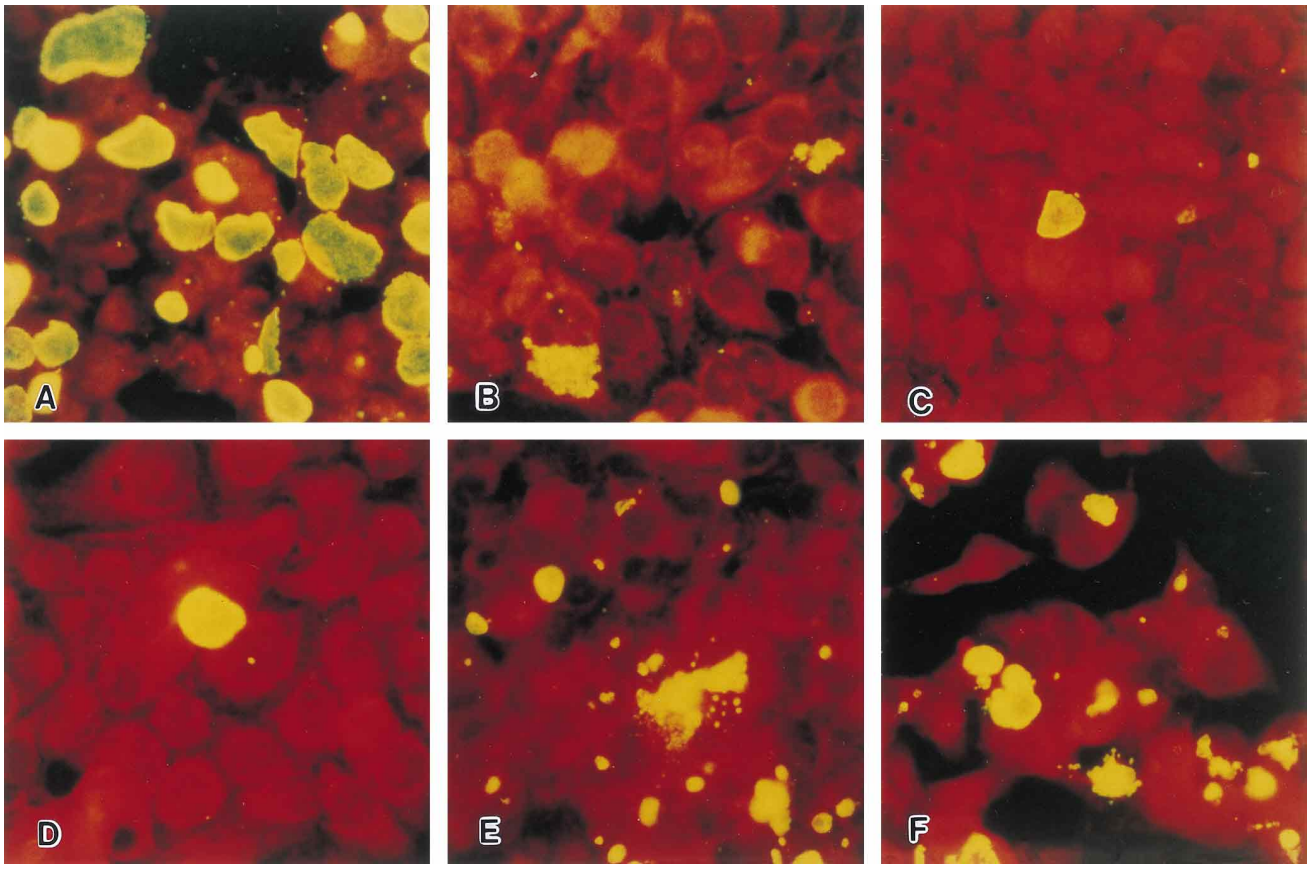


FIG. 1. C31G activity on purified *C. trachomatis* EB and on *C. trachomatis*-infected HEC-1B cells. EB infectivity for HEC-1B cells results in the development of numerous inclusions by 48 hpi at 35°C (control) (A). Infectivity of chlamydial EB preexposed to 0.1% C12/C12 (B) or C14/C16 (C) for 1 h at 4°C resulted in a considerably reduced number of inclusions at 48 hpi. Alternatively, HEC-1B cells were inoculated with purified EB (D through F). At 1 hpi (E) and 24 hpi (F) the DMEM was replaced with medium containing 0.0001% C31G, pH 7.2. At 48 hpi, the number of chlamydial inclusions was noticeably reduced. Passage at 72 hpi of chlamydiae from C31G-exposed infected cells (from inclusions in E or F) to fresh HEC-1B cell monolayers resulted in rare chlamydial inclusions (D). Magnification, ×1,400.

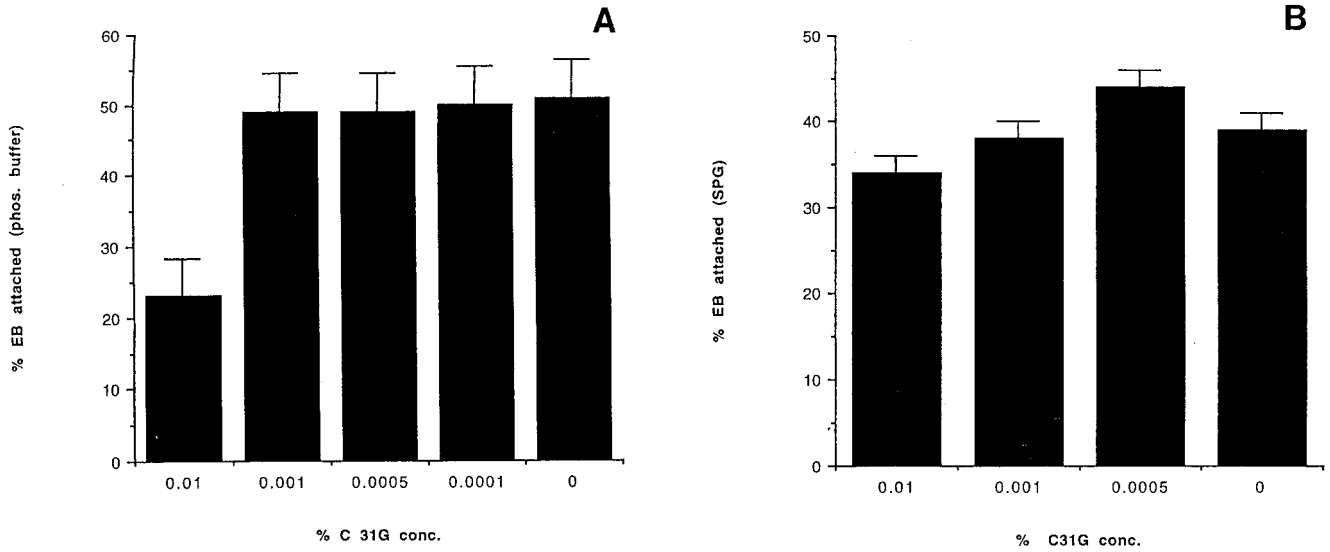
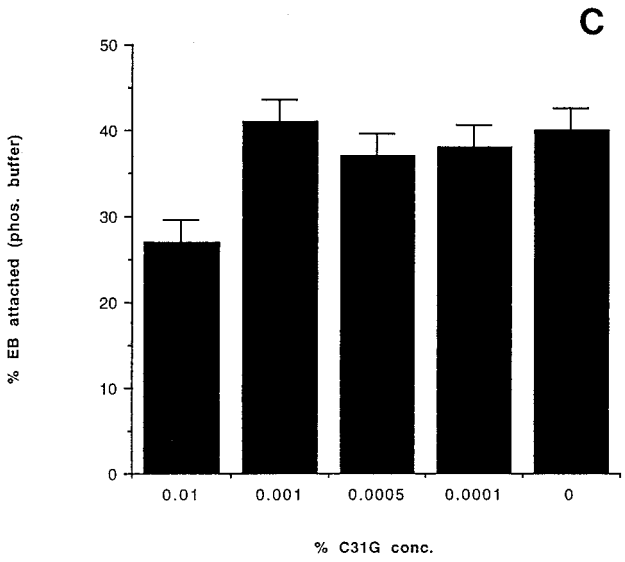


FIG. 2. Binding of C31G-exposed ^{35}S-EB to HEC-1B cells. ^{35}S-EB were preexposed to various concentrations (conc.) of C31G formulation C14/C16 and were prepared in phosphate (phos.) buffer adjusted to pH 7.2 (A), SPG buffer adjusted to pH 7.2 (B), or phosphate buffer adjusted to pH 5.7 (C), and the percent EB bound to HEC-1B cells after 1 h at 35°C was determined by scintillation counting. Attachment of microbicide-exposed EB, suspended in phosphate buffer, was reduced at the 0.01% C31G concentration, whereas there was little or no reduction in attachment of microbicide-exposed EB suspended in SPG. Error bars indicate standard errors of the means.



exposed to the C14/C16 preparation at pHs 7.2 and 5.7 for 1 h at 4°C, and the amount of microbicide-exposed EB bound to HEC-1B cells was determined by scintillation counting. A representative example of the data from two separate experiments is shown in Fig. 2. The binding of microbicide-exposed EB, suspended in phosphate buffer, was reduced by 55.6% at pH 7.2 (Fig. 2A) and 32.5% at pH 5.7 (Fig. 2C) at the 0.01% microbicide concentration. In contrast, there was little reduction in binding of microbicide-exposed EB to HEC-1B cells if the EB were suspended in SPG, the usual chlamydial cryoprotectant storage buffer (Fig. 2B). Perhaps the presence of small amounts of sucrose affected the ability of the surfactant to form micelles.

Examination of the supernatant and wash fluids from the pelleted microbicide-exposed EB used in the attachment assays revealed a release of radiolabeled material, suggesting a possible extraction of envelope components by C31G. Therefore, the microbicide-exposed EB were subjected to SDS-PAGE, and the gels were examined by autoradiography or were stained with silver for LPS (Fig. 3) (12) or with Coomassie blue for protein; the material on duplicate gels was electrotransferred to nitrocellulose for immunoblotting. Exposure of purified EB to the higher concentrations of C31G revealed a release of chlamydial envelope components, particularly LPS. In addition, proteins with masses of 40 and 60 kDa, as well as higher-molecular-mass proteins, appear to be released (data not shown). These proteins likely represent MOMP, the large cysteine-rich structural protein, and other EB envelope proteins. Thus, the mechanism of C31G activity may involve a general destabilization of chlamydial envelope integrity, perhaps by intercalation of the C31G alkyl chains into the outer membrane. There are two noteworthy observations: (i) the C14/C16 formulation of C31G appears to be more efficient in extracting LPS than the C12/C12 formulation, and (ii) both formulations illustrate higher activity at the lower exposure pH of 5.7 (Fig. 3B and C). A similar effect was observed for release of total protein (data not shown.)

C31G activity on the development of *C. trachomatis* serovar E within HEC-1B cells. The activity of C31G on the intracellular development of chlamydial inclusions containing dividing RB was examined by immunofluorescence and transmission electron microscopy. Monolayers of HEC-1B cells were inoculated by adsorption with EB and incubated at 35°C. At 1 and 24 hpi, the culture medium was replaced with medium containing noncytotoxic concentrations of C31G (pH 7.2). At 48 hpi, the infected monolayers were fixed with methanol, stained with a pool of fluorescein-conjugated monoclonal antibodies generated against *C. trachomatis* MOMP, and the fluorescent inclusions in 500 host cells were counted from each of three coverslip preparations. The data are recorded in Table 2, and representative fluorescence photographs are illustrated in Fig. 1E and F. In a duplicate set of samples, the EB progeny from these inclusions were harvested at 72 h and titrated for infectivity in fresh HEC-1B cell monolayers; only rare chlamydial

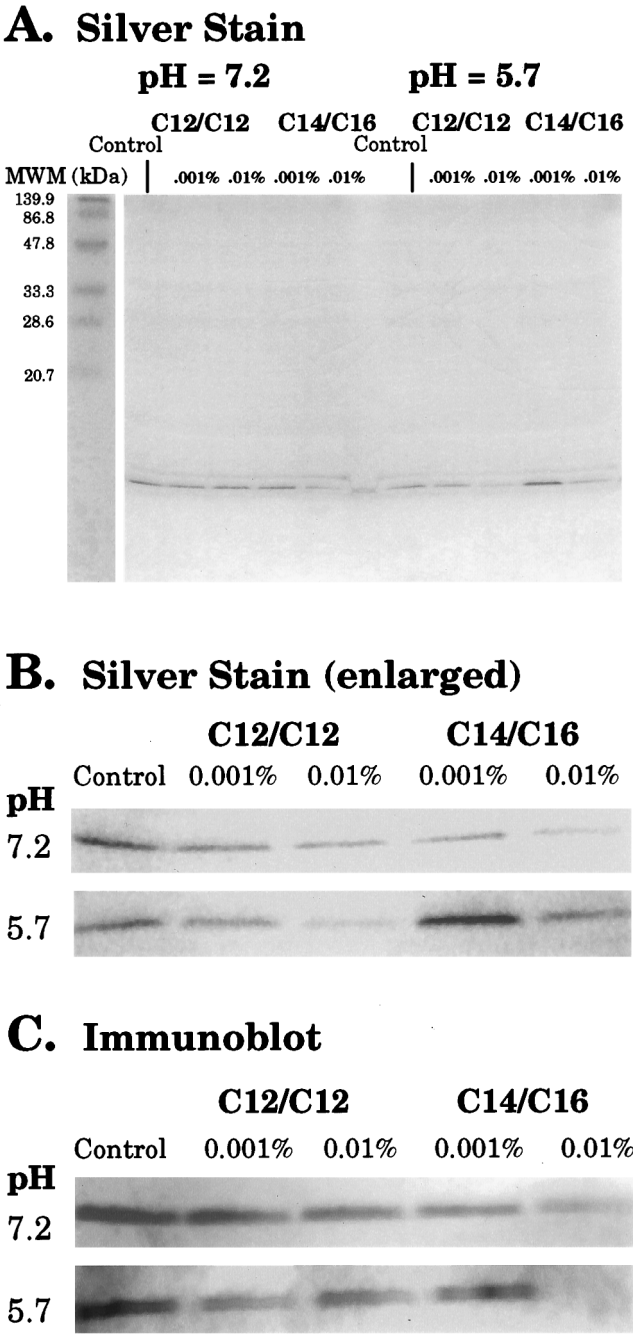


FIG. 3. Extraction of LPS from *C. trachomatis* EB exposed to C31G. Purified EB were not exposed (control) or were exposed to 0.001% or 0.01% C12/C12 or C14/C16 at pH 7.2 or 5.7 for 1 h at 4°C, followed by centrifugation at 12,000 × *g* for 15 min at 4°C. Each pelleted EB sample, containing 9.8×10^8 particles (~25 μg of protein), was solubilized in lysis buffer for resolution by SDS-PAGE (15% polyacrylamide), and the gel was subjected to silver staining (A and B) transferred to nitrocellulose for immunoblotting (C), using a primary monoclonal antibody specific for chlamydial LPS and an alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody as a reporter probe. There was an absence of LPS from EB preexposed to 0.01% C14/C16, pH 5.7. MWM, molecular mass markers.

inclusions could be found (Fig. 1D). These data indicate (i) there was a dose-dependent reduction in inclusion formation with increasing concentrations of C31G, and (ii) a much lower C31G concentration was effective against chlamydial RB in comparison with that observed for EB.

In order to closely examine ultrastructural activities of C31G on *C. trachomatis* inclusions and RB, samples were processed for high-contrast electron microscopy in Epon resin and for postembedding staining immunoelectron microscopy in Lowicryl resin (32). C31G concentrations of 0.0005 and 0.0001% were used, and duplicate samples were stained with fluorescein diacetate and propidium iodide to confirm that these C31G concentrations were below the threshold of cytotoxicity for HEC-1B cells. In Fig. 4A and B, the normal progressive maturation of metabolically active RB via intermediate bodies (IB) to metabolically inert, infectious EB in Epon-embedded samples of infected epithelial cells at 48 hpi is shown. In 48-h-infected cells exposed to 0.0001% C31G, there were two subtle differences in the chlamydiae in the inclusions, shown in Fig. 4 for C12/C12-exposed cells: a delayed conversion of RB into IB and EB (Fig. 4C) and an apparent loosening of the RB envelope or separation of the outer and inner membranes (Fig. 4D). By increasing the concentration of C31G from 0.0001 to 0.0005% and extending the chlamydial infection from 48 to 60 hpi, the microbicide-induced differences were made more obvious. Control infected cells contained distinct IB and EB (Fig. 5A and B), whereas there was a dearth of these forms in both C12/C12-exposed (Fig. 5C and D) and C14/C16-exposed (Fig. 5E and F) infected cells. The damage to RB was also more notable (Fig. 5D and F).

While these data clearly illustrate damaging activity of C31G against intracellular chlamydial forms, it was difficult to assess alterations to the integrity of the surrounding inclusion membrane by examination of morphology alone. Therefore, thin sections of samples processed in Lowicryl were exposed to primary polyclonal anti-RB antibody and primary monoclonal antibody directed against chlamydial hsp60 and were subsequently exposed to 20-nm-gold-conjugated second-affinity antibody in order to examine the potential release of chlamydial antigen beyond the confines of the inclusion membrane. Important controls included thin sections of infected cells stained with gold-conjugated second-affinity antibody alone and uninfected cells stained with both primary and secondary antibodies (data not shown). Figure 6A illustrates a normal chlamydial inclusion containing RB in infected HEC-1B cells not exposed to microbicide; exposure of this sample to chlamydial hsp60

TABLE 2. Inhibition of *C. trachomatis* exposed to C31G during intracellular growth

Time C31G added to culture medium[a]	C31G concn (% [vol/vol])	% Inhibition of chlamydial inclusions by:[b]	
		C12/C12	C14/C16
1 hpi	0.0005	68.0 ± 23.0	71.5 ± 24.5
	0.0001	16.0 ± 6.0	18.5 ± 9.5
	0.00001	19.5 ± 11.5	12.5 ± 2.5
	0.000001	3.0	3.0
	0	0	0
24 hpi	0.0005	ND[c]	ND
	0.0001	28.6 ± 7.8	25.6 ± 6.2
	0.00001	18.3 ± 6.4	17.0 ± 12.7
	0.000001	9.0 ± 2.5	0
	0	0	0

[a] *C. trachomatis* EB were adsorbed to HEC-1B monolayers, and at 1 and 24 hpi the culture medium was replaced with one containing C31G (pH 7.2). The infected monolayers were incubated for a total of 48 h before staining.

[b] Each number represents the average of the results obtained for 500 cells per each of three monolayers in two experiments for 1 hpi and three experiments for 24 hpi. Results are expressed as means ± absolute deviations from the means.

[c] ND, not done.

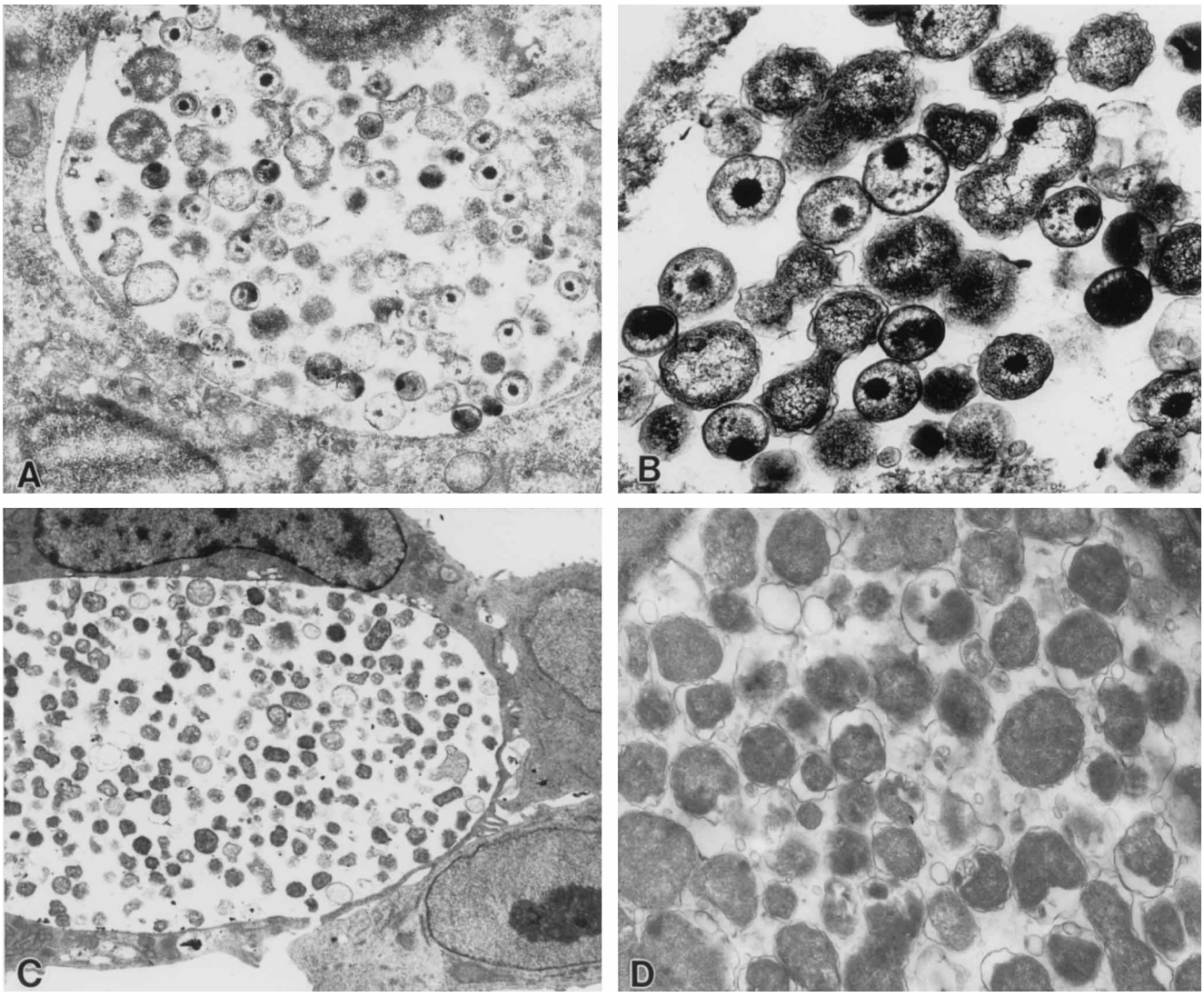


FIG. 4. Action of C31G on *C. trachomatis*-infected HEC-1B cells at 48 hpi. For the control, infected epithelial cells were not exposed to C31G and maturation of RB to EB occurred normally (A and B). HEC-1B cells at 24 hpi were exposed to 0.0001% C31G (C12/C12-exposed cells are shown) and incubated for an additional 24 h (C and D). By 48 hpi there was a delayed maturation of RB to EB (C) and an apparent loosening of the RB envelope (D). Magnifications: ×12,000 (A), ×23,828 (B), ×4,698 (C), and ×13,945 (D).

monoclonal antibodies and gold-conjugated secondary antibodies revealed that there was no apparent escape of chlamydial hsp60 antigen beyond the confines of the inclusion by 36 hpi (Fig. 6B). By comparison, exposure of this sample to primary polyclonal antibodies generated against purified RB revealed labeled RB as well as some RB antigens beyond the confines of the inclusion membrane (Fig. 6C). We have previously shown that chlamydial antigens can be exocytosed from normal inclusions (32). However, the localization of RB antigens in 36- and 48-h-infected HEC-1B cells exposed to 0.0005% C12/C12 (Fig. 6D and E) and C14/C16 (Fig. 6F and G) suggests that the amount of chlamydial antigen escaping from the inclusion in microbicide-exposed cells was greater than that escaping in the non-C31G-exposed infected cell samples.

DISCUSSION

It is well recognized that the genital *C. trachomatis* serovars D through K are responsible for epidemic sexually transmitted infections worldwide. Indeed, these chlamydiae are the leading cause of bacterially acquired sexually transmitted infections in North America and Europe. In women, the initial site of infection is the columnar epithelium lining the endocervical canal. Without antibiotic therapy, the chlamydiae can spread canalicularly to the endometrium, the fallopian tubes, and into the peritoneal cavity. *C. trachomatis* is responsible for 250,000 to 500,000 of the estimated one million cases per year of pelvic inflammatory disease—defined as endometritis, salpingitis, and ectopic pregnancy and sterility as a result of tubal scarring and/or impaired ovum transportation. Clearly, chlamydial diseases constitute significant primary, secondary and tertiary health care concerns in which women bear a special burden because of their increased risk of adverse reproductive consequences (5, 29). Safe, effective, female-controlled topical microbicides are urgently needed to help prevent the transmission of and to help control sexually transmitted infections (1, 5, 26).

How long it takes chlamydiae to find a susceptible epithelial

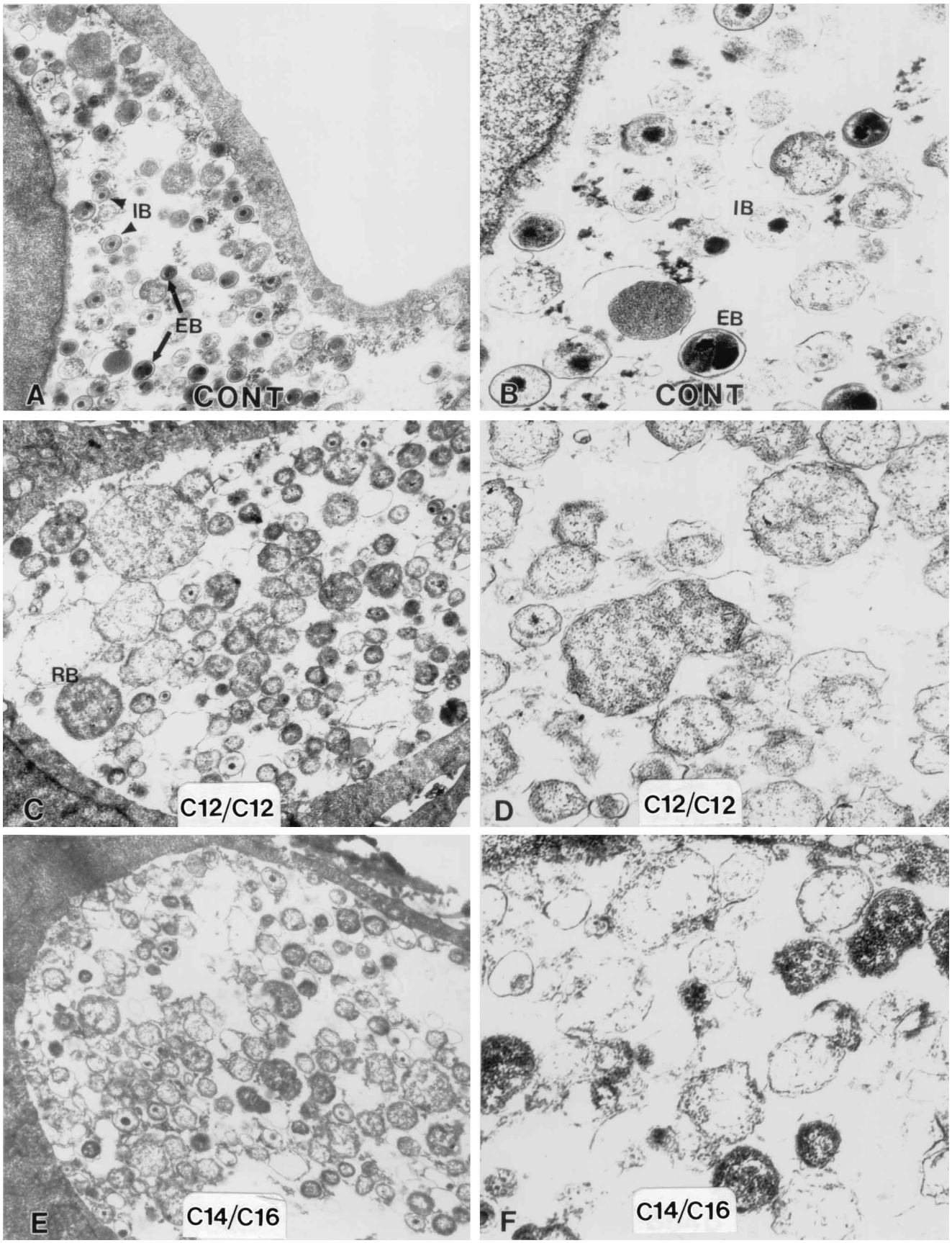


FIG. 5. Action of C31G on *C. trachomatis*-infected HEC-1B cells at 60 hpi. Control (CONT), non-microbicide-exposed infected epithelial cells contained IB and EB (A and B). HEC-1B cells at 24 hpi were exposed to 0.0005% C12/C12 (C and D) or C14/C16 (E and F) and incubated for an additional 48 h. By 60 hpi, there was delayed inclusion development due to obvious damage to RB (D and F) and consequently, a noticeable reduction in EB. Magnifications: ×9,716 (A), ×27,000 (B), ×9,313 (C), ×16,764 (D), ×9,000 (E), and ×15,050 (F).

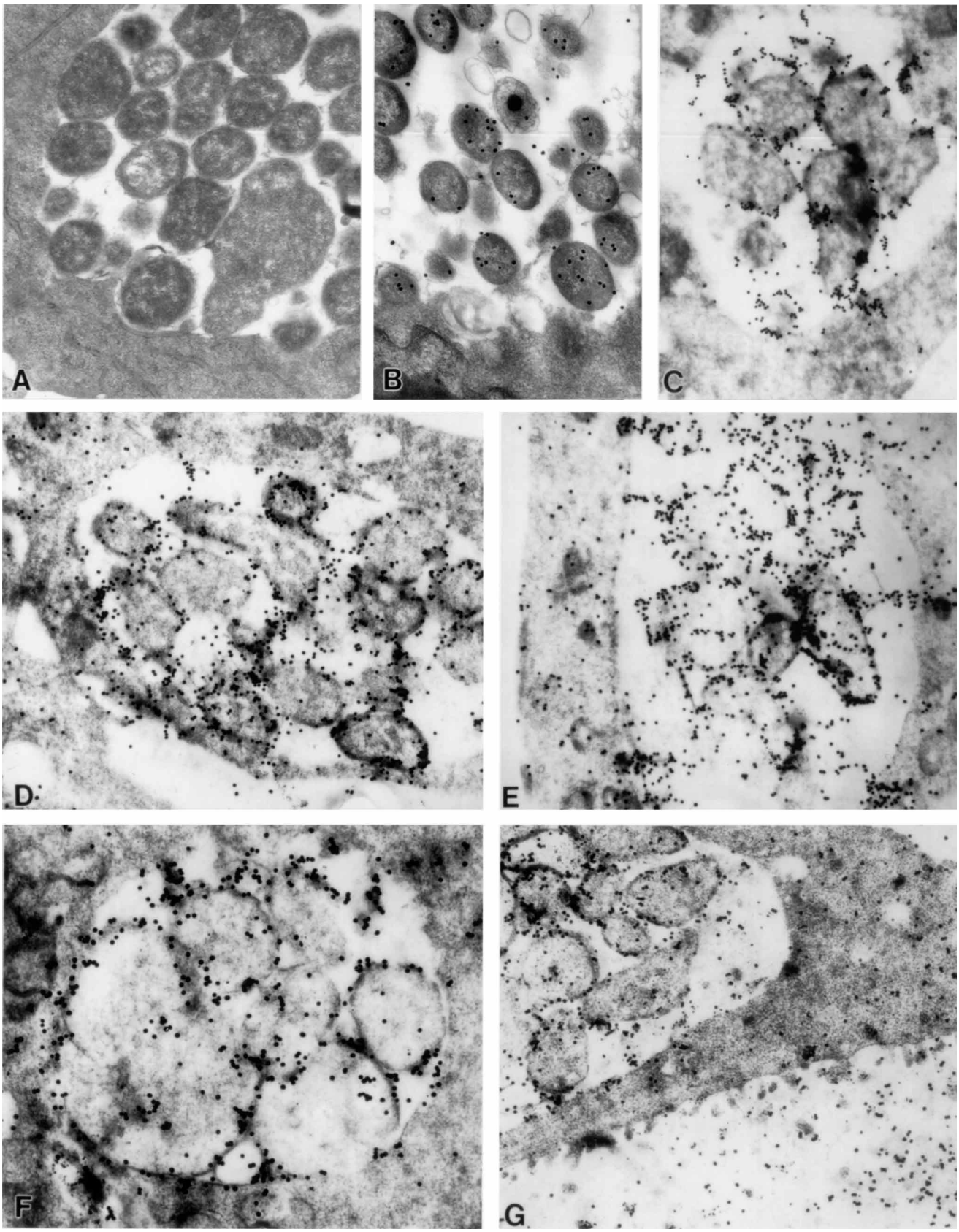


FIG. 6. Localization by immunoelectron microscopy of chlamydial antigens in infected HEC-1B cells exposed to C31G. A normal chlamydial inclusion containing RB in a 36-h-infected HEC-1B cell not exposed to C31G (control) is shown (A to C). Duplicate thin sections reacted with primary monoclonal antibodies directed against the chlamydia-specific hsp60 and gold-conjugated second-affinity antibodies (B) or primary polyclonal antibodies generated against purified RB and gold-conjugated second-affinity antibodies (C). Localization of RB antigens in 36- and 48-h-infected HEC-1B cells exposed to 0.0005% C12/C12 (D and E, respectively) and 0.0005% C14/C16 (F and G, respectively) is shown. Magnifications: ×17,600 (A), ×17,600 (B), ×16,172 (C), ×16,320 (D), ×13,125 (E), ×22,875 (F), and ×13,800 (G).

cell following discharge into the vaginal vault is not known. In our previous studies, infectivity of *C. trachomatis* serovar E for in vitro-cultured primary squamous epithelial cells taken from the ectocervix is poor, approximately 5% (19), compared with that for columnar epithelial cells from the endometrium, which is 30 to 50% for cells in the progesterone-dominant phase and >80% for cells in the estrogen-dominant phase (17). Squamocolumnar cells from the endocervical canal that have migrated onto the ectocervix (ectopy) may be intermediate in susceptibility to chlamydiae and probably serve as more accessible target host cells for an initial infectious nidus. It has been reported that chlamydiae can be found in the peritoneal cavity within seconds to a few minutes from discharge, prompting speculation, albeit controversial (21), that the nonmotile chlamydiae attach to spermatozoa for transport to the upper female genital tract (11, 30). Once the chlamydia do attach to the apical surface of a susceptible epithelial cell, entry is rapid.

So a first obvious issue is whether a microbicide perturbs the infectious EB during its putatively short sojourn in the vaginal vault, affecting its ability to attach to and enter an epithelial cell and convert into its metabolically active RB form once inside the host cell. Indeed, exposure of EB to high concentrations of C31G did reduce EB attachment as well as subsequent host cell infectivity. The mechanism of C31G activity appeared to involve a general destabilization of chlamydial envelope integrity, perhaps by intercalation of the alkyl chains into the outer membrane, which resulted in the release of LPS and protein. Although these data are qualitative, there are some interesting implications. First, it is not so surprising that the C14/C16 formulation was more effective than the C12/C12 formulation since the majority of chlamydial fatty acid chain lengths are 15, 16, and 18 (20). Thus, the longer alkyl chains of C14/C16 more closely resemble native chlamydial phospholipids in size. Secondly, and perhaps more importantly, the enhanced activity at a low pH (5.7 compared to 7.2) may reflect a reduced charge repulsion or enhanced charge-charge interactions at the EB surface and/or enhanced activity due to protonation of C31G or target chlamydial surface molecules. These activities, however, required concentrations of the microbicide that were cytotoxic to the host eukaryotic cell under our in vitro conditions.

Importantly, when the host epithelial cells were infected with chlamydiae and were then exposed to C31G at quite dilute concentrations which did not alter epithelial cell viability, chlamydial infectivity was still markedly reduced. Extremely low concentrations of microbicide were able to gain access into the developing chlamydial inclusion to cause damage or destruction to growing RB. These findings are particularly significant in that they imply that in individuals already infected with chlamydiae, some microbicides may be able to penetrate eukaryotic cells to act on intracellular chlamydiae. In addition, there was an apparent alteration by C31G of the inclusion membrane, which in turn resulted in premature escape of chlamydial antigen from the inclusion. Whether or not the chlamydial antigen is released free in the epithelial cell cytoplasm, with access to the proteosome and the major histocompatibility complex class I processing pathway, or incorporated into inclusion membrane "pinched-off" vesicles and secreted to the infected-cell surface is not known at this time. In any case, the secreted chlamydial antigen may serve as an early signal for the host surveillance system to mount an immune response, hopefully a protective one. Studies are under way to determine if polymorphonuclear leukocyte chemotaxis occurs sooner and is more intense towards C31G-exposed, chlamydiae-infected cells than non-microbicide-exposed, infected cells.

In conclusion, these studies have shown that the broad-spectrum antibacterial microbicide C31G also has antichlamydial activity, for both EB and RB forms. Further, some aspects of the mechanism of action of C31G on both the bacterium alone as well as on the host infected cell have been presented. We believe it is vitally important to have a sound scientific understanding of microbicide specificity in order to evaluate more effectively the results from animal model studies, which are now in progress for C31G.

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