New View of the Surface Projections of *Chlamydia trachomatis*

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Two kinds of surface specializations of chlamydiae have been described: hemispheric projections and spikelike rods. We undertook the present studies to demonstrate chlamydial ultrastructure in greater detail in conventional thin-sectioned specimens. *Chlamydia trachomatis* (LGV strain L2/434/Bu), cultured for 40 h in L929 mouse fibroblasts, was fixed in glutaraldehyde-acrolein, *p*-formaldehyde-glutaraldehyde, or glutaraldehyde-osmium tetroxide mixtures, postfixed in osmium tetroxide, stained in uranyl acetate, dehydrated in ethanols, and embedded in Epon. By the use of fixatives that penetrate and fix rapidly, chlamydial outer and plasma membranes were clearly revealed. Our results indicate that the hemispheric projections are specializations of the plasma membrane of elementary bodies. The spikelike projections are found in intermediate forms, originate beneath depressions of the plasma membrane, and extend through the periplasmic space and outer membrane to end with pointed tips. Improved preservation of chlamydiae provides a new, informative view of their complex structure. Significant interactions between chlamydiae and host cells might be influenced by the surface structures shown in this study.

Chlamydia trachomatis, a bacterial parasite of epithelial cells, produces serious human diseases of the genital tract and eye. The organisms have a complex life cycle consisting of reticulate bodies (RBs), the intracellular replicative form, and elementary bodies (EBs), the extracellular infective stage, as well as intermediate developmental forms (IFs) (10). EBs and RBs meet different environmental conditions. For survival of the parasites, therefore, the two stages require different adaptations. For example, the metabolically inactive EBs must be internalized by host cells, suggesting that EBs might have surface properties that promote uptake by epithelial cells, which are not highly phagocytic. RBs, which require energy from the host cell for growth and division, are intimately associated with the inclusion membrane, perhaps for the uptake of essential nutrients. It is evident, then, that functions critical for the survival and replication of the parasites are invested in their surfaces. It is therefore of particular interest that specialized surface structures have been detected on chlamydiae.

Previous investigators (1, 4, 9) have described projections of two kinds on the surfaces of chlamydiae. Hemispheric projections approximately 30 nm in diameter and clustered in one area of the EB surface have been detected by scanning electron microscopy (SEM) (1, 9). Spikelike rods that extend from the cytoplasmic membrane through the periplasmic space and the outer membrane have been described in chlamydiae isolated from cell cultures, extracted, and treated in various ways before thin sectioning or negative staining (6, 8). Thus far, however, neither of these structures has been demonstrated by electron microscopy of conventionally prepared thin sections, the technique that allows the most precise resolution of structural relationships. We undertook electron-microscopic studies to demonstrate these and other ultrastructural features of *C. trachomatis* in greater detail by three different methods of fixation.

MATERIALS AND METHODS

C. trachomatis (LGV, strain L2/434/Bu) was grown in L929 mouse fibroblasts suspended in minimum essential medium

with Hanks salts and 5% fetal calf serum. After 40 h of growth in culture, when many of the chlamydiae had developed into EBs, the cell suspension was divided into three portions, each of which was centrifuged and fixed in one of the following ways.

Some specimens were fixed in a mixture of glutaraldehyde and osmium tetroxide for 90 min on ice. The fixative was composed of 1% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, Pa.) plus 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate hydrochloride buffer (final pH 7.4). Other samples were fixed in a mixture of glutaraldehyde and acrolein (Aldrich Chemical Co., Milwaukee, Wis.) for 2 h on ice. The fixative contained 1.5% glutaraldehyde and 2% acrolein in 0.1 M sodium cacodylate hydrochloride (pH 7.4). The remaining specimens were fixed for 4 h at room temperature in dilute *p*-formaldehyde-glutaraldehyde fixative composed of 1% *p*-formaldehyde (Sigma Chemical Co., St. Louis, Mo.) and 3% glutaraldehyde in 0.1 M sodium cacodylate hydrochloride (final pH 7.4).

After initial fixation by one of these three methods, all specimens were pelleted in a microfuge (Beckman Instruments, Palo Alto, Calif.), postfixed for 2 h at 4°C in 2% osmium tetroxide in 0.1 M sodium cacodylate hydrochloride (pH 7.4), and left overnight at 4°C in 1.5% glutaraldehyde in 0.1 M sodium cacodylate hydrochloride-1% sucrose (pH 7.4). They were then stained in block with Kellenberger uranyl acetate (3) plus 4% sucrose for 2 h at room temperature, dehydrated in ethanols, and embedded in Epon. Thin sections were cut on a Sorvall MT-5000 ultramicrotome, stained with aqueous uranyl acetate and Reynolds lead citrate, and examined in a Siemens 1A electron microscope operating at 80 kV with a 50- μ m objective aperture.

RESULTS

After 40 h of growth in culture, the chlamydial inclusions occupied most of the cytoplasm of the host cells. The chlamydiae were in all stages of development, from EBs to RBs, with EBs predominating. Scattered glycogen particles of various sizes were interspersed among the chlamydiae. In many instances, the inclusion membrane was disrupted and

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FIG. 1. EB fixed with glutaraldehyde and acrolein showing that the hemispheric projections (arrow) that cover one pole of the bacterium are specializations of the plasma membrane (pm). Bar, 100 nm.

FIG. 2. EB fixed with glutaraldehyde-osmium tetroxide showing that the ultrastructure of the projections (arrows) appears essentially the same as with glutaraldehyde and acrolein fixation. The periplasmic space (s) between the two limiting membranes is dense. Bar, 100 nm.

chlamydiae were scattered in the damaged cytoplasm of the host cell. Occasionally, the entire cell was broken open.

Hemispheric projections. The hemispheric projections, named by Gregory et al. (1), were found in EBs, which were distinguished from other forms by their small size (300 nm) and dense cytoplasm (Fig. 1 and 2). The hemispheric projections were found in only a few EBs and were formed by well-defined differentiations of the plasma membrane (Fig. 1 and 2). The projections of the plasma membrane altered the shape of the overlying outer membrane so that the surface of

the EB at that pole of the organism appeared to be molded into rounded domes. When viewed in section, each projection of the plasma membrane had a rounded apex and straight sides; the base was in continuity with the cytoplasm. The diameter of the projections at the base was approximately 40 nm, their height was 30 nm, the center-to-center spacing was 65 nm, and the distance between projections was approximately 25 nm. The projections were separated by small depressions with flat bases. The dense periplasmic space that separated the outer membrane from the plasma

FIG. 3. IFs fixed in glutaraldehyde-acrolein showing surface projections (arrows) extending from depressions of the plasma membrane (pm) across the periplasmic space and through the outer membrane (om), best seen at the upper right-hand corner. The depressions may appear shallow or deep, depending on their orientation in the section. The projections are thin, needle-like spikes that taper at the tip. Note the extra segments of membrane (arrowheads) between the inner and outer membranes. Bar, 100 nm.

FIG. 4. Slightly disrupted IF fixed in glutaraldehyde-acrolein. The bases of the spikes are revealed in a lucent area of the cytoplasm beneath the plasma membrane (pm). The root (r) of one spike inside the cell and the segment crossing the periplasmic space are thick, whereas the part projecting beyond the outer membrane (om) is thin and tapers to a point. Bar, 100 nm.

membrane measured only 6 nm wide over the projections but was approximately 20 nm wide over the depressions. Although the contour of the outer membrane conformed roughly to that of the plasma membrane, its outline varied considerably. In the specimens fixed with glutaraldehyde-

acrolein, the outer membrane was more closely apposed to the segments of plasma membrane that formed the hemispheric projections (Fig. 1) than in specimens prepared with another fixative (Fig. 2). In those preparations, the outer membrane was irregular in shape, with occasional curved segments.

Spikelike projections. In specimens prepared by our methods, the spikelike projections were found only in IFs; IFs were larger and less dense than EBs and lacked hemispheric projections (Fig. 3). The spikes originated beneath rounded depressions of the plasma membrane (Fig. 4), traversed the periplasmic space, extended through and beyond the outer membrane, and ended in pointed tips (Fig. 3 and 4). Their total length in optimally prepared specimens measured 90 nm, of which 25 to 35 nm projected beyond the surface of the chlamydiae. Occasionally, the spikes were preserved in organisms that had lost some of their content (Fig. 4). In such cells, the bases of the spikes could be easily seen in areas of decreased cytoplasmic density (Fig. 4).

Membranes. The cytoplasmic membranes of the IFs were occasionally modified by complex infoldings (Fig. 5). In one instance, a complex of membranes was organized into a closely packed parallel array suggestive of a membranous organelle (Fig. 5). In other instances, membranes were not well defined and followed a more meandering course through the peripheral cytoplasm of the chlamydiae (Fig. 3 and 5). Occasionally, it appeared that excess membrane was situated in the periplasmic space (Fig. 3), and its continuity with

FIG. 5. IF fixed in glutaraldehyde-acrolein showing an elaborate proliferation of the plasma membrane, consisting of regularly spaced infoldings (double arrows) in one area and random segments (arrows) in other regions of the cytoplasm. Note the thickened outer leaflet of membrane at the surface of the organism (arrowhead), the location of the glycocalyx. Bar, 100 nm.

FIG. 6. Diagram of part of an EB bearing hemispheric projections (h) formed by the plasma membrane (pm). The outer membrane (om) is rounded over them. Between the projections are the craters (c) described by Louis et al. (4).

FIG. 7. Diagram of part of an IF bearing spikes (s). The spikes originate beneath small depressions in the plasma membrane (pm) and extend across the periplasmic space and through the outer membrane (om).

either the outer or the plasma membrane was not clearly evident.

In IFs, depending on their degree of development, the periplasmic space was either electron-lucent, like that of RBs, or electron-dense, like that of EBs. When the two limiting membranes of IFs were separated by a lucent periplasmic space, it could be seen that the outer membrane was thicker (7 nm) in some places than the plasma membrane (5 nm) (Fig. 3 and 5). This was in part because the external leaflet of the outer membrane had a surface coat, or glycocalyx (6). Even so, the hydrophobic, lipid-rich interior of the outer membrane clearly appeared to be wider than that of the plasma membrane. It was difficult to measure total membrane thickness in the dense EBs because only the lipoidal interiors of the membranes were clearly delineated. The inner leaflet of the outer membrane and the outer leaflet of the plasma membrane were not discernible in the dense periplasmic space (Fig. 1 and 2).

General comments. The two kinds of projections were not found at the same stage of chlamydial development in specimens prepared by our techniques. The EBs with hemispheric projections did not have spikelike projections. Conversely, the IFs with spikelike projections did not have

hemispheric projections. Both forms, however, had what appeared to be rigidly formed depressions of the plasma membrane. The depressions of the IF plasma membrane were the regions through which the spikelike projections extended (Fig. 3). The depressions of the EBs were positioned between the hemispheric projections (Fig. 1 and 2). The EB depressions might be specialized sites from which the spikes originate at another stage of development. Neither kind of projection was seen in RBs with our methods.

All three methods of fixation gave similar results. The spikelike projections were preserved best, however, by glutaraldehyde-acrolein fixation (Fig. 3) and were seen only incompletely or rarely in samples preserved with the other two fixatives.

DISCUSSION

Projections. In this investigation, the hemispheric projections of chlamydiae were demonstrated for the first time by electron microscopy of thin-sectioned specimens. Their presence was originally discovered by SEM (9). Although SEM is a useful technique, it provides limited resolution and shows only surface contours without revealing the underly-

FIG. 8 and 9. Line diagrams illustrating the approximate dimensions (in nanometers) of the structures described in this study.

ing structures. Therefore, how the inner and outer membranes contribute to the structure of the hemispheres had not been clearly shown previously. By our methods, the hemispheres were revealed to be primarily specializations of the plasma membrane. Their outlines were impressed upon the overlying outer membrane, however, so that they would be visible by SEM as projections of the bacterial surface. It seems likely that critical-point drying during specimen preparation for SEM may have accentuated the rounded appearance of the domes by drying the outer membrane down tightly over the plasma membrane. The structure of the hemispheres is evidently labile, because they were seen in only a few EBs in our study. Gregory et al. (1) have also indicated that there is some lability of the hemispheric projections of *C. trachomatis*; they were less frequently observed than those of *C. psittaci* in their investigations.

Using *C. psittaci* for analysis, Matsumoto and Higashi (9) were the first to demonstrate the hemispheric projections by SEM. In the same investigation, they also found rosettes in the plasma membrane by freeze-fracture techniques. Their SEM results were confirmed and extended by Gregory et al. (1), who examined both *C. psittaci* and *C. trachomatis* and found similar hemispheres in the two species. Matsumoto (7) later found that the hemispheric projections had a substructure composed of nine leaflets after freeze-fractured specimens were deep-etched. According to his interpretation, the hemispheric projections and the rosettes were the same structures. Louis et al. (4), however, discovered in their freeze-fracture studies of *C. psittaci* with complementary replicas that the rosettes were not projections, but depressions. They correctly described them as craters of the plasma membrane. Our conception of an EB bearing hemi-

spheric projections and the craters of Louis et al. (4) is shown in Fig. 6 and 8.

The spikes have been shown only by Matsumoto (5, 6, 8) in his studies of *C. psittaci*. Our demonstration of the spikes in the present study is therefore the first evidence of this specialization in *C. trachomatis*, as well as the clearest demonstration of both projections in conventionally prepared thin-sectioned specimens. Apparently, these two chlamydial species, although not closely related (10), have similar surface structures, which might represent adaptations that equip them for their similar life cycles. We plan to conduct additional studies comparing the two chlamydial species.

Our findings on the structure of the spikes are in close agreement with those of Matsumoto. His information about the spikes was largely derived from chlamydiae that were purified from infected cells and subsequently treated in various ways. Perhaps his best views of the spikes were obtained from specimens that were treated with the negative stain phosphotungstic acid (5). He also used repeated freeze-thawing, followed by digestion with substances such as trypsin and DNase, to reveal the spikelike projections (6). He correctly indicated that the spikes originate from the centers of the rosettes or craters (6), where Louis et al. had previously described pores (4). Our interpretation of the structure of the spikes is shown in Fig. 7 and 9.

In our studies, the two kinds of projections were seen not only at different locations on the bacterial surfaces but also at different stages of the chlamydial life cycle. The hemispheres were seen only on EBs, in agreement with the reports of previous investigators (1, 9). However, we found the spikes only on IFs, contrary to the results of Matsumoto, who found the spikes on both EBs and RBs (5, 6, 8). This lack of agreement may be the result of differences between *C. trachomatis* and *C. psittaci* or of differences in the techniques that were employed in the two laboratories. It should also be noted, however, that some bacteria labeled as EBs in studies by both Louis et al. and Matsumoto (4, 8) are considerably larger (0.5 to 0.6 μm) than classical EBs (0.3 μm) (1), suggesting that EBs were not carefully distinguished from IFs. We plan to pursue additional studies concerning the RBs, a form that we have not yet analyzed to our satisfaction.

Functions of the projections. The functions of the two kinds of projections are not yet known. Gregory and colleagues (1) have suggested that the hemispheric projections of EBs may play a role in the attachment of EBs to prospective host cells, but they were unable to obtain evidence to support their conjecture. An additional possibility is that the folding of the plasma membrane into hemispheric projections may provide a reservoir of membrane that is readily available for the rapid expansion of the EB during transformation into an IF. Matsumoto has suggested that the spikes may be connections between the host cell and the parasite (8). He advanced this hypothesis after examining negatively stained preparations of isolated inclusions. In those specimens, he found that spikes originating from rosettes on the chlamydiae protruded beyond the inclusion membrane. His earlier studies with negative stains were interpreted as indicating that the spikes are hollow (6). He therefore suggested that the spikes might connect RBs directly to the host cytoplasm (8). Louis et al. found no spikes but suggested that the central pores of the craters may play a role in the transfer of substances into or out of chlamydiae (4).

Techniques. Several factors contributed to our success in preserving and demonstrating surface specializations of

chlamydiae in thin-sectioned specimens. First, the chlamydiae were fixed while still inside their host cells, so that delicate structures exposed at the surfaces of the organisms were not damaged during the disruptive procedures used to isolate EBs and RBs. Second, improved methods of fixation were used. We found that the mixture of glutaraldehyde-acrolein, although not commonly used, gave the best structural preservation of chlamydiae, particularly of their spikes. The use of acrolein in addition to the traditional use of glutaraldehyde had the advantage of providing both rapid penetration and rapid fixation (2). Greater contrast of membranes and other structures was achieved by doubling our usual time of osmication and by staining in block with Kellenberger uranyl acetate (3). In short, improved fixation preserved structure with fidelity and postfixation and staining methods improved structural visibility.

The improved definition of chlamydial structure thus obtained has established a firm background for future studies. The use of immunocytochemical techniques to localize various constituents of the plasma and outer membranes may enable us to determine the functions of the surface specializations demonstrated in this study.

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