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Unique Ultrastructure in the Elementary Body of *Chlamydia* sp. Strain TWAR

EMIL Y. CHI,[1] CHO-CHOU KUO,[2]* AND J. THOMAS GRAYSTON[2,3]

Departments of Pathology,[1] Pathobiology,[2] and Epidemiology,[3] University of Washington, Seattle, Washington 98195

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The ultrastructure of two prototype strains (TW-183 and AR-39) of *Chlamydia* sp. strain TWAR was described. The TWAR elementary body (EB) demonstrated a unique morphology and structure distinct from those of other chlamydial organisms. It was pleomorphic but typically pear shaped. The average size was 0.38 μm, with a long axis of 0.44 μm, a short axis of 0.31 μm, and a ratio of the long to the short axes of 1.42. The cytoplasmic mass was round, with an average diameter of 0.24 μm. There was a large periplasmic space. Small, round electron-dense bodies (0.05 μm in diameter), which were attached to the cytoplasm by a stringlike structure, were seen in the periplasmic space. These features are in contrast to those of other chlamydiae, which are typically round with a narrow or barely discernible periplasmic space. The TWAR reticulate body (RB) was morphologically and structurally similar to those of other *Chlamydia* species, having an average diameter of 0.51 μm and being circular in shape. The ultrastructural observations of the intracellular growth of TWAR in HeLa cells revealed that TWAR underwent the same developmental cycle as do other chlamydiae, i.e., transformation of EB to RB, multiplication by binary fission, and maturation by transformation of RB to EB via the intermediate-form stage.

We recently described and named TWAR, a new *Chlamydia* strain associated with human acute respiratory diseases (8, 13). TWAR organisms form intracytoplasmic inclusions in HeLa cells that contain no glycogen and are morphologically more related to *Chlamydia psittaci* than to *Chlamydia trachomatis* (8, 13). Immunological analysis with various chlamydia-specific monoclonal antibodies revealed that TWAR strains belong to the genus *Chlamydia* and are distinct from *C. trachomatis* and from eight avian and mammalian strains of *C. psittaci* tested. The nine TWAR isolates tested so far are serologically identical (8, 13). In this report, we describe an ultrastructural study of purified organisms of two prototype TWAR strains, TW-183 and AR-39, and of organisms developing in HeLa cells in comparison with other chlamdyiae from the two species.

MATERIALS AND METHODS

Organisms. The following strains were studied: TWAR strains TW-183 (ocular isolate), and AR-39 (pharyngeal isolate) (8, 13); *C. trachomatis* B/TW-5/OT, H/UW-4/Cx, and L2/434/Bu (20, 25, 26); and *C. psittaci* 6BC (7), meningopneumonitis (5), and sheep abortion. The sheep abortion strain is a local isolate obtained from Pamela Dilbeck, Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman, Wash. All these strains have been adapted to grow in HeLa 229 cells. The organisms were grown to high titers in HeLa 229 cell culture (15), harvested 3 days postinoculation, and frozen at −75°C in 1-ml aliquots for use as inocula or purified by a meglumine diatrizoate (Hypaque-76; Winthrop-Breon Laboratories, Div. of Sterling Drug, Inc., New York, N.Y.) linear gradient (12) and frozen in 1-ml aliquots for ultrastructural studies.

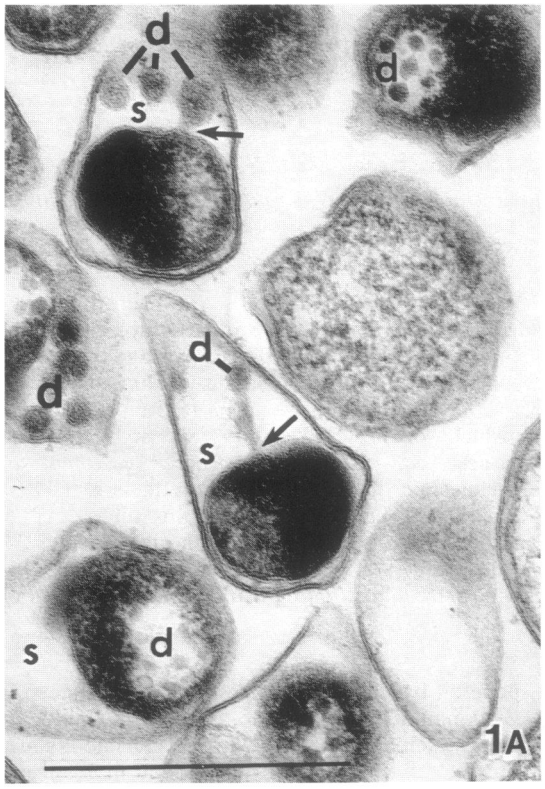
Ultrastructural studies. (i) TEM of purified organisms. An aliquot of organisms in suspension containing 10^8 to 10^9 inclusion-forming units was placed in a Microfuge tube, pelleted by centrifugation with a centrifuge (Microfuge 11; Beckman Instruments, Inc., Palo Alto, Calif.), fixed with 2% glutaraldehyde buffered with 0.1 M cacodylate at pH 7.4, postfixed with 1% OsO_4, embedded in 1% agar, and finally processed by the standard method for transmission electron microscopy (TEM; 3).

(ii) Immunogold labeling of surface antigens. Detection of surface antigens specific to TWAR was done by immune electron microscopy by using a TWAR-specific monoclonal antibody (8, 13) with the immunogold stain (14). Briefly, 1.0 ml of chlamydia suspension was placed in a Microfuge tube, pelleted by centrifugation in the Microfuge, and washed once with phosphate-buffered saline. The organisms were reacted with TWAR-specific monoclonal antibody RR-402 (ascitic fluid at a 1:100 dilution) at 37°C for 30 min. After the reaction, the organisms were pelleted, washed twice, suspended in phosphate-buffered saline, and reacted with colloidal gold (particle size, 15 nm) conjugated to anti-mouse immunoglobulin G (Janssen Pharmaceutica, Piscataway, N.J.) at a 1:15 dilution for 30 min at room temperature. Finally, the organisms were pelleted, washed once with phosphate-buffered saline, pelleted, fixed with glutaraldehyde, and processed for TEM.

(iii) Observations of intracellular growth. The ultrastructure of inclusions was studied by inoculating HeLa 229 cells which had been grown in a 24-well (well diameter, 16 mm) culture plate (Costar, Cambridge, Mass.). The plates were centrifuged at 900 x *g* for 60 min at 22°C. The inoculated cells were incubated with Eagle minimum essential medium containing 10% fetal calf serum and 0.5 μg of cycloheximide per ml at 35°C in a 5% CO_2 atmosphere. At 24, 48, 72, and 96 h postinoculation, the medium was removed. The cell monolayers were washed once with Hanks balanced salt solution and fixed with 2% glutaraldehyde. Cells were postfixed with 1% OsO_4 in the culture plate by in situ processing and embedding for TEM.

Size measurement. The size of the organism was calculated as the average of the two largest perpendicular diameters (10).

* Corresponding author.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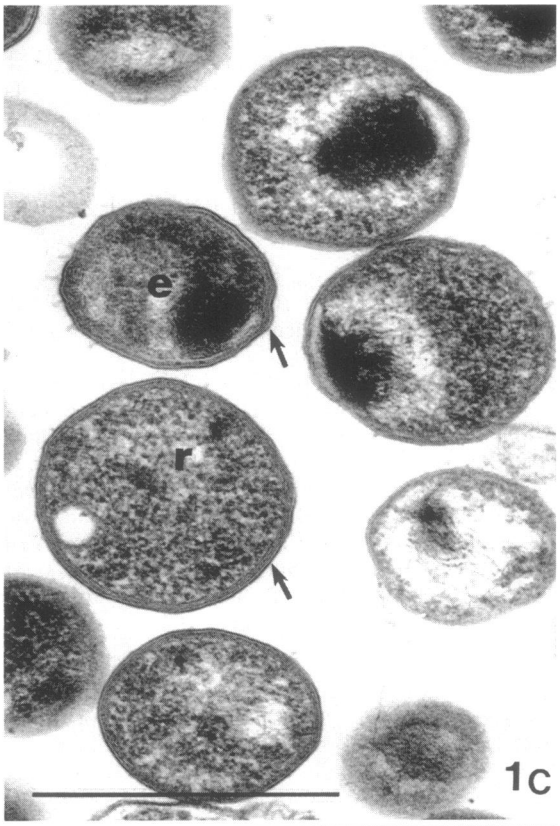
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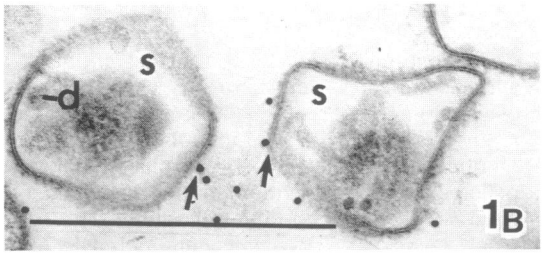
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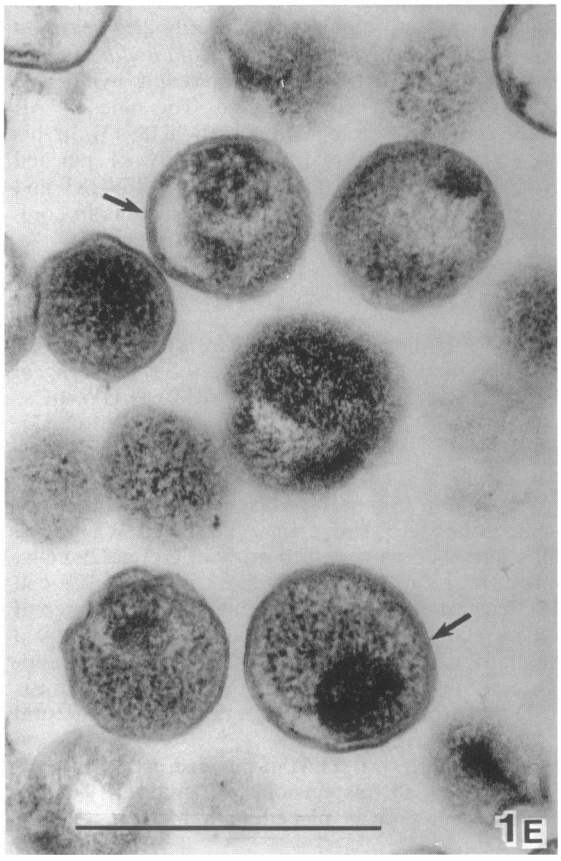
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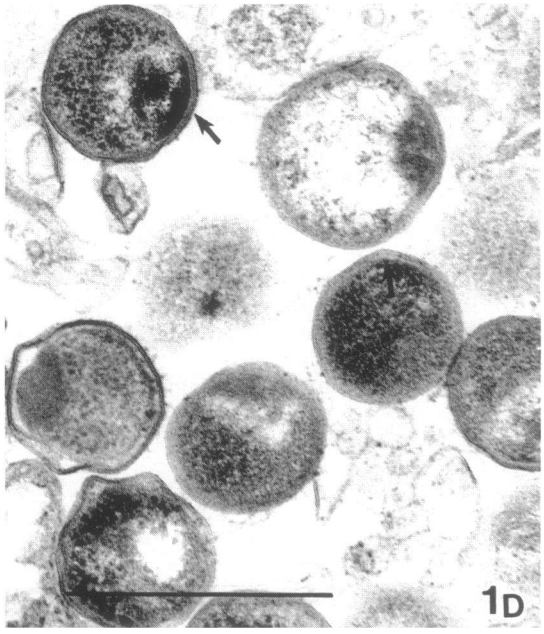
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RESULTS

Unique morphology of TWAR. The morphology of the elementary bodies (EBs) of the two TWAR prototype strains (TW-183 and AR-39) was unique. It was pleomorphic and typically pear shaped (Fig. 1A; see Fig. 3). The size of an EB in an inclusion was 0.38 ± 0.05 μm (mean ± standard deviation) in diameter (an average of 190 EBs). The long axis was 0.44 μm, the short axis was 0.31 μm, and the long-to-short-axis ratio was 1.42. The size was comparable to that of the purified organisms, which was 0.36 ± 0.05 μm (an average of 77 EBs). However, the cytoplasm was usually round, with an average diameter of 0.24 ± 0.03 μm; the long axis was 0.25 μm, the short axis was 0.22 μm, and the long-to-short-axis ratio was 1.14 (an average of 190 EBs). Both the outer and plasma membranes were composed of a bilayer structure. There was a large periplasmic space (Fig. 1A; see Fig. 3). Small (0.05 μm) electron-dense round bodies (miniature bodies) were observed in the periplasmic space (Fig. 1A). These miniature bodies seemed to attach to the cytoplasmic mass by a thin peduncle. These features were distinctly different from those of other chlamydiae studied, which were round with a narrow or a barely discernible periplasmic space (Fig. 1C, D, and E).

The morphology of TWAR reticulate bodies (RBs) was round and similar to that of other chlamydiae studied (Fig. 2 and 3). The diameter of TWAR RBs in the inclusion was 0.51 ± 0.15 μm (an average of 181 RBs); the long axis was 0.56 μm, the short axis was 0.47 μm, and the ratio of the two axes was 1.19. Unlike EBs, the outline of the cytoplasm was in conformity with the outer membrane. There was a narrow but conspicuous periplasmic space (about 0.01 μm wide). The bilayer structure of the outer and plasma membranes was clearly discernible.

Another difference was noted in the composition of RBs and EBs in the purified preparations from 3-day cultures. There was an increase in the percentage of immature forms with TWAR. The EB-to-RB ratio was 33 to 47% with TWAR and 80 to 10% with other chlamydiae. The intermediate form constituted the remaining percentage.

Immunogold stain of TWAR surface antigens. Because the TWAR organism has an unusual morphology, we next determined whether the structure revealed as the outer membrane in electron micrographs is the organism itself by using the immunogold stain. It was shown that the TWAR-specific monoclonal antibody RR-402 recognized the surface antigens on the outer membrane of the TW-183 and AR-39 strains (Fig. 1B). The reaction was negative with *C. psittaci* meningopneumonitis.

Ultrastructural observations of intracellular development. The ultrastructural observations of the development of TWAR (TW-183 and AR-39) in HeLa cells showed that at 24 h, inclusions contained only RBs (Fig. 2A). Some degenerating extracellular organisms unassociated with HeLa cells could be seen. At 48 h, the inclusions had grown. They were located near the host cell nucleus at the area of the Golgi apparatus (Fig. 2B). The population consisted of 49% RBs, 23% intermediate forms, and 28% EBs. RBs were seen dividing by binary fission. Widening of the periplasmic space started to develop when the RB transformed into the intermediate form. As the intermediate form transformed into the EB, the cytoplasmic mass was condensed and reduced in size, while the outer membrane circumference was not reduced proportionally, creating an enlarged periplasmic space. Dividing intermediate forms were often seen.

The typical EB in longitudinal section looked like a pear-shaped bag lined by the outer membrane (Fig. 3). Inside was a round cytoplasmic mass located at the wide base of the bag. The miniature bodies could be observed in the enlarged periplasmic space. Outer membrane blebs (without cytoplasmic contents), 0.07 μm in diameter, as described by Stirling and Richmond (22), were observed in inclusions on days 2, 3, and 4 (Fig. 3). However, the miniature cells (with cytoplasmic contents) described by Tanami and Yamada (24) and Stirling and Richmond (22) were not seen. Both are produced from RBs by a process similar to budding.

Maturation of the organism progressed slowly. The composition of the organisms was 47% RBs, 19% intermediate forms, and 34% EBs at 72 h; it was 38% RBs, 11% intermediate forms, and 51% EBs at 96 h. At 96 h, some inclusions were found collapsed, the vacuolar structure was oblitered, and the inclusion membrane was not identifiable. These may represent inclusions in dying cells. Small inclusions containing only RBs, similar to those seen at 24 h, could be found. These may represent a second cycle. In contrast, development of the meningopneumonitis agent of *C. psittaci* was twice as fast as development of TWAR. At 24 h, all organisms were RBs. However, 45% of them had transformed into EBs at 48 h, and 75% had transformed at 72 h. Second-cycle inclusions were observed at 72 h. Outer membrane blebs and miniature cells were also seen in 72-h inclusions. The morphology of intracellular organisms was similar to that of the purified organisms.

DISCUSSION

Our previous reports have identified TWAR as a new chlamydial agent (8, 13). The electron microscopic study has shown that the morphology of the TWAR RB is similar to that of other chlamydiae and the sizes of the TWAR RB and EB are within the range of the *Chlamydia* species (16). However, the electron microscopic observations have demonstrated that the structure of the TWAR EB is distinct from those of other clamydiae. TWAR EBs are typically pear shaped and have a large periplasmic space. These features are in contrast to many reports of similar findings that chlamydia EBs are round with a narrow or barely discernible periplasmic space in host tissues, tissue cultures, or purified preparations. These reports include studies of avian *C. psittaci* strains in parakeets (6BC) (4, 11) and pigeons (18); the mammalian *C. psittaci* strains in mice (meningopneu-

FIG. 1. Ultrastructural characteristics of purified TWAR elementary bodies in comparison with those of other chlamydiae. (A) TWAR strain TW-183. The EB is pleomorphic but typically pear shaped. The bilayer structure of the outer membrane is clearly seen. There is a large periplasmic space (s). Small (0.05 μm), round electron-dense bodies (d) are located in the periplasmic space. Some of them are attached to the cytoplasm by a stringlike structure (arrows). (B) Detection of TWAR-specific surface antigens on the outer membrane of TW-183 by immune electron microscopy by using a TWAR-specific monoclonal antibody in the immunogold stain. Arrows indicate gold particles bound to the outer membrane. (C) *C. trachomatis* serovar B strain. The EBs (e) and RBs (r) are round. No periplasmic space is seen. The bilayer structure of the outer membrane is clearly seen. The outer and plasma membrane layers are in close approximation (arrows). (D) *C. psittaci* sheep abortion strain. The organism is round. There is little or no periplasmic space. The outer membrane is in close approximation with the cytoplasm (arrows). (E) *C. psittaci* meningopneumonitis strain (mouse). The organisms are round. No periplasmic space is seen. The outer and plasma membrane structures are clearly demonstrated (arrows). Bar, 0.5 μm.

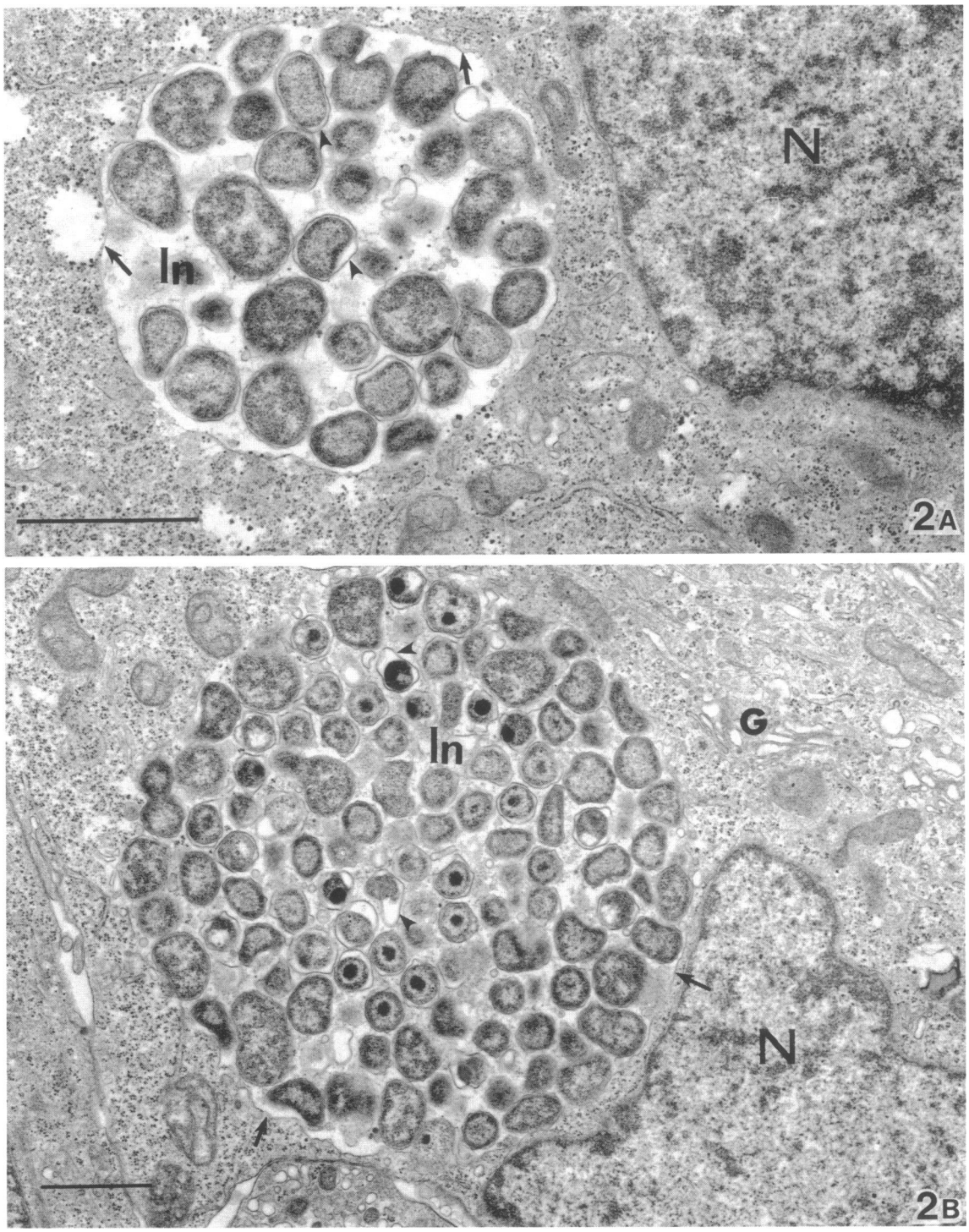

FIG. 2. Micrographs illustrating the intracellular growth of TWAR (TW-183) organisms in HeLa cells. (A) At 24 h, the inclusion (In) is clearly membrane bound (arrows) and contains only RBs. The periplasmic space is begining to widen in some organisms (arrowheads). N, HeLa cell nucleus. (B) At 48 h, the inclusion is enlarged. Pear-shaped elementary bodies have appeared (arrowheads). Three developmental forms, i.e., RB, intermediate form, and EB, are seen. However, the majority of them are RBs. Arrows indicate the inclusion membrane. G, Golgi apparatus; N, HeLa cell nucleus. Bars, 1 μm.

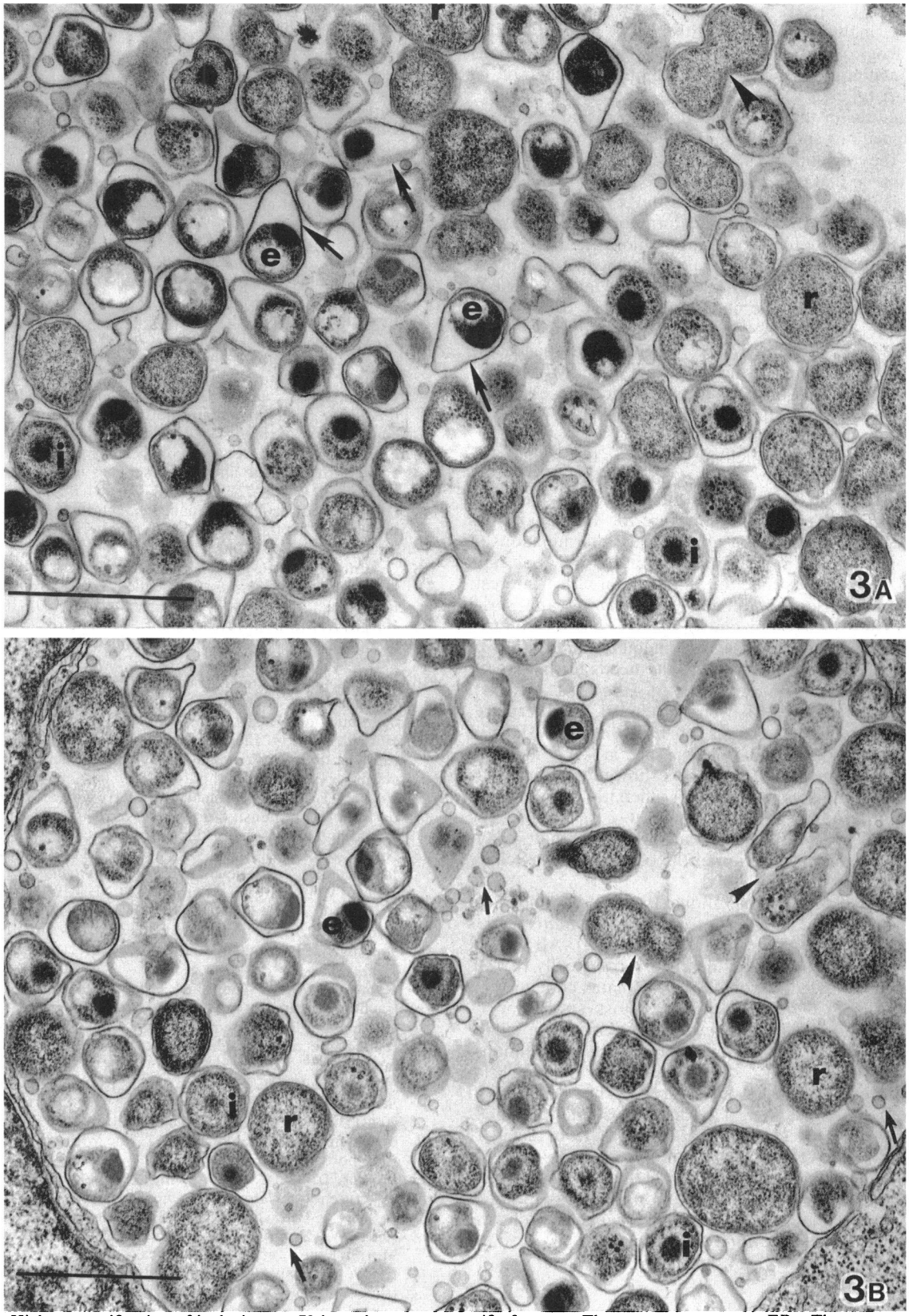

FIG. 3. Higher magnification of inclusions at 72 h to show some specific features. There are more mature EBs. They are typically pear shaped, with a large periplasmic space (arrows in panel A). However, a substantial proportion (about 50%) of them are still in the RB stage (see text). Dividing RBs and intermediate forms are seen (arrowheads). Many outer membrane blebs (0.07 μm) are seen inside the inclusion (arrows in panel B). r, RB; i, intermediate form; e, EB. Bars, 1 μm.

monitis; 6, 23), cats (a gastric strain; 9), and guinea pigs (guinea pig inclusion conjunctivitis; 1); a *C. psittaci*-like strain in frogs (19); the murine biovar of *C. trachomatis* (mouse pneumonitis; 17); and the human biovars of *C. trachomatis* types E (21) and L2 (2).

Explanations for the pear-shaped structure and large periplasmic space are speculative. It may be caused by disturbance in the regulation of outer membrane synthesis or degradation, so that excess outer membrane continues to be made or is not cleaved off when the cytoplasmic mass shrinks during the RB-to-EB transformation. Another possibility is that the outer membrane is more rigid than with other chlamydiae. The rigidity may prevent the membrane from collapsing on the shrinking cytoplasmic mass. This may suggest a difference in the chemical structure of the outer membrane between TWAR and other chlamydiae.

The developmental process of TWAR in the phagosome appears to be similar to that of other chlamydiae, i.e., transformation of EB into RB after entering the host cell, multiplication by binary fission, and maturation of RB to EB through an intermediate-form stage (16).

The miniature bodies which are located in the periplasmic space and are attached by a thin peduncle (which probably originates from the cytoplasmic membrane) to the main cytoplasmic mass is a unique structure for TWAR and has not been described before for chlamdyia. Determinations of its structure, whether it is a unique entity or a cytoplasmic membrane bleb, and its function should be of future interest.

We have experienced difficulty in isolating and serial passaging TWAR in cell or chick embryo yolk sac cultures (8, 13). Whether the unique outer membrane structure of TWAR EB has any effect on infectivity needs further study. Although the maturation of RBs to EBs was retarded, adequate numbers of EBs were produced. Furthermore, there was a continuous increase in EBs through 4 days of culture. While this suggests that passage at 4-day intervals might be more effective, experiments showed that the yields of infectious organisms were greater when the cultures were harvested at 3 days than they were at 4 days (13). The toxicity of TWAR to host cells, causing cell death at 4 days of culture (13) and inactivation of the organisms, may cause this phenomenon.

Immunological analysis has shown that TWAR belongs to the genus *Chlamydia*, is distinct from *C. trachomatis*, and is serologically unique among *C. psittaci* (8, 13). All TWAR strains isolated so far appear identical serologically (8, 13). DNA analysis showed that the TWAR isolates contained no plasmid, unlike *C. trachomatis* and most strains of *C. psittaci*. The endonuclease restriction patterns were identical among TWAR isolates, which were readily differentiated from *C. trachomatis* and *C. psittaci*. The Southern hybridization with cloned TWAR DNA probes showed identical patterns of homology among TWAR isolates, and the probes were TWAR specific (L. A. Campbell, C.-C. Kuo, and J. T. Grayston, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D163, p. 99). The unique ultrastructural findings with TWAR organisms will be an important criterion in consideration of the proper classification of the TWAR organisms, including the possibility of a new *Chlamydia* species.

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