INFECTION AND IMMUNITY, July 1989, p. 2214–2222
0019-9567/89/072214-09$02.00/0

Vol. 57, No. 7

Cellular Events and Intracellular Survival of *Campylobacter jejuni* during Infection of HEp-2 Cells

MERIVANE A. DE MELO,[1]* G. GABBIANI,[2] AND JEAN-CLAUDE PECHÈRE[1]

Département de Microbiologie[1] and Département de Pathologie,[2] Centre Médical Universitaire, CH-1211 Geneva 4, Switzerland

Received 30 January 1989/Accepted 5 April 1989

Invasion and intracellular survival of *Campylobacter jejuni* in HEp-2 cells were analyzed by transmission electron microscopy and by viable counts after killing of extracellular bacteria by gentamicin. During the first 30 min after challenge, no bacteria were seen in association with the host cell. After 1 h, campylobacters apparently attached to the cell membrane, with areas of close appositions. In these areas, an intracellular network of actin-like filaments was seen beneath the plasma membrane. Other bacteria were included into endocytic vacuoles. After 3 h, an intense lysosomal response was observed in the host cells, as determined by the presence of myelinic forms and acid phosphatase activity. After 9 h, bacteria still contained in vacuoles showed signs of degradation with a change from spiral to coccal forms. Morphological evidence of phagosome-lysosome fusion was also seen, and these observations by transmission electron microscopy correlated well with a decrease in bacteria viability 9 h after challenge, as determined from separate kinetics studies. Inhibitors of phagocytosis were observed to reduce markedly the entry of *C. jejuni* into the cells at concentrations which apparently did not affect bacterial viability. These results suggest that the campylobacters were successively attached to the HEp-2 cell membrane, internalized by a phagocytic-like mechanism, and digested after phagosome-lysosome fusion.

Campylobacter jejuni is regarded as a major cause of diarrhea, both in industrialized countries and in the Third World (1). The mechanism by which the bacterium produces the disease is not fully understood, but clinical evidence exists for intestinal epithelial invasion in cases of *Campylobacter* enteritis, with bloody diarrhea and inflammatory cells in the stools. The ability to penetrate into the enterocytes has been confirmed in experimental models in which *C. jejuni* was orally administered to infant chickens (6), mice (19), and hamsters (10). In addition, invasion potential has been shown in mammalian cell lines (5, 13), and this property has been associated with pathogenicity in humans (5). Recently, we have shown that pretreatment of HEp-2 cells (a human epidermal cancerous cell line) with mucin enhances internalization of fresh isolates of *C. jejuni* (4). However, little is known about the mechanism of penetration and about the intracellular fate of *C. jejuni*. Ultrastructural observations of epithelial cells infected with *C. jejuni* have indicated that bacteria associate with the cell membrane and are internalized within endocytic vacuoles (5, 13). Here, we provide further information on the internalization process and intracellular fate of *C. jejuni*. We also provide evidence that these organisms are unable to survive for extended periods within HEp-2 cells.

MATERIALS AND METHODS

Bacterial strains. *C. jejuni* 5223, 5265, 5387, and 5425 were fresh isolates obtained from patients suffering from diarrheal disease with erythrocytes and leukocytes present in the stools and were kindly provided by R. Auckenthaler, Hôpital Cantonal de Genève, Switzerland.

The *C. jejuni* isolates were cultured on blood agar selective-medium plates (Virion, Cham, Switzerland) and incubated at 42°C with an atmosphere of 85% N_2, 10% CO_2, and 5% O_2 for 24 h. Their identity was confirmed by their typical spiral morphology, mobility, positive oxidase and catalase test results, sensitivity to nalidixic acid, and resistance to cephalothin. All strains were also sensitive to gentamicin. The strains grew in 1% glycine medium and did not grow in 3.5% NaCl medium. Upon arrival in our laboratory, strains were passaged once or twice before being frozen at −70°C in thioglycolate broth with 30% glycerol and 5% dimethyl sulfoxide for use as stock cultures. When required, stock cultures were thawed, passaged in thioglycolate broth, and grown on blood agar selective-medium plates as described above. Colonies were harvested and suspended in Hanks minimum essential medium (MEM) (Seromed, Basel, Switzerland). The number of CFU was determined after 24 h of growth on blood agar with appropriate dilutions.

Host cells. HEp-2 cells, obtained from a human epidermal carcinoma, were supplied by C. Lyons, Queens University, Belfast, United Kingdom. Cells were maintained in MEM containing 5% fetal calf serum, 50 U of penicillin G, 50 μg of streptomycin, and 2.5 μg of amphotericin B per ml. Cells were routinely subcultured on plastic tissue culture flasks at 37°C in an atmosphere containing 5% CO_2. Confluent stock cultures were trypsinized and adjusted to approximately 2×10^4 cells per ml before being transferred into 24-well microdilution plates (Nunc, Basel, Switzerland). Bovine submaxillary mucin (Boehringer, Rotkreuz, Switzerland) was added to the growth medium at 100 μg/ml and was incubated until the cells reached confluence (4×10^5 to 5×10^5 cells per well), i.e., 24 h after seeding.

Invasion assay and kinetics of bacterial intracellular killing. Confluent HEp-2 cells, grown on a 24-well culture plate, were prepared for infection by removing the medium saline solution. The bacterial suspension (1 ml) was added to each well and incubated at 37°C under 5% CO_2 for 3 h. After the infection period, extracellular bacteria were removed by three washings with MEM saline solution.

In long-term experiments, intracellular survival of *C. jejuni* was monitored after an additional incubation with

* Corresponding author.

fresh medium containing 200 μg of gentamicin per ml for 3 h. This incubation was followed by several washings and replacement with fresh medium containing 50 μg of gentamicin per ml for 9, 12, 24, and 36 h of incubation. HEp-2 cell survival during the incubations was monitored by phase-contrast microscopy. Under the conditions described, no cell detachment or cytotoxicity effect was observed. The monolayer appeared to be intact. Controls were made without using gentamicin during the incubations. At intervals, medium was changed, cells were washed, and intracellular bacteria were released with 0.1% Triton X-100. Suspensions were diluted and plated onto blood agar to calculate viable bacteria.

Treatment of HEp-2 cells with phagocytosis inhibitors. Confluent HEp-2 cells were prepared as described above. Cytochalasin B (Sigma, Zurich, Switzerland) was prepared as a 5-mg/ml stock solution in dimethyl sulfoxide. This solution was diluted to 10 μg/ml in MEM, added to the monolayer, and maintained for 30 min before the infection period. The bacterial inoculum was suspended in MEM and was applied to cell monolayers for the standard 3-h infection period. A similar procedure was used with iodoacetate (5 mM) and dinitrophenol (2 mM).

TEM. Confluent monolayers were prepared in petri dishes (6 cm) (Falcon, Basel, Switzerland) and processed for transmission electron microscopy (TEM) by standard techniques. *C. jejuni* 5425 inoculum (5.4×10^9 CFU/ml) was used to infect cells at 5, 15, and 30 min and 1, 3, and 9 h before being processed for TEM. Monolayers were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 to 3 h at 4°C and postfixed with 1% osmium tetroxide for 15 min. Monolayers were stained with 2% uranyl acetate, dehydrated in a graded series of ethanol washes before being infiltrated and embedded in a fresh mixture of Epon (Fluka, Geneva, Switzerland), and then cured at 60°C for 48 h. Sections were prepared with a diamond knife on an ultramicrotome (Reichert, Vienna, Austria), counterstained with uranyl acetate and lead citrate, and examined in a Philips 400 electron microscope operating at 80 kV.

Acid phosphatase cytochemistry. The presence of the lysosomal enzyme marker acid phosphatase was determined histochemically by the Gomori technique (7). After the bacterium-host cell incubation (1, 3, and 9 h) described above, the cells were fixed at 4°C for 30 min or 1 h in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde to which 0.1% dimethyl sulfoxide (E. Merck, Darmstadt, Federal Republic of Germany) was added. They were then treated with 0.88 M sucrose for 15 min at 4°C before they were incubated for 30 min at 37°C in a solution containing 0.01 M sodium β-glycerophosphate (Sigma) and 3.6 mM lead nitrate in 0.05 M acetate buffer (pH 5.0). This assay was controlled by incubating the cells for 30 min at 37°C in the same solution with added 0.01 M NaF. This was followed by washing three times in cacodylate buffer containing 5% (wt/vol) sucrose. Cells were processed for electron microscopy as described above.

RESULTS

TEM. Ultrastructural studies of HEp-2 cells infected with *C. jejuni* 5425 provided information on the series of events which occurred in the hours following the infectious challenge. During the first minutes after infection, bacteria were neither associated nor internalized within HEp-2 cells. However, examination at 1 h postinfection showed bacteria apparently associated with the HEp-2 cells (Fig. 1C). Most of these bacteria were observed at the microvillus sites or apparently adhering to the cell membrane with areas of close apposition between the bacterial surface and the host cell surface. Some campylobacters were engulfed into cellular invaginations (Fig. 1A), which were associated with a dense intracellular network of actin-like filaments beneath the plasma membrane (Fig. 1A and B). Other bacteria were apparently internalized into cytoplasmic vacuoles (Fig. 2). No connection was ever seen between these vacuoles and the extracellular environment when successive sections in a given tissue block were examined. The membranes of the vacuoles containing bacteria were morphologically similar to the HEp-2 cell plasma membrane and were occasionally similar in shape to the bacteria (Fig. 2A). At this stage, number and morphology of lysosomes were similar to those of uninfected control preparations, and no acid phosphatase activity could be demonstrated. Evaluation of the extent of infection at this stage, based on a count of the number of bacteria observed in 100 cells from six different grids, indicated that 25% of the cell monolayer was infected.

By contrast, 3 h after challenge the lysosomal response was clearly evident in the infected cells, although it was still absent in the controls. Myelinic forms were numerous, and acid phosphatase activity was detected both in the lysosomes and at the surface of internalized bacteria (Fig. 3A). The characteristic spiral shape of *Campylobacter* spp. was rarely seen in the HEp-2 cells 9 h after challenge (Fig. 3B). Most of the bacteria showed signs of degradation as indicated by a change from spiral to coccal form. The electron density of these bacteria was diminished compared with that observed earlier, and lysosomes were generally localized nearby (Fig. 3B). At this stage, we commonly observed a heterologous assembly of lysosomes showing a pronounced electron-dense reaction (Fig. 3B). A clearer demonstration of this phagosome-lysosome fusion is shown in Fig. 4. At no time after challenge were free bacteria ever observed in the cytoplasm, and no evidence of intracellular replication was ever seen.

Lysis assay. As an alternative means of analyzing bacterial internalization and quantitating bacterial survival at 6, 9, 12, 24, and 36 h postinfection, we performed a lysis assay essentially as outlined above, except that the gentamicin concentration was reduced to 50 μg/ml after 6 h to alleviate any potential problems caused by excessively large intracellular antibiotic concentrations. Experiments performed with one strain in the absence of gentamicin in the fresh incubation medium produced the same number of CFU as those treated with 50 μg/ml of gentamicin as a control (data not shown).

All four strains showed the same intracellular behavior after the serial incubations (Fig. 5). After 6 h of incubation, we saw no further increase in viable counts recovered from HEp-2 cells. By 12 h we observed a considerable decrease in the number of CFU of internalized bacteria. In terms of the timing of intracellular events, these results are consistent with the TEM observations in that the decrease in bacterial viability correlates with the period when phagosome-lysosome fusion was seen to occur and is consistent both with subsequent bacterial degradation and with the absence of bacterial intracellular replication.

Lysis assays were also carried out after pretreatment of the HEp-2 cells with different inhibitors of phagocytosis (Fig. 6). Pretreatment with cytochalasin B (10 μg/ml), iodoacetate (5 mM), and dinitrophenol (2 mM) resulted in a significant reduction in the number of bacteria protected from gentamicin killing compared with results of control

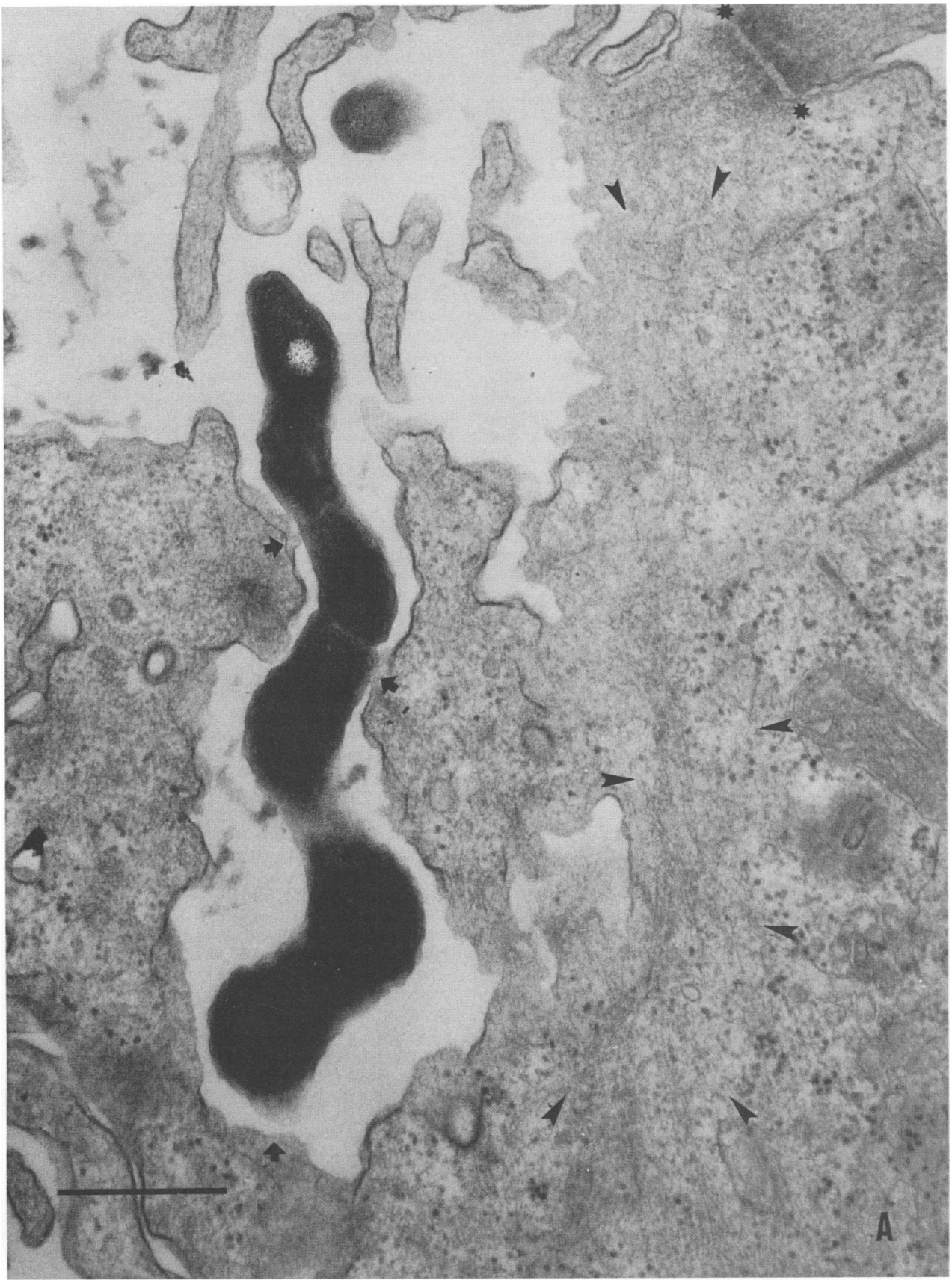

FIG. 1. TEM micrographs showing entry of *C. jejuni* into HEp-2 cells 1 h after challenge. Arrowheads indicate cytoskeletal filaments underneath the plasma membrane, and arrows indicate areas of close apposition between the bacterial and host-cell membranes (A and B). Note the nascent vacuole and the absence of coat material beneath the host-cell membrane. Desmosome (*) can also be seen (A). Interactions between bacteria and the microvilli of HEp-2 cells are indicated (*) (C). Bar, 0.5 μm.

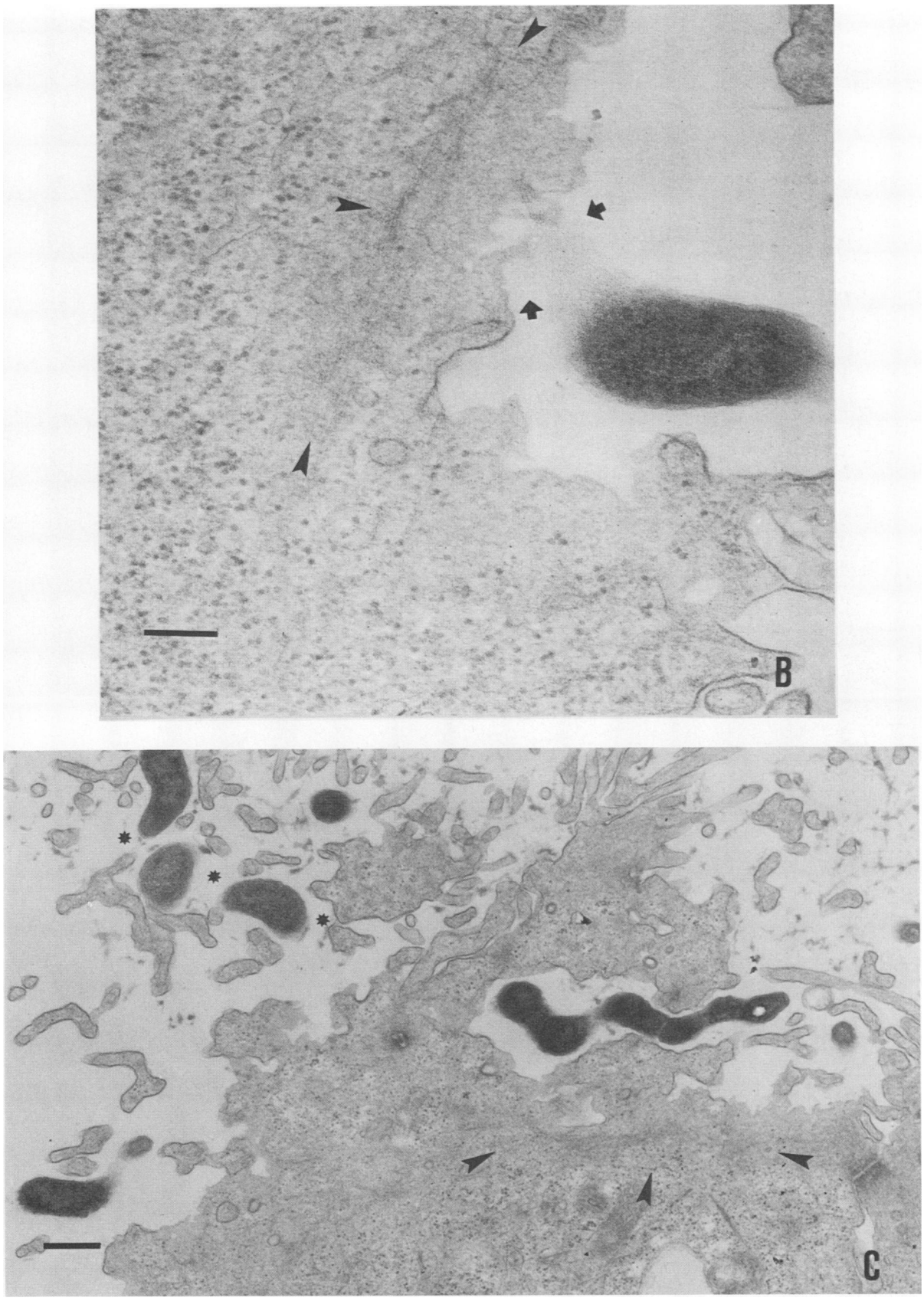

FIG. 1—*Continued.*

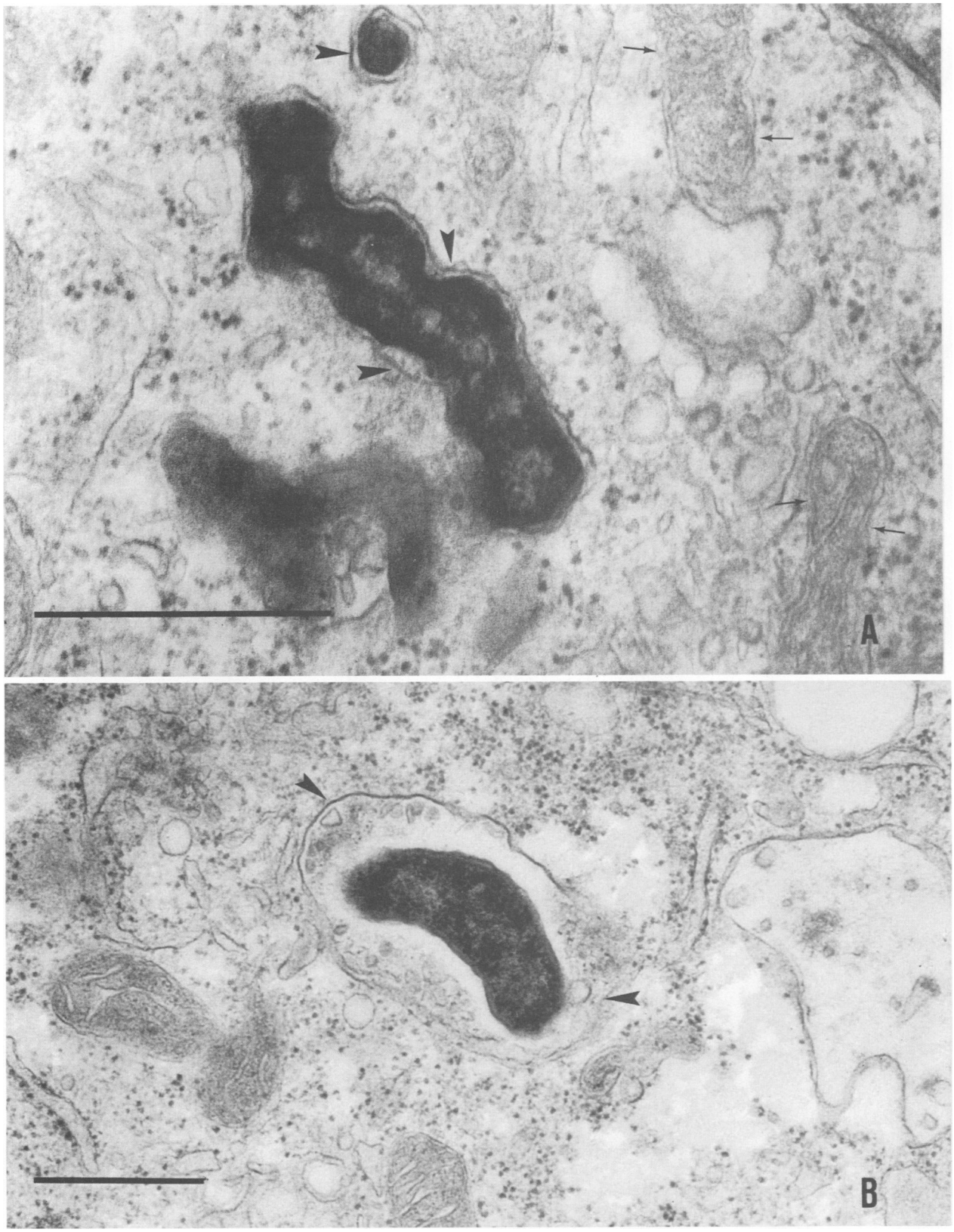

FIG. 2. HEp-2 cells infected with *C. jejuni*. 1 h after challenge. At this stage, phagocytic vesicles surrounding the bacteria are already present and bacteria appear to be free inside the vesicle. Arrows point to altered mitochondria surrounding the bacterial vesicles and several swollen vesicles. Arrowheads show details of bacteria contours around membrane vacuoles. Bar, 0.7 μm.

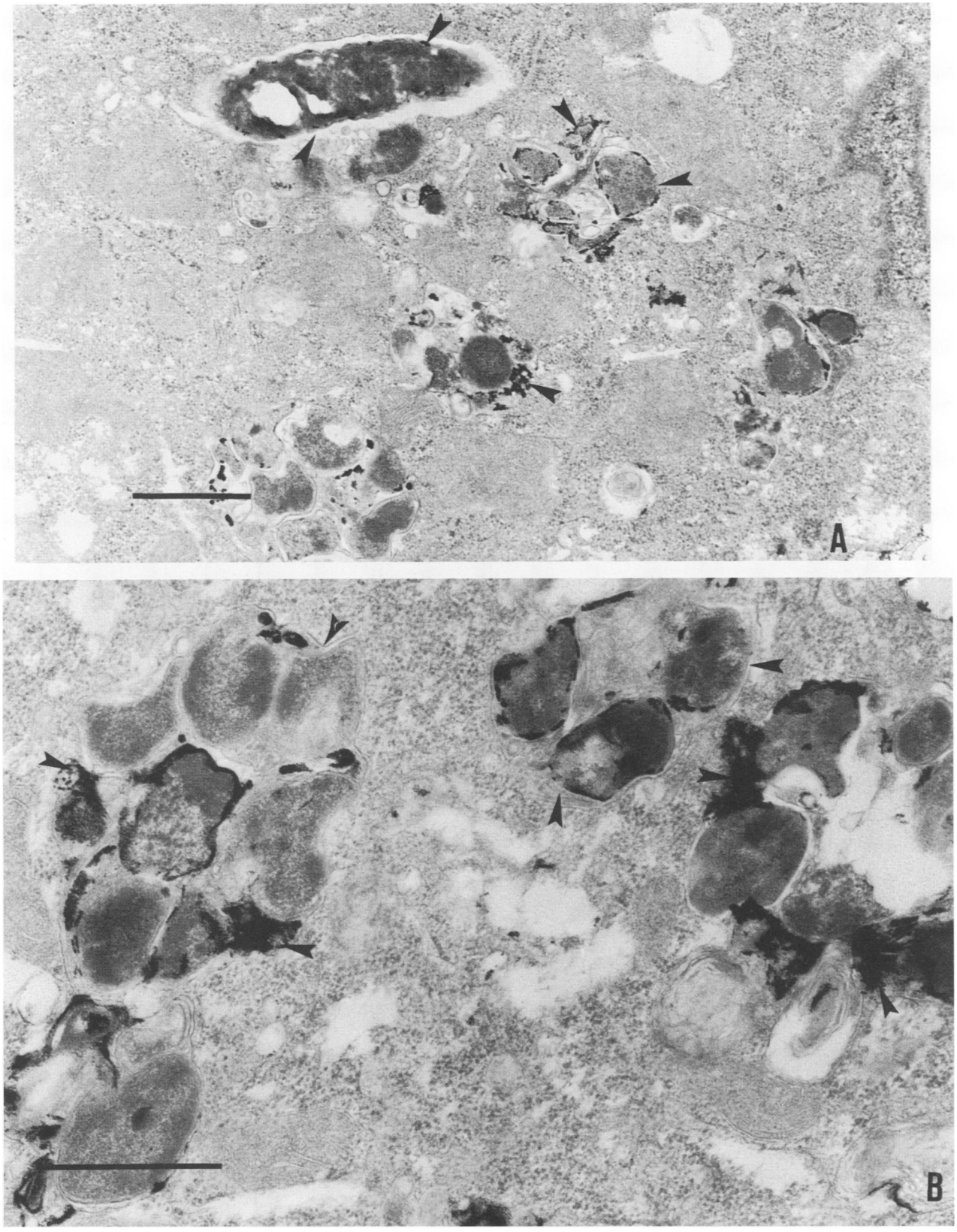

FIG. 3. Acid phosphatase activity in HEp-2 cells after 3 h (A) and 9 h (B) of challenge. First signs of acid phosphatase activity are seen along the bacterial membrane (arrows in panel A). Abundant secondary lysosomes with electron-dense reaction are also visible (arrows). Electron-dense reactions and lysosomal assembly in the cytoplasm with intraphagocytic coccal forms of *C. jejuni* are also shown (B). Bars, 1 μm.

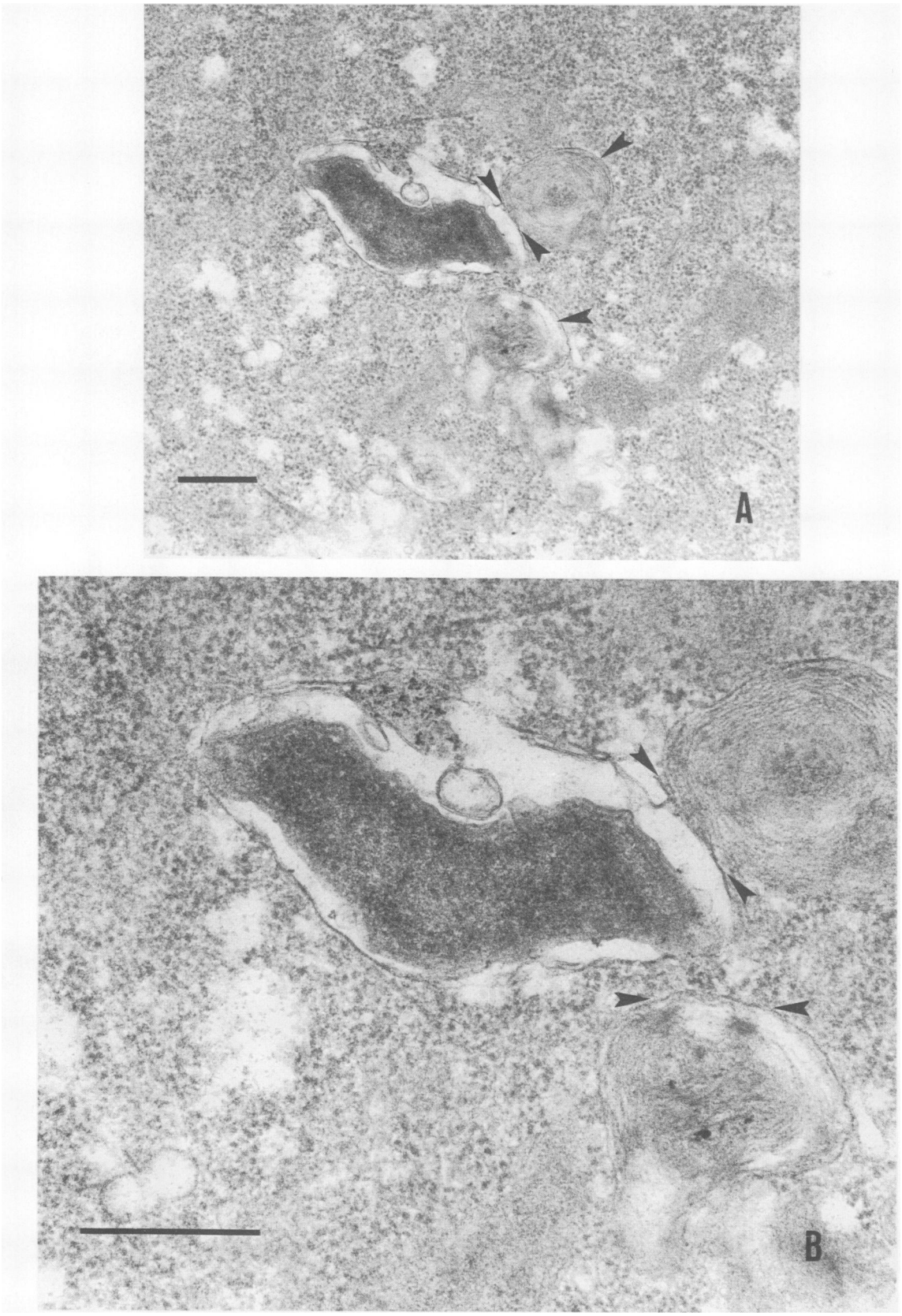

FIG. 4. Phagolysosome fusion in HEp-2 cells infected with *C. jejuni*, 9 h after challenge. Note detail of the fragmented lysosome membrane surrounding the endocytic membrane (arrows in panel B) at high magnification. Bars, 0.5 μm.

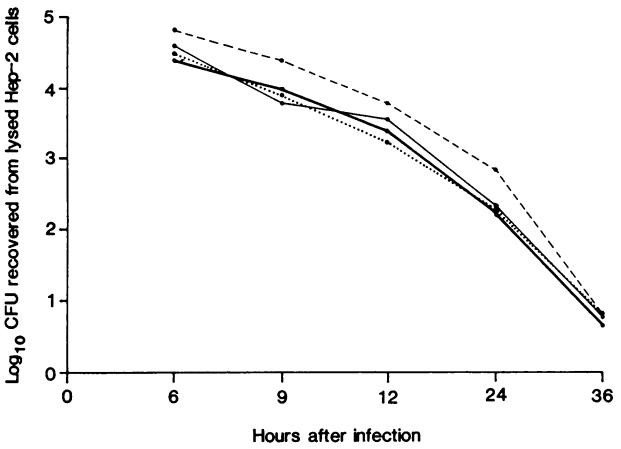

FIG. 5. Kinetics of intracellular survival of *C. jejuni* within HEp-2 cells. - - - -, 5223; ▬, 5425; ——, 5265; . . . . . , 5387. Each point represents the mean of four experiments.

experiments performed without inhibitors. Other results (data not shown) indicated that these inhibitors had no apparent antibacterial effects against our strains at the concentrations used.

DISCUSSION

The experiments described above extend our studies on *C. jejuni* invasion of mammalian cells with the model described previously (4). Such a tissue culture model can assess the ability of an enteroinvasive pathogen to initiate infection, but this article is unable to address the question of virulence determinants or to allow an evaluation of the role of the host defense mechanism that must be overcome by the bacteria in the intact intestine. However, using this system we were able to demonstrate the entry process, the intracellular location, and the intracellular survival of *C. jejuni* on the basis of TEM observations and kinetic studies of viable counts of internalized bacteria, respectively. Adhesion of *C. jejuni* to epithelial cells has been shown in different tissue culture models (5, 13). The formation of areas of close apposition between *Campylobacter* spp. and the host cell, as seen here and elsewhere (5), may represent localized binding of bacterial surface adhesins to receptors on the epithelial cell. Studies have demonstrated that in vitro adherence of *C. jejuni* to various cells could be mediated by flagella (13). The present study does not support this view, since we have never seen close apposition between flagella and the plasma membrane. However, other adhesins which might be involved in this process have been identified (18). Partial engulfment and areas of close appositions have been observed in HeLa cells infected with *Shigella* spp. (8). These observations may correspond to sequential receptor-ligand binding, which engulfs the bacteria into an endocytic vacuole in a type of "zipper-up" mechanism (3, 8). In this fashion, once campylobacters are attached to the cell membrane by binding, they can be internalized. The fact that we have observed neither adherence nor internalization during the first minutes of infection suggests that this process is delayed, indicating that the initial interactions take place slowly. Electron micrographs revealed that only after 1 h were interactions between the microvilli and the bacterial cell surface observed. Invasion with other enteroinvasive bacteria such as *Shigella*, *Yersinia*, and *Salmonella* spp. has also been shown to be delayed 1 to 2 h, even after infection by the centrifugation procedure (15, 17). Therefore, it does not seem that the invasion process is accelerated by this procedure (17).

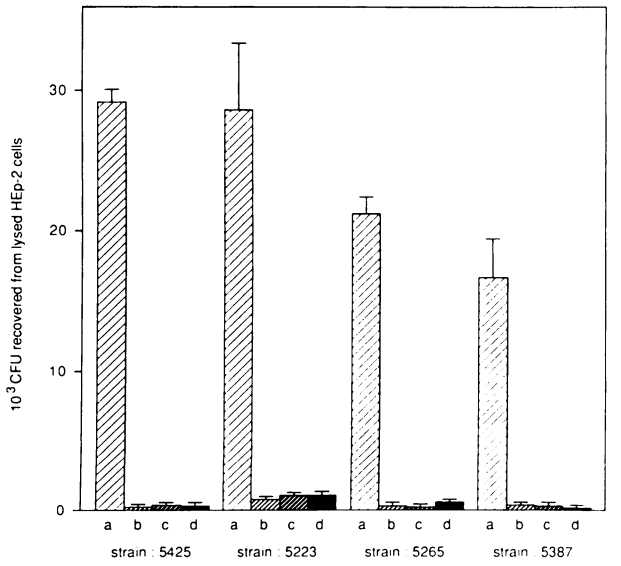

FIG. 6. Effect of phagocytosis inhibitors on the infection of *C. jejuni* into HEp-2 cells (3 h after challenge). a, Positive control; b, cytochalasin B (10 μg/ml); c, iodoacetate (5 mM); d, dinitrophenol (2 mM). Values are means and standard deviations of four independent experiments.

C. jejuni penetration occurred with evidence of actin filament assembly at the site of entry. TEM data also showed microfilament assembly underneath the plasma membrane at the sites of close apposition. Several lines of evidence support the view that internalization of *Campylobacter* sp. proceeds by a phagocyte-like mechanism (5, 13). The fungal metabolite cytochalasin B, which inhibits endocytosis by causing actin depolymerization (2, 9), significantly decreased the number of *C. jejuni* protected from gentamicin killing. Similar findings were obtained with iodoacetate and dinitrophenol, which block glycolysis and the Krebs cycle, respectively, and which consequently inhibit phagocytosis, an energy-dependent process. *Shigella* spp. (9, 15) and *Salmonella* spp. (12) similarly invade HeLa cells by an endocytic process which does not rupture the plasma membrane and which requires both host cell energy production and microfilament function (3, 9). During the invagination process, the host cell membrane is seemingly devoid of coated material, although in a previous study using TEM, Hale (8) occasionally observed coated pits in nascent endocytic vacuoles forming around invading *Shigella flexneri*. Additional, more-specific studies will be necessary to examine any possible role of coated pits in the penetration of *C. jejuni*.

Once internalized, like *Salmonella* spp. (12, 14) and *Yersinia pseudotuberculosis* (16) but unlike *Shigella* spp. (8), ingested campylobacters seemed to remain enveloped within endocytic vacuoles in the cytoplasm of the host cell. Our observations indicated that phagolysosome fusion subsequently occurred and could be correlated with the decrease in viable internalized bacteria observed after 9 h of incubation in separate kinetic studies (Fig. 5). A heterogenous population of lysosomes, most pronounced at 9 h postinfection, assembled and colocalized in the cytoplasmic matrix (Fig. 4B). The resulting lysosomal environment of the bac-

teria could explain the morphological change from spiral to coccal form observed at this stage of the infection; this could consequently explain the loss of bacterial viability.

We now presume that the virulent capacity of *C. jejuni* is attenuated by the lysosome system so that the bacteria can neither survive nor proliferate within the hostile environment of phagolysosome fusion in cultured HEp-2 cells.

Comparative studies have revealed that in general, the intracellular fate of any organism is a product of the organism itself rather than a result of cell type; i.e., all cells respond in a similar fashion to bacterial invasion, but each bacterial species has evolved a different way of overcoming this cellular response. However, in the case of *C. jejuni*, an earlier study indicated long-term survival of the bacteria in mononuclear phagocytes, with the transition from spiral to coccal form occurring only after 6 to 7 days (11). It would therefore appear that in this instance HEp-2 cells provide a less favorable environment for bacterial survival or for replication. It is also difficult to assess how extensively these in vitro observations can be applied to the situation in vivo, i.e., in the intestinal epithelium. In particular, we have no findings which help elucidate the mechanism of portal entry of *C. jejuni* through the intestinal epithelium to the lymph nodes (10, 19). Thus, the picture of cellular events which occur during *C. jejuni* invasion of HEp-2 cells is far from complete.

ACKNOWLEDGMENTS

This work was supported by a grant from the Fond National Suisse de la Récherche Scientifique (3.536.086).

We thank M. C. Peclet for his expert assistance with the electron microscope.

LITERATURE CITED

1. **Blaser, M. J., D. J. Duncau, and P. F. Smith.** 1984. Pathogenesis of *Campylobacter* infection: clearance of bacteremia in mice. Microecol. Ther. **14**:103–108.
2. **Bukholm, G.** 1984. Effect of cytochalasin B and dihydrocytochalasin B on invasiveness of entero-invasive bacteria in HEp-2 cell culture. Acta. Pathol. Microbiol. Immunol. Scand. Sect. B **92**:145–149.
3. **Clerc, P., and P. J. Sansonetti.** 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. Infect. Immun. **55**:2681–2688.
4. **De Melo, M., and J. C. Pechère.** 1988. Effect of mucin on *C. jejuni* association and invasion on HEp-2 cells. Microb. Pathog. **5**:71–76.
5. **Fauchere, J. L., A. Rosenau, M. Veron, E. N. Moyen, S. Richard, and A. Pfister.** 1986. Association with HeLa cells of *Campylobacter jejuni* and *Campylobacter coli* isolated from human feces. Infect. Immun. **54**:283–287.
6. **Field, L. H., V. L. Headley, J. L. Underwood, S. M. Payne, and L. J. Berry.** 1986. The chicken embryo as a model for campylobacter invasion: comparative virulence of human isolates of *Campylobacter jejuni* and *Campylobacter coli*. Infect. Immun. **54**:118–125.
7. **Gomori, G.** 1952. Microscopic histochemistry principles and practice, p. 189–194. University of Chicago Press, Chicago.
8. **Hale, T. L.** 1986. Invasion of epithelial cells by *Shigellae*. Ann. Inst. Pasteur Microbiol. **137A**:311–314.
9. **Hale, T. L., E. R. Morris, and P. F. Bonventre.** 1979. *Shigella* infection of Henle intestinal epithelial cells: role of the host cell. Infect. Immun. **24**:887–894.
10. **Humphrey, C. D., D. M. Montoy, and F. E. Pittman.** 1985. Experimental infection of hamsters with *Campylobacter jejuni*. J. Infect. Dis. **151**:485–493.
11. **Kiehlbauch, J. A., R. A. Albach, L. L. Baum, and K.-P. Chang.** 1985. Phagocytosis of *Campylobacter jejuni* and its intracellular survival in mononuclear phagocytes. Infect. Immun. **48**:446–451.
12. **Kihlstrom, E., and L. Nilsson.** 1977. Endocytosis of *Salmonella typhimurium* 395 MS and MR10 by Hela cells. Acta. Pathol. Microbiol. Scand. Sect. B **85**:322–328.
13. **Newell, D. G., and A. Pearson.** 1983. The invasion of epithelial cell lines and the intestinal epithelium of infant mice by *Campylobacter jejuni/coli*. J. Diarrhoeal Dis. Res. **2**:897–902.
14. **Popiel, I., and P. C. B. Turnbull.** 1985. Passage of *Salmonella enteritidis* and *Salmonella thompson* through chick ileocecal mucosa. Infect. Immun. **47**:786–792.
15. **Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier.** 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51**:461–469.
16. **Small, P. L. C., R. R. Isberg, and S. Falkow.** 1987. Comparison of the ability of enteroinvasive *Escherichia coli*, *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* to enter and replicate within HEp-2 cells. Infect. Immun. **55**:1674–1679.
17. **Vesikari, T., J. Bromirska, and M. Mäki.** 1982. Enhancement of invasiveness of *Yersinia enterocolitica* and *Escherichia coli* in HEp-2 cells by centrifugation. Infect. Immun. **36**:834–836.
18. **Walker, R. I., M. B. Caldwell, E. C. Lee, P. Guerry, T. J. Trust, and G. M. Ruiz-Palacios.** 1986. Pathophysiology of *Campylobacter* enteritis. Microbiol. Rev. **50**:81–94.
19. **Youssef, M., G. Corthier, H. Goossens, C. Tancrede, M. Henry-Amar, and A. Andremont.** 1987. Comparative translocation of enteropathogenic *Campylobacter* spp. and *Escherichia coli* from the intestinal tract of gnotobiotic mice. Infect. Immun. **55**:1019–1021.