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Growth and Effects of Ureaplasmas (T Mycoplasmas) in Bovine Oviductal Organ Cultures

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Ureaplasmas isolated from the human genital tract and from the genital and respiratory tracts of cattle were grown in association with organ cultures of bovine oviduct (uterine tube). All strains of ureaplasmas multiplied in organ cultures, stopped ciliary activity, and caused histological lesions. Most strains grew well, and 10^8 to 10^9 color-changing units were determined 18 to 144 h after inoculation. Twenty-four to 144 h after inoculation with ureaplasmas, ciliostasis was complete. Ciliostasis was also caused by additions of nonviable cultures at pH 8.8 (or adjusted to 7.4) or washed disrupted cells (100 μg of protein/ml); it occurred in 48 to 96 h. The cilia-stopping effect of nonviable cultures was diminished by heating (56 C for 30 min) and was abolished by boiling. When added to fresh medium in amounts exceeding 25%, nonviable ureaplasmal cultures completely inhibited ureaplasmal growth. By light, scanning, and transmission electron microscopy, cilia-stopping effect was correlated with collapse and sloughing of the cilia (the initial lesion was "bent" cilia), with bulging and vacuolization of secretory and ciliated cells, and finally with disorganization of the epithelium, necrosis, and desquamation.

Ureaplasmas (formerly known as T mycoplasmas) (19) have been isolated from a wide variety of apparently normal animals (25) and from the lungs of pneumonic cattle (6, 13, 18). There is evidence that they may cause pneumonia in calves (8) and mastitis in cows (7, 10). Although ureaplasmas are commonly found in the genital tract of cows and semen of bulls (22, 25), nothing is known of their pathogenicity for the bovine reproductive system or of their role in reproductive diseases of animals. In this study, we have examined the effects of ureaplasmas on small pieces of the bovine oviduct maintained in vitro as we did in earlier studies with mycoplasmas and bacteria (20). We observed the cilia-stopping effect (CSE) of ureaplasmas and studied the histopathological lesions of the oviduct.

MATERIALS AND METHODS

Ureaplasmas. Strain P108 was isolated from a vaginal swab in the Institute of Hygiene, Palerno, Italy (15), and obtained from Paul Smith, University of South Dakota Medical School, Vermillion, S.D. Strain VMRI was isolated from a bovine vaginal swab by M. L. Frey and furnished by T. Koski, Animal and Plant Health Inspection Service, National Animal Disease Center, Ames, Iowa. Strain 96 was isolated from bovine lung (13) and supplied by C. W. Livingston, Jr., Texas A&M University Agricultural and Research Center, San Angelo, Tex. NADC strains 5, 7139, and 7147 were isolated from swabs of bovine vaginal mucus from normal cattle maintained at this laboratory. The ureaplasmas were maintained in modified Hayflick medium or by storage at −70 C.

Medium. Modified Hayflick medium (13) consisted of PPLO broth (Difco, Detroit, Mich.; 21 g/liter) supplemented with 15% unheated horse serum, 10% of a yeast extract preparation, 1,000 U of penicillin per ml, 0.002% phenol red, and 0.1% urea. The pH was adjusted to 6.0 to 6.5 with 1 N HCl.

Organ cultures. Oviducts were obtained from healthy cows at necropsy or slaughter. The genital tract was removed, and the fimbriae and adjoining 1 cm of the infundibular part of the uterine tube were excised and placed in a test tube containing 20 ml of mycoplasma growth medium (12). The specimens were promptly transferred to the laboratory and incubated for 1 to 4 h at 37 C to inhibit the growth of opportunistic bacteria and to test for the presence of mycoplasmas and ureaplasmas (22). After the oviduct was opened and spread flat on a sterile surface, sections (3 by 3 mm) were excised and placed on the surface of preconditioned agar (21). Agar (2%) was dissolved in boiling water, autoclaved, and cooled to 50 C, and 4 ml was placed in a 50-mm plastic petri dish. When it solidified, 3 ml of medium (13) was added and the dish was incubated at 37 C in a desiccator jar. Sixteen to 24 h later, medium was decounted and five tissue sections were placed on each plate. A part of each agar plate was removed to form a reservoir, and 3 to 4 ml of modified Hayflick medium was added. The organ cultures were incubated

overnight at 37 C in an environment of 5% CO_2 in air. The medium was removed and the organ cultures were examined microscopically for ciliary activity by using phase contrast optics at ×240. Edges of each culture were examined for characteristic ciliary movements, and each culture was scored as positive or negative (no detectable activity). Any culture without detectable ciliary activity was discarded (20).

Exposure of organ cultures. Modified Hayflick medium was removed and replaced with cultures of ureaplasmas diluted with modified Hayflick medium. Titers of viable ureaplasmas were determined by a tube dilution method (7) that depends upon the ability of these organisms to produce ammonia from urea with an alkaline change in the pH.

Oviductal organ cultures were also exposed to nonviable cultures of ureaplasmas (i.e., after growth, alkaline shift, and loss of viability upon attempts to subculture) and to preparations of disrupted ureaplasmas. Strain 96 cells were produced in modified Hayflick medium. A 200-ml amount of culture was added to 800 ml of medium and incubated until it was slightly red. The ureaplasmas were sedimented by centrifugation (27,000 × *g*) at 5 C for 30 min and washed once in saline (0.85%). They were sedimented again, resuspended in 5 ml of modified Hayflick medium, and subjected to 10 cycles of freeze-thaw. After the protein concentration was estimated (biuret tests), the preparation was stored at −70 C until added to organ cultures.

Histopathology. Cultures of bovine oviduct were exposed to a young, growing culture of ureaplasma strain 96 and incubated for 1, 10, 24, 48, 72, 96, and 144 h. The microscopic lesions were observed by light and electron microscopy and compared with control organ cultures maintained in modified Hayflick medium. For studies with the light microscope, two organ cultures at each time interval were fixed in Bouin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissues for scanning electron microscopy (SEM) were processed as described previously (21). For transmission electron microscopy (TEM), cultures were rinsed overnight in cacodylate buffer (pH 7.4), fixed in 1% OsO_4 in cacodylate buffer, and rinsed again in buffer. After dehydration in alcohol, the tissues were embedded in epoxy resins (15). Ultrathin sections were cut on an ultramicrotome, stained with lead citrate and uranyl acetate (27), and examined with a Philips 200 electron microscope.

RESULTS

Ureaplasmal growth in organ cultures. All strains grew in modified Hayflick medium and in the presence of oviductal cultures (Table 1). Growth curves for strain 96 are shown (Fig. 1). The number of color-changing units (CCU) per milliliter was 1 to 2 logs lower in organ cultures. Similar growth responses were observed with strains P108, VMRI, and NADC 5, but strains NADC 7139 and NADC 7147 grew poorly in that maximum CCU per milliliter were only 10^3 to 10^4. After 24 to 48 h of ureaplasmal growth, the pH of the medium changed from 6.0–6.4 to 7.8–8.8. During continued incubation for an additional 2, 3, or 4 days, the pH decreased and stabilized from 7.2 to 7.8.

TABLE 1. *Growth and CSE of ureaplasmas in bovine oviductal organ cultures*

Strain	Expt no.	Inoculum (CCU/ml)	Maximum growth (CCU/ml)	CSE (days)
96	I	10^7	10^8	3
	II	10^8	10^9	2
	III	10^3	10^9	6
P108	I	10^7	10^8	4
	II	10^8	10^8	3
VMRI	I	10^6	10^9	3
	II	10^7	10^9	2
NADC 5	I	10^2	10^2	Neg[a]
	II	10^7	10^8	4
	III	10^8	10^8	3
NADC 7139	I	10^2	10^2	6
	II	10^4	10^4	6
NADC 7147	I	10^1	10^1	Neg
	II	10^3	10^4	5

[a] Neg, Not detected by 7 days.

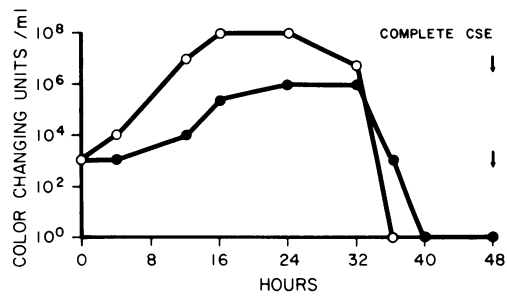

FIG. 1. *Growth curves of ureaplasma strain 96 in modified Hayflick medium (○) and in Hayflick medium in the presence of bovine oviductal cultures (●). Complete CSE occurred after 48 h of incubation (arrow).*

CSE of ureaplasmas in oviductal cultures. All strains of ureaplasmas stopped ciliary activity of oviductal cultures. Complete CSE was recorded from days 2 to 6 of incubation (Table 1, Fig. 1). CSE was related to the number of ureaplasmas added to oviductal cultures (Fig. 2) and to the maximum number of ureaplasmas attained during incubation (Table 1). The average time of CSE for 15 experiments was 74 h. Control organ cultures in modified Hayflick medium displayed normal ciliary activity for 7 days.

CSE of nonviable ureaplasmal cultures and of disrupted ureaplasmas. Nonviable cultures of strain 96, 5 or 6 days old, with pH values of 8.4 to 8.8, had complete CSE in 48 h. The CSE

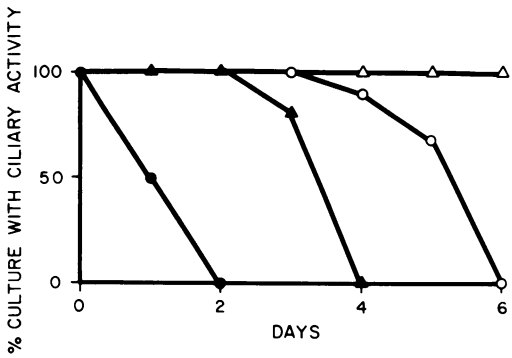

FIG. 2. *Percentage of bovine oviductal cultures with ciliary activity after exposure to different numbers of ureaplasma strain 96. Percentage of ciliary activity was calculated by dividing the total number of organ cultures with full or reduced ciliary activity by the total number of cultures observed. Symbols: ●, $>10^8$ CCU of inoculum per ml; ▲, 2×10^6 CCU of inoculum per ml; ○, $<10^2$ CCU of inoculum per ml; △, uninoculated control.*

was not diminished by adjusting the pH to 7.4; it was diminished by heating (56 C for 30 min) and was abolished by boiling (Table 2). The CSE of spent culture was diminished by dialysis for 72 h against 4 volumes of modified Hayflick medium so that complete CSE was not observed before 6 days of incubation. The dialysate caused complete CSE at 6 days.

Disrupted ureaplasmas had complete CSE. As little as 100 μg of cellular protein had CSE in 96 h (Table 2). Control organ cultures displayed normal ciliary activity.

Growth inhibition by nonviable cultures. Dialyzed nonviable culture of strain 96 inhibited the growth of strain 96 (Table 3); in medium containing 25 or 50% nonviable culture, attempts to recover ureaplasmas 24 h after inoculation failed. Lesser amounts of nonviable culture (12.5 and 6.2%) inhibited but did not prevent ureaplasmal growth. Color changes were noted after 48 and 72 h in medium with added nonviable culture, whereas color changes were noted at 24 h in control medium. Although the growth responses were delayed, the maximum number of ureaplasmas was almost the same (10^9 CCU/ml) in the presence of spent medium as in control medium (2×10^9 CCU/ml).

Histopathology. Ciliary activity of bovine oviductal tissue ceased after exposure to ureaplasma strain 96 for 48 h. After 5 days, severe damage was observed by light microscopy. There was desquamation of the epithelial surface and clumping and margination of the chromatin of cells in the submucosa, whereas control (nonexposed) oviductal tissue remained normal in appearance and well differentiated for 6 days. When tissue was examined by SEM, ciliary morphology was normal after incubation for 6 days although some debris was present on the cilia (Fig. 3). When the cellular morphology of the same control cultures was examined by TEM, the luminal surface of the epithelium was parallel to the basement membrane, and small villi were observed between the bases of the erect cilia.

The effects of exposure for intermediate periods of incubation (1 to 72 h) were evaluated by electron microscopy. Ciliary and cellular morphology was normal 1 h after exposure to ureaplasma strain 96 (Fig. 4), but after exposure for 10 h the cilia were bent and clumped and nodular irregularities were present on the surface (Fig. 5). By TEM, irregular dilations were observed in the plasma membrane of parts of some cilia, and some epithelial cells bulged into the lumen (Fig. 6). After 24 h, cilia were absent from many cells, and large cytoplasmic blebs protruded from the surface. The cytoplasm in these blebs was electron translucent and contained degenerate organelles (Fig. 7).

After 48 h of exposure to ureaplasmas, ovi-

TABLE 2. *CSE of disrupted ureaplasmas and nonviable cultures in bovine oviductal cultures*

Treatment	CSE (days)
Disrupted ureaplasmas, 10 μg of protein/ml	Neg[a]
Disrupted ureaplasmas, 100 μg of protein/ml	4
Disrupted ureaplasmas, 1,000 μg of protein/ml	4
Nonviable culture, pH 8.8	2
Nonviable culture, pH 7.2	2
Nonviable culture, heated (56 C for 30 min)	4
Nonviable culture, boiled (15 min)	None

[a] Neg, Not detected by 4 days.

TABLE 3. *Growth inhibition of ureaplasma strain 96 by nonviable ureaplasma culture*

Addition of spent culture[a]	Growth response[b]	
	Expt I	Expt II
50	Neg[c]	Neg
25	Neg	Neg
12.5	2	3
6.2	2	2
None, control	1	1

[a] Seven-day-old nonviable culture of strain 96 was dialyzed for 48 h against modified Hayflick medium and added to fresh medium in percentages shown. The medium was inoculated with 10^5 CCU/ml.

[b] Day of color change.

[c] Neg, Not detected by 4 days.

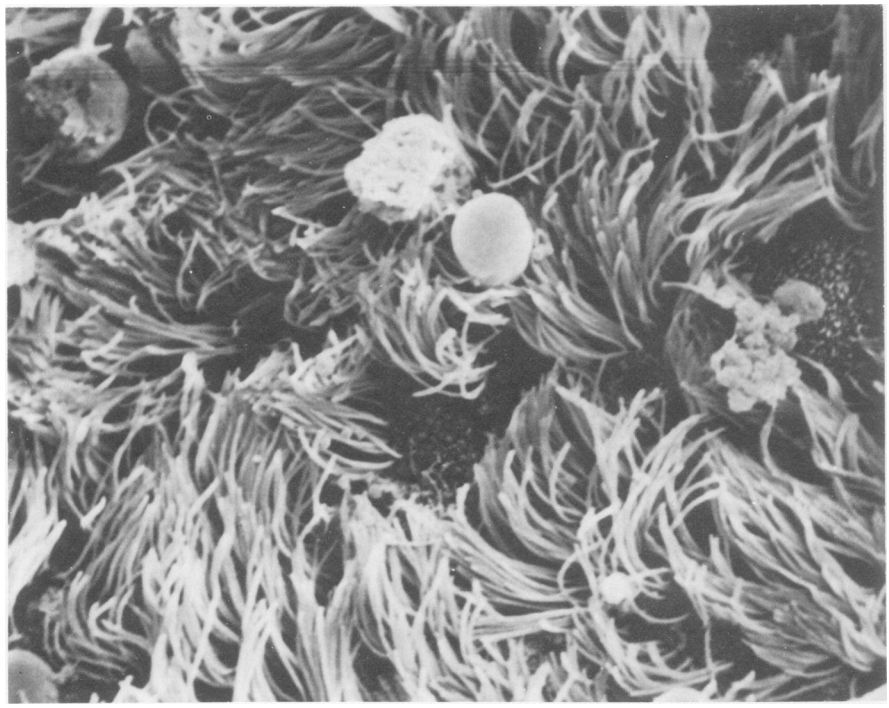

FIG. 3. *Control (nonexposed) oviductal organ culture after 144 h in modified Hayflick medium. The cilia have normal morphology. SEM, ×2,800.*

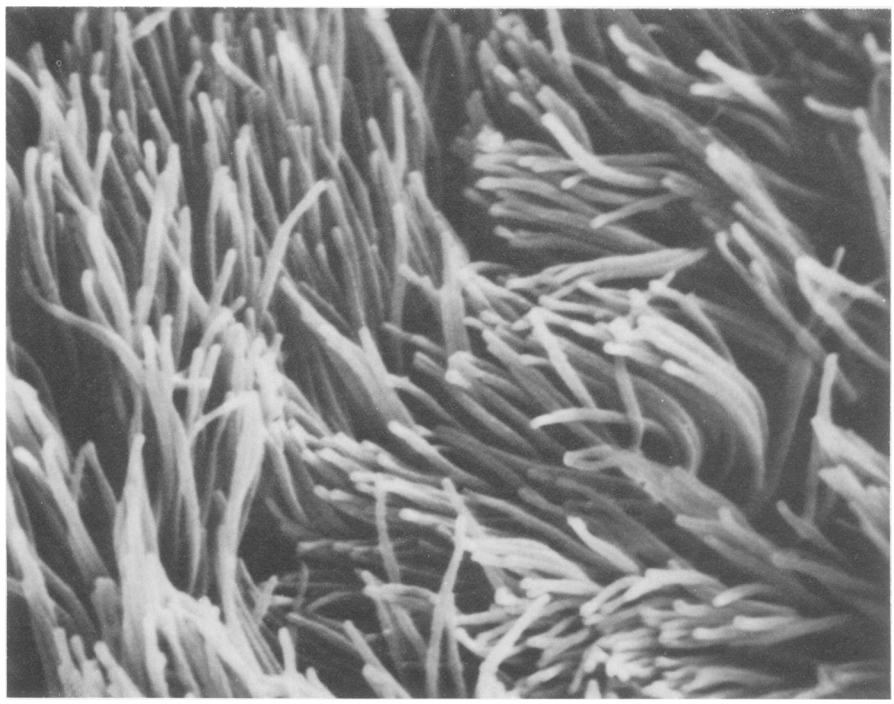

FIG. 4. *After exposure of oviductal organ culture to ureaplasma strain 96 for 1 h, cilia have normal morphology. SEM, ×6,000.*

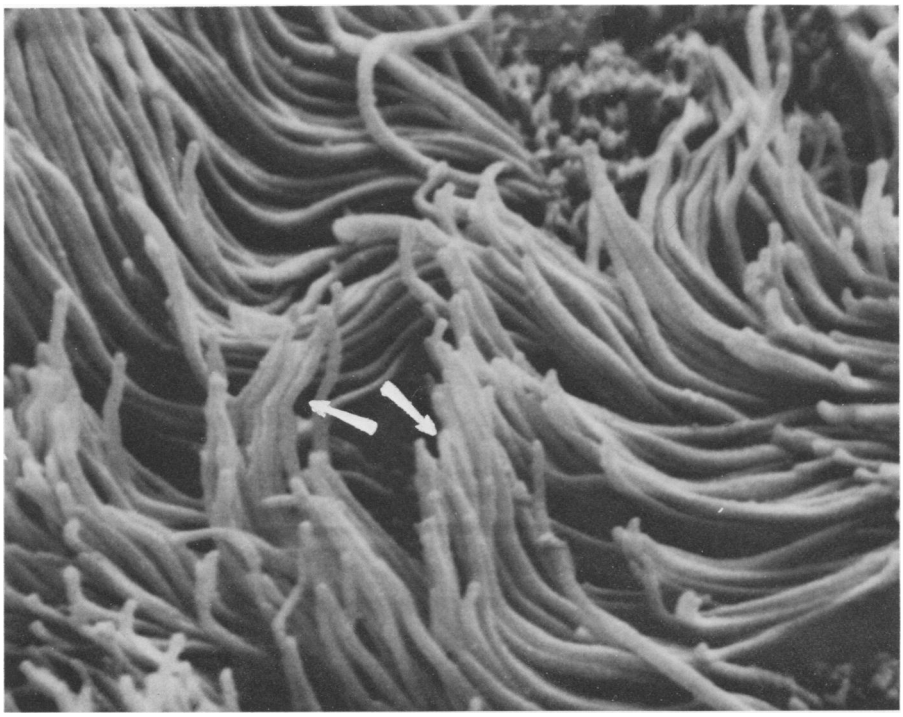

FIG. 5. *After exposure of bovine oviductal organ culture to ureaplasma strain 96 for 10 h, cilia are bent and are in clusters (arrows). The external contours of some cilia are irregular. SEM, ×6,600.*

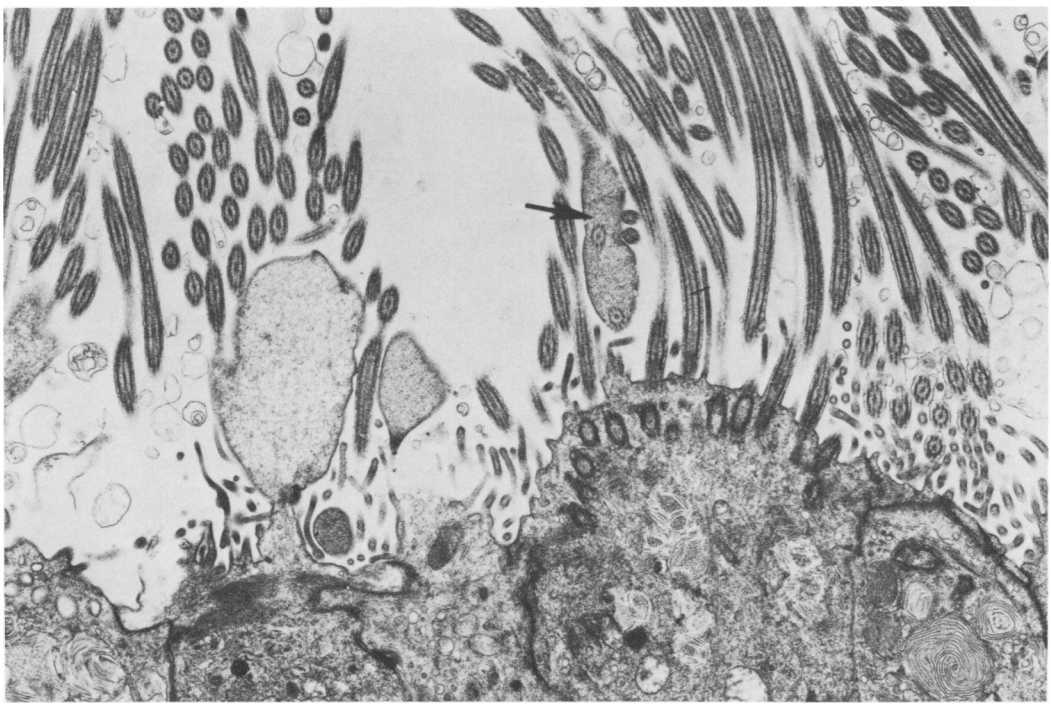

FIG. 6. *Part of ciliated epithelial cells of bovine oviduct after exposure to ureaplasma strain 96 for 10 h. Cells are bulged into lumen; some cilia have irregular dilations (arrow). TEM, ×6,300.*

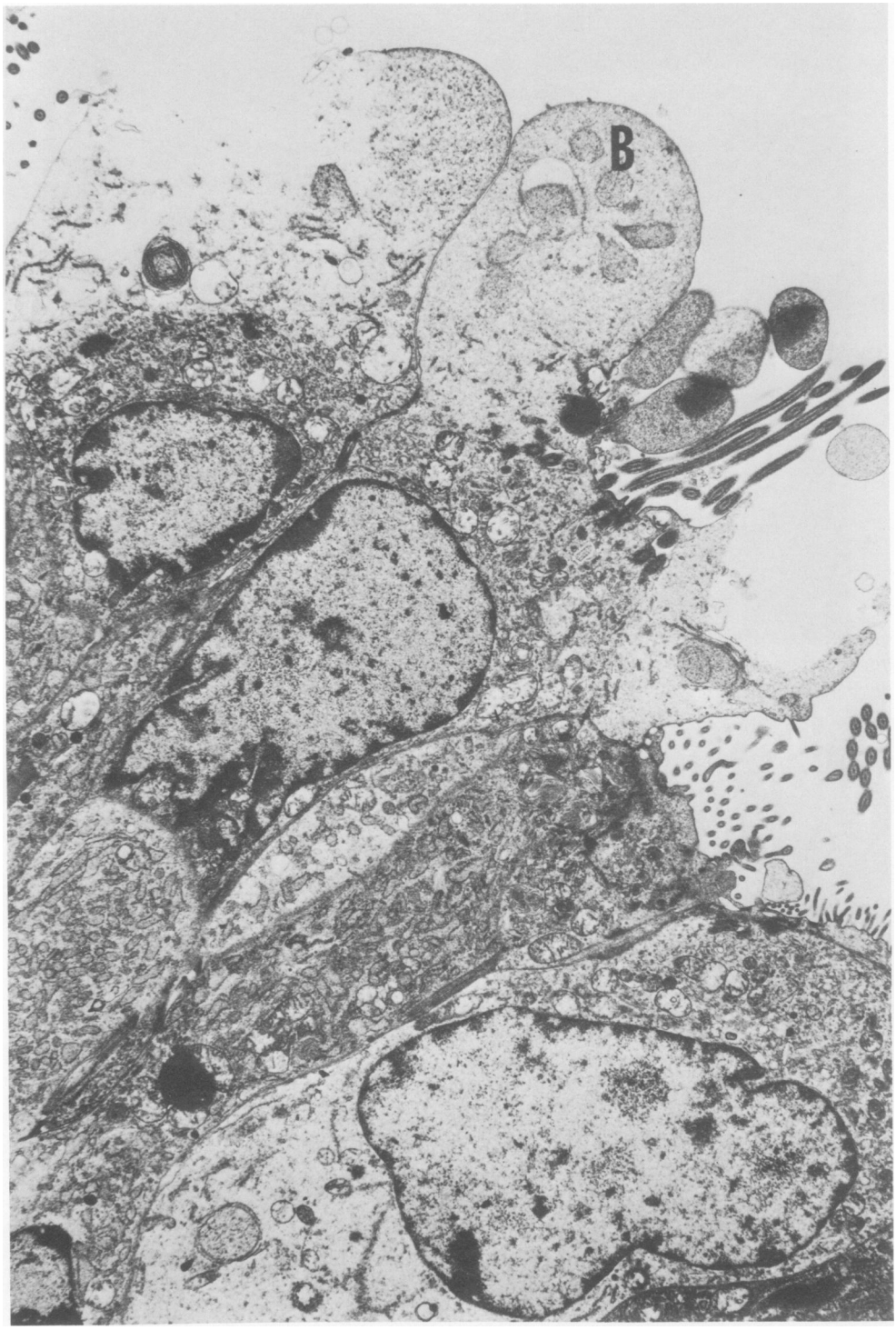

FIG. 7. *After exposure of oviductal organ culture to ureaplasma strain 96 for 24 h, cilia are absent from some epithelial cells of the oviduct, and large blebs (B) of cytoplasm protrude from the luminal surface. TEM, ×10,000.*

ductal cilia were severely damaged, and cellular detritus overlaid epithelial cells that protruded into the lumen (Fig. 8 and 9).

After 72 h of exposure, most epithelial cells were deciliated. The surface of most cells contained hemispheric protrusions covered by villous projections (Fig. 10). By TEM, we could see that the endoplasmic reticulum was dilated and that autophagic vacuoles were present in the cytoplasm (Fig. 11). The chromatin of some nuclei was clumped and marginated. After 144 h of exposure, most epithelial cells had been sloughed, and the basement membrane was thus exposed. Similar histopathological lesions were observed in organ cultures exposed to ureaplasma strains NADC 5 and 7147.

DISCUSSION

Although organ cultures of ciliated epithelium have been used to study a broad range of microorganisms (2, 3, 16, 20, 28), this work represents the first successful model system for studying ureaplasmas in relation to differentiated host cells. The in vitro system is free from the responses of host, the microbial populations can be monitored and controlled, ciliary activity is hormone independent (24), cilia will continue to beat for several days or weeks (20), and suitable cultures may be prepared from oviducts collected from cows regardless of the stage of the estral cycle (20).

When grown in the presence of oviductal organ cultures, ureaplasmas isolated from the human genital tract or those of bovine genital or pulmonary origin stopped ciliary activity and caused severe lesions of the epithelium. The CSE coincided with the appearance of "debris" in the culture medium and cytopathology. The initial lesion was "bent" cilia followed by deciliation and epithelial necrosis. The lack of CSE reported by other investigators (24, 26) may have been due to poor growth of the ureaplasmas in Eagle medium without serum (11).

The same pathological sequence was caused by a substance in nonviable cultures of ureaplasmas. The dialyzable, heat-labile, toxic factor was produced by growing ureaplasmas and released into the medium when they matured. In high concentrations, this factor inactivated ureaplasmas; in lower concentrations, it temporarily inhibited their growth. Similar reactions were reported earlier (4).

The chemical nature of the cytotoxin is unknown. The copious production of ammonia by ureaplasmas results in a strongly alkaline environment. Romano et al. (17) suggested that

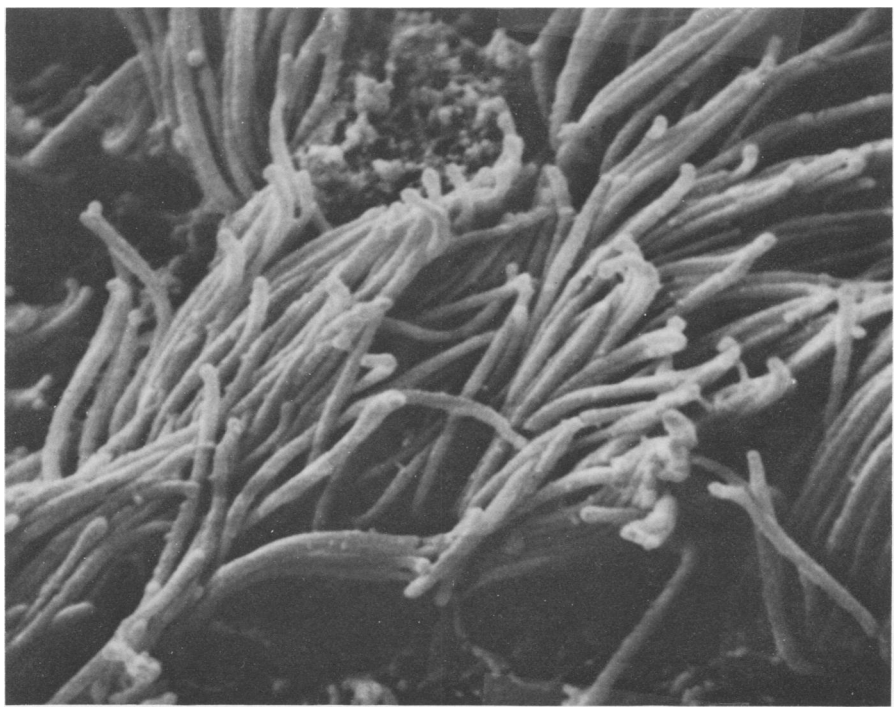

FIG. 8. *After exposure of oviductal organ culture to ureaplasma strain 96 for 48 h, the ends of some cilia are bent, and small nodules are present on the surface. SEM, ×6,600.*

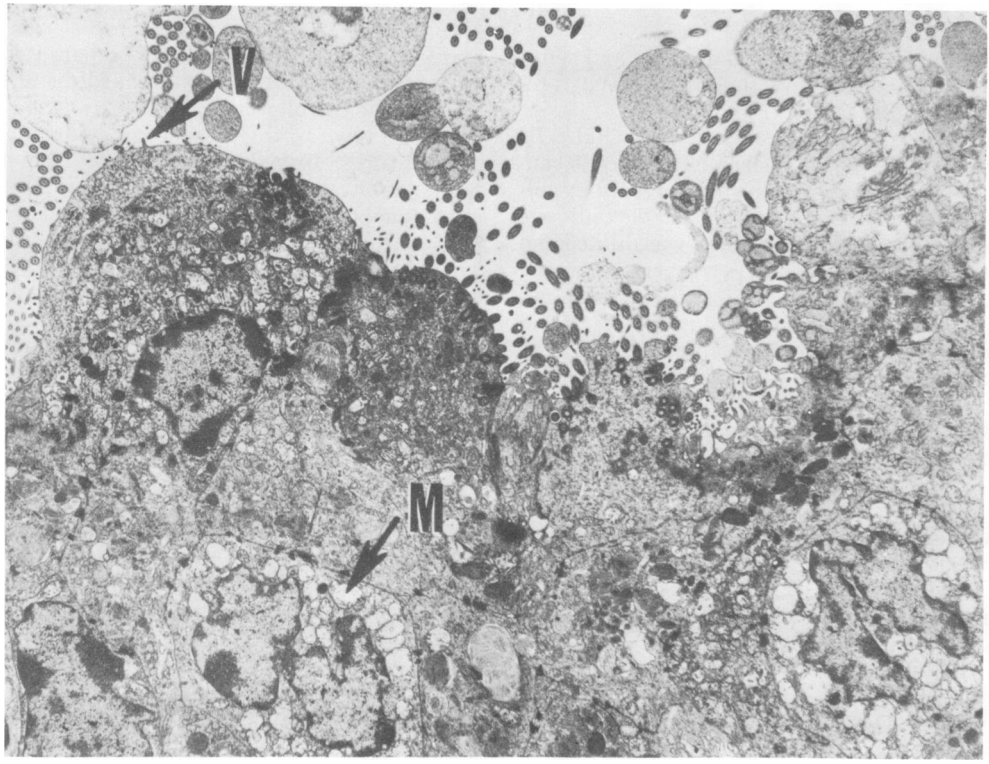

FIG. 9. *After exposure of oviductal organ culture to ureaplasma strain 96 for 48 h, epithelial cells protrude into the lumen of the oviduct, and cellular detritus is present. Cilia are absent from some cells; only small villi (V) are present in others. Note swollen mitochondria (M) and clumped, marginated chromatin of epithelial cells. TEM, ×5,000.*

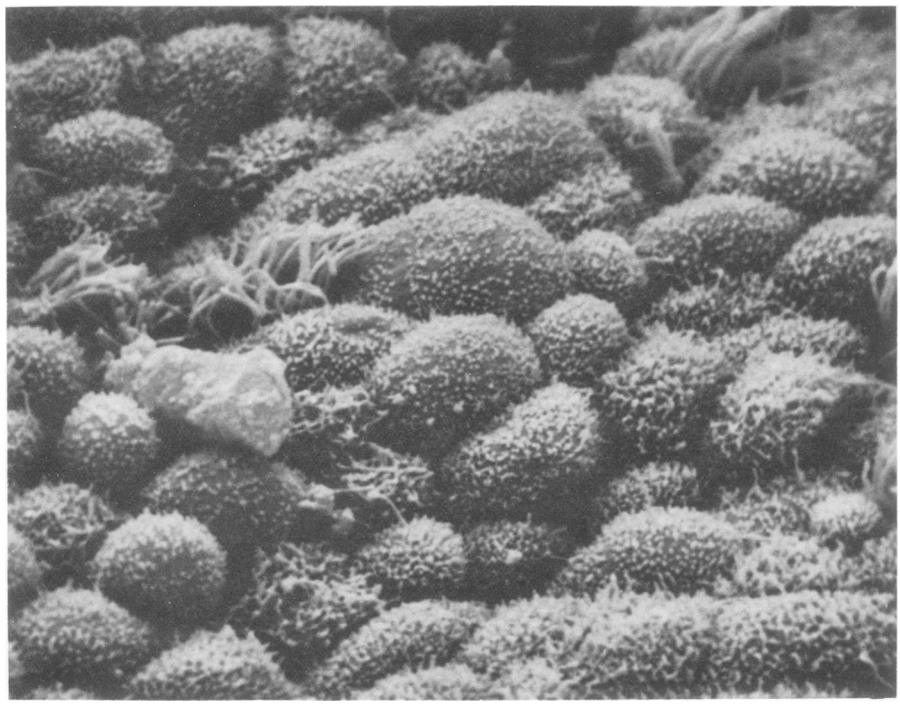

FIG. 10. *After exposure of bovine oviductal organ culture to ureaplasma strain 96 for 72 h, deciliation of most cells was revealed; small villi remain on bulging epithelial cells of bovine oviduct. SEM, ×2,200.*

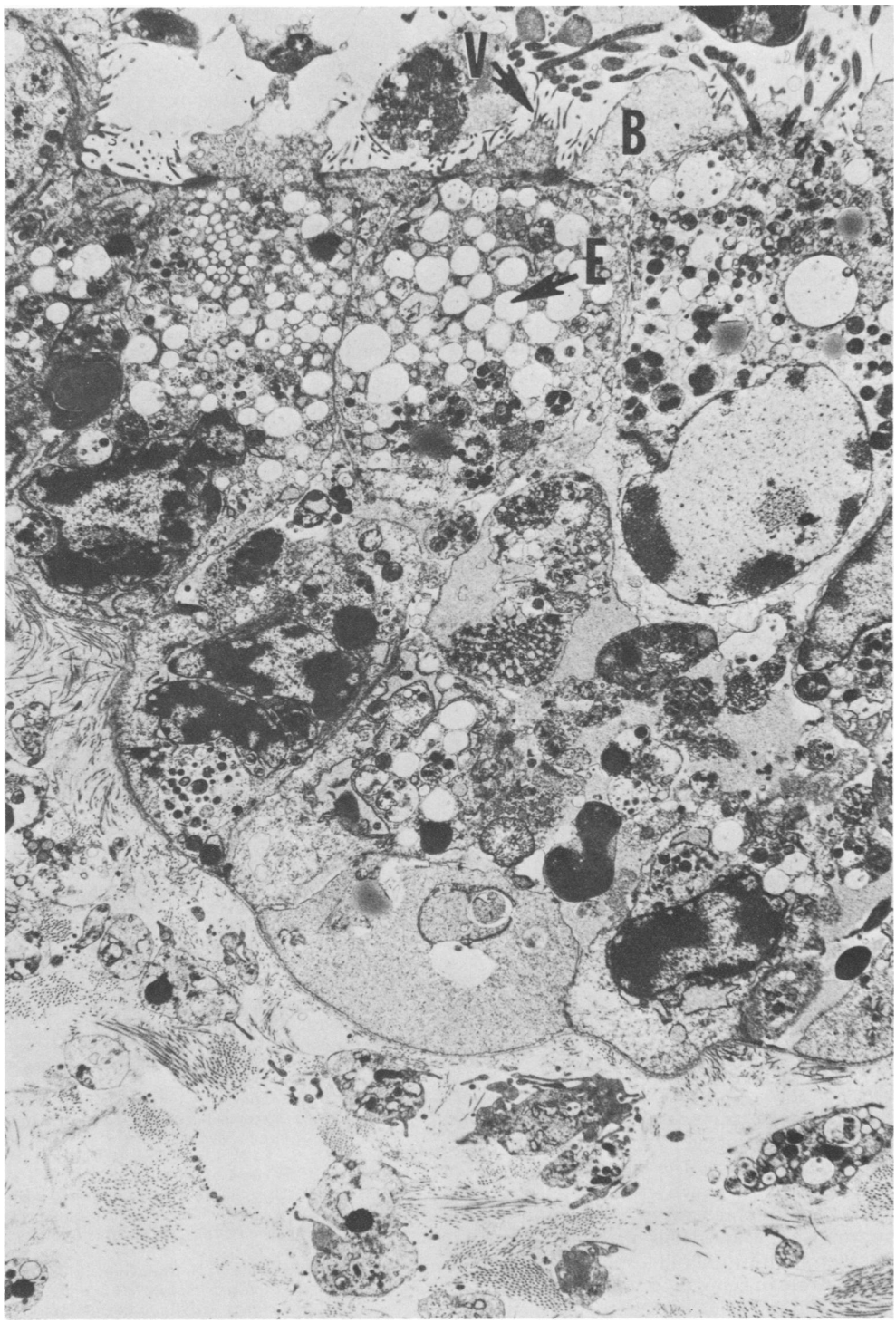

FIG. 11. *Histopathology of bovine oviduct exposed to ureaplasma strain 96 for 72 h. Cellular detritus is present in the lumen (top). Most cells have undergone deciliation; blebs (B) of cytoplasm and small villi (V) protrude from the luminal surface. The endoplasmic reticulum (E) is dilated, the chromatin is clumped, and autophagic vacuoles are present in the cytoplasm. The nuclear membrane has irregular involutions. TEM, ×5,000.*

ureaplasmas control the integrity of their membrane by compensatory production of strongly acidic lipids such as phosphatidic acid and free fatty acids. If such radicals are part of the cytotoxin, they were not inactivated at neutral pH values.

Ureaplasmas have been associated with cases of unexplained infertility and reproductive failure in humans (1, 5, 9) and animals (23), but there has been no clear evidence as to the pathogenic mechanism. One possible mechanism suggested by Horne et al. (9) was subacute endometrial inflammation characterized as a focal accumulation of inflammatory cells most commonly found beneath the surface epithelium. There was 68.5% positive correlation between the lesion and genital ureaplasmosis in a series of 38 infertile women patients. The results of this study suggest an investigation of oviductal deciliation in cases of salpingitis and as a cause of occlusion of the fallopian tubes. In addition, one can speculate that the severe losses due to reproductive failure, particularly in dairy cows that are bred artificially, are due in part to very subtle deviations from the normal physiological state of the cilia of the genital tract from the cervix to the fimbriae. An absence of ciliary activity or even a decrease in the rate or vigor of the beat might interfere with conception and early embryonic development at any one of several crucial events, such as migration of the sperm and ovum, capacitation, fertilization, movement of the blastomere from the oviduct, or uterine implantation. Suboptimal health of the cilia would reflect subtle disease of the epithelium, which might be associated with slight but significant changes in the amount or composition of secretions. Perhaps a combination of decreased ciliary activity and abnormal epithelial secretions at a site of a crucial reproductive event might cause more infertility than one or several quite obvious histological lesions in a noncritical tissue, e.g., chronic interstitial salpingitis (14). Therefore, in addition to gross and histological pathology, studies on the causes of bovine infertility should include cultural examinations for microbial etiological agents and evaluations of the health of the mucosal epithelium as revealed by secretory and ciliary activities. Finally, the predilection of ureaplasmas for the genital tract of cows and bulls and their pathogenicity for oviductal epithelium should foster further studies on their role in reproductive diseases and on their elimination from semen to be used for artificial insemination.

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