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Ultrastructural pathology of the upper respiratory tract of rabbits experimentally infected with *Pasteurella multocida* A:3

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SUMMARY

Twenty-four 8 to 9 week-old *Pasteurella multocida*-free rabbits were divided into three equal groups, the first group was pretreated with hydrocortisone and inoculated intranasally with *pasteurella multocida* serotype A:3. The second group was inoculated intranasally with *P. multocida* without hydrocortisone treatment. The third group was inoculated with phosphate buffered saline only and used as a control group. *Pasteurella multocida* was isolated from the nasal cavity of all infected rabbits in group 1 and 2 and from the trachea of seven rabbits in group 1 and five rabbits in group 2. This study was conducted to observe the ultrastructural changes of the upper respiratory tract of hydrocortisone treated and non-treated rabbits infected with *P. multocida* serotype A:3. The ultrastructural changes detected in infected rabbits were ciliary destruction and deciliation of the ciliated epithelial cells, cellular swelling, goblet cell hyperplasia and endothelial cell damage. *Pasteurella multocida* was observed attached to the degenerated cilia, microvilli and mucus. *Pasteurella multocida* infection was associated with inflammatory responses, which may have caused tissue damage. It is possible that hydrocortisone modulates the severity of infection as an immune suppressor and an inhibitor of goblet cell secretion.

PASTEURELLOSIS caused by *Pasteurella multocida* is one of the most common and serious diseases of rabbits (Flatt 1974). Type A was reported to be the most predominant serotype associated with pasteurellosis in rabbits (Lu et al 1978, DiGiacomo et al 1983, Al-Haddawi et al 1998). Following experimental pathogenicity studies of *P. multocida* in rabbits, serotype A:3 was found to be more virulent than A:12 (DiGiacomo et al 1983, Lu and Pakes 1981), and stress factors were reported to influence the course of the disease (Lu et al 1982, Corbeil et al 1983). Upper respiratory tract infections (rhinitis and sinusitis) are recognised as the most frequent clinical manifestations, often leading to pneumonia and septicaemia (Manning et al 1989).

Bacterial adherence to the mucosal surface and to the cells of target tissues is an important initial step in the infection process. This process involves specific interaction between bacterial adhesins and host receptors (Beachey 1981). Various infective agents have evolved mechanisms to utilise mucosal surface receptors to enhance their colonisation (Laux et al 1986, Mouricout and Julien 1987). It has been reported that *P. multocida* has an affinity for porcine respiratory tract mucus (Letellier et al 1991), adheres well to non-ciliated respiratory epithelial cells (Pijoan and Trigo 1990), and increases production of mucus (Dugal et al 1992, Al-Haddawi et al 1997). This study reports the ultrastructural changes associated with *P. multocida* interaction at the mucosal surface and intracellular changes of the upper respiratory tract of rabbits infected with *P. multocida* A:3.

The objective of this study was to investigate the morphological changes of the nasal mucosa and trachea of hydrocortisone treated and non-treated rabbits infected with *P. multocida* serotype A:3.

MATERIALS AND METHODS

Animals

Twenty-four 8 to 9 week-old New Zealand White rabbits (both sexes) were obtained from the Animal Resource Center, Universiti Putra Malaysia. These rabbits were negative for *P. multocida* on three occasions of nasal culture, which were collected at 4-day intervals two weeks before the inoculation. Rabbits were housed individually in stainless steel cages and provided with water and rabbit pellet *ad libitum*.

Pasteurella multocida *inoculum*

The *P. multocida* serotype A:3 used in this experiment was isolated from a naturally infected rabbit with signs of mucopurulent rhinitis. The isolate was maintained on nutrient agar slant (Oxoid) at 25°C and was subcultured on 5 per cent horse blood agar before one colony was grown in brain heart infusion broth (BHI) (Oxoid) at 37°C for 18 hours. A mouse was inoculated intraperitoneally with 0·1 ml of the cultured broth before the micro-organism was reisolated from the heart blood of the mouse after 24 hours. The isolate was identified as type A by the Staphylococcal hyaluronidase decapsulation test (Carter and Rundell, 1975) and somatic serotyping was conducted by Dr. Thula Wijewardena at the Veterinary Research Institute, Sri Lanka. The bacteria were quantitated by diluting a 1·0 ml sample of the broth (BHI growth) culture 10-fold in sterile PBS, pH 7·4. Each dilution was streaked onto 5 per cent horse blood agar and the colonies were counted after incubation for 24 hours at 37°C. Samples for inoculation were diluted in PBS to $1{\cdot}75 \times 10^8$ colony forming unit per ml (cfu ml^{-1}).

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Experimental design

The 24 *P. multocida*-free rabbits were divided into three equal groups. Rabbits in group 1 were treated with hydrocortisone intramuscularly at the rate of 25 mg kg^{-1} (hydrocortisone sodium succinat, Rotex Medica GMBH, Germany) daily for three consecutive days. One day after the last hydrocortisone treatment, all rabbits in group 1 were inoculated intranasally with *P. multocida* at a dose of $1{\cdot}75 \times 10^8$ cells in 1 ml of PBS. Rabbits in group 2 were inoculated with *P. multocida* without hydrocortisone treatment, while group 3 was inoculated with 1 ml PBS, pH 7·4 and act as control. The rabbits were inoculated by inserting the end of a 1-ml syringe without needle into the nares of rabbits and pushing the inocula gently. On days 14 and 21 p.i., four rabbits respectively from each group were killed by severing the jugular vein following anaesthesia with 0·5 ml Ketavet (Ketamin hydrochloride 100 mg ml^{-1}. Delta Vet. Laboratories Pty. Ltd. Australia) and 0·2 ml Romazine (Xylazine 20 mg ml^{-1}. Jurox Pty. Ltd. Australia) intramuscularly.

Bacteriology

Nasal swabs were collected at 3-day intervals for bacterial isolation during the time of the experiment. Swabs from nasal and tracheal mucosa were collected also for bacterial isolation at post-mortem.

Pathology

Samples from three portions (cranial, middle and caudal) of nasal mucosa (both sides) and middle part of trachea that yielded heavy to pure growth of *P. multocida* were collected for transmission electron microscopy (TEM). The samples were collected from six rabbits from both infected groups. Samples from four rabbits in group 3 were also collected. The samples of nasal mucosa were fixed for 8 hours with 2·5 per cent glutaraldehyde and of trachea were fixed with 4 per cent glutaraldehyde, using 0·1 M sodium cacodylate buffer. Samples were washed three times in buffered for 10 minutes each, postfixed for 2 hours with 1 per cent osmium tetroxide. Fixed samples were rinsed, dehydrated in a graded acetone series and embedded in resin. Ultrathin sections were cut on Ultracut E (Reichert-Jung, Austria) microtome and mounted on 200-mesh copper grids. The sections were stained with uranyl acetate and lead citrate and examined using Hitachi transmission electron microscope H 7100.

RESULTS

Bacterial isolation

Pasteurella multocida was isolated from the nasal cavity of all the killed rabbits from groups 1 and 2 on 14 and 21 days p.i., from the trachea of seven rabbits in group 1 and from the trachea of five rabbits in group 2. Heavy to pure growth of *P. multocida* were obtained from the nasal swabs of six and seven infected rabbits in group 1 and 2 respectively. In addition, tracheal swabs cultures of six and five rabbits in group 1 and 2 respectively yielded to heavy to pure growth of *P. multocida*. *P. multocida* was not isolated from rabbits in group 3. Other bacteria were also isolated from the nasal cavity prior and post-inoculation from rabbits of groups 1, 2, and 3. These bacteria were *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Citrobacter spp, Bacillus subtilus, Aeromonas spp and Pseudomonas aeroginosa*

Clinical signs

None of the live rabbits died during the experiment. Four rabbits in group 1 and two in group 2 had slight increase in rectal temperature at different times during the experiment. Two days p.i. all rabbits in groups 1 and 2 were depressed and showed loss of body weight. Mucopurulent nasal discharge was evident in seven rabbits from group 1 and seven rabbits in group 2. Clinical signs were not observed in rabbits from group 3.

Gross findings

Congestion of the nasal mucosa with mucopurulent exudate in the nasal cavity was observed in all four rabbits from group 1 killed 14 days p.i.. Slight congestion of the trachea was observed in two rabbits. Similar changes were present in the nasal cavity of three rabbits and the trachea of two rabbits killed 21 days p.i.. The gross findings in rabbits of group 2 killed 14 days p.i. were congestion of the nasal mucosa with mucopurulent exudate in the nasal cavity of one rabbit and catarrhal exudate mixed with yellowish threads of pus in three cases. No significant lesion was observed in the trachea of rabbits from group 2 that were killed on either day 14 or 21 p.i. No gross lesions were observed in nasal and tracheal mucosa of all the rabbits from group 3.

Transmission electron microscopy

Nasal mucosa The nasal mucosa of infected rabbits from groups 1 and 2 which were killed 14 days p.i., had severely swollen epithelial cells with vacuolated cytoplasm. The mitochondria of these epithelial cells were swollen and lacked cristae or the cristae were disorganised or distended or osmiophilic (Fig 1). The rough endoplasmic reticulum was dilated and appeared as irregular-shaped cytoplasmic vacuoles. The nucleus of degenerated epithelial cells had a distended nuclear space and an irregular nuclear membrane (Fig 2). Loss of cilia was frequently observed in degenerated cells whereas the necrotic cells had lost cilia and cell membranes had broken down. Multi-layered balloon-like structures had formed on the sides of cilia. Multi-layered hemispherical structures were present in the balloon-like structures (Fig 3), possibly the result of coiling of the axial microtubules complex within the swollen portion of cilia. Some of the microvilli of the epithelial cells also had focal multilayered balloon-like structure on their tips. Bacterial cells were seen attached to the microvilli and deformed cilia (Fig 4). Gaps had formed between the basal cells, possibly caused by oedema. Polymorphonuclear (PMN) leukocytes at different stages of maturation had infiltrated between the degenerated epithelial cells. These cells contained a single or multilobed nucleus with at least two types of cytoplasmic granules (Fig 5). Eosinophils that had large crystalloid cytoplasmic granules (Fig 6) were also observed infiltrated

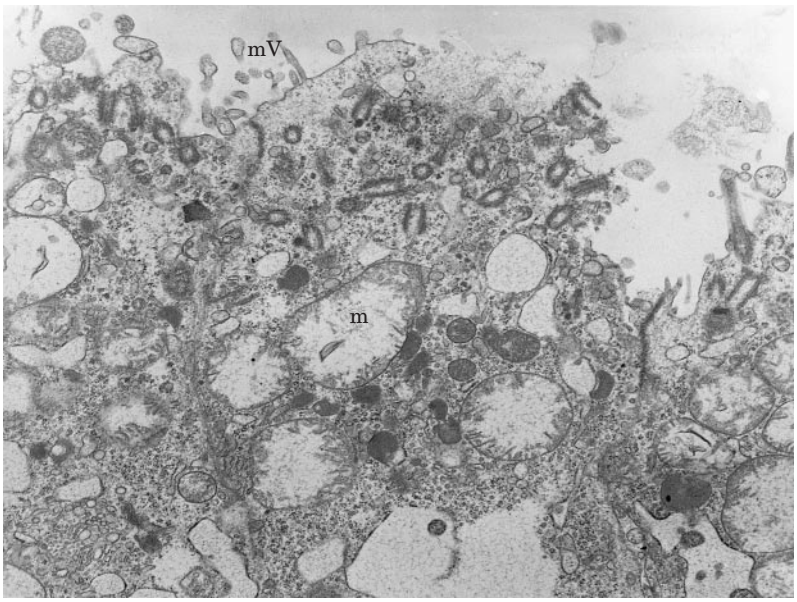

FIG 1: Electron micrograph. Nasal respiratory epithelium from a rabbit in group 1 infected with *Pasteurella multocida* intranasally and killed 14 days p.i.. Note loss of cilia, shortened microvilli (mV) and swelling of mitochondria (m). Mitochondria lack cristae or they are disorganised or ballooned and may contain opaque particles in their matrix. TEM × 9200.

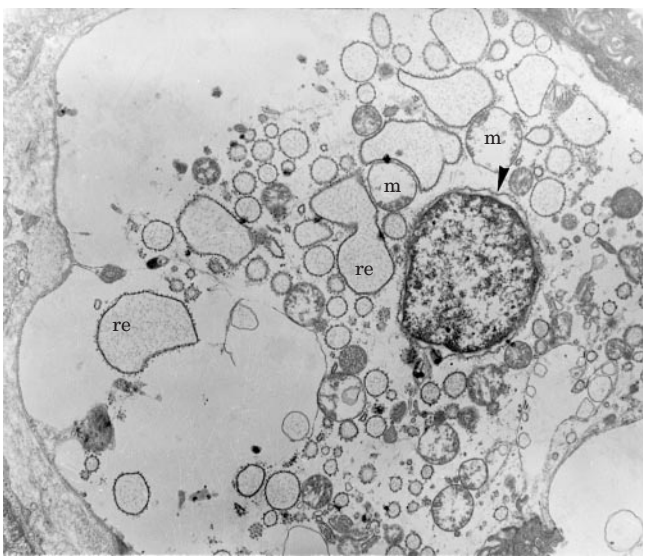

FIG 2: Electron micrograph. Nasal epithelium from an infected rabbit in group 2 that was killed 14 days p.i.. Note dilatation of rough endoplasmic reticulum (re), swelling of mitochondria (m) and irregular nuclear membrane (arrow head). TEM × 6750

adjacent to plasma cells and heterophils in the nasal mucosa of rabbits from group 1. Polymorphonuclear leukocytes and macrophages were seen attached to the endothelial cells and some had migrated between the endothelial cells. Intravascular fibrin deposition was found in the nasal mucosa of rabbit from group 1 killed 14 days p.i., and this deposition was adjacent to the endothelial cells and macrophages (Fig 7). Additionally, in rabbits from both groups 1 and 2 killed 21 days p.i. sloughed degenerated cell were observed in the nasal cavity leaving only one layer of basal cells. Hyperplasia and hypertrophy of goblet cells was evident and excessive secretion of mucus was noted. Hypertrophied cells contained numerous mucous granules, causing the cells to appear more elongated and larger than normal cells and the cell membranes were disrupted. Bacterial cells were present near goblet cells and were observed attached to the secreted mucus in the nasal cavity. Degenerated pmns with karyorrhexic nuclei, marginated chromatin and damaged cell membranes were observed. Many macrophages and plasma cells had infiltrated the submucosal layer. The plasma cells contained extensive rough endoplasmic reticulum (RER) and globulin precipitates in their cytoplasm. Collagen fibres were found in subepithelial area and beneath the detached cells. Enlarged endothelial cells and precipitation of electron dense material in the wall of blood vessels were noted in rabbits in groups 1 and 2 killed 14 and 21 days p.i.. The endothelial cells had large nuclei, foamy cytoplasm, and intracytoplasmic osmiophilic dense particles. Destruction of endothelial cell membrane was also noticed. In rabbits from groups 1 and 2 killed 14 and 21 p.i., many platelets were seen attached to or engulfed by intravascular macrophages.

Trachea The ultrastructural changes seen in the trachea in rabbits from group 1 killed 14 days p.i. were swelling of the non-ciliated cells with presence of large vacuoles inside the cytoplasm. Most of the organelles had disappeared and the rest were scattered into the cytoplasm (Fig 8). Balloon-like structures were observed on the cilia and microvilli of the ciliated cells. The cross sections of many cilia showed disorganisation of their microtubules (Fig 9). Bacterial cells were seen attached to the balloon-like deformation of the microvilli. Hyperplasia of goblet cells was also observed. Many PMN leukocytes and a few macrophages had infiltrated the subepithelial area. In rabbits from the same group killed 21 days p.i., sloughing of degenerated cells and infiltration by macrophages, plasma cells and a few heterophils were observed in the subepithelial layer. Fibroblast proliferation and collagen fibres formation were also noticed. In rabbits from group 2 killed 14 days p.i., the changes were milder in

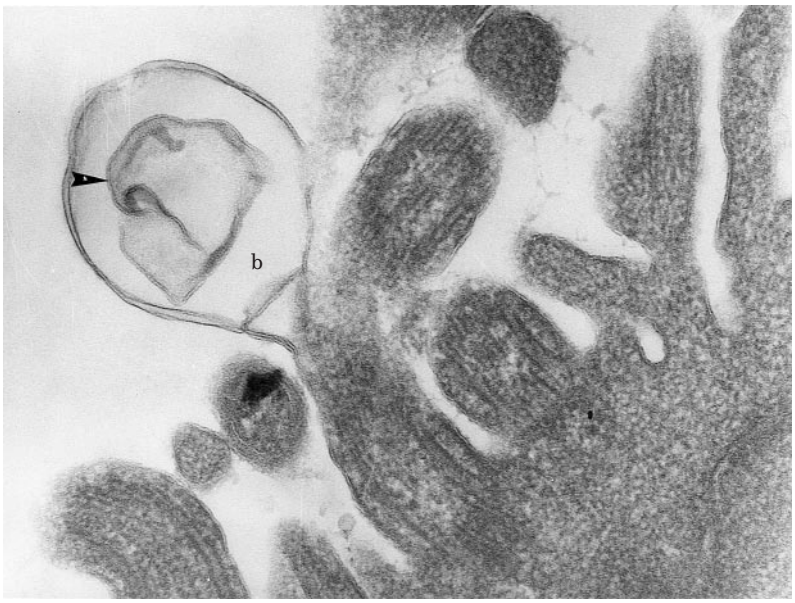

FIG 3: Electron micrograph. Nasal respiratory epithelium from a rabbit in group 1 infected with *P. multocida* and killed 21 days p.i.. Note presence of balloon-like (b) deformation on the lateral aspects of cilia with multilayered hemisphere structure (arrow head). TEM × 73,200

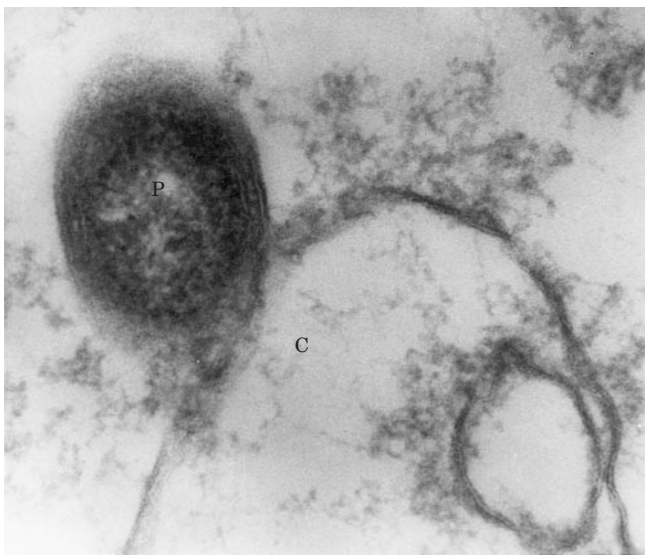

FIG 4: Electron micrograph. Nasal epithelium from a rabbit in group 2 infected with *Pasteurella multocida* and killed 21 days p.i.. Note *Pasteurella multocida* (P) attached to the swollen end of deformed cilia (C) × 100,000.

comparison to changes in group 1. Gaps between the basal cells was observed as a result of oedema. Many heterophils were seen that had infiltrated between the epithelial cells. In rabbits of the same group killed 21 days p.i., there were degenerative changes in the epithelial cells, focal loss of cilia and swelling of microvilli. Focal sloughing of degenerated cells leaving only one layer of basal cells was observed. Macrophages, plasma cells and a few heterophils had infiltrated the subepithelial area. In the blood capillaries in both groups, swelling of the endothelial cells and vacuolation of their cytoplasm had occurred. These cells contained intracytoplasmic osmiophilic particles and large nuclei. Intravascular platelets were numerous and some were attached to the endothelial cells or had been engulfed by macrophages. No ultrstructural changes were observed in the trachea of rabbits from group 3.

DISCUSSION

The clinical signs and gross pathological findings in our study agree with the previous experimental and natural infection studies of snuffles caused by *P. multocida* in rabbits (Lu et al 1982, DiGiacomo et al 1983).

Transmission electron microscopy (TEM) results showed ciliary destruction and deciliation of the ciliated epithelial cells, cellular swelling and goblet cell hyperplasia. These results agree with the results from our scanning electron microscopy study of the upper respiratory tract of rabbits infected with *P. multocida* type A (Al-Haddawi et al 1997).

In this study, *P. multocida* attached to the microvilli and to the mucus adjacent to the hyperplastic goblet cells. It is suggested that following attachment to the microvilli and mucus, *P. multocida* and its toxic products cause the cilia to deformed by inducing blebbing and thinning and rupturing of their membranes; subsequently deciliation occurs. These findings are in agreement with the results of previous studies, which reported that *P. multocida* adheres closely to the nasal respiratory non-ciliated epithelium (Pijeon and Trigo 1990). We found that some bacterial cells were attached to the deformed cilia, which may indicate the ability of this microorganism to adhere to the deformed rather than healthy cilia. The micro-organism can produce endotoxin and its component lipopolysaccharide (LPS) which has been reported to cause mucus secretion by goblet cells (Shimizu et al 1996) and to adhered to specific receptors on the mucus (Letellier et al 1991).

In the natural disease, the diminished ability of cilia to clear the pathogen as well as secretions from the respiratory tract during infection may provide an optimal environment

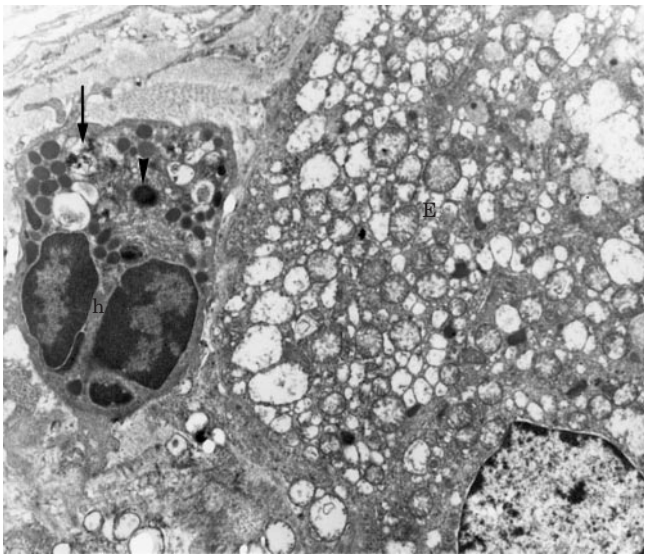

FIG 5: Electron micrograph. Nasal mucosa from a rabbit in group 2 infected with *P. multocida* and killed 21 days p.i.. Note nasal epithelial cells (E), with swollen mitochondria that have lost their cristea and a heterophil (h) between the epithelial cells. Note immature granules with a nucleoid (arrow head) or multicored content (arrow). TEM × 5600.

for increased colonisation by the organism during the course of disease. Thus, the deciliation of epithelial cells and induction of mucus secretion from goblet cells by *P. multocida* and its products resulted in a decrease ciliary clearance of the mucus. These changes led to impairment of bacterial clearance from the nasal cavity and provided an environment conducive for colonisation of the nasal cavity by *P. multocida*.

The TEM findings in the present study showed that the lesions of the nasal mucosa in group 1 were more severe than group 2. However, the hyperplasia of goblet cells was not evident in rabbits in group 1 killed at 14 days p.i which were pre-treated with hydrocortisone. These findings are consistent with the inhibitory effect of hydrocortisone on the endotoxin-induced intraepithelial mucus secretion as reported recently by Takahashi et al (1997). The deciliation caused by the organism and the depressive effect of hydrocortisone on phagocytic and killing activities of polymorhonuclear cells (Mandell et al 1970) may enhanced the rapid and heavy multiplication of *P. multocida* in the nasal cavity and subsequently severe damage to the tissue. Shimizu et al (1996) also reported that endotoxin-induced changes of goblet cells were significantly inhibited in neutrophil-depleted rats.

The ultrastructural changes in mitochondria and RER of the epithelial cells were either the result of toxic affects of *P. multocida* components or due to inflammatory reaction. In the present study, infiltration of heterophils in the subepithelial layer and between epithelial cells was observed. It is known that endotoxin is the most potent agent capable of inducing a neutrophil influx (Movat et al 1987). The presence of heterophils in the lesion may have been due to *P. multocida* and/or its products which are known to act as a chemotactic factors for leukocytes (Galdiero et al 1998, Latimer et al 1990). Furthermore, the presence of heterophils in different stages of maturation may be due to the role of LPS in accelerating the release of these cells from bone marrow pools (Klut et al 1998). On the other hand, the activated or fragmented heterophils in the inflammatory process may have released cytotoxic products that can disorganise the normal architecture of the tissue (Ganey et al 1994, Barnett et al 1998). Thus, the presence of these cells in inflammatory process may have been harmful to the nasal epithelium and cilia.

Plasma cells laden with globulins were observed in the nasal mucosa in rabbits of both infected groups, that indicates the immune response against *P. multocida*. Eosinophils were also found infiltrated near the plasma cells in the nasal mucosa of infected rabbits from group 1. The

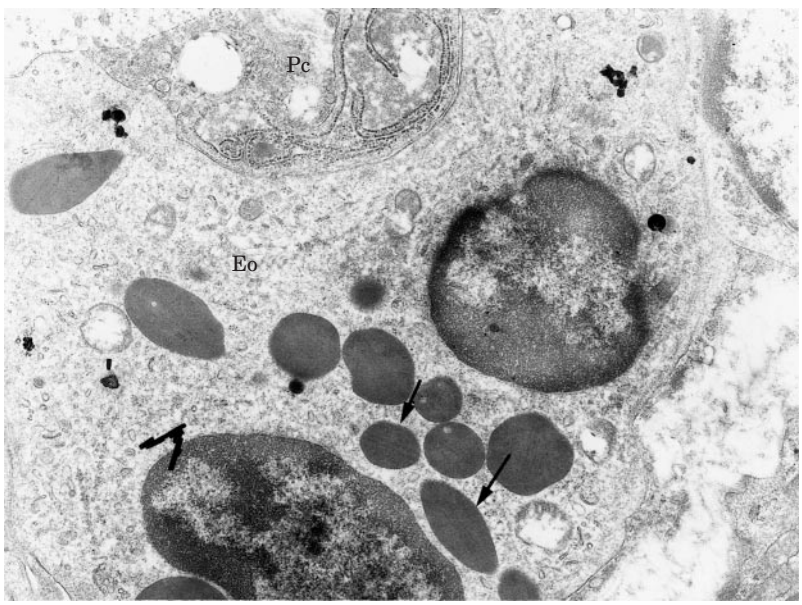

FIG 6: Electron micrograph. Section through eosinophil (Eo) adjacent to plasma cell (Pc) in nasal mucosa from a rabbit in group 1 infected with *P. multocida* and killed 21 days p.i.. Note the large crystalloid cytoplasmic granules (arrows). TEM × 11,000.

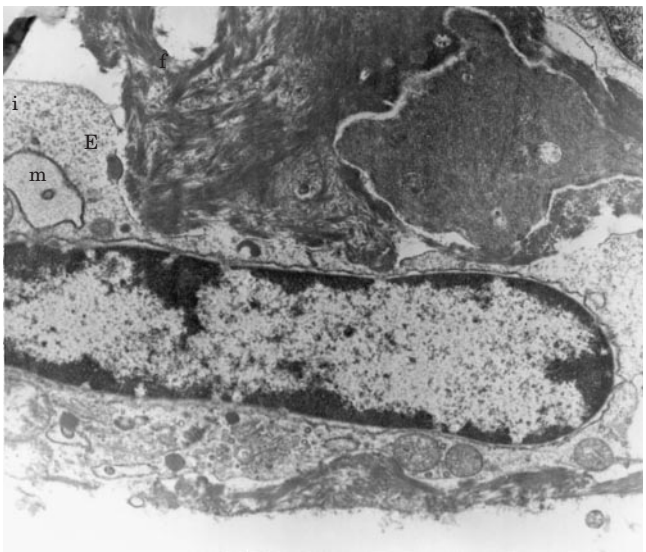

FIG 7: Electron micrograph. Section through a capillary in the nasal mucosa from a rabbit in group 1 infected with *P. multocida* and killed 14 days p.i.. Note intravascular fibrin (f) deposition adjacent the endothelial cell (E). The endothelial cell is swollen and has dilated mitochondria (m). TEM × 9400

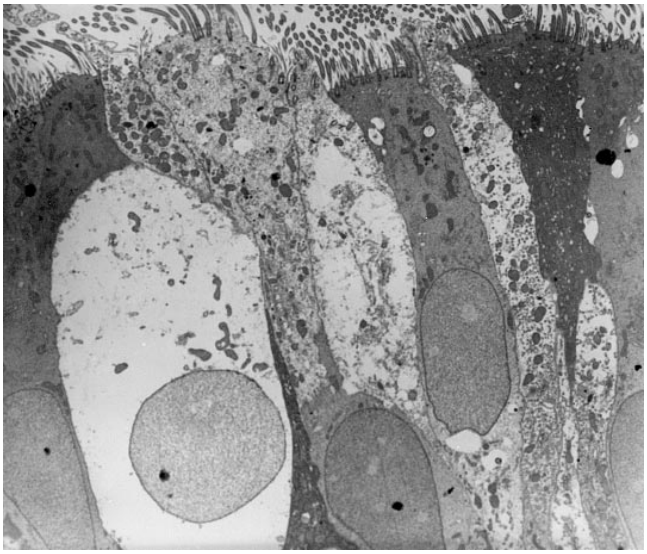

FIG 8: Electron micrograph. Tracheal mucosa from a rabbit in group1 infected with *P. multocida* and killed 14 days p.i.. Note the swelling of the epithelial cells and vacuolation of their cytoplasm. The organelles are scattered inside the cytoplasm. TEM. × 3000.

presence of eosinophils may be explained by an in-vitro study that has shown that antigen-antibody (Ag–Ab) complex can activate the complement (C) system to produce factors which are chemotactic agent to eosinophils (Walls et al 1971, Ward 1969). Eosinophils contain specific receptors for C and Ab on their surface (James and Colley 1978) and it has been reported that eosinophils have a curious appetite for Ag–Ab complex (Litt 1963).

Ultrastructural changes similar to that in the nasal mucosa were observed in the tracheal mucosa. Excessive secretion of mucus and loss of cilia were the prominent changes. The inhibition of goblet cell secretion in rabbits from group 1 was not observed. This may be because the late invasion of trachea by *P. multocida* occurred after the disappearance of hydrocortisone effect.

Some capillaries in the nasal and tracheal mucosa contained swollen endothelial cells, which had damaged cell membranes. Their cytoplasm was vacuolated and had osmiophilic particles. Lipopolysaccharide can cause detachment and altered morphology of bovine endothelial cells in maintained culture (Gartner et al 1988) and increased turnover of endothelial cells in rat aortic endothelium (Reidy and Schwartz 1983). Stern et al (1985) suggested that endothelium participates in the regulation of inflammatory/immunologic phenomena. Administration of LPS has been demonstrated to induce the secretion of several

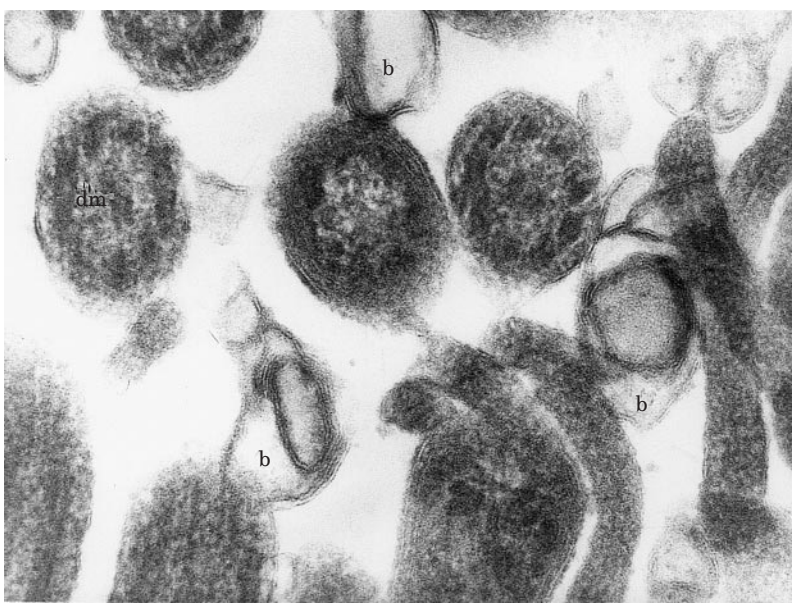

FIG 9: Electron micrograph. Tracheal epithelium from a rabbit in group 2 infected with *P. multocida* and killed 14 days p.i.. Note the focal blebbing (b) of cilia tips and cross section of cilia with disorganised microtubules (dm). TEM × 80,500.

cytokines by macrophages including IL-1, IL-6, IL-8, tumour necrosis factor (TNF) and platelets activating factor (PAF) (Salyers and Whitt 1994). The IL-1 subsequently can promote endothelial cells synthesis and expression of tissue factor (Bevilacqua et al 1984). This factor acts as a cofactor in the initiation of coagulation (Rashid et al 1997). Once activation of coagulation is initiated, the endothelium can promote an entire pathway of coagulation leading to fibrin formation (Stern et al 1985). The release of cytokines from the activated macrophages will subsequently cause damage of endothelial cells through the activation of complement, coagulation cascades and production of prostaglandin (Proctor et al 1980). It has been reported that phagocytosis of bacteria by neutrophils is associated with the release of oxygen radicals and lysosomal proteases from the neutrophils. These products can cause microvascular injury (Movat et al 1987).

Aggregation of platelets was also evident in the microvessels of nasal mucosa and trachea in infected rabbits, in association with intravascular macrophages. The association of platelets with intravascular macrophages was reported with endotoxin of *P. hemolytica* in the lungs of calves (Whiteley et al 1991). The endotoxin-activated macrophages produced platelets activating factor which enhances the aggregation of platelets.

From this experiment, it is clear that *P. multocida* of heavy and pure growth caused damage to the epithelial and endothelial cells, hyperplasia of goblet cells and resulted in inflammatory responses, which subsequently may have caused tissue damage. Deciliation and stagnation of mucus promoted colonisation of *P. multocida* by induction of ciliastasis.

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