FULL PAPER *Pathology*

Epithelial Cell Kinetics in the Inflammatory Process of Chicken Trachea Infected with Infectious Bronchitis Virus

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ABSTRACT. All stages of degeneration and regeneration in chicken tracheal epithelium were studied morphologically following an intratracheal inoculation of infectious bronchitis virus (IBV). Viral antigen was detected in the cytoplasm of tracheal epithelium from 1 to 7 days post-inoculation (d.p.i.) with a peak on 3 d.p.i. At 1 d.p.i., almost all epithelial cells were involved in the degeneration. At this time, labelling index of bromodeoxyuridine (BrdU) in the basal cells showed significantly high value compared with control. At 2 and 3 d.p.i., a great number of basal cells were recognized, but the BrdU labelling index tended to decrease. At 4 and 5 d.p.i., the BrdU labelling index of basal cells significantly decreased than 1 d.p.i., and a few number of regenerated immature ciliated epithelia appeared. At 6 to 11 d.p.i., the ciliated columnar epithelia increased rapidly in number, and returned to the normal appearance except for non-ciliated patch by 13 d.p.i. These results suggested that the tracheal epithelial cells infected with IBV degenerated within 24 hours and proliferating activity of basal cells functioned immediately, and 3 to 4 days later, these basal cells were differentiated to the ciliated epithelia.—KEY WORDS: bromodeoxyuridine (BrdU), chicken, infectious bronchitis virus, regeneration, trachea.

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Since the respiratory system communicates directly with the outside of the body, foreign substances such as microorganisms and dust are always inhaled. However, the mucociliary system of the respiratory tract is able to clear the inhaled agents [19]. This mucociliary system is destroyed by infection with various pathogenic microorganisms and inhalation of harmful gases. Studies on the repairing process of injured tracheal mucosa have been reported on the mechanical wound in rats [9] and hamsters [10, 11], inhalation of formaldehyde in rats [18], ozone in monkeys [24] and toxic cotton smoke in sheep [23].

Infectious bronchitis (IB) is one of the most important respiratory diseases in chickens. The respiratory system is the primary multiplication site of IB virus (IBV), thereafter the virus distributes to other organs [8]. Death of chicken with IB occurs due to renal failure in the nephrotropic strain [2, 26], but tracheal lesion repairs almost completely within 2 or 3 weeks after infection when the infected chicken survived [6, 14, 16, 17, 25]. Therefore the chicken trachea infected with IBV would be a useful model to study the regenerating process of virally injured trachea.

The present study investigated morphologically repairing process of the IBV-induced tracheitis by light and scanning electron microscopy, and the kinetics of epithelial cell proliferating activity using bromodeoxyuridine (BrdU) for detection of DNA replication.

MATERIALS AND METHODS

Chickens: Thirty-nine 21-day-old specific-pathogen-free (SPF) White Leghorn chickens (Line LMG) that were kindly provided by a certain breeder in Aburahi of Shiga prefecture were used in this study.

Virus: The A1 strain of IBV (IBV-A1) was used in this experiment. This virus was isolated from nephritic kidneys of a 30-day-old chicken of a broiler flock in Hyogo prefecture, and it has been passaged six times in embryonated eggs and twice in chicken kidney cell culture.

Experimental infection and autopsy: Thirty-six chickens were inoculated intratrachealy with 0.2 m*l* of viral inoculum containing 3.2×10^3 EID_{50}/0.2 m*l* of IBV-A1. As controls, three chickens were autopsied before IBV inoculation. Clinical signs were recorded twice a day in the morning and evening. At 1–5, 7, 9, 11, 13, 17 and 21 days post-inoculation (d.p.i.), three chickens were killed by pentobarbital anesthesia and the larynx and trachea were immediately removed. One hour before killing, BrdU (5-bromo-2'-deoxyuridine; Sigma Chemical) was injected subcutaneously (100 mg/kg body weight). The dorsal part of the trachea was cut vertically, and the mucous membrane was examined grossly. The tracheal specimens for light microscope (LM), for detection of viral antigen and scanning electron microscope (SEM) were collected in the respective order, 2 cm, 0.5 cm and 0.5 cm in length from the anterior part of trachea.

Detection of viral antigen: The viral antigen in trachea was detected by a direct immunofluorescent method. Frozen sections, fixed with cold acetone, were incubated with fluorescein isothiocyanate (FITC)-conjugated chicken anti-IBV antibody in a moist chamber at 4°C overnight and specific fluorescence was examined under a fluorescent

microscope.

Scanning electron microscopy: The tracheal specimens for SEM were washed several times with phosphate buffered saline (PBS) at 4°C, fixed in 1% phosphate buffered glutaraldehyde for 3 hr at 4°C, washed overnight in PBS, post-fixed 1% osmium tetraoxide for 1 hr at 4°C, washed in PBS, dehydrated in graded series of ethanol, critical-point dried, coated with gold, and examined under the SEM (Hitachi S-800) at 10 kV.

Histopathology and assay for labelling index: The tracheal specimens for LM were fixed with Bouin's fluid, embedded in paraffin, sectioned at 3 μm and stained with hematoxylin and eosin (H-E). The proliferating cells (S-phase cells) whose nuclei incorporated BrdU were visualized using anti-BrdU monoclonal antibody by an immunohistochemical technique [7, 20]. Tracheal sections were de-paraffinized, inactivated by 0.3% H_2O_2 in methanol for 20 min, washed in PBS, treated in 4N HCl to denature double strand DNA for 20 min, washed in PBS, incubated with monoclonal anti-BrdU antibody (DAKO) diluted 1/400 in PBS for 1 hr, washed in PBS, incubated with peroxidase-conjugated rabbit anti-mouse IgG (DAKO) diluted 1/800 for 1 hr, washed in PBS, reacted by adding chromogenic substrate solution containing diaminobenzidine, counterstained whole nuclei with hematoxylin. The labelling index was determined by counting 1,200 epithelial nuclei in each ventral and dorsal tracheal mucosa, and expressed as the percentage of labelled/total nuclei. The values of each group were compared with the control or 1 d.p.i. group by Student *t*-test. Statistic significance was at $P<0.05$.

RESULTS

Clinical signs: Moderate to severe respiratory signs, such as rales and sneeze, and depression were recognized from 1 to 5 d.p.i. and continued slightly by 7 d.p.i.

Gross findings in the trachea: Moderate or severe catarrhal tracheitis accompanied by hyperemia and hypertrophy of the mucosa and increased mucus in the lumen was observed on 1 to 6 d.p.i., and mild catarrhal tracheitis continued until 7 to 9 d.p.i.

Viral antigen: The specific fluorescence of IBV antigen was observed in the cytoplasm of tracheal epithelia and desquamated epithelia from 1 to 7 d.p.i. with a peak on 3 d.p.i. (Figs. 1, 2). On 7 d.p.i., only a few positive cells were detected. After 9 d.p.i., no specific fluorescence of IBV was observed.

Scanning electron microscopic changes in the trachea: The surface of normal trachea was covered with abundant cilia and some goblet cells (Fig. 3A). On 1 d.p.i., cilia of the tracheal mucosa disappeared almost completely in the whole area and the surface of epithelial cells was covered with rough and indistinct microvilli (Figs. 1, 4A). On 2 and 3 d.p.i., the surface of mucosa was covered by the various sized degenerated epithelial cells which had indistinct microvilli. Desquamated epithelial cells, inflammatory cells and erythrocytes were observed on the surface of mucosa

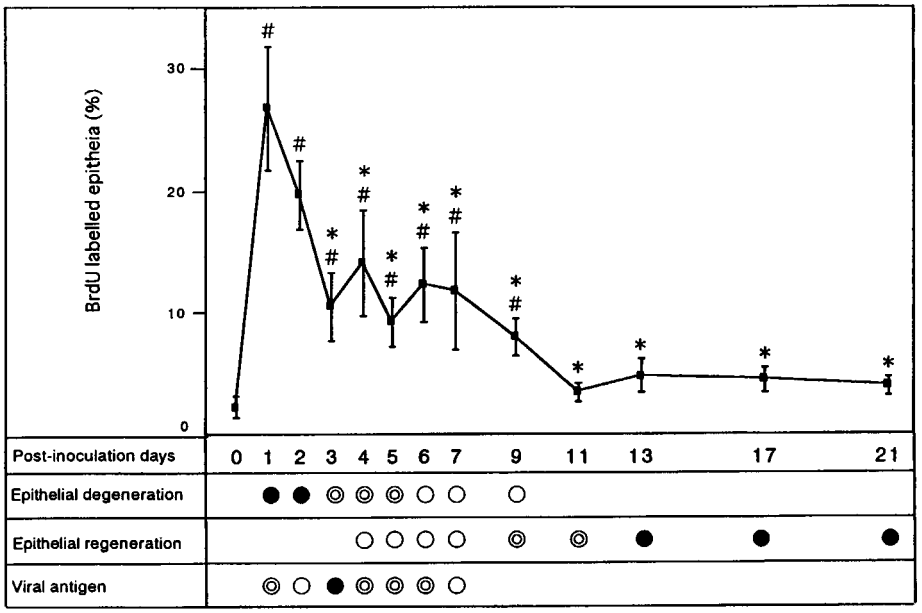

Fig. 1. Chronological changes of the bromodeoxyuridine (BrdU) labelling index, degeneration, regeneration and specific viral antigen in the tracheal epithelium infected with infectious bronchitis virus (IBV). *: significantly ($P<0.05$) different from control, #: significantly ($P<0.05$) different from group of 1 day post-inoculation (d.p.i.) (Student *t*-test). ○, ◎, ●: slight, moderate and remarkable changes in scanning electron microscopy (SEM) and immunofluorescent method.

(Fig. 5A). On 4 and 5 d.p.i., there were a few regenerated epithelial cells which had distinct and abundant microvilli with a small number of short cilia (Fig. 6A). On 6 to 11 d.p.i., ciliated cells were increased in number and the length and density of cilia were also increased (Fig. 7A). After 13 d.p.i., the surface of tracheal mucosa was covered with cilia except for small plaques covered with nonciliated flat epithelium, which were observed until 21 d.p.i., with the area gradually decreasing (Figs. 8A, 8B).

Histopathology: Normal trachea had a few BrdU positive basal cells (Fig. 3B). On 1 d.p.i., ciliated columnar epithelial cells of trachea degenerated toward non-ciliated cubic or flat cells, and goblet cells disappeared (Fig. 4B). Basal cells were markedly increase in number. A great number of BrdU positive nuclei were observed in the basal cells (Fig. 4C). There were severe hyperemia and edema in the submucosa and infiltration of heterophils and lymphocytes into the tracheal lumen and submucosa. On 2 and 3 d.p.i., large vacuolar spaces with inflammatory exudates were formed between the degenerated epithelial cells and proliferating basal cell layer. BrdU positive nuclei were detected in many basal cells (Fig. 5B), but they were significantly decreased on 3 d.p.i. from 1 d.p.i. (Fig. 1). On 4 and 5 d.p.i., although degenerative epithelial cells,

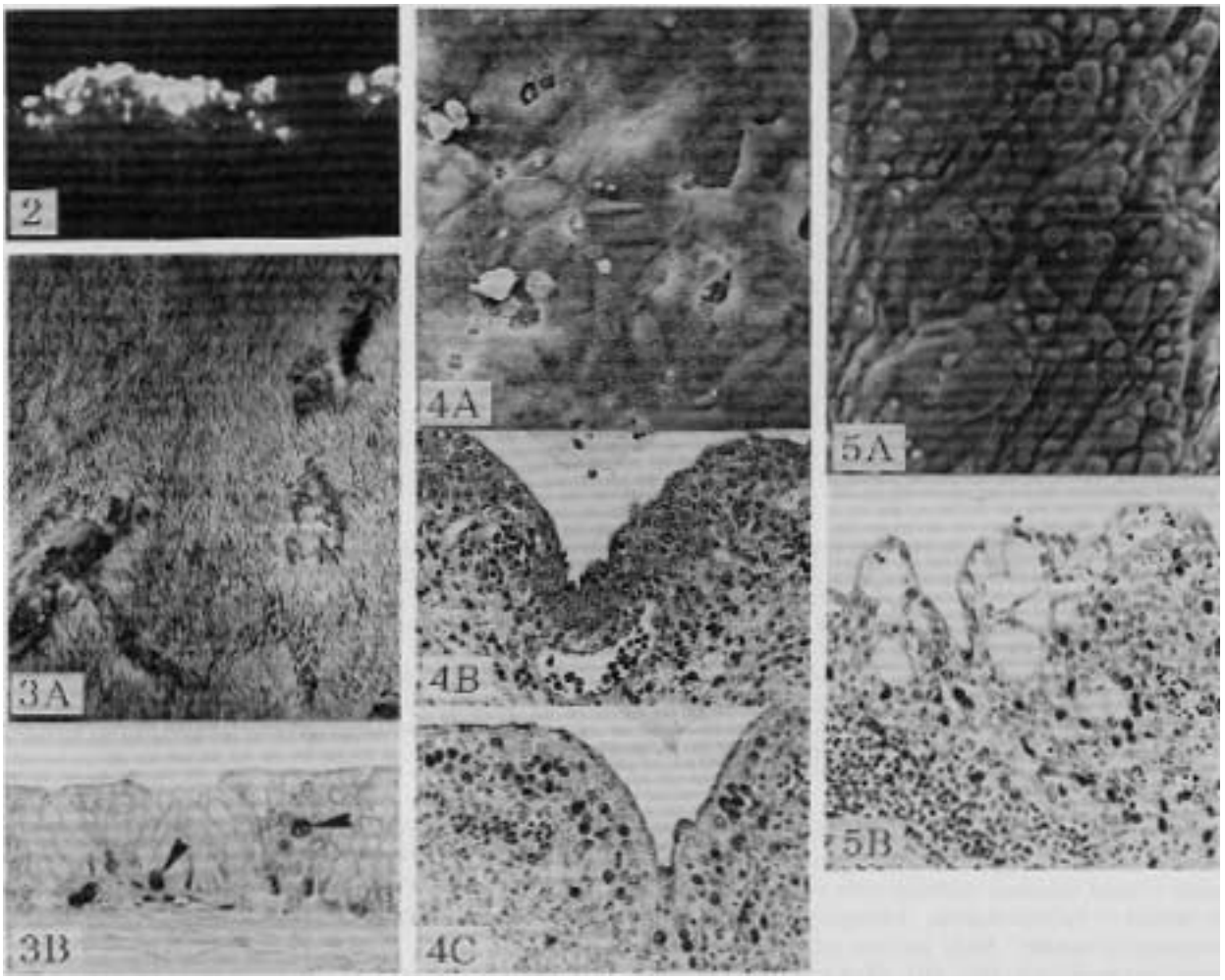

Fig. 2. IBV specific antigen in the cytoplasm of tracheal epithelium on 3 d.p.i. Direct immunofluorescent method. × 260.

Fig. 3. Trachea of control chicken. 3A: Surface of trachea is covered with abundant cilia. SEM. × 630. 3B: BrdU positive nuclei are detected in a few basal cells (arrow heads). Immunohistochemistry for BrdU. × 350.

Fig. 4. Tracheal mucosa on 1 d.p.i. infected with IBV. 4A: Marked deciliation in almost all area. SEM. × 630. 4B: Epithelial cells have been degenerated to cuboidal or flattened in shape and the basal cells have already begun to proliferate. Infiltration of heterophils and lymphocytes, congestion and edema are observed in the submucosa. Hematoxylin and eosin stain. × 240. 4C: Many nuclei of basal cells are labelled by BrdU. Immunohistochemistry for BrdU. × 240.

Fig. 5. Tracheal mucosa on 2 d.p.i. infected with IBV. 5A: All cilia have disappeared, and the inflammatory cells are seen on the degenerated epithelial cells. SEM. × 630. 5B: Large edematous spaces with inflammatory exudate are recognized between the degenerated epithelia and basal cells. BrdU positive nuclei of basal cells existed, showing a tendency to decrease as compared with those of 1 d.p.i. Immunohistochemistry for BrdU. × 240.

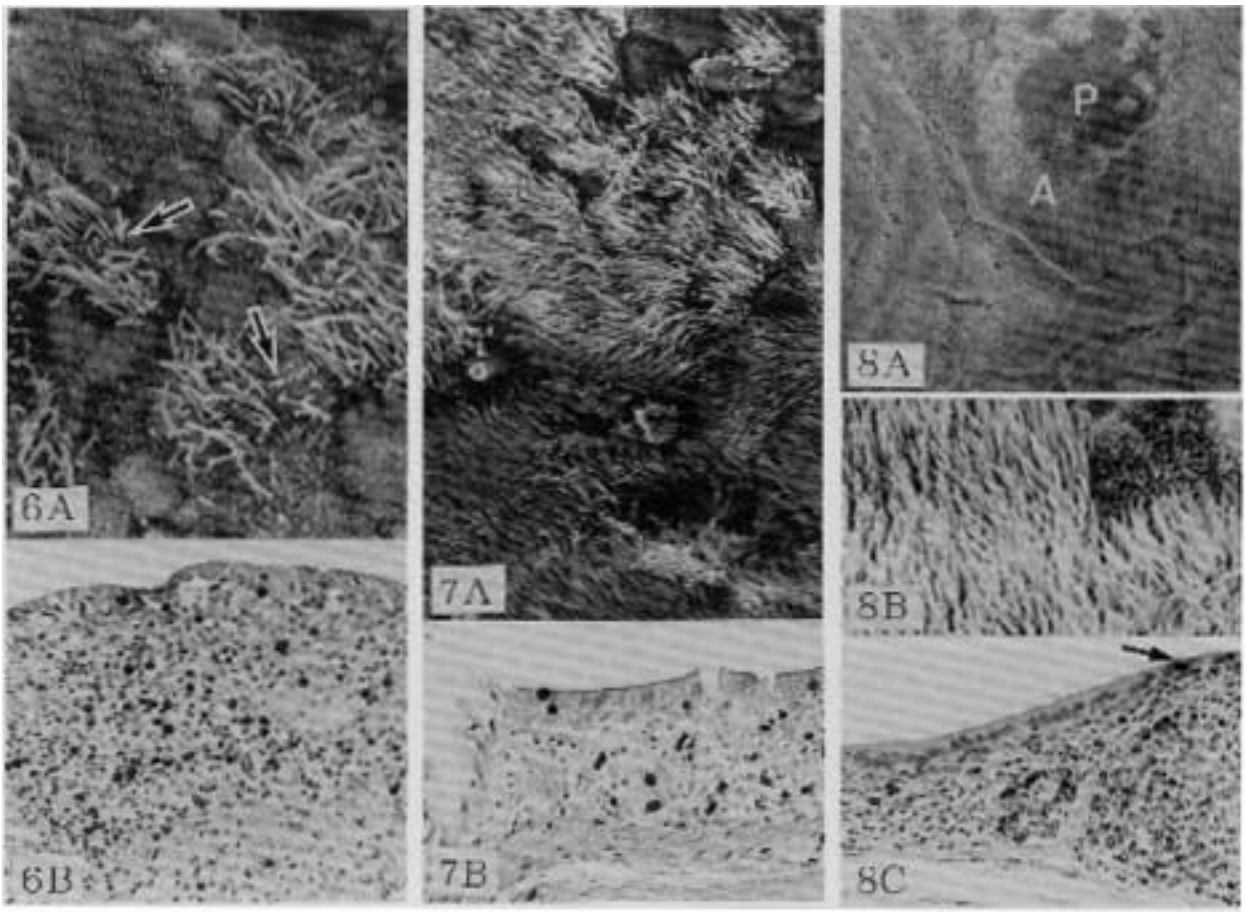

Fig. 6. Tracheal mucosa on 5 d.p.i. infected with IBV. 6A: Immature regenerated epithelial cells have a few short cilia (arrows). SEM. × 2,100. 6B: BrdU positive basal cells are recognized in moderate number. Immunohistochemistry for BrdU. × 240.

Fig. 7. Tracheal mucosa on 11 d.p.i. infected with IBV. 7A: Ciliated area extended by regeneration. SEM × 1,020. 7B: Columnar ciliated epithelial cells are arranged on the surface of mucosa. BrdU positive basal cells are decreased in number. Immunohistochemistry for BrdU. × 240.

Fig. 8. Tracheal mucosa on 21 d.p.i. infected with IBV. 8A: Complete regeneration of cilia is seen except for non-ciliated plaque (P) (See Fig. 8B for site A). × 220. 8B: High-power magnification of site A in Fig. 8A. Boundary area of non-ciliated plaque and ciliated area. SEM. × 1,860. 8C: BrdU positive basal cells are very few (arrow). Some BrdU positive lymphocytes are seen in lymphoid follicle in submucosa. Immunohistochemistry for BrdU. × 240.

stratified basal cells and vacuolar spaces in the epithelial layer were still recognized, some regenerated cubic and faintly ciliated columnar epithelial cells were recognized on the surface of tracheal mucosa. Infiltrated heterophils were decreased in number. BrdU positive epithelial cells were recognized moderately (Fig. 6B). On 6 to 11 d.p.i., ciliated columnar epithelial cells increased in number rapidly. Moderate or small numbers of BrdU positive cells were recognized (Fig. 7B). On 13 to 21 d.p.i., the surface of tracheal mucosa was almost covered with regenerated columnar ciliated epithelium and there were no proliferation of basal cells and subepithelial infiltration of inflammatory cells (Fig. 8C). In the area with dense accumulation of lymphocytes, plasma cells and monocytes, the mucosa was covered by the nonciliated flat epithelial cells. Lymphoid follicles were recognized in the subepithelial layer after 13 d.p.i. BrdU positive nuclei were observed in a few epithelial cells (Fig. 8C). Some lymphocytes were positively stained with BrdU in the germinal center of lymphoid follicles (Fig. 8C).

Labelling index of BrdU in the tracheal epithelium: Figure 1 shows the chronological change of the BrdU labelling index in the tracheal epithelium. The BrdU labelling index of normal chicken trachea was 2.8 ± 0.8% (mean ± SD). A dramatic increase in the labelling index of BrdU positive epithelium occurred at 1 d.p.i. (27.0 ± 5.0%). Although the labelling index decreased gradually after 2 d.p.i. (20.2 ± 2.8%), it still exceeded 10% in the epithelium until 7 d.p.i., and reverted to normal by 11 d.p.i. (4.0 ± 0.8%).

DISCUSSION

Replication of IBV occurs in the ciliated epithelium and mucous cell within 24 hr after intratracheal or aerosol inoculation, and viral particles are confined to small vacuoles of cytoplasm [3, 16, 22]. Following aerosol inoculation of IBV, virus is recovered from the trachea at 24 hr and through 7 or 8 days [8]. In our experiment, IBV antigen was detected in the tracheal epithelium from 1 to 7 d.p.i. Degeneration of tracheal epithelia was observed from 1 to 9 d.p.i., proliferation of basal cells started from 1 d.p.i. and regeneration of epithelial cells began from 4 d.p.i. There have been many reports on tracheitis infected with IBV. Although the pathology on the affected trachea is varied due to different inoculation route, strain and inoculum size of IBV and age of chicken infected, chronological tracheal changes are essentially the same [3, 6, 14, 17, 25]. The tracheal epithelial changes were classified as 3 stages; 1) acute desquamation or degeneration, 2) hyperplasia, and 3) regeneration or recovery stages [14, 17]. In our experiment, tracheal epithelial changes were similar to those described previously.

The autoradiographic and immunocytochemical techniques using tritiated thymidine ([^{3}H]TdR) and BrdU, respectively, are both well known procedures for labelling cells in the S-phase of the cell cycle [1, 7, 9, 12, 13, 18]. In recent years the BrdU labelling technique is widely used to investigate replicating cell kinetics *in vivo* because it is quicker and safer than [^{3}H]TdR. The results both techniques are not significantly different from each other [1]. The synthetic activity has been shown to occur in rat tracheal basal cells which had been arrested in G_1-phase [12]. In this study, BrdU positive basal cells suddenly increased in number from 1 d.p.i. and significant increase in number continued until 9 d.p.i. as compared with those in controls. The highest value of BrdU labelling index was attained on 1 d.p.i., and the value significantly lowered after 3 d.p.i. than that on 1 d.p.i. After the mechanical injury in rat trachea, a dramatic increase of the labelling index of BrdU occurred in the epithelium between 12 and 18 hr. Although the frequency decreased gradually after 18 hr, it exceeded 10% in the epithelium until 5 days after injury, and normalized within 7 days [9]. In mild mechanical injury in vitamin A deficiency rats, proliferative cells passed through a period of DNA synthesis with the greatest numbers of thymidine incorporating cells at 22 hr after injury. A peak of cell division occurred at 32 hr and there was no further DNA synthesis or cell division [12].

In response to a mechanical injury of hamster tracheal epithelium, viable basal cells migrated into the denuded wound sites, covering the small wounds by 12 hr and larger wounds by 48 hr [10]. By 27 hr a peak of mitotic rate was reached in small wound sites, but mitotic activity in the large wound sites did not peak until 33 hr [10]. A very few pre-ciliated cells appeared first at 33 hr and they peaked in numbers at 3 days in both wound sites. Ciliated cell numbers were restored to control level in the small wound sites by 7 days, but only to 8.4% of control values in the larger wound sites, were epidermoid metaplasia persisted [10]. In the inhalation of toxic cotton smoke in sheep, there was a stratified reparative epithelium over the basal lamina at day 4, and returned to normal number and size and columnar shape by day 18 [23]. In our study, regeneration of immature ciliated epithelia was observed after 4 d.p.i., and after 13 d.p.i., the surface of trachea was almost covered with regenerated columnar ciliated epithelia. These results indicated that the epithelial cells infected with IBV degenerated within 24 hr and proliferating activity of basal cells functioned immediately, and that the basal cells differentiated to the immature ciliated epithelia at 3 days later. In experiments causing mechanical wounds in the tracheal mucosa in the rat and hamster, the injurious stimulus was only once [9, 10, 12]. However, the tracheal injury with IBV infection continued for several days. Therefore, in the case of viral infection, the epithelial degeneration coexisted with regeneration for several days postinoculation and the repairing process was lingered.

Several cell adhesion molecules including gicerin, N-CAM, cadherin, selectin and integrin have been reported to be involved in the various tissue regeneration [4, 5, 15, 21]. Furthermore, extracellular matrices such as neurite outgrowth factor (NOF), fibronectin and tenascin, maybe interacted in the morphogenesis of various tissues [9, 21]. In our previous report, expressions of gicerin and NOF molecules were observed in the proliferating basal cell layer of trachea at 1 to 4 d.p.i. of IBV [21]. These findings may contribute to further investigation for pathogenesis, especially on the repairing process of tracheitis caused by IBV.

Pathological examination of tracheal mucosa infected with IBV is important to investigate the pathogenesis of IB as an avian disease. On the other hand, it might be a useful animal model to observe the repairing process of tracheal lesions in the viral infection.

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