STUDIES ON THE NASAL HISTOLOGY OF EPIDEMIC INFLUENZA VIRUS INFECTION IN THE FERRET

II. The Resistance of Regenerating Respiratory Epithelium to Reinfection and to Physicochemical Injury

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Plates 40 and 41

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Iontophoresis of the nose, using a weak galvanic current and a solution of 1 per cent zinc sulfate, has been shown to cause a disintegration of the nasal mucosa, an inflammatory reaction in the submucosa, and hyperemia of the venous sinuses (1, 2). Boling (3) also showed that if zinc iontophoresis was applied in sheep to a portion of the nasal mucosa which included an area of epithelium regenerating after traumatic removal, this area alone was uninjured. He concluded that the stratified cells covering a repairing mucosa were resistant to injury and were even stimulated to more rapid development as evidenced by an increased number of mitoses. Because of the similarity of the repair process following influenza virus infection (4) and that described by Boling, it was of interest to study the resistance in the former instance to specific and non-specific injury. Using a technique similar to that employed by Boling, the effect of intranasal zinc iontophoresis was studied in normal ferrets and in ferrets convalescent from an attack of influenza.

Experimental Methods

The procedure used was to anesthetize the ferret with one or more intraperitoneal injections of evipal[1] (5.0 cc. of a 1 per cent solution was usually sufficient),

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[1] Sodium salt of *n*-methylcyclohexenylmethyl barbituric acid.

and then to instil a few drops of 2 per cent cocaine into each nostril. The anode, a fine platinum-iridium wire 1 mm. in diameter, was inserted into one nostril and a gentle stream of 1 per cent zinc sulfate solution was introduced by gravity from a reservoir into the other nostril through a fine intravenous needle inserted into the nostril. The cathode, a sheet of copper covered with gauze and moistened with saline, was fastened over a clipped area of skin on the back. After several trials with different strengths of current the following procedure was adopted: A current of 3 milliamperes at between 8 and 10 volts was passed for 2½ minutes; the anode and irrigating needle were then reversed and the current was again passed for a further 2½ minutes. In this way both sides of the nose received identical treatment. Aspiration of the irrigating solution into the lung was minimized by inserting a bent glass tube over the back of the tongue so as to drain the pharynx. The ferrets usually regained consciousness ½ to 1 hour after the operation.

The dissection of the nose and the preparation of sections were carried out as in the preceding paper (4). The tests for resistance to reinfection with influenza virus were made by the intranasal inoculation of a 10 per cent suspension of PR8 strain of virus.

The Effects of Ionization on the Normal Nasal Mucous Membrane

In previously untreated ferrets sacrificed 3 or 4 hours after intranasal ionization the turbinates appeared hemorrhagic and darkened. Microscopically almost the entire epithelium of the nose, both respiratory and olfactory, showed a coagulative necrosis. At this stage, the epithelium appeared to be lifted off the basement membrane and had the hazy outline of coagulated tissue. The basal cell layer, however, was intact. The submucosa showed intense hyperemia and diapedesis of polymorphonuclear leucocytes, and a purulent exudate was already present in the nasal passages.

In the following series of ferrets, the nose was usually examined on the 1st day after ionization because the coagulated epithelium had then been desquamated and removed so that the gross appearance was less obscured by the dead tissue. At this stage the changes in the respiratory area (Fig. 1) were very like those present in the nose on the 2nd day of influenza. The high power view (Fig. 2) shows the exudate, the desquamated epithelium with intact basal layer, and the inflammatory changes of the submucosa. In contrast, however, to the absence of damage to the olfactory epithelium as a result of infection with the PR8 strain of influenza virus, zinc ionization resulted in a complete desquamation of the entire epithelium in the olfactory area. The congestion and inflammatory changes were the same as those seen in the respiratory area and Bowman's glands were stimulated to increased secretion. Having thus established the picture produced by ionization in normal ferrets, the same technique was next employed in ferrets convalescent from influenza virus infection.

The Effect of Ionization or Reinfection on the Respiratory Mucous Membrane of Convalescent Ferrets

A series of ferrets was subjected to intranasal ionization with zinc sulfate at various intervals during convalescence from experimental influenza, and sections were prepared from the turbinates 24 hours later (Table I). Since the primary purpose was to test the resistance of the regenerating respiratory epithelium, the earliest tests were carried out in ferrets 7 to 8 days after infection, at which time a well developed transitional or stratified squamous type of epithelium covers the respiratory area. At this stage of convalescence reinoculation of influenza virus into the nose elicited no detectable reaction either clinically or anatomically. In

TABLE I

Results of Ionization on the Nasal Mucosa of Ferrets at Different Intervals after Infection with Influenza Virus

Ferret No.	Day after infection	Result
11-06*	7th	No effect
11-18	7th	" "
10-95	8th	" "
10-94	15th	Partial necrosis of respiratory mucosa; complete necrosis of olfactory mucosa
10-78	21st	Partial necrosis of respiratory epithelium; complete necrosis of olfactory epithelium
11-11	28th	Almost complete necrosis of respiratory epithelium; complete necrosis of olfactory epithelium
11-03	38th	Almost complete necrosis of respiratory epithelium; complete necrosis of olfactory epithelium

* Examined immediately. All other ferrets examined 24 hours after ionization.

Figs. 3 and 4 is shown a section from the turbinate of a ferret (11-01) tested with virus intranasally on the 8th day and sacrificed on the 11th day after the original infection. The epithelium revealed no evidence of new damage but presented a state of repair characterized by stratified columnar epithelium with early cilia formation. The state of repair was quite typical of that seen on the 10th and 11th days after a single inoculation. A control ferret (10-99) in its 8th day after primary infection with virus was sacrificed for comparison at the same time the previous animal was given its second virus inoculation. It revealed a repair process (Figs. 5 and 6) typical of the 8th day of convalescence with epithelium in the phase of change from transitional to stratified columnar type.

Since it was shown that the abnormal transitional epithelium of the convalescent nasal respiratory mucous membrane was completely

resistant to reinoculation of the virus, it was of further interest to determine whether this resistance was specific for the virus. If, however, the resistance of the regenerating epithelium should be effective against destructive agents of an entirely different nature, such as that furnished by ionization with zinc sulfate, it would represent an instance of tissue immunity or refractoriness unrelated to resistance in the ordinary immunological sense. Consequently ferrets were tested by intranasal ionization at different periods in convalescence from influenza virus infection.

Ferrets treated by ionization on the 7th and 8th days after infection with influenza virus behaved just like those receiving a second inoculation of virus. There was no evidence of damage to the nasal mucosa as a result of the procedure. Sections of ferret 10-95 (Figs. 7 and 8) revealed a picture quite comparable to the non-ionized control (Figs. 5 and 6) except that repair in the latter seemed slightly less advanced due probably to a difference of 24 hours in the time of death. Moreover, in contrast to the widespread damage to the olfactory epithelium created by ionization in the normal ferret, the convalescent ferret on the 7th to 8th day showed complete absence of reaction in the olfactory area.

In order to exclude the possibility that ionization at this stage of repair of the influenzal nasal lesion might produce only a slight transient damage which would be repaired in the 24 hour period between ionization and the removal of the turbinates, the nasal passages of 2 ferrets (11-06 and 11-18) were subjected to ionization on the 7th day of influenzal infection. One was sacrificed immediately, the other after 24 hours. Sections of neither showed evidence of recent necrosis or damage, while the repair of the influenzal virus lesion was in both instances in approximately the same stage.

These results show clearly that the resistance of the abnormal epithelium present in the respiratory area of the nose on the 7th to 8th day after influenza virus infection is extremely effective and is non-specific. The refractory state of the epithelium withstands further damage by the original destructive agency and furthermore is completely resistant to a physicochemical agent which in the normal ferret creates a widespread destruction.

After this stage in the convalescence from influenza virus infection different effects were noted.

While the epithelium of the nose was unaffected by reinoculation of virus during the 3rd and 4th weeks of convalescence (see ferrets 10-43, 10-44, 10-46, 10-50, and 10-52 in the third paper of this series (5)), varied degrees of damage were occa-

sioned by the ionization process. Thus on the 15th day of convalescence, when the non-ionized control (10-98, Fig. 9) revealed a stratified columnar ciliated epithelium, ionization resulted in a partial desquamation of the epithelium (10-94, Fig. 10). While the residual epithelium was two, three, or four cells thick the ionization seemed to have effected the removal of most of the superficial columnar cells, leaving the intermediate and basal cells intact (Fig. 11). Nevertheless, comparatively large numbers of ciliated cells escaped.

Ionization on the 21st day after infection produced a picture similar in some places to that at 15 days. Desquamation of the superficial ciliated cells had occurred although one or two of the deeper cell layers remained intact. In other areas, however, the destruction was almost as extensive as in the normal ferret after ionization in that the epithelium had been shed leaving only the basement membrane and pavement epithelium. Similar effects were obtained in a ferret subjected to ionization 28 days after virus infection (Fig. 12) in which the response resembled that of the normal ferret. The epithelium had been shed, the submucosa was infiltrated with inflammatory cells, and an exudate had formed. There were, however, both in this ferret and in the one treated on the 38th day of convalescence surviving patches of epithelium of a ciliated columnar type which had not been affected by ionization. While it is true that in the previously untreated ferrets occasional patches of ciliated columnar epithelium remained intact after the ionization process was applied, they were never as extensive as in the convalescent influenza ferrets treated in the same manner.

These observations reveal clearly that on the 8th day of convalescence from influenza virus infection, the polygonal transitional or stratified squamous epithelium which covers the nasal respiratory area is resistant to reinfection and to physicochemical injury. In the next weeks, however, while still resistant to reinfection with virus, an increasing susceptibility of the respiratory mucous membrane to chemical injury is noted until the 4th to 5th weeks when the damage induced by ionization is nearly as severe as that produced in the normal ferret.

During the period between the 8th and 15th days in addition to the mere aging a distinct maturation of the respiratory epithelium takes place in which the rather primitive transitional stratified epithelium is giving way to the highly differentiated ciliated columnar cells. These facts plus the observation that the latter are the cells most definitely affected suggest that it is the ciliated columnar cell which is the one susceptible to ionization and that resistance is related to the primitive undifferentiated cell type.

The Effect of Ionization on the Olfactory Mucous Membrane of Convalescent Ferrets

A curious effect was produced on the olfactory epithelium of the convalescent ferret by ionization. It has already been stated that the PR8 strain of influenza virus produces no visible effect on this epithelium and therefore if resistance to ionization were a property of certain types of cells, it might be expected that the olfactory epithelium of the convalescent ferret would be as susceptible to the necrotizing effect of ionization as the olfactory epithelium of a normal ferret. In fact, the olfactory epithelium of the 7 and 8 day convalescent ferrets was unharmed by ionization, but that of the convalescent ferrets ionized on the 15th day or after was completely necrotized by the ionization. In the convalescent ferret so long as no damage due to ionization occurred in the respiratory area, the olfactory mucosa escaped; but at a time when the respiratory mucosa again became susceptible to damage, the olfactory mucosa responded in the same way as that of a normal animal. Two explanations may be suggested for this phenomenon. First, it may be that destruction of the superficial cells of the respiratory mucous membrane exposes the contiguous olfactory cells which are then destroyed, whereas when the respiratory epithelium is resistant, this phenomenon exerts a protective effect upon the olfactory epithelium. On the other hand, since the WS strain of influenza virus in one instance did cause damage to the olfactory epithelium, it may be that the PR8 strain attacks the cells without causing obvious injury but thereby inducing a refractory state protective against chemical injury.

The Effect of Zinc Sulfate Alone on the Nasal Mucous Membrane

Further confirmation of the results obtained by the technique of zinc ionization was found in a study of the effect of prolonged intranasal irrigation with zinc sulfate solution. The 1 per cent solution used during ionization was itself damaging to the nasal mucosa of the normal ferret, and irrigation alone under anesthesia for 15 minutes caused as complete a desquamation of the epithelium as ionization. When the nostrils of convalescent ferrets were irrigated for 15 minutes, the repairing epithelium was unattacked by the zinc sulfate on the 8th and 9th days, partially damaged on the 16th and 19th days, and more extensively damaged on the 28th and 29th days. In these latter ferrets whose noses were irrigated 1 month

after infection, foci of resistant ciliated epithelium were found in the respiratory area although the olfactory epithelium was completely destroyed as early as the 16th day.

DISCUSSION

In the first paper of this series (4) it was shown first, that influenza virus infection causes a complete destruction of the respiratory epithelium in the ferret's nose, and second, that during the period of repair from the 6th to 14th day after infection a strikingly abnormal type of epithelium varying from an undifferentiated low transitional type to a stratified squamous or columnar variety constitutes the respiratory epithelium. This gradually matures and differentiates until after 3 weeks a relatively normal ciliated columnar epithelium with numerous goblet cells is again seen.

In the present paper it has been found that at the time the repairing respiratory epithelium is most abnormal it is resistant not only to reinfection with influenza virus but to severe chemical stimuli which in the normal animal destroy both the respiratory and the olfactory epithelium. During the subsequent 3 to 4 weeks as the respiratory epithelium returns to normal, it again becomes susceptible to chemical injury although showing no reaction to reinfection. The first signs of loss of the refractory state appear with the return of the ciliated columnar cells.

The series of events observed, although distinctly more rapid, is quite parallel to that observed in studies of the mechanism of resistance to chemical injury which has been investigated by MacNider (6–12). This author demonstrated that the acquired immunity of an organ to functional and structural damage by a chemical poison could be correlated with an altered morphological appearance and functional response of the cells of that organ. MacNider found that immunity to uranium poisoning in dogs following a single large dose of uranium nitrate was correlated with repair of the liver and kidneys and replacement of the normal hepatic and renal tubule cells by others less specialized in appearance. Repair, which was accomplished by regeneration of cells similar in appearance to normal hepatic and renal cells and which usually occurred after a small dose of the toxic agent, failed to confer any resistance to a second dose of the poison. The resistant cells were elongated and flattened with

deeply staining nuclei and a tendency to fuse together to form syncytial strands. Senile but otherwise normal dogs might possess an inherent immunity to uranium and the liver of such animals showed irregular cords of flattened cells like those seen in younger dogs after the development of acquired resistance to uranium. MacNider also showed that resistance acquired as a result of damage by a chemical agent was not specific for that agent, but in the case of the liver recovery from uranium poisoning was followed by resistance to chloroform poisoning. In the case of the kidney acquired resistance to mercury bichloride poisoning was accompanied by resistance to uranium.

Much experimental work has been done on the regeneration of the nasal mucosa after injury. Boling (3) showed that during the repair process in the nasal mucosa there was a definite cycle of changes and that at one stage an abnormal stratified and many layered epithelium was present which was resistant to the necrotizing effect of zinc iontophoresis.

Whatever may be the nature of the resistance of the nasal membrane of the convalescent ferret to ionization, the demonstration that such a resistance exists is proof that the repairing epithelium differs from the normal both morphologically and functionally. This altered reaction is effective not only against the original infectious agent but against non-specific physicochemical injury as well. The return of the normal columnar ciliated cells is accompanied by a return of susceptibility to chemical injury although even 1 month after the attack of influenza there is a less complete susceptibility than in the normal ferret.

It is difficult to determine what relation the resistance herein described bears to immunity to virus infection. Both the morphological abnormality of the epithelium and the functional resistance to severe chemical injury are much more short lived than the resistance to second virus inoculation. In the latter instance circulating antibodies represent an uncontrolled factor which makes it impossible to measure the duration of tissue resistance alone, while in the case of ionization this factor is eliminated. On the basis of the present experiments, despite the factor of circulating antibodies, it seems not unreasonable to believe that in the first 2 or 3 weeks of convalescence

after an initial attack of influenza, the respiratory tissues possess a refractory state wholly independent of specific antibodies. Subsequently it seems likely that a balance between tissue reactivity and specific antibodies comes into play. This concept will be discussed more fully in the following paper.

It may be of interest in passing to suggest that reactions of the type herein described play some rôle in the type of resistance described as "the interference phenomenon." Moreover, procedures such as resistance to chemical injury may possibly be of value in measuring variations in the susceptibility of specific cells to different infectious agents.

SUMMARY

Because of the marked morphological abnormality of the nasal respiratory epithelium in ferrets recovering from epidemic influenza virus infection, attempts were made to determine whether the anatomical changes were associated with functional changes in the epithelial cells. It was found that on the 7th or 8th day after infection, at which time an immature transitional type of epithelium covers the respiratory area, the cells are resistant not only to reinfection with influenza virus but to a severe physicochemical stimulus supplied by iontophoresis or prolonged irrigation with zinc sulfate. Later, as the ciliated columnar cells return, susceptibility to physicochemical injury returns although resistance to influenza virus persists. The ciliated columnar cells are the ones which are damaged by the physicochemical agent while the deeper cells in the regenerating area remain unaffected. 5 weeks after infection the epithelium is anatomically normal but tissue resistance to zinc sulfate is still present to some degree as evidenced by foci of undamaged cells remaining after ionization.

The olfactory epithelium which is undamaged by the PR8 strain of epidemic influenza virus also becomes resistant to ionization after infection. As soon as the respiratory epithelium exhibits any loss of resistance to zinc sulfate the chemical produces complete necrosis of the olfactory area.

The refractory state to physicochemical agents exhibited by the regenerating nasal mucosa of the ferret after influenza virus infection

is thought to be a non-specific resistant state, significant for a time at least, in the mechanism of immunity to influenza virus.

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EXPLANATION OF PLATES

All sections stained with hematoxylin and eosin.

PLATE 40

FIG. 1. Normal ferret subjected to intranasal zinc ionization and sacrificed 24 hours later. Anterior turbinate. × 80. The epithelium has been desquamated, the submucosa is wider than normal, and an exudate is present in the air passages.

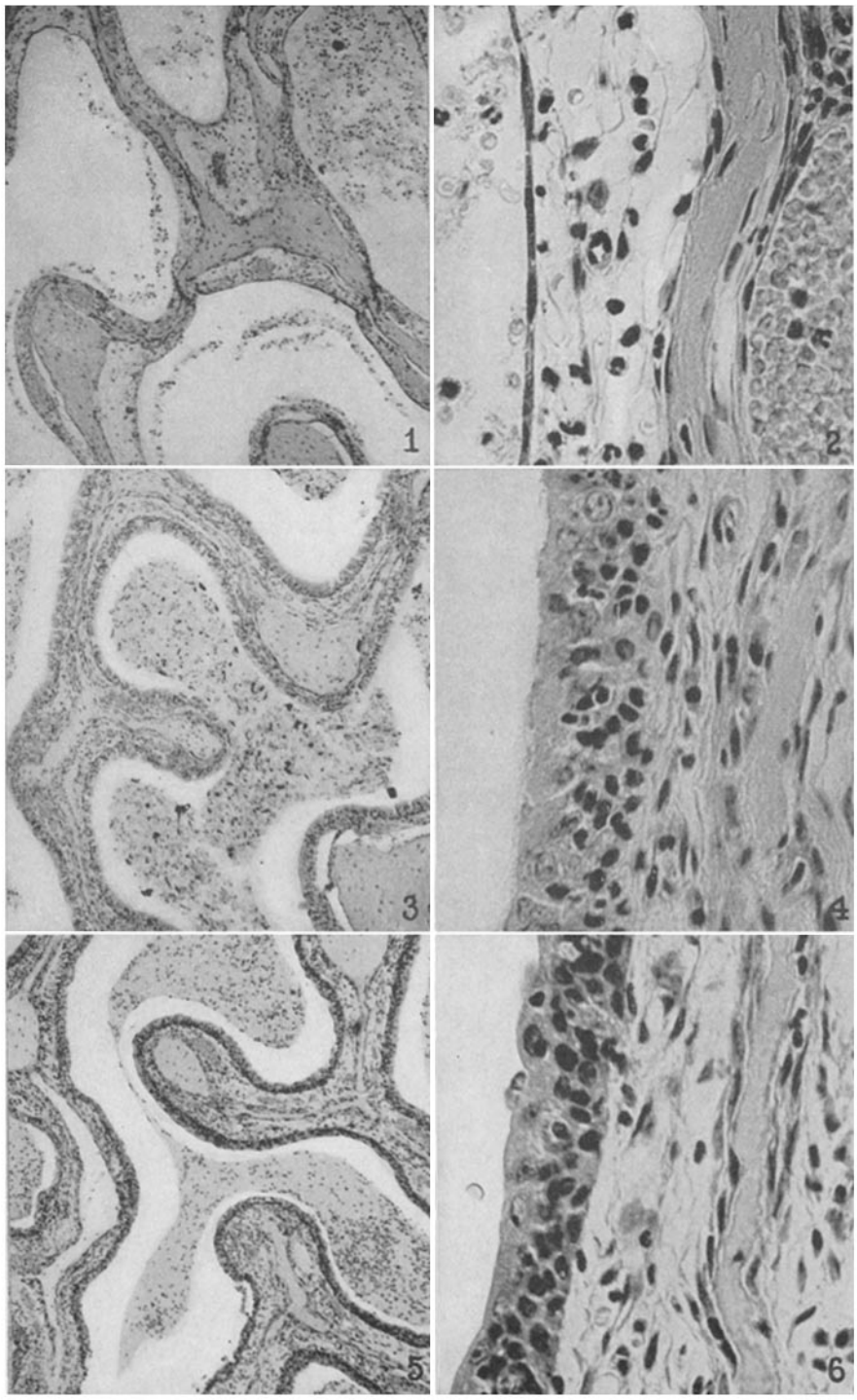
FIG. 2. High power view of Fig. 1. × 570. The epithelium to the left is composed of a flattened layer of cells incorporated with the basement membrane. The submucosa is edematous with congested vessels and polymorphonuclear leucocytic infiltration. The exudate contains leucocytes and cell debris.

FIG. 3. Convalescent influenza ferret 11-01 reinoculated with virus on the 8th day after the first infection. Sacrificed 3 days later. Anterior turbinate. × 80. The epithelium is of the stratified columnar type, and there is no sign of necrosis since the second inoculation. The exudate in the air passages is structureless.

FIG. 4. High power view of Fig. 3. × 570. The epithelium, here cut obliquely, is regular, stratified columnar and ciliated. There is no sign of recent necrosis.

FIG. 5. Convalescent influenza ferret 10-99 sacrificed on the 8th day. Anterior turbinate. × 80. The epithelium is partly stratified transitional and partly stratified columnar.

FIG. 6. High power view of Fig. 5. × 570. The epithelium is stratified and transitional in type. The stage of regeneration is clearly earlier than in ferret 11-01.



Photographed by Joseph B. Haulenbeek

(Stuart-Harris and Francis: Nasal histology of influenza infection. II)

PLATE 41

FIG. 7. Convalescent influenza ferret 10-95 subjected to intranasal zinc ionization on the 8th day and sacrificed 24 hours later. Anterior turbinate. × 80. The epithelium is comparable with that in 10-99 and of stratified transitional or early columnar type. There is no sign of recent necrosis since the ionization. There is a little mucus in the air passages. The olfactory epithelium of this ferret was normal in appearance.

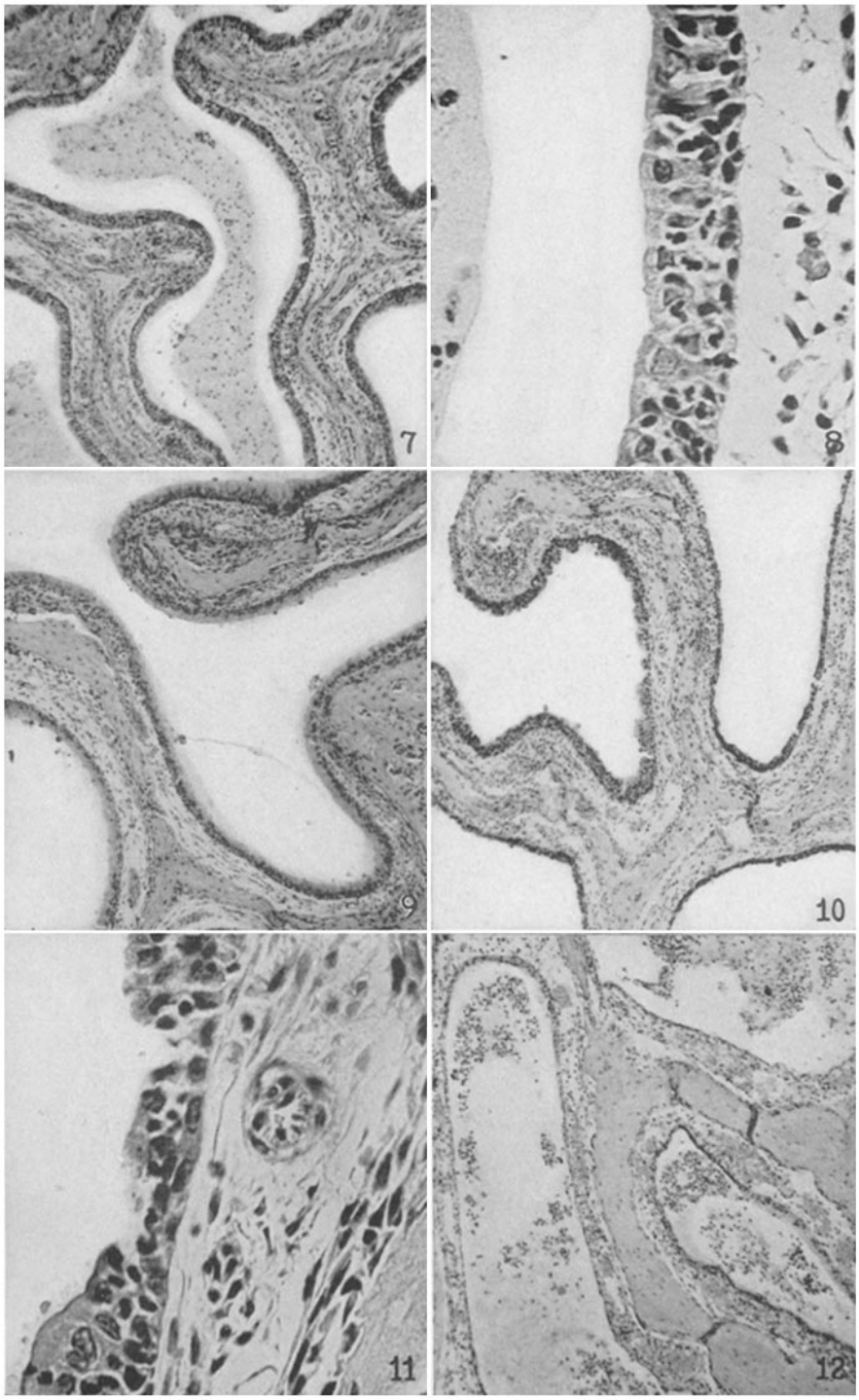
FIG. 8. High power view of Fig. 7. × 570. The epithelium is stratified columnar in type and shows leucocytic infiltration. There is no sign of necrosis since the ionization, however, and the submucosa shows no congestion.

FIG. 9. Convalescent influenza ferret 10-98 sacrificed on the 15th day. Anterior turbinate. × 80. The epithelium is regular, partly simple ciliated columnar, and partly stratified ciliated columnar.

FIG. 10. Convalescent influenza ferret 10-94 subjected to intranasal zinc ionization on the 15th day and sacrificed 24 hours later. Anterior turbinate. × 80. There has been a partial desquamation of the epithelium which varies from a narrow layer 2 cells deep to a partly ciliated layer in which some of the cells have been removed. The olfactory epithelium of this ferret was completely desquamated.

FIG. 11. High power view of Fig. 10. × 570. The epithelium is partly destroyed but the basal cells are still present and toward the bottom of the figure some ciliated cells which have resisted destruction are seen.

FIG. 12. Convalescent influenza ferret 11-11 subjected to intranasal zinc ionization on the 28th day and sacrificed 24 hours later. Anterior turbinate. × 80. The epithelial destruction is almost as severe as in a normal ferret after ionization. Note the exudate and inflammatory reaction. An area of undestroyed ciliated columnar cells is visible toward the right hand corner of the figure, and there were several areas of such epithelium in this turbinate. The olfactory epithelium was completely destroyed.



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(Stuart-Harris and Francis: Nasal histology of influenza infection. II)