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

III. Histological and Serological Observations on Ferrets Receiving Repeated Inoculations of Epidemic Influenza Virus

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After a single intranasal inoculation of influenza virus, the ferret becomes for a time solidly immune to reinfection and shows no clinical signs of illness when reinoculated. Accompanying this immunity, neutralizing antibodies develop in the blood and, as has been shown previously (1, 2), morphological changes occur in the infected tissues so that for a time the epithelium which covers the nasal mucosa is abnormal both structurally and functionally. It seems probable on the basis of the foregoing observations that the tissue changes which occur during repair of the nasal mucosa may of themselves confer some protection against an infection which inflicts its primary injury upon the respiratory tract. In this case there may exist a short-lived refractory state of the nasal mucous membrane which subsequently is reinforced by the humoral changes. With this possibility in mind, a study was made of ferrets which had received repeated inoculations of influenza virus.

Materials and Methods

Estimations of the antibody content of the blood were made from time to time by the method of Francis and Magill (3) with the observation period prolonged to 10 days. The ferrets were killed at a time when the titer of circulating anti-

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bodies was known, and sections were prepared from the nasal fossae of the ferrets in the manner previously described (1). In this way it was hoped to correlate the histological findings in the ferret's nasal mucosa with the clinical reaction to a final test dose of virus intranasally at a time when the content of circulating antibodies was known. Some of the ferrets received subcutaneous injections of virus as well as intranasal ones, but no attempt was made to study the reaction of the nasal mucosa in ferrets which received only vaccinating doses of virus.

One group comprised 24 ferrets which, after varying numbers of intranasal or subcutaneous inoculations of virus at previous times, were given a final intranasal inoculation, observed clinically for 2 or 3 days, and then sacrificed so that histological preparations of the nasal mucosa could be obtained. A second group of 11 ferrets were subjected to repeated inoculations in a manner similar to that employed with the preceding ferrets but were sacrificed without a final intranasal test. Sections from these animals furnished histological material which served to control the findings in the first series.

The PR8 ferret passage strain of human influenza virus was used for most of the inoculations including all the final tests, but other strains of human influenza virus were used from time to time. All intranasal inoculations were given under ether anesthesia.

Histological Findings in Control Ferrets

Table I summarizes the histories of the control ferrets. It will be seen that all received one or more intranasal inoculations and 5 had received subcutaneous inoculations in addition during a period of observation varying from 6 weeks to 10 months in duration. Only 1 ferret (9-56) suffered from more than one clinical attack of influenza during this time. The ferrets were sacrificed at periods varying from 14 days to 4 months after their last intranasal inoculation of virus. As this final inoculation was not always accompanied by clinical signs of infection, the table also includes the interval from the last inoculation followed by a clinical response to the day of autopsy. None of the ferrets showed any gross abnormality of lungs or turbinates when sacrificed.

Two ferrets were sacrificed on the 14th day, 1 on the 34th day, and the rest 4 months after the last inoculation of virus which had elicited a clinical response. The histological appearance of the turbinates differed from that of normal ferrets in certain particulars. The 2 ferrets sacrificed 14 days after an attack of influenza showed the characteristic features of epithelium and cartilage regeneration seen after influenza virus infection described in the first paper of this series (1). One of them (10-53) exhibited more advanced epithelial repair than is usually observed at this stage of convalescence, the epithelium having already reached a columnar ciliated stage although areas of flattened pseudostratified epithelium covered the tip of the anterior turbinates. The second (10-57) showed the hyperplastic

TABLE I

Histological and Serological Findings in Previously Infected Ferrets Not Reinoculated at Time of Autopsy

Ferret No.	Total period of observation	Total number of intranasal inoculations of virus	Interval from last inoculation to day of autopsy	Number of clinical attacks	Interval from last clinical attack to day of autopsy	Titer of neutralizing antibodies	Histology of turbinates
10-53*	6 wks.	1	14 days	1	14 days	—	Epithelial irregularities; pseudostratified and stratified columnar epithelium. New cartilage
10-57	6 "	2	14 "	1	14 "	—	Epithelial irregularities; pseudostratified and stratified columnar epithelium
10-45*	2 mos.	1	34 "	1	34 "	—	Epithelial irregularities. Lymph follicle in gland
9-56	7 "	2	4 mos.	2	4 mos.	950	Normal epithelium
10-27	4 "	2	3½ "	1	4 "	35	Pseudostratified epithelium at tip
10-37	4 "	2	3½ "	1	4 "	35	Epithelium normal. Dilated venous sinuses. Lymph follicle in gland
9-57	7 "	3	3½ "	1	4 "	150	Pseudostratified epithelium at tip
9-08	10 "	3	3½ "	0	Subclinical infection	400	Pseudostratified epithelium at tip. Dilated venous sinuses
10-07*	4 "	1	4 "	1	4 mos.	100	Normal epithelium
9-46†	7 "	2	4 "	1	4 "	400	Chronic changes: fibrosis, squamous and stratified columnar epithelium
7-75†	10 "	2	4 "	1	4 "	280	Pseudostratified epithelium at tip. Scanty exudate

* Received virus subcutaneously prior to first intranasal inoculation.

† Received virus subcutaneously between first and second intranasal inoculations.

stratified columnar ciliated epithelium which is usually found on the 14th day after an attack of influenza.

In all but one of the ferrets which were killed at later periods after their last clinical response to inoculation, the turbinates were normal in the gross. However, microscopically these showed the pseudostratified epithelium at the tip of the anterior turbinates and the slight increase in fibrous tissue in the submucosa which have been found to persist for some months after an attack of influenza. The turbinates of 2 ferrets (10-37 and 9-08) showed a dilatation of the venous sinuses, 2 (10-37 and 10-45) exhibited a well developed lymph node in the submucosa of the lateral nasal gland, and 1 (7-75) showed a normal epithelium but contained an excess of mucus with polymorphonuclear leucocytes in one portion of the air passages. The last of this group of control ferrets (9-46) presented a definitely abnormal structure of the turbinates. On one side of the nose the mucous membrane of the turbinates was fibrotic; the epithelium was hyperplastic and of stratified columnar ciliated type. On the other side the turbinate tissue was normal except for three laminae which were fibrotic, showed areas of hemorrhage into the submucosa, and were lined by a low flattened squamous epithelium. As this ferret had not had an inoculation for 4 months previously, it is apparent that chronic changes can persist in the turbinates for long periods of time after influenza, possibly as a result of a superadded bacterial infection.

In general, among these control ferrets the nasal mucosa was found to have returned to normal in spite of repeated intranasal inoculations of virus even when these were accompanied by clinical attacks of influenza. Certain features, such as a pseudostratified arrangement of the epithelium at the tip of the nose, irregularities of the venous sinuses, increase in fibrous tissue of the submucosa, and lymphocytic infiltration of the lateral nasal gland, suggested residual abnormalities of infection and regeneration in these ferrets. Some of them would undoubtedly have been clinically responsive to intranasal influenza virus at the time of sacrifice but others would probably have been immune. The essential fact, however, is that in most instances it has been impossible to demonstrate without further manipulation any simple histological alteration which differentiates the animal completely recovered from repeated inoculations of epidemic influenza virus from ferrets which have never been subjected to infection. Repeated stimuli have not resulted in a permanent anatomical change in the epithelium of the respiratory mucous membrane approaching that seen temporarily in the period of repair from primary infection.

Histological Findings in Ferrets Recently Reinoculated

The animals in this group of 24 ferrets were reinoculated 2, 3, or 4 days before sacrifice and according to their clinical response may be classified into three groups: (*a*) those which developed undoubted clinical signs of illness after the test inoculation; (*b*) those which were clinically immune in that no fever, nasal discharge, or other signs of infection were detected; and (*c*) those which developed doubtful signs of illness such as fever only or symptoms only.

Ferrets Responsive Clinically to the Test Inoculation.—The first group includes 9 ferrets all of which responded to the test inoculation of virus with signs of illness of varying severity as listed in Table II. In each case the final illness represented the second clinical attack of influenza virus infection although 1 ferret had received two previous intranasal inoculations of virus. It will also be seen that 1 ferret received a subcutaneous inoculation of virus, but the two clinical attacks of influenza occurred at a later date than the vaccination so that the final histological findings were probably not influenced by the subcutaneous inoculation. Intervals of from 3 to 14 months had elapsed between the two clinical attacks of influenza in the various ferrets but here again the histological findings bore no clear relation to the length of the interval between attacks. The majority of the ferrets were sacrificed on the 3rd day after inoculation but 2 were sacrificed on the 2nd and 1 on the 4th day after inoculation.

Table II also shows the clinical features of the attack of influenza induced by the test dose of virus. All of the ferrets showed fever, all developed nasal signs, such as nasal discharge and sneezing, but only 2 ferrets showed definite abnormalities of respiration. At post mortem the modification of infection as measured by pulmonary involvement in these ferrets compared with that seen in normal ferrets infected for the first time was obvious. The lungs of 5 of the ferrets were normal, the lungs of 3 others showed mottled areas without true consolidation, and only 1 ferret showed the typical lung lesions of influenza virus infection. Yet with this particular strain of virus, inoculation under an anesthetic invariably caused the production of extensive lung lesions in normal ferrets. There was no doubt, therefore, that the infection was modified clinically in these ferrets during their second attack of influenza.

With regard to the nasal reaction, the turbinates macroscopically appeared glistening and injected as in the case of turbinates during a first attack of influenza. One ferret (10-05) exhibited frank pus in the nasal passages but in the others only a watery exudate was observed.

Microscopically the turbinates from 3 ferrets showed lesions typical of those seen during a first attack of influenza virus infection between the 2nd and 4th days of illness. There was epithelial necrosis and desquamation; the submucosa was infiltrated with polymorphonuclear leucocytes and showed edema and congestion; and the air passages were occupied by an exudate of mucus, debris, and leucocytes. The epithelium in the respiratory area consisted of a single layer of basal cells which was closely incorporated with the basement membrane.

TABLE II

Histological and Serological Findings in Previously Infected Ferrets Again Clinically Responsive to Test Inoculation

Ferret No.	Total period of observation	Total number of intranasal inoculations	Number of clinical attacks	Interval between attacks	Severity of last clinical attack			Interval from last inoculation to day of autopsy	Titer of neutralizing antibodies at time of test inoculation	Histology of turbinates
					Fever	Nasal symptoms	Respiratory symptoms			
9-66	5 mos.	2	2	5 mos.	++	±	0	3 days	+ 5 mos. before	Necrosis, foci of normal epithelium, accelerated repair
9-67	5 "	2	2	5 "	++	+	0	3 "	+ 5 " "	Necrosis without repair
9-69	6 "	2	2	5 "	++	+	±	3 "	<50	Necrosis, foci of normal epithelium, accelerated repair
9-98	4 "	2	2	4 "	++	++	+	3 "	0	Necrosis, accelerated repair
8-28	14 "	2	2	14 "	++	±	+	2 "	2	Necrosis, foci of normal epithelium, accelerated repair
8-35	14 "	2	2	14 "	++	+	0	2 "	150	Necrosis without repair
8-39	14 "	2	2	14 "	++	+	±	4 "	100	Necrosis, accelerated repair
10-25	4 "	3	2	4 "	++	+	0	3 "	18	Necrosis, accelerated repair; residual foci of polygonal cells
10-05*	3 "	2	2	3 "	++	±	0	3 "	—	Necrosis without repair; some foci of normal epithelium

* Received virus subcutaneously prior to first intranasal inoculation.

The turbinates of the 6 other ferrets which exhibited clinical evidence of infection showed significant differences from those seen in the acute stage of a first attack of influenza. Epithelial necrosis and desquamation were present in all 6 turbinates but the residual respiratory epithelium instead of being a flattened pavement layer was in many places two or three cells deep (Figs. 1 and 2), thus resembling the transitional epithelium seen on about the 6th to 8th days of a first attack of influenza. Moreover, the epithelial necrosis was not uniform, and in 1 ferret (9-66) there were in the anterior turbinates extensive areas of unaffected ciliated columnar epithelium immediately adjacent to areas of necrosis (compare Figs. 3 and 4 from ferret 9-47). The submucosa showed a richer infiltration of cells than ordinarily observed even in a first attack although mononuclear cells preponderated over the polymorphonuclear leucocytes. It was evident, therefore, that the cellular reaction to the virus in these latter ferrets was sharply different from that in previously uninfected normal ferrets.

The epithelial reaction is of particular interest since at the time the respiratory area of the nasal mucous membrane of a previously untreated ferret would be stripped to the basement membrane, in the present animals a multilayered epithelium was observed. Moreover, frequent areas of relatively normal epithelium had escaped damage. Two possible explanations for the presence of multilayered epithelium as early as the 3rd day after infection are suggested. The first is based upon the observations reported in the preceding paper of this series (2) that in the regenerating epithelium a return to susceptibility to chemical injury is paralleled by the return of the normal ciliated columnar cell. Hence if only the superficial columnar cells were susceptible to repeated virus infection the underlying cells would remain undamaged. This explanation would of necessity indicate that the respiratory epithelium of the ferret before reinfection is stratified columnar in type. The appearance of the turbinates in 1 animal (10-25, Figs. 5 and 6) in which exceptionally well developed areas of stratified cells were present on the 3rd day after reinoculation lends support to this view. Furthermore, it has previously been observed (1) that pockets of stratified epithelium may persist after a primary infection. On the other hand, in control animals of the present series, in repeatedly inoculated ferrets no suggestion of persistence of a widespread stratification of cells in the respiratory epithelium was found.

The other explanation suggested for the presence of a many

layered epithelium by the 3rd day after infection is that an acceleration of the repair process occurs in animals receiving repeated injuries. In the first place, in these animals the basal layer of cells was cubical or polygonal in shape with large nuclei. Such cells were found even in the basement membrane of the turbinates of the 3 ferrets described above in which the appearance was otherwise similar to that in a first attack of influenza. In the second place, numerous mitoses were present in the more superficial cells and the appearance of the whole epithelium suggested an area of active regeneration. Furthermore, the stratified form of epithelium was not observed in the control animals not subjected to reinoculations of virus, while in at least 6 of the 9 ferrets examined during the acute stage of a second attack of influenza the residual epithelium was distinctly of the transitional type. The evidence suggests then that as a result of infection with epidemic influenza virus a conditioning of the basal cells of the epithelium occurs, so that when a sufficient decrease in immunity permits the induction of a second clinical attack the whole process of repair is greatly accelerated.

Ferrets Immune to the Test Inoculation.—11 ferrets showed no clinical signs of illness after the final inoculation (Table III). They were considered clinically immune and were sacrificed on the 3rd day after inoculation in order that histological preparations could be obtained from their nasal turbinates. At post mortem the lungs of 7 of the ferrets were normal, 1 showed lesions atypical of influenza, and 3 showed lesions suggesting healing influenza virus lesions. Macroscopically the turbinates were normal in 8 ferrets, but in 2 ferrets pus was present, and in 1 other the inferior portion of the anterior turbinate was dark red and hemorrhagic in appearance. Microscopically none of the turbinates showed lesions suggesting a recent necrosis such as might have been induced by the test inoculation of virus.

In the group were 5 ferrets sacrificed 3 days after the last test dose of virus but 3 weeks after the previous dose which elicited clinical infection. All of these animals had been given virus subcutaneously prior to the first intranasal inoculation. The turbinates of 4 of them showed epithelial irregularities and cartilage regeneration typical of the 3rd week of repair after an original infection. The other (10-44) presented an extraordinary degree of fibrosis of the turbinates together with a hyperplastic stratified columnar epithelium and leucocytic infiltration. Frank pus was detected in the nasal passages of the latter animal at post mortem, and the histological picture was thought to represent the result of a superadded bacterial infection secondary to the influenza virus infection 3 weeks previously.

TABLE III

Histological and Serological Findings in Previously Infected Ferrets Clinically Immune to Test Inoculation

Ferret No.	Total period of observation	Total number of intranasal inoculations	Number of clinical attacks	Interval from last attack to day of autopsy	Interval from last test to day of autopsy	Gross appearance at autopsy		Titer of neutralizing antibodies at time of test inoculation	Histology of turbinates
						Lung	Nose		
9-11	9 mos.	3	1	9 mos.	3 days	Normal	Normal	400	Pseudostratified epithelium at tip
9-53	7 "	4	1	4 "	3 "	"	Inferior part dark red	280	Normal but for 3 laminae with transitional epithelium and leucocytic infiltration
9-03	9 "	4	2	3 "	3 "	"	Normal	—	Pseudostratified epithelium at tip; fibrosis of submucosa; new cartilage
9-60	6 "	4	1	3 "	3 "	"	"	—	Normal but for fibrosis of a few laminae with squamous epithelium and leucocytic infiltration
6-45	18 "	4	2	15 "	3 "	Atypical in left upper lobe	Pus in nasal passages	400	Normal but for epithelial cysts and leucocytes
9-45*	7 "	3	1	4 "	3 "	Old lesion	Normal	150	Epithelial irregularities; lymphocytic infiltration of gland
10-43†	6 wks.	2	1	28 days	3 "	Normal	"	400	Slight epithelial irregularity; lymph follicle in gland
10-44†	6 "	2	1	21 "	3 "	Old lesion	Pus in nasal passages	400	Fibrosis; stratified columnar epithelium; leucocytic infiltration
10-46†	6 "	2	1	21 "	3 "	" "	Normal	560	Normal epithelium; new cartilage
10-50†	6 "	2	1	21 "	3 "	Normal	"	960	Pseudostratified epithelium at tip; new cartilage
10-52†	6 "	2	1	21 "	3 "	"	"	1400	Epithelial irregularities; new cartilage

* Received virus subcutaneously between first and second intranasal inoculations.

† Received virus subcutaneously prior to first intranasal inoculation.

One other ferret received virus subcutaneously in the interval between the first and second intranasal inoculations. The nasal mucosa of this animal (9-45) was normal.

The remaining 5 ferrets received only intranasal inoculations of virus, the last being 3 days before death, and they form a group comparable with the control ferrets. 2 of these ferrets (9-11 and 9-03) exhibited no abnormalities in the tissues except for the presence of pseudostratified epithelium at the tip of the nose and a slight increase in fibrous tissue in the submucosa. One ferret (6-45) showed a normal nasal mucosa except for a small area with cysts lined by columnar epithelium and containing leucocytes. Frank pus had been found in the nasal passages of this ferret at post mortem. The remaining 2 ferrets (9-53 and 9-60) showed abnormalities of the turbinates resembling those seen in ferret 9-46 of the uninoculated control series. Some of the laminae were fibrotic, infiltrated with red blood cells, and covered by a low flattened squamous epithelium with leucocytic infiltration. The only feature suggesting that these lesions were different etiologically from those seen in ferret 9-46 and that they were due to the recent inoculation of virus was the leucocytic infiltration of the epithelium.

Summarizing the findings among ferrets which were clinically immune to their final test dose of virus, none showed definite lesions of the nasal mucosa resembling recent virus lesions. Some of the ferrets showed an abnormal appearance of the turbinates and the possibility that these abnormalities represented focal necrotic lesions of virus etiology could not be excluded. However, the fact that 1 of the control ferrets not recently inoculated had shown somewhat similar lesions was thought to indicate that the abnormalities in the recently inoculated immune ferrets were due to chronic changes, possibly the result of superadded bacterial infection.

Ferrets with Doubtful Clinical Reaction to the Test Inoculation.—Finally, there were 4 ferrets in which clinical signs of doubtful significance developed following the final reinoculation. 2 (9-47 and 10-06) developed nasal symptoms without elevation of temperature, and 2 (9-59 and 7-51) developed a rise of temperature without nasal symptoms or other signs of illness. At autopsy the lungs of both 9-47 and 10-06 showed small lesions which were atypical of influenza virus lesions in appearance, and the lungs of 9-59 and 7-51 were normal. 3 of the ferrets, as shown in Table IV, had received a subcutaneous inoculation of virus at some time in the past. The histological findings suggested that ferrets 9-47 and 10-06 were undergoing reinfection of the turbinates by influenza virus but that 9-59 and 7-51 were not. Thus the turbinates of 9-47 and 10-06 exhibited widespread epithelial necrosis with desquamation, accelerated repair of the basal layer of cells, and es-

cape of some areas of ciliated epithelium adjacent to areas of necrosis (Figs. 3 and 4 from ferret 9-47). The turbinates of 9-59 and 7-51, on the other hand, showed an absence of epithelial necrosis and were for the most part normal. However, a few of the laminae of 9-59 were fibrotic and covered by a squamous epithelium as in the case of some of the clinically immune ferrets described above (9-53 and 9-60). Nearly all of the turbinate epithelium was normal in 7-51 but there was a

TABLE IV

Histological and Serological Findings in Previously Infected Ferrets with Doubtful Clinical Reaction to Test Inoculation

Ferret No.	Total period of observation	Total number of intranasal inoculations	Interval from last attack to day of autopsy	Interval from last test to day of autopsy	Reaction to test inoculation			Titer of neutralizing antibodies at time of test inoculation	Histology of turbinates
					Fever	Nasal symptoms	Respiratory symptoms		
9-47*	7 mos.	3	4 mos.	3 days	0	+	0	100	Necrosis, foci of normal epithelium; accelerated repair
10-06†	4 "	2	4 "	3 "	0	+	0	100	Necrosis, foci of normal epithelium; accelerated repair
9-59	7 "	4	7 "	3 "	+	0	0	150	Normal but for fibrosis of a few laminae with squamous epithelium and leucocytic infiltration
7-51*	11 "	3	4 "	3 "	+	0	0	400	One small area of transitional epithelium and leucocytic infiltration

* Received virus subcutaneously between the first and second intranasal inoculations.

† Received virus subcutaneously prior to the first intranasal inoculation.

tiny focus of transitional type of epithelium infiltrated with leucocytes. It is possible, therefore, that the abnormalities in the turbinates of these 2 ferrets represented focal lesions.

The Relation between Antibody Titer and Resistance

Histological studies in animals which were completely immune to reinfection several months after one or more previous inoculations

with epidemic influenza virus have yielded no evidence that a new type of resistant epithelium develops in the nasal respiratory area as a result of repeated inoculations with epidemic influenza virus. Abnormalities considered to be reflective of earlier infection were seen both in the uninoculated controls and in the animals which were clinically immune at the time of the final test. In the controls, however, changes of this type were irregularly distributed both in animals which would most certainly have been resistant to test inoculation and in others which would with equal probability have been susceptible. The abnormalities observed did not involve the entire epithelium but consisted of small cyst-like areas in the epithelium, persistence of pseudostratified epithelium at the anterior tip of the turbinates, epithelial irregularities and fibrous changes in the submucosa and cartilage. It was not possible, therefore, with any confidence to correlate the immunity or susceptibility of ferrets after repeated virus inoculations with mere structural changes in the epithelium. Accordingly, titrations were made of neutralizing antibodies in the serum of 19 ferrets immediately prior to the immunity test in order to ascertain whether a relationship existed between resistance to reinfection and the level of circulating antibodies.

The animals included in the series had received previous inoculations of virus from 21 days to 15 months before the present test. Serum was obtained 3 to 5 days before the test and the titrations were done without knowledge of the clinical response or the histological evidence. Thus the clinical, the histological, and the serological results were arrived at by observers entirely independent of each other. The results are presented in Chart 1.

The vertical columns at the head of which the ferret numbers are given represent the titers of circulating antibodies. At the side of the column is shown the interval since previous inoculation with virus, and above, a brief statement of the histological findings. The letters *I* and *S* indicate that the animals were clinically immune or susceptible to the test inoculation.

On the extreme left of the chart are animals which received a fresh inoculation of virus only 3 weeks subsequent to an acute attack and which would be expected to be completely immune. They were immune, possessed the highest titers of neutralizing antibodies, and at the same time revealed no pathological changes which could not be fully accounted for by the earlier infection.

The next group to the right comprises 6 animals, which were probably clinically

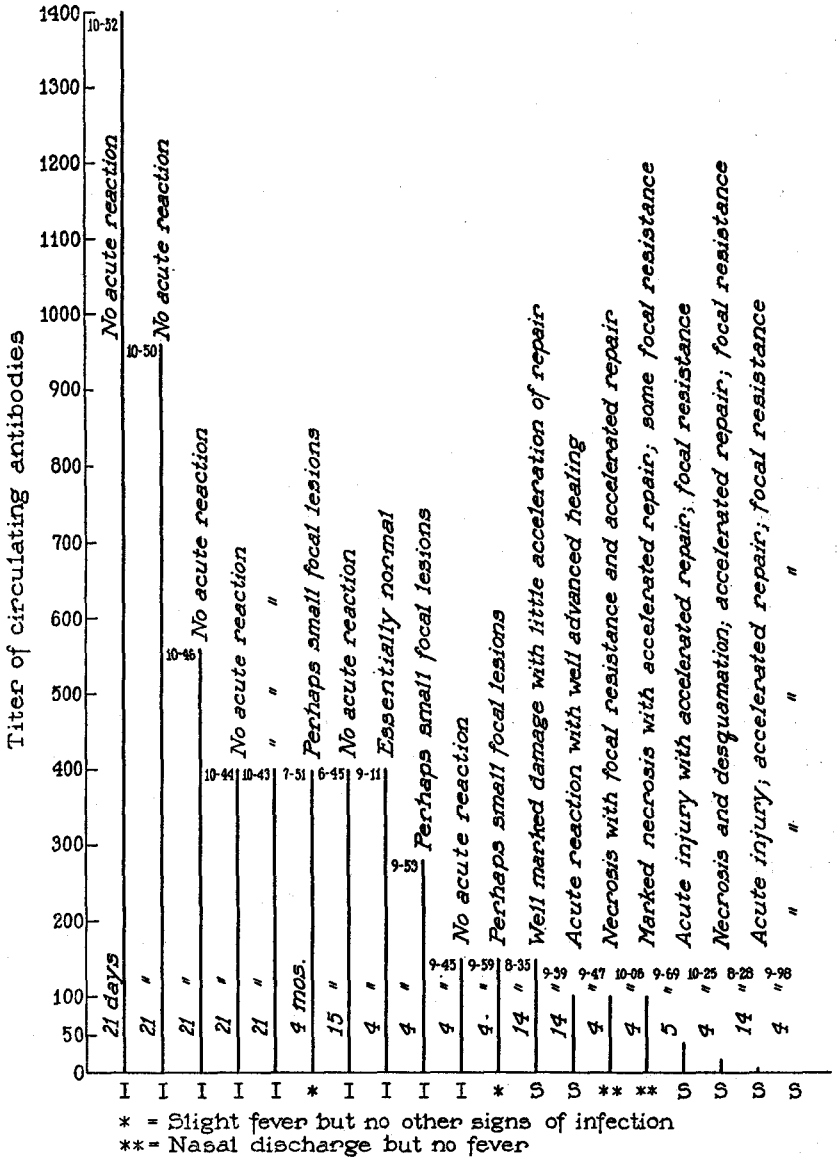

CHART 1. Relation of titer of circulating antibodies to clinical and histological reaction in reinoculated ferrets.

resistant, but in 2 of which (7-51 and 9-59) a slight, brief rise of temperature occurred without other clinical signs of infection. One had been allowed to wait 15 months since previous test, the others 4 months. Histological study of the turbinates of all 6 animals failed to reveal evidence of acute injury to the nasal epithelium although 3 of them showed small foci of abnormal epithelium possibly related to the virus inoculations.

Beyond this point a different sequence of events occurs since all of the 8 animals at the right of the chart exhibited acute nasal reactions to the reinoculation of virus. Of the 8, 3 had not received virus for 14 months, 1 for 5 months; the other 4 had been tested 4 months previously. The antibody titers varied from 1:150, a level which in 2 other ferrets was associated with immunity, to a complete absence of antibody in 1 animal. In 2 animals the clinical reaction was extremely mild; there was no rise of temperature to the ordinary febrile level but nasal discharge developed. Associated with lower antibody titers and clinical susceptibility was the fact that necrosis of the respiratory epithelium was observed in each case. In most instances the tissue damage was marked but despite the extensive necrosis of the epithelium, areas of resistant epithelium, normal in appearance, were invariably present. Furthermore, in all but one a marked acceleration of the repair process, as compared with previously untreated ferrets, was observed. This acceleration, previously mentioned, is indicated by the rapid proliferation of the basal cells of the respiratory epithelium producing by the 3rd day after virus inoculation a stage of repair equivalent to that observed on the 8th day after a primary infection.

The results clearly suggest that in ferrets 4 months or more after previous virus inoculation a parallelism tends to prevail between the height of antibody titer to the virus, the resistance of the nasal respiratory mucosa to reinfection, and the clinical response to reinoculation. It seems that the antibody titer supplies a factor for estimating resistance to reinoculation (at least, in animals repeatedly inoculated) which could not be derived from histological studies of animals before inoculation. That a level of antibody exists which separates immune from susceptible ferrets has been indicated by the observations of Smith (4). The results of studies in the present group of ferrets suggest that the dividing line occurs at a titer of about 1:100 to 1:150. The animals with titers above this level show little or no clinical evidence of infection and exhibit no significant damage to the respiratory epithelium while below this range of antibody titer nasal damage and clinical infection are the rule. In the intermediate zone variability of response occurs.

DISCUSSION

The previous papers of this series (1, 2) have presented evidence to show that influenza virus inflicts a specific injury which destroys the respiratory epithelium in the nose of the ferret. In the process of repair a modified epithelium develops which between the 6th and 8th days is of a transitional immature type. This abnormal epithelium is resistant not only to the virus causing the original damage but to ionization or irrigation with zinc sulfate, a procedure which causes extensive destruction of the entire nasal epithelium in the normal ferret. The period during which the abnormal, resistant epithelium persists is, however, transitory and after 21 days a relatively normal respiratory epithelium is encountered. At that period the tissue is again susceptible to physicochemical injury although still resistant to influenza virus. The demonstration of the nonspecific tissue resistance in an abnormal cell type indicates clearly that in the early period of convalescence resistance to reinfection may bear no relation to the immunological activity of the blood or the tissues. The resistance of the epithelium, however, as measured by its immunity to physicochemical damage is of brief duration and it has not been possible to measure the duration of only the cell refractoriness to the virus since circulating antibodies cannot be eliminated from consideration.

It seemed possible, nevertheless, that with repeated exposures to influenza virus a more permanent structural change might be induced in the respiratory mucous membrane and that the persistence of immunity might be closely related to the length of time through which the modified epithelium covered the normally vulnerable area. The observations in the present paper were made for the purpose of determining if such an alteration did exist. While certain limited changes reflected previous infection there was no evidence that as a result of repeated insults with epidemic influenza virus the respiratory mucous membrane develops a morphologically different epithelium persisting beyond the period of acute injury. In general, the histological appearance of the epithelium differs in no well defined manner from that of the normal untreated ferret.

On the other hand, there is evidence that a conditioning of the

respiratory epithelium occurs as a result of infection, perhaps accentuated by repeated inoculation of virus. This is reflected in animals which have lost sufficient immunity to render them clinically reactive to a fresh virus exposure. In these cases, while extensive necrosis of the respiratory epithelium is again produced there are considerable areas of ciliated columnar epithelium which completely escape injury. There is also a marked acceleration of the repair processes so that a well developed multilayered transitional epithelium is seen in the damaged areas as early as the 3rd day, whereas at a similar time in the animal experiencing its first attack, complete desquamation is observed without any evidence of repair. The comparable stage in the latter animals is not reached until the 6th to 8th days.

Thus, contrary to expectations, the anatomical changes do not suffice to explain a continued complete immunity. However, resort to the serological evidence has resulted in the impression that a parallelism exists between neutralizing antibody titer and resistance to reinfection as determined by clinical signs and histological studies. Above a titer of 1:150 resistance was uniformly observed, while below this level distinct evidence of infection was invariably present. Although in the early stages of convalescence a non-specific tissue immunity is associated with an abnormal type of epithelium and in animals previously infected a conditioning of the epithelium results in a greatly exaggerated reparative capacity in later infections, the conclusion seems inescapable that after a period of months and in animals receiving repeated inoculations, at least, immunity is closely associated with the presence of an effective amount of circulating antibody which tends to protect the cellular structures.

It is of interest to attempt the application of these observations to the problem of human influenza. The complete destruction of the respiratory epithelium seen early in infection creates an ideal opportunity for bacterial invasion, particularly if pathogenic organisms are already resident in the injured areas. Later in convalescence the regenerating epithelium is highly resistant to injury of all types, thus reducing the possibilities of relapses or secondary infections. By this time circulating antibodies are developed in sufficient amount to furnish an additional protective mechanism. With the passage of time and the return of the epithelium to normal the resistance of the

tissues is lost and the serological factors assume a more important rôle in the prevention of reinfection. With repeated exposures, however, the vulnerable epithelium has developed the capacity to initiate regenerative processes extremely soon after damage has occurred. Hence, antibodies prevent too extensive injury and the conditioned cells hasten the repair so that together a distinct modification of the infection results.

It seems probable that complete immunity may be a product of the interaction of these forces. Repeated inoculations probably result in a more stable antibody level than is the case after a single attack. A further acceleration of the repair processes would then result in a period of injury and symptomatology so brief as to be considered subclinical or of an entirely different nature.

The implications of the observations in the problem of resistance to infectious disease in general and to other respiratory disease, especially the common cold, are obvious. Moreover, the accelerated reaction of repair tends to substantiate the impression gained in earlier studies (5) that in animals which have lost a certain degree of immunity the response to second infection resembles an accelerated immune reaction.

SUMMARY

A study of the respiratory mucous membrane was made in the turbinates of ferrets which had received repeated inoculations of influenza virus. There was no evidence that persistent immunity is related to the presence of a structural modification of the respiratory epithelium. In fact, the respiratory epithelium in fully immune animals differs histologically only in minor respects from that of the normal, untreated ferret. On the other hand, a functional difference exists between the normal and the previously infected animals as evidenced by a marked acceleration of the repair process in the latter.

Serological studies at the time of reinfection, 4 months or more after the previous attack, indicate that a relation exists between the height of antibody titer and resistance. The degree of immunity is probably a product of serological immunity and the rate of tissue repair.

The implications of these studies to the problem of influenza in man are discussed.

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EXPLANATION OF PLATE 42

All sections stained with hematoxylin and eosin.

FIG. 1. Influenza in a previously inoculated ferret. Day 3. Ferret 9-69. Anterior turbinate. × 80. The ciliated cells of the epithelium have been desquamated; there is exudate and inflammatory reaction. The residual epithelium is several cells deep and corresponds to that seen on about the 6th day after a first attack of influenza.

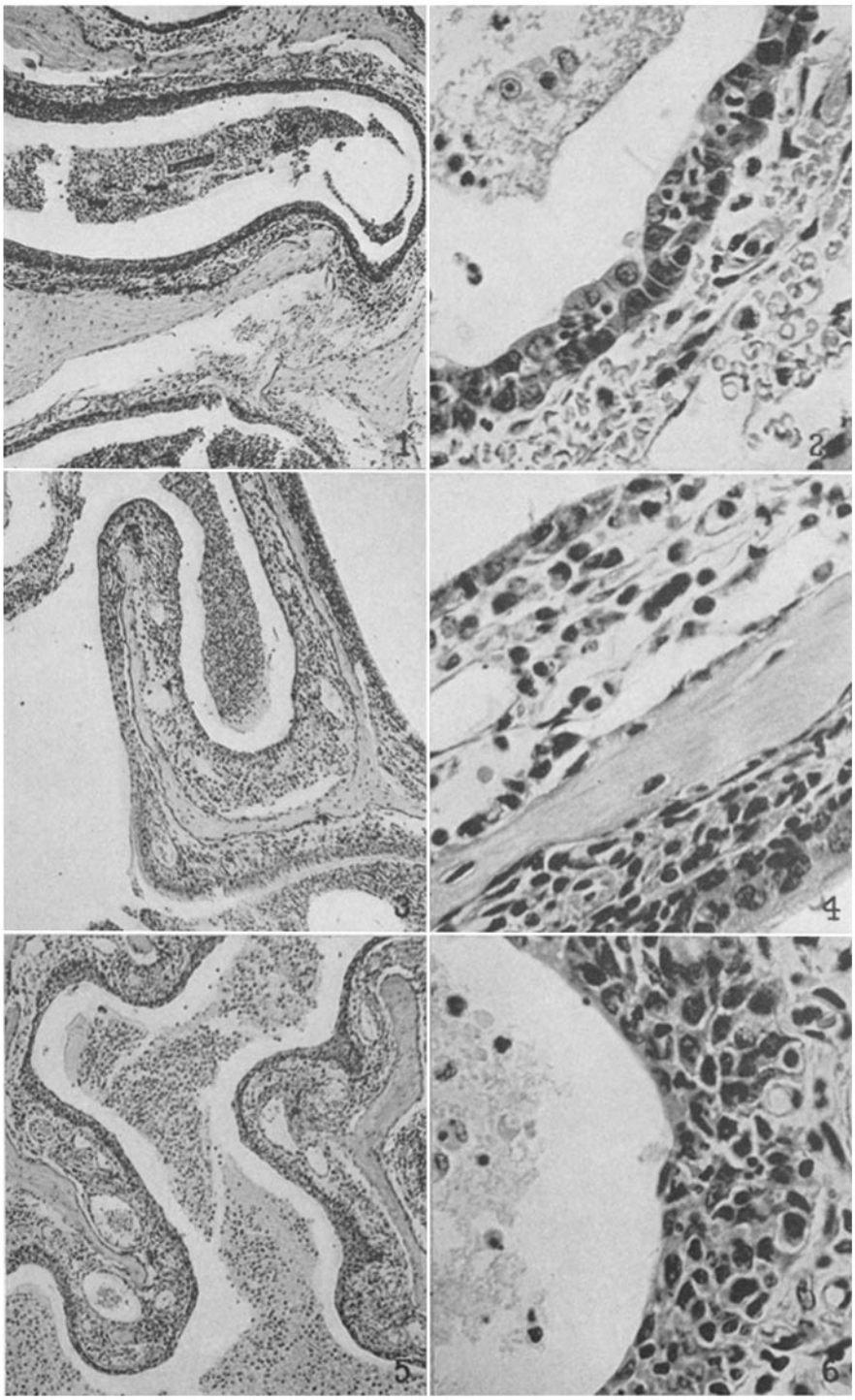
FIG. 2. High power view of Fig. 1. × 570. The epithelium is of a transitional type and shows leucocytic infiltration. The basal cells are cubical in shape and the appearance suggests a regenerative process.

FIG. 3. Influenza in a previously inoculated ferret. Day 3. Ferret 9-47. Anterior turbinate. × 80. Illustrates the presence of resistant areas of epithelium. On one side of the lamina the epithelium has been desquamated to a single layer of cells and on the other side it is normal in appearance. There is exudate and inflammatory reaction.

FIG. 4. High power view of Fig. 3. × 570. The epithelium covering the lamina on one side is necrotic and desquamated (above) and on the other is unaffected (below). The submucosa is abnormal beneath the desquamated epithelium but unaffected beneath the normal epithelium.

FIG. 5. Influenza in a previously inoculated ferret. Day 3. Ferret 10-25. Anterior turbinate. × 80. Epithelial desquamation, exudative and inflammatory reactions are present but the residual epithelium varies in thickness and has pockets of stratified cells.

FIG. 6. High power view of Fig. 5. × 570. A pocket of stratified cells with some leucocytic infiltration which suggests that here an area of stratified epithelium remained after the first attack of influenza and that this was partially resistant to reinfection.



Photographed by Joseph B. Haulenbeek

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