Comparison of Overt and Inapparent Influenzal Infection in Ferret

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ABSTRACT

In relation to the size of viral inoculum, influenza infection in ferrets caused by the inoculation of a mouse-adapted subline of PR 8 strain of type A influenza virus was studied. The results are summarized as follows;

1) When ferrets were inoculated with a highly diluted virus \(10^{-7}\), a small proportion of them experienced inapparent infections and the rest of them escaped the infection.

2) With the increased size of viral inocula, there was a good correlation between the size of infecting doses and the frequency of overt infections in ferrets.

3) Nasal tissues were the main locus of viral multiplication in ferrets at 72 hours after viral inoculation. Viral multiplication in nasal tissues was demonstrated only in a small proportion of ferrets which were inoculated with a \(10^{-7}\) dilution of virus; however, when the size of viral inoculum was increased above this level, all ferrets had viral growth in their nasal tissues.

4) The involvement of pulmonary tissues, viral growth in those tissues and the development of gross lung lesions were significantly rare. There was no clear-cut relationship between the size of infecting doses and the frequency of such pulmonary involvements in ferrets.

INTRODUCTION

It is generally agreed that a large proportion of influenza infections in man is inapparent, and this has been proven serologically (Brown et al. 1960, Keogh et al. 1958). In human influenza epidemics, the clinical manifestations of the infection is well characterized by sudden onset of fever, generalized muscular pains and rapid recovery in uncomplicated cases. Significant pneumonic complications among cases from the general population in usual epidemics scarcely exceed 1 per cent of the total (Francis and Maassab 1965).

Since ferrets are known to react with fever and with an efficient immune response to experimental infection by certain strains of influenza virus, studies were made on the occurrence of overt and inapparent influenzal infections in ferrets in relation to the size of infecting doses. In this study, the criteria of infection in ferrets were as follows: 1) Overt infection is the one in which the ferret had fever during the acute phase of the infection and produced a high level of antibody (HI) in its serum which could be detected during the convalescent stage. and 2) inapparent infection is that indicated by antibody production as in the case of overt infection but without fever during the course of the infection.

MATERIALS AND METHODS

Ferrets: Twelve to 18-month old female ferrets, weighing from 700 to 1000 grams, were purchased from Gilman Marshall (N.Y., U.S.A.). Two ferrets were kept in a single metal cage and fed with canned dog food. From one week
before the experiments rectal temperature was measured, twice daily at 10:00 A.M. and 2:00 P.M., with Thermistemp Telethermometer (Model TK, Yellow Springs Inst. Co., Yellow Springs, Ohio, U.S.A.) by inserting 1.5 cm. length of a metal probe into the rectum. Rectal temperature was well stabilized before experiments were started. A sharp rise in rectal temperature to 102°F or more after virus inoculation was considered as fever.

For virus inoculation the ferret was anesthetized by intraperitoneal injection of 15 to 20 mg of Sodium Nembutal (veterinary, Abbott Labs.) and then 0.5 ml of an appropriately diluted virus in Standard Medium (S.M.) (Fazekas de St Groth and White 1958) was given intranasally to the ferret.

Following virus inoculation, each ferret was placed in an individual cage and kept in the isolation room in which normal ferrets were kept. Room temperature of normal animal and isolation room was maintained between 74 to 78°F.

When an experiment was completed, the ferret was killed by intraperitoneal injection of 40 to 50 mg of Nembutal. Lung and trachea were removed aseptically and nasal tissues including mucous membrane and turbinates were thoroughly scraped out after cutting the frontal part of maxilla through the eye-to-eye line. To obtain nasal washing 5 ml of S.M. was injected into the trachea which was still attached to the larynx and nasal cavity, and the wash fluid coming out from the anterior nostril was collected.

A paired serum, namely pre-and post-infection, was obtained by cardiac puncture under intraperitoneal Nembutal anesthesia. Pre-infection serum was obtained when the temperature measurement was started, and post-infection serum when the ferret was killed after completing the experiment. All sera were stored at −48°C and heat-inactivated at 56°C for 30 minutes before use.

Virus: A mouse-adapted subline of PR 8 strain of type A influenza virus used in the previous study (Yang and Evans 1961) was further passaged 8 times in BALB/c mice with 3-day intervals. At the end of the 15th passage a 10% lung homogenate of infected mice was made with S.M. and 0.1 ml of a 10⁻⁴ dilution of the homogenate was inoculated intra-allantoically into 10-day embryonated eggs. After 45 hours incubation at 37°C, infected allantoic fluid was pooled, centrifuged at 2500 rpm for 10 minutes and stored at −48°C.

One ml of the pooled virus had a titer of 10⁴.⁰ EID⁵₀ and 320 HA units. In a preliminary experiment, this virus was shown to be insensitive to the Chu inhibitor (Chu 1951).

Virus titration: Viral contents in nasal tissues and lung of virus inoculated ferret were determined by egg infectivity titration using four of 10⁻¹ or 11-day embryonated eggs per dilution, and titer was expressed in terms of EID⁵₀ per ml of the tissue homogenate.

For the titration, a 10% homogenate of ferret’s tissues was made separately with S.M., centrifuged at 2500 rpm for 10 minutes and the supernatants were used. All titrations were carried out on the same day when ferrets were killed.

Hemagglutination-inhibition test of sera and nasal washings: Hemagglutination-inhibiting antibody (HI) in a ferret’s serum was determined by Salk’s method (Salk 1944). None of the pre-infection sera contained a detectable amount of HI antibody. “Lipid-insensitive” chicken red blood cells were used for the titration of HI antibody in nasal washings (Fazekas de St Groth and Donnelley 1950).

EXPERIMENTAL RESULTS

1. The effect of size of viral inoculum on the development of overt or inapparent infection: In two experiments ferrets were inoculated with a series of dilutions of virus and the incidence of overt, and inapparent infections was determined. In the first experiment 2 ferrets
were inoculated with each of 3 dilutions of virus, $10^{-3}$, $10^{-5}$ and $10^{-7}$. Two ferrets served as controls: one was inoculated with S.M. and the other received only an intraperitoneal injection of anesthetic. On the 8th day the ferrets were killed and HI antibody titers in nasal washings and post-infection sera were determined. The results are shown in Fig. 1. Tests for virus of nasal washings and lung homogenates were negative in all animals.

All ferrets inoculated with $10^{-5}$ and $10^{-3}$ dilutions of virus had fever and all of their post-infection sera contained a high level (1:2560) of HI antibody. Nasal washings from these ferrets contained HI antibody (1:8 to 1:32), considerably below the level found in the serum.

The two ferrets inoculated with a $10^{-7}$ dilution of virus showed no fever. However, one of them, i.e., ferret #6, experienced an inapparent infection, as a significant amount (1:640) of HI antibody was found in its post-infection serum. No HI antibody was detected in nasal washings of these two animals.

From these results, it was evident that inapparent influenza infection could be produced in ferrets, with the virus strain employed, only by using small viral inocula. Six ferrets were then inoculated with a $10^{-7}$ and 4 ferrets with a $10^{-4}$ dilution of virus, and the course of the infection was followed as in the preceding experiment. As a positive control, 2 ferrets received a $10^{-3}$ dilution of virus. None of the ferrets used in this experiment contained any detectable amount of virus in their nasal or in lung homogenates when they were killed on the 8th day after the virus inoculation. The results of this experiment are summarized in Table 1.

By combining the results of the 2 experiments, as shown in Fig. 1 and Table 1, a general picture emerges of the relationship of size of

**Fig. 1.** Size of viral inoculum and development of overt or inapparent infection in ferrets.

*Controls: ferret #7 was inoculated with S.M. and ferret #8 received only an intraperitoneal injection of Nembutal.
**HI antibody in the post-infection serum and in nasal washing.
Table 1. Relationship between the size of viral inocula and the frequency of overt infections

<table>
<thead>
<tr>
<th>Size of inoculum</th>
<th>No. of ferrets with fever*</th>
<th>No. of ferrets produced HI antibody in serum**</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-3}$</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>3/4</td>
<td>3/3*</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>1/6</td>
<td>2/6</td>
</tr>
</tbody>
</table>

*Ferrets which had sharp rises in rectal temperature to or above 102°F during 8 days after virus inoculation.
**Post-infection serum which was obtained on the 8th day after virus inoculation.
One ferret in this $10^{-6}$ group was killed on the 4th day after virus inoculation, and therefore not included.

Viral inoculum to the nature of the infection it elicited.

A $10^{-7}$ inoculum approximated 1 ferret infectious dose (ID₅₀) as 3 of 8 ferrets given this amount of virus were infected. Two of the three ferrets infected with this amount of virus experienced inapparent infections. All four of those inoculated with $10^{10}$ (10⁻⁷ dilution of virus) were infected and one of them had an inapparent infection. Of 6 ferrets given 100 ID₅₀ or more (10⁻⁷ or 10⁻⁵) 100% were infected and there were no inapparent infections.

Fever curves observed in these experimental groups of ferrets were usually of the "single-spike" type as seen in ferret #2 of Fig. 1. Two ferrets, one in the $10^{-3}$ group and the other in the $10^{-4}$ group, had rather typical diphasic fever curves as shown in ferret #1 of Fig. 1.

Of 18 ferrets used in these 2 experiments there were 3 ferrets which developed lung lesions recognizable at autopsy, one ferret in the $10^{-3}$ group and 2 in the $10^{-4}$ group. In all cases the gross lung lesions covered only a small area of lower lobe of either right or left lung.

2. The extent and site of viral multiplication in overt and inapparent infection: The extent and site of viral multiplication in the case of inapparent infections was determined by titrating viral contents of nasal and lung homogenate of ferrets inoculated with a $10^{-7}$ dilution of virus and killed at 72 hours after virus inoculation.

Table 2. The extent and site of viral multiplication in ferrets in relation to the size of viral inocula

<table>
<thead>
<tr>
<th>Size of inoculum</th>
<th>Viral growth in</th>
<th>No. of ferrets</th>
<th>No. of ferrets with gross lung lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nose</td>
<td>Lung</td>
<td></td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>4/4</td>
<td>1/4</td>
<td>4/4</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>3/3</td>
<td>1/3</td>
<td>2/3</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>2/8</td>
<td>1/8</td>
<td>0/8</td>
</tr>
</tbody>
</table>

All ferrets were killed at 72 hours after virus inoculation.

At the same time several ferrets were inoculated with $10^{-6}$ and $10^{-5}$ dilutions of virus to observe the effects of increased size of viral inoculum on the patterns of viral multiplication in their tissues. Table 2 summarizes the results of this experiment.

Tests of 8 ferrets inoculated with a $10^{-7}$ dilution of virus showed that viral multiplication occurred in 3 ferrets, 2 in nasal tissues and 1 in the lung. None of the ferrets in this $10^{-7}$ group had fever and none developed gross lung lesion.

When the size of viral inoculum was increased by 10 and 100-fold, all 7 ferrets had viral multiplication in their nasal tissues, and 6 of them had fever.

In general, the extent of viral multiplication in nasal tissues of ferrets at 72 hours after virus inoculation was extremely high, ranging from $10^{4.3}$ to $10^{5.7}$ EID₅₀ per ml of the homogenates. There appeared to be no significant difference in the amount of virus produced in nasal tissues of ferrets which were inoculated with $10^{-7}$, $10^{-6}$ and $10^{-5}$ dilutions of virus respectively.

Of the total of 15 ferrets used in this experiment, viral growth in lungs was demonstrated only in 3 ferrets, one in each $10^{-7}$, $10^{-6}$ and $10^{-5}$ group (Table 3). The viral titers observed, $10^{3.7}$, $10^{4.1}$ and $10^{5.3}$ respectively, were lower than those in nasal tissues.

At 72 hours after virus inoculation, none of the ferrets had a detectable amount of HI antibody in their sera.
Table 3. The frequency of pulmonary involvements in relation to the size of viral inocula

<table>
<thead>
<tr>
<th>Size of inoculum</th>
<th>Days after virus inoculation</th>
<th>No. of ferrets with gross lung lesion</th>
<th>No. of ferrets showed viral growth in lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-3}$</td>
<td>3 days</td>
<td>N.D.*</td>
<td>N.D.</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>1/4</td>
<td>0/4</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>3</td>
<td>1/4</td>
<td>1/4</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>8</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>0/8</td>
<td>1/8</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>3</td>
<td>0/8</td>
<td>0/8</td>
</tr>
</tbody>
</table>

*N.D.: Not done.

**DISCUSSION**

Studies on experimental influenza in ferrets indicate the occasional occurrence of inapparent infections (Smith et al. 1933, Shope 1934, Burnet 1937, Taylor and Dreguss 1941, Liu 1955). In this study it was observed that when ferrets were inoculated with a highly diluted virus ($10^{-7}$) many of them escaped influenza infection and that a small proportion had the infection, some of which took the course of inapparent infections. In support of these observations, viral multiplication was demonstrated in 3 of 8 ferrets inoculated with such a highly diluted virus, 2 in their nasal tissues and one in the lung at 72 hours after viral inoculation. Even though ferrets were inoculated with larger amounts of virus such as a $10^{-4}$ dilution of virus, it was still possible to observe the occurrence of inapparent infection among them. However, it was unsuccessful to produce such a model of inapparent influenza infection in ferrets with regularity.

There was a good correlation between the size of viral inoculum and the frequency of overt infection in ferrets when they were inoculated with larger amounts of virus above the level of a $10^{-7}$ dilution of virus. At 72 hours after virus inoculation with large amounts of virus such as $10^{-4}$ and $10^{-5}$ dilution of virus, viral multiplication was demonstrated in nasal tissues of all ferrets. These findings strongly suggest that fever was ordinarily accompanied by viral multiplication limited to the upper respiratory tracts.

From the data shown in Table 3, it is evident that nasal tissues rather than pulmonary, were the main locus of viral multiplication in ferrets which were inoculated with $10^{-7}$, $10^{-4}$ and $10^{-3}$ dilutions of virus respectively and tested 72 hours after the inoculation.

It is also apparent that the involvement of pulmonary tissues of ferrets, i.e., viral multiplication in lungs and the development of gross lung lesions, was significantly rare. Table 3 clearly shows that with increasing amounts of virus in the inoculum there was no concomitant increase in the number of ferrets which had viral growth in the lungs, and no increase in numbers showing gross lung lesions at 72 hours after the inoculation. These results are in contrast to the earlier reports on "ferret influenza" that showed high incidences of pulmonary involvement (Shope 1934, Liu 1955, Francis 1934, Hull and Loosli 1951). The difference may be due to the smaller volume of viral inoculum used in this study which might help to confine the infection in the upper respiratory tracts (Bang 1961), or due to the effect of the "lowered ferret virulence" of the virus as shown by others (Taylor and Dreguss 1941, Sugg and Nagaki 1955, Horsfall et al. 1940).

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**REFERENCES**


