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# IMMUNITY TO INFLUENZA IN FERRETS

## I. RESPONSE TO LIVE AND KILLED VIRUS

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**Summary.—Ferrets were found to react with a sharp febrile response to intranasal infection with influenza virus A2/Hong Kong/3/68. Virus was recovered from nasal washings taken 3 days after infection, and virus antibody was found in serum specimens taken 21 days after virus infection. Virus infection produced a pronounced rhinitis; the protein concentration in nasal washings was found to increase three to five-fold with peak levels occurring on day 7, post-infection. Concomitant with the increased protein levels, detectable levels of HI and neutralizing antibody were found in the nasal washings. However, nasal washings taken 13 days or more after influenza virus infection did not contain either increased levels of protein or detectable antibody. These ferrets were immune to re-infection with homologous virus inoculated 5 weeks after primary infection. Thus, ferrets showed no febrile response; virus was not recovered from nasal washings; serum antibody titres did not increase; no increase in protein levels was found in nasal washings; and HI antibody was not found in nasal washings.**

**Using these criteria to assess susceptibility or immunity to influenza virus infection, infection with attenuated influenza virus A2/Hong Kong/1/68 produced immunity to re-infection with virulent virus. Ferrets infected with influenza virus B/England/13/65 or immunized with killed A2/Hong Kong virus did not induce any immunity to infection with influenza virus A2/Hong Kong/3/68.**

THE evaluation of vaccines against influenza would greatly benefit from studies in experimental animals which possess the same tissue tropisms for virus infection as seen in man. These studies would allow a comparison of immunizing procedures; an opportunity to challenge immunized animals with fully virulent viruses under controlled conditions; and provide an experimental system to investigate cross-immunity with variant virus strains. Influenza virus has been shown to be transmissible to a variety of animals, but it is the disease in ferrets which most closely approximates that of influenza in man (Smith, Andrewes and Laidlaw, 1933; Shope, 1934; Lui, 1955; Sugg and Nagaki, 1955; Haff, Shriver and Stewart, 1966). In addition, the size of ferrets is convenient for studies of both systemic and local antibody production.

Previous studies have described the susceptibility of ferrets to influenza, and showed that infected animals are subsequently resistant to re-infection. Thus, ferrets re-infected with influenza viruses fail to show the febrile response and

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physical signs which characterize the primary infection (Smith *et al.*, 1933; Francis and Stuart-Harris, 1938). In the present study, we report the temperature response, virus recovery and changes in serum and nasal antibody which follow influenza virus A2/Hong Kong infection of normal and immunized ferrets. The results serve to establish the reactions of immunized and unimmunized animals to infection, and help to define the necessary criteria for the evaluation of future immunization procedures in ferrets.

## MATERIALS AND METHODS

### *Viruses*

Influenza virus A2/Hong Kong/3/68, which had received 5 egg passages, was kindly supplied by Dr G. C. Schild, National Institute for Medical Research, Mill Hill, London. A virus pool was prepared from the allantoic fluids of 10-day embryonated eggs inoculated with $10^{5\cdot0}$ $EID_{50}$ and incubated for 48 hours at 36°. This virus pool had an infectivity titre of $10^{8\cdot5}$ $EID_{50}$/ml and was stored at − 80° in 2·0 ml aliquots. Ferrets were anaesthetized with ether and inoculated intranasally with $10^{6\cdot5}$ $EID_{50}$ of virus in 0·5 ml of Hanks' saline. This inoculum reproducibly produced a sharp rise in temperature 24–48 hours after inoculation, and the physical signs of influenza in the ferret described previously (Smith *et al.*, 1933; Lui, 1955; Haff *et al.*, 1966). The inoculum did not produce visible lung lesions in ferrets killed 7 days after virus infection, and did not cause death or lung lesions in mice. The virus is referred to as the virulent virus for ferrets, and was used for all tests of ferret immunity.

The strain of influenza virus A2/Hong Kong attenuated for man by low temperature egg passage (Beare and Bynoe, 1969), was obtained from Dr A. S. Beare, Common Cold Research Unit, Salisbury, Wilts. The influenza virus strain B/England/13/65, also attenuated for man (Beare, Bynoe and Tyrrell, 1968), was obtained from Dr D. C. Breeze, Evans Medical Ltd., Speke. Groups of ferrets were each inoculated intranasally under ether anaesthesia with $10^{6\cdot5}$ $EID_{50}$ of one of these human attenuated viruses in 0·5 ml of Hanks' saline.

### *Virus vaccine*

Formalin-killed, monotypic influenza virus A2/Hong Kong was kindly supplied by Dr D. C. Breeze. Ferrets were each inoculated intramuscularly with 8000 HA units of this vaccine in an 0·5 ml volume.

### *Experimental design*

Adult ferrets, aged 4–6 months and weighing between 800–1200 g, were obtained from accredited dealers and were housed in individual cages for 7 days prior to virus infection or immunization. During this time nasal wash specimens and temperatures were taken to establish the normal values for each ferret. Following immunization, ferret temperatures were taken twice daily for 4 days, and subsequently daily for 3 days. Nasal washings were collected 3 days after infection for virus titration, and on alternate days from the fifth to the fifteenth day for protein and antibody determinations. Ferrets were inoculated intranasally with virulent virus 35 days after immunization, and temperature readings and nasal washings were obtained as described following immunization. Blood specimens were collected by cardiac puncture 24–48 hours prior to immunization and to challenge, and a third specimen was taken 28 days after challenge.

### *Temperatures*

Ferret temperatures were taken *per rectum* using a clinical thermometer. Base-line temperatures for individual ferrets varied less day-by-day than the temperatures of different animals. Thus mean pre-immunization temperatures (mean of 7 daily readings) varied from 38·1° ± 0·3° to 39·4° ± 0·3°. For this reason, the significance of the temperature response observed in ferrets following immunization or infection with virus was gauged by two parameters. Since a temperature of 40·0° or more was never recorded for a normal ferret, 2 readings of 40·0° or greater occurring 24–72 hours after virus infection was recorded as a significant temperature, regardless of the mean pre-infection temperature. Two temperature readings of 1·0° or greater above the mean pre-inoculation temperature and occurring 24–72 hours after virus infection was recorded as a significant increased temperature.

*Nasal washings*

Nasal washings were collected from ferrets for virus isolation and for protein and antibody determinations. A volume of 0·3–0·4 ml of phosphate buffered saline, pH 7·4 (PBS) was instilled with a syringe into one nostril. After 1–3 seconds the ferret forcibly expressed the PBS which was collected in a Petri dish; this was due to the natural sneeze reflex of the ferret (Shope, 1934). Larger volumes of PBS were frequently swallowed when injected into the nostril while volumes of less than 0·3 ml were not consistently expressed. The nostrils were washed out alternately with a total of 10 ml of PBS. Specimens of nasal washings for virus isolation were stored at — 80° with bovine serum albumin (2·0 per cent final concentration) and antibiotics (250 unitsl/ml of penicillin and 200 $\mu$g/ml of streptomycin). Nasal washings for antibody studies were shaken with glass beads, centrifuged at 3000 rpm/10 min, and the supernatant fluid was concentrated ten-fold by dialysis against 30 per cent carbowax and then stored at — 20°.

*Virus isolation*

Serial, logarithmic dilutions of nasal washings were made in mixture " 199 " and 0·1 ml volumes inoculated into 10-day embryonated eggs by the allantoic route. After incubation at 36° for 72 hours, the allantoic fluids were harvested and tested for virus haemagglutinin using 0·5 per cent fowl cells. The titre of virus in nasal washings was estimated using the methods of Reed and Muench (1938). The identity of the virus recovered from each ferret was established by haemagglutination inhibition tests using specific ferret antisera (Machin, Potter and Oxford, 1970).

*Serological tests*

(a) *Haemagglutination inhibition (HI) tests.*—HI tests were performed in Perspex plates (WHO, 1953). Before testing, sera or nasal washings were incubated for 18 hours at 37° with four volumes of cholera filtrate (Burroughs Wellcome Ltd.) and subsequently heated at 56° for 1 hour. A 0·2 ml volume of serum or nasal washing was incubated with an equal volume of virus containing 8 haemagglutinating units (50 per cent end point) for 10–15 min at room temperature. After this time 0·2 ml of fowl erythrocytes (0·5 per cent suspension in PBS) was added and the HI tests read after the cells had settled at room temperature. The antibody titre was expressed as the highest dilution which caused a 50 per cent reduction of virus haemagglutination.

(b) *Neutralization tests.*—Primary rhesus monkey kidney cells were kindly supplied by the Biological Standards Division of the National Institute for Medical Research. The cells were grown in Eagle's minimal essential medium (MEM) containing 10 per cent inactivated calf serum and maintained in mixture " 199 " without added serum. Influenza virus A2/Hong Kong/3/68 was titrated in rhesus monkey cells and the haemadsorption end-point titre ($TCD_{50}$) calculated after 72 hours' incubation at 37°; the virus titre was separately established for each batch of cells prior to their use in neutralization tests. Dilutions of nasal washings were mixed with an equal volume of virus containing 100 $TCD_{50}$ of virus and incubated for 1 hour at room temperature. After this time 0·2 ml of virus-nasal wash mixture was inoculated on to rhesus monkey kidney cells. After incubation for 72 hours at 36° the tubes were tested for haemadsorption by the quantitative haemadsorption (QH) technique (Finter, 1967). The neutralization titre of antibody was expressed as the highest dilution of nasal wash which reduced virus haemadsorption by 50 per cent ($QH_{50}$) compared with that measured in virus controls.

*Protein determination*

The protein concentration of ten-fold concentrated nasal washings was estimated by the method of Lowry *et al.* (1951).

## RESULTS

### 1. *Infection and subsequent re-infection of ferrets with virulent influenza virus A2/Hong Kong/3/68*

(a) *Response to infection.*—Five ferrets were inoculated with $10^{6·5}$ $EID_{50}$ of virulent influenza virus A2/Hong Kong/3/68 by the intranasal route under ether

TABLE I.—*Response of Ferrets to Infection and Subsequent Re-infection with Influenza Virus A2/Hong Kong/3/68*

| | Response to infection | | | | | | Response to re-infection | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temperature | | | | Change in nasal antibody† | | Temperature | | | | Change in nasal antibody | |
| Ferret No. | ⩾ 40·0° | ⩾1·0° rise | Virus isolation (titre)* | Change in serum HI titre | HI | Neut.‡ | ⩾ 40·0° | ⩾ 1·0° rise | Virus isolation | Serum HI titre | HI | Neut. |
| 104 | + | + | + (5·25) | <5–1280 | <5–20 | <2–30 | — | — | — | 480 | <5–<5 | <2–<2 |
| 105 | + | + | + (5·25) | <5–1280 | <5–10 | <2–25 | — | — | — | 480 | <5–<5 | <2–<2 |
| 88 | + | + | + (NT) | <5– 960 | <5–15 | <2–25 | — | — | — | 240 | <5–<5 | <2– 5 |
| 106 | + | + | + (4·25) | <5–2560 | <5–15 | <2–50 | — | — | — | 480 | <5–<5 | <2– 5 |
| 89 | + | + | + (NT) | <5–1280 | <5–10 | <2–15 | — | — | — | 480 | <5–<5 | <2–<2 |

* $Log_{10}$ $EID_{50}$/ml.
† Change from pre-infection titre to peak post-infection titre.
‡ Neutralization test.
NT, not tested.

anaesthesia. This inoculum produced a sharp increase in temperature 36–48 hours after inoculation which then subsided. A second peak of fever was observed 5 days after virus inoculation in 3 of the ferrets, after which time the temperature fell to pre-inoculation levels. The temperature response for 2 of the ferrets is shown in Fig. 1. For all 5 ferrets, a significant temperature (2 readings of

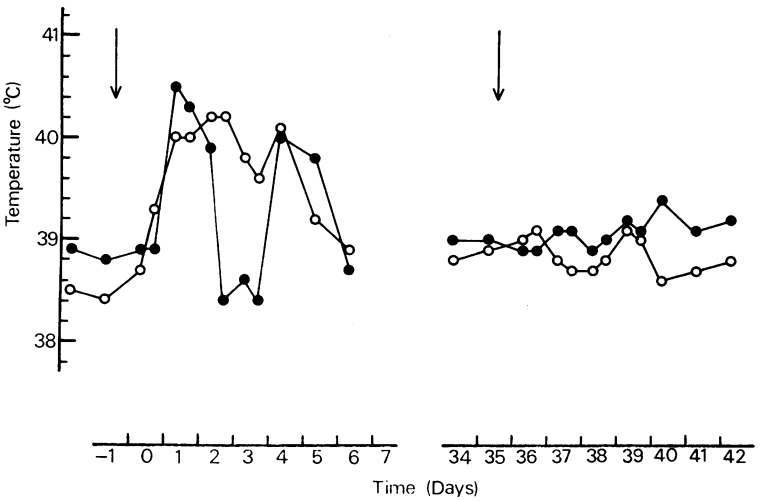


FIG. 1.—Temperature response of ferrets No. 88 and 106 to infection with influenza virus A2/Hong Kong/1/68 and later re-infection with homologous virus. Arrows indicate virus infection (day 0) and re-infection (day 35).

$\geqslant$ 40·0°) and a significant increased temperature (2 readings of $\geqslant$ 1·0° rise above the mean pre-inoculation level) were recorded in the period 24–72 hours after virus inoculation.

Nasal washings taken 3 days after virus inoculation yielded virus from all 5 animals; the titre of virus recovered in the nasal washings of 3 of the ferrets was $10^{4\cdot25}$–$10^{5\cdot25}$ $EID_{50}$/ml (Table I). Following infection, all animals were found to have serum HI antibody to A2/Hong Kong influenza virus.

Tests on nasal washings, which had been concentrated ten-fold, indicated the presence of both HI and neutralizing antibody in specimens from all the ferrets (Table I). Haemagglutination-inhibiting antibody was not detected in nasal washings until 7 days post-infection, at which time it was detected in all the animals. Antibody was also found in all specimens taken 9 days after infection, but at day 11 antibody was detected in the nasal washings of only 2 of 5 animals. No HI antibody was detected in nasal washings taken 13 or more days after infection. Similar results were obtained in titrations of nasal washings for neutralizing antibody, though this test was more sensitive than the HI test. Nasal washings taken 5–7 days post-infection contained three to five-fold greater concentrations of protein than pre-infection washings. The protein and antibody changes which were found in serial nasal washings from one of the ferrets is shown in Fig. 2.

(b) *Response to re-infection.*—Thirty-five days after infection with influenza virus A2/Hong Kong/3/68, the 5 ferrets were re-infected with $10^{6\cdot5}$ $EID_{50}$ of the

same virus. This time the virus did not produce an elevated temperature response in any of the animals (the temperature recordings for 2 of the ferrets are shown in Fig. 1). Virus was not recovered from any of the ferret nasal washings taken 3 days after re-infection (Table I). Serum samples taken 28 days after re-infection showed levels of HI antibody which were one-third to one-fifth of the pre-infection titre.

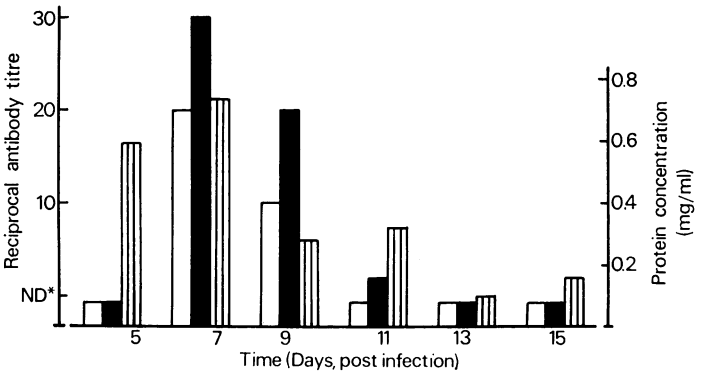


FIG. 2.—Protein and antibody levels in serial nasal washings from ferret No. 104 following infection with influenza virus A2/Hong Kong/3/68.

* Antibody not detected.
□ HI antibody titre.
■ Neutralizing antibody titre.
▥ Protein concentration.

Haemagglutination-inhibiting antibody was not detected in nasal washings taken 5–15 days after re-infection (Table I) and no increase was found in the protein concentration of nasal washings. However, in a single nasal wash specimen from each of 2 ferrets taken 7 days after challenge there was a detectable level of neutralizing antibody (titre 1 : 5) but specimens from the same ferrets taken 5 days and 9 or more days after challenge did not reveal antibody. All specimens from other ferrets were negative (titre $< 1 : 2$) for neutralizing antibody to influenza virus A2/Hong Kong/3/68.

2. *The response to inoculation with attenuated A2/Hong Kong virus, and subsequent inoculation with virulent influenza virus A2/Hong Kong/3/68*

(a) *First infection.*—Four ferrets were inoculated intranasally with $10^{6.5}$ $EID_{50}$ of attenuated A2 Hong Kong virus. This virus produced a marked increase in temperature in 2 ferrets, a modified response in one animal and no temperature increase in the remaining animal. The temperature response for 2 of the ferrets is shown in Fig. 3. Virus was recovered from nasal washings taken 3 days after immunization from all 4 animals. The titre of virus in the nasal wash specimens was $10^{3.25}$–$10^{4.25}$ $EID_{50}$/ml (Table II).

Serum HI antibody was demonstrable in all 4 ferrets following inoculation with attenuated virus (Table II). Neutralizing antibody and HI antibody was found in nasal washings from 3 of the ferrets taken 7 and 9 days after infection, but not in subsequent specimens. In the remaining ferret a low titre of neutral-

TABLE II.—*Response of Ferrets to Infection with A2/Hong Kong/1/68 and Subsequent Re-Infection with Influenza Virus A2/Hong Kong/3/68.*

| | Response to infection | | | | | | Response to re-infection | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temperature | | Virus | Change in | Change in nasal antibody† | | Temperature | | | | Change in nasal antibody | |
| Ferret No. | ⩾ 40·0° | ⩾1·0° rise | isolation (titre)* | serum HI titre | HI | Neut.‡ | ⩾ 40·0° | ⩾ 1·0° rise | Virus isolation | Serum HI titre | HI | Neut. |
| 99 | + | + | + (3·75) | <5–320 | <5–<5 | <2–5 | — | — | — | 480 | <5–<5 | <2–<2 |
| 100 | − | − | + (4·25) | <5–320 | <5–10 | <2–20 | — | — | — | 480 | <5–<5 | <2–<2 |
| 101 | + | + | + (4·25) | <5–640 | <5–10 | <2–40 | — | — | — | 480 | <5–<5 | <2–<2 |
| 102 | − | + | + (3·25) | <5–240 | <5–5 | <2–20 | — | — | — | 480 | <5–<5 | <2–5 |

* $Log_{10}$ $EID_{50}$/ml.
† Change from pre-infection titre to peak post-infection titre.
‡ Neutralization test.

izing antibody (titre 1 : 5) was found only on day 7 after infection, and no nasal specimens from this animal contained detectable HI antibody (Table II).

(b) *Response to re-infection.*—The ferrets were inoculated with virulent virus A2/Hong Kong/3/68 35 days after the first infection with attenuated A2/Hong Kong virus. Re-infection did not produce a rise in temperature in any of the animals (the temperature response for 2 of the ferrets is shown in Fig. 3). Virus was not recovered from nasal washings collected 3 days after inoculation (Table II). Serum HI antibody levels were not significantly altered following re-infection; the titres were unchanged in 3 animals, and increased two-fold in the remaining ferret. Nasal washings taken 5–15 days after re-inoculation did not show

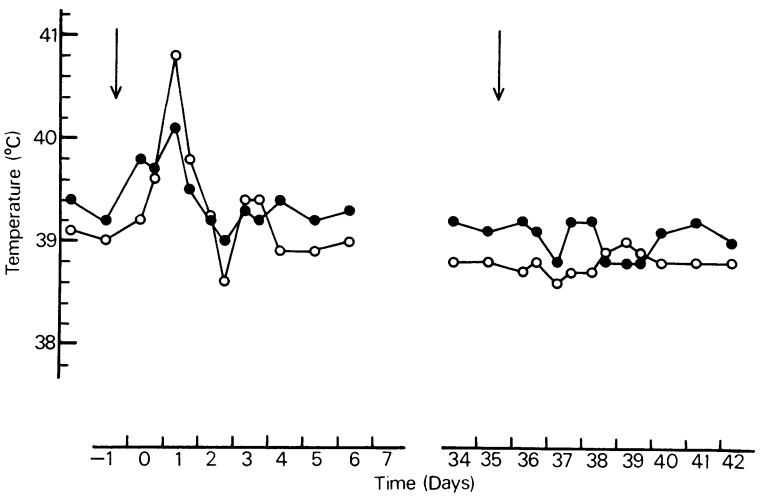


FIG. 3.—Temperature response of ferrets No. 100 and 102 to infection with attenuated influenza virus A2/Hong Kong/1/68, and later re-infection with influenza virus A2/Hong Kong/3/68. Arrows indicate virus infection (day 0) and virus challenge (day 35).

increased protein concentrations and HI antibody was not detected in any of the specimens; only one specimen from a single ferret contained demonstrable neutralizing antibody. Thus, the reaction of these ferrets to re-inoculation was very similar to that found following challenge of ferrets previously infected with live virulent influenza virus A2/Hong Kong/3/68 (Table I).

3. *Infection with attenuated influenza virus B/England/13/65, and re-infection with virulent influenza A2/Hong Kong/3/68*

(a) *Response to infection with B/England/13/65.*—Four ferrets were infected intranasally with $10^{6 \cdot 5}$ $EID_{50}$ of influenza virus B/England/13/65. This infection caused a significantly increased temperature in 2 of the 4 ferrets but none of the animals showed a significantly high temperature (Table III). Virus was recovered from all 4 ferrets from nasal washings taken 3 days after infection. Nasal washings showed a relatively small increase in protein concentration for day 7, post-infection, and none of the specimens contained detectable levels of HI or neutralizing antibody to influenza virus B/England/13/65. Serum specimens taken 5 weeks after infection showed HI antibody to influenza virus B/England/13/65 for all 4 ferrets, but no antibody to influenza virus A2/Hong Kong/3/68.

TABLE III.—*Response of Ferrets to Infection with Influenza Virus B/England/13/65*

| Ferret No. | Temperature ⩾ 40·0° | Temperature ⩾ 1·0° rise | Virus isolation (titre)* | Change in serum HI titre | Change in nasal antibody† HI | Change in nasal antibody† Neut.‡ |
|---|---|---|---|---|---|---|
| 91 | — | — | + (4·25) | <5–160 | <5–<5 | <2–<2 |
| 92 | — | + | + (4·5) | <5–120 | <5–<5 | <2–<2 |
| 93 | — | + | + (4·25) | <5–320 | <5–<5 | <2–<2 |
| 94 | — | — | + (3·5) | <5–320 | <5–<5 | <2–<2 |

* $Log_{10}$ $EID_{50}$/ml.
† Change from pre-infection titre to peak post-infection titre.
‡ Neutralization test.

(b) *Response to re-infection with A2/Hong Kong/3/68.*—The ferrets immunized with attenuated influenza virus B/England/13/65 were infected 35 days later with live, virulent influenza virus A2/Hong Kong/3/68. This inoculation caused a marked rise in temperature in all animals; the temperature charts for 2 of the ferrets are shown in Fig. 4. All 4 ferrets exhibited both a significantly high temperature and a significant rise in temperature (Table IV). Virus was recov-

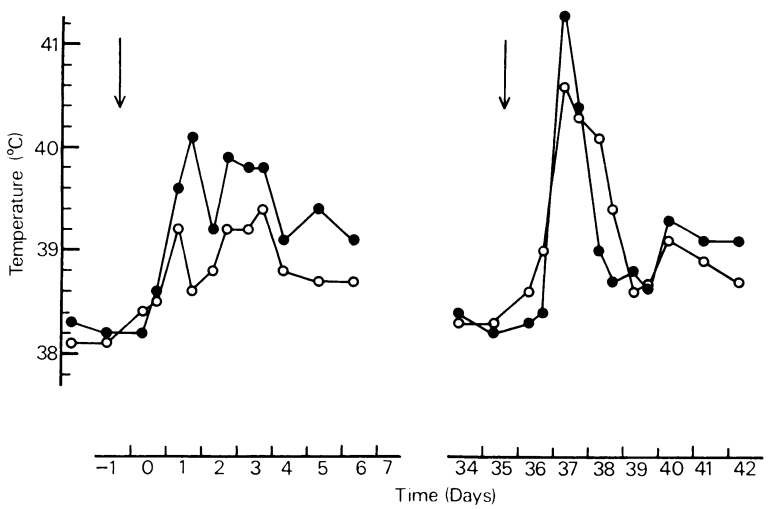


FIG. 4.—Temperature response of ferrets No. 92 and 93 to infection with influenza virus B/England/13/65, and later re-infection with influenza virus A2/Hong Kong/3/68. Arrows indicate virus infection (day 0), and virus challenge (day 35).

ered from nasal washings taken 3 days after challenge from all 4 ferrets, and the titre of virus in these specimens was similar to that found in non-immunized animals (Table I).

Haemagglutination-inhibition tests on serum specimens taken before and after re-infection showed a fall in HI titre to influenza virus B/England/13/65 and a rise in HI titre to influenza virus A2/Hong Kong/3/68 (Table IV) Nasal washings showed a three to four-fold increase in protein for specimens taken 7 and 9 days after re-infection; after this time the protein content of the nasal secretions fell to prechallenge levels. Concomitant with the appearance of

TABLE IV.—*Response of Ferrets to Re-infection with A2/Hong Kong/3/68 after Infection with B/England/13/65*

| Ferret No. | Temperature | | Virus isolation (titre)* | Change in serum HI to: | | Change in nasal HI to:† | | Change in nasal neut. antibody to:‡ | |
|---|---|---|---|---|---|---|---|---|---|
| | ⩾ 40·0° | ⩾ 1·0° rise | | A2/HK/3/68 | B/Eng/13/65 | A2/HK/3/68 | B/Eng/13/65 | A2/HK/3/68 | B/Eng/13/65 |
| 91 | + | + | + (4·75) | <5–640 | 160–60 | <5–15 | <5–10 | <2–30 | <2–5 |
| 92 | + | + | + (5·5) | <5–480 | 120–60 | <5–10 | <5–<5 | <2–15 | <2–2 |
| 93 | + | + | + (5·25) | <5–480 | 320–80 | <5–5 | <5–<5 | <2–10 | <2–<2 |
| 94 | + | + | + (4·75) | <5–480 | 320–120 | <5–30 | <5–5 | <2–60 | <2–5 |

* $Log_{10}$ $EID_{50}$/ml.
† Change from pre-infection titre to peak post-infection titre.
‡ Neutralization test.

TABLE V.—*Response of Ferrets to Immunization with Killed A2/Hong Kong, and Subsequent Infection with Influenza Virus A2/Hong Kong/3/68*

| | Response to immunization | | | | | Response to infection | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temperature | | Change in serum HI titre | Change in nasal antibody† | | Temperature | | Virus isolation (titre)* | Serum HI titre | Change in nasal antibody | |
| Ferret No. | ⩾ 4°·0° | ⩾ 1·0° rise | | HI | Neut.‡ | ⩾ 40·0° | ⩾ 1·0° rise | | | HI | Neut. |
| 90 | — | — | <5–<5 | <5–<5 | <2–<2 | + | + | + (4·75) | 480 | <5–15 | <2–45 |
| 95 | — | — | <5–<5 | <5–<5 | <2–<2 | + | + | + (4·5) | 960 | <5–30 | <2–50 |
| 96 | — | — | <5–<5 | <5–<5 | <2–<2 | + | + | + (5·5) | 960 | <5–20 | <2–20 |
| 97 | — | — | <5–80 | <5–10 | <2–30 | — | — | — | 1920 | <5–<5 | <2–<2 |
| 110 | — | — | <5–<5 | <5–<5 | <2–<2 | + | + | + (5·5) | 240 | <5–15 | <2–30 |
| 111 | — | — | <5–<5 | <5–<5 | <2–<2 | + | + | + (5·25) | 240 | <5–20 | <2–60 |
| 115 | — | — | <5–<5 | <5–<5 | <2–<2 | — | + | + (5·75) | 640 | <5– 5 | <2–20 |

* $Log_{10}$ $EID_{50}$/ml.
† Change from pre-infection titre to peak post-infection titre.
‡ Neutralization test.

relatively high levels of protein in nasal washings, the specimens contained demonstrable HI and neutralizing antibody for A2/Hong Kong virus (Table V). In addition, nasal washings from 2 of the 4 and from 3 of the 4 ferrets contained demonstrable HI and neutralizing antibody, respectively, to influenza virus B/England/13/65.

4. *Immunization of ferrets with killed A2/Hong Kong virus, and subsequent infection with virulent influenza virus A2/Hong Kong/3/68*

(a) *Response to immunization.*—Seven ferrets were each inoculated by the intramuscular route with 8000 HA units of killed A2/Hong Kong influenza virus vaccine. The results are shown in Table V. Immunization did not cause a febrile response in any of the animals. For 6 of the 7 animals, no detectable HI antibody was found in serum specimens taken 4 weeks after immunization, and

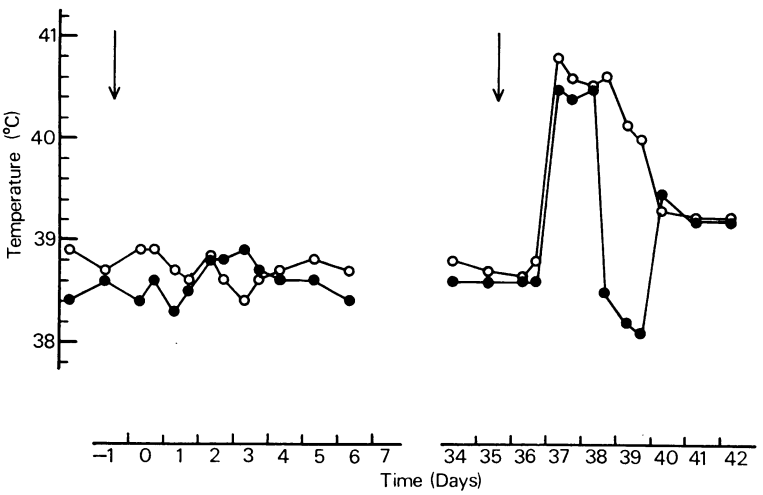


FIG. 5.—Temperature response of ferrets No. 95 and 111 to killed A2/Hong Kong vaccine and later infection with influenza virus A2/Hong Kong/3/68. Arrows indicate virus inoculation (day 0) and virus challenge (day 35).

nasal washings taken 5–15 days after immunization did not contain demonstrable HI or neutralizing antibody. Tests on specimens from the remaining ferret showed a rise in serum HI antibody (< 5 to 1 : 80) following immunization, and both HI antibody (maximum titre 1 : 10) and neutralizing antibody (maximum titre 1 : 30) was detected in nasal washings taken 7 days after immunization; however, HI and neutralizing antibody was not detected in specimens taken 11 or more days after immunization.

(b) *Response to infection.*—Five weeks following immunization with killed virus vaccine, all ferrets were infected with virulent influenza virus A2/Hong Kong/3/68. For the single ferret (No. 97) which produced a serum HI antibody response and a detectable nasal antibody response following immunization, no febrile response was observed after infection, nor was virus recovered from nasal washings taken 3 days after virus inoculation (Table V). In addition, no

HI or neutralizing antibody was detected in nasal washings taken 5–15 days after challenge, but the serum HI titre rose 24-fold.

The 6 remaining ferrets all showed a febrile response equal to that in non-immunized animals following infection by the virulent A2/Hong Kong/3/68 virus. The response for 2 typical ferrets is shown in Fig. 5. Virus was recovered from the nasal wash specimens taken 3 days after infection from all 6 ferrets (Table V), and the titre of virus present was $10^{4.5}$–$10^{5.75}$ $EID_{50}$/ml; these titres were similar to that found in specimens from unimmunized ferrets (Table I). Studies of nasal washings showed a three to five-fold increase in protein content on day 7 and 9 post-infection which subsequently fell. Neutralizing and HI antibody was detected in specimens taken 7 and 9 days after infection of all 6 ferrets, and the peak titres are shown in Table V. In one instance (Ferret No. 115) antibody was detected only in the nasal wash specimen taken 9 days after infection and no HI or neutralizing antibody was found in nasal specimens taken 11 days or more after virus infection.

## DISCUSSION

The results of the present study showed that ferrets responded to experimental infection by virulent influenza virus A2/Hong Kong/3/68 with a sharp rise in temperature, an increase in protein in nasal washings, and a rise in both serum and nasal wash antibody. The temperature response, as a criterion of infection, has been reported by numerous workers (Smith *et al.*, 1933; Shope, 1934; Haff *et al.*, 1966), and the height of temperature peak, the duration of fever and the variably reported diphasic nature of the temperature response are probably characteristics which vary for different virus strains and for different methods of virus inoculation (Shope, 1934). Two criteria of an increased temperature were recorded; however, in practice, most ferrets which responded to experimental infection with A2/Hong Kong/3/68 virus by producing serum and nasal antibody, by showing an increase in nasal protein, and from whom virus was recovered, showed both a significant fever and a significant temperature rise. The 2 measurements of temperature were not always correlated in other studies of immunized ferrets (Potter *et al.*, 1972).

An examination of nasal wash specimens from ferrets which were susceptible to A2/Hong Kong virus showed an increased protein content following infection. Serial specimens from individual ferrets indicated that the amount of protein increased three-fold or greater above pre-infection levels at 5–9 days after infection; the concentration returned to normal levels by day 11, post-infection.

The rise in serum antibody in ferrets infected with influenza viruses has been reported by many workers (Haff *et al.*, 1966; Francis and Stuart-Harris, 1938; Andrewes, Laidlaw and Smith, 1935; Marois *et al.*, 1971), and in the present study invariably occurred in ferrets which were judged susceptible to infection by other criteria. Antibody was also detected in nasal secretions. Haemagglutination-inhibition tests indicated that antibody was present in nasal washings taken 7 and 9 days post-infection; occasionally antibody was detected in specimens taken 11 days after infection, but never in specimens taken after this time. The results of neutralization tests, which were more sensitive in detecting virus-specific antibody in nasal secretions, agreed closely with the results of HI tests.

Ferrets infected with virulent influenza virus were immune to re-infection with the same virus 5 weeks later. Thus, ferrets showed no febrile response to

re-infection; virus was not recovered from nasal washings taken 3 days after re-infection; serum HI antibody titres were observed to fall; and no detectable increase in protein or detectable HI antibody was found in nasal washings. In 2 of 5 immune ferrets, however, low levels of neutralizing antibody were detected in nasal washings taken 7 days after re-infection. Using these criteria for assessing susceptibility or immunity to re-infection, it was observed that immunization with attenuated influenza virus A2/Hong Kong (Beare and Bynoe, 1969) resulted in an immunity comparable to that observed following infection with virulent virus. Ferrets previously immunized with attenuated influenza virus B/England/13/65 were shown to be as susceptible to challenge with influenza virus A2/Hong Kong/3/68 as were unimmunized animals. Of 7 ferrets immunized with killed influenza A2/Hong Kong vaccine, only one animal responded by producing detectable levels of serum and nasal HI antibody. This ferret was immune to infection with virulent A2/Hong Kong virus. Thus, following infection, no febrile response was found, virus was not recovered from nasal washings and no antibody or increased protein content was detectable in nasal secretions. This animal was the only one to produce nasal antibody following immunization with killed virus. A further 34 ferrets have been immunized with killed A2/Hong Kong vaccine in single doses, in many cases with adjuvants that stimulated serum HI antibody, and in no case has detectable HI antibody been found in nasal secretions. The results for ferret No. 97 are therefore suspect, and may be due to an accidental and undetected infection with live virus. Of the remaining 6 ferrets none responded to immunization by producing detectable serum or nasal antibody, and on challenge all these animals were fully susceptible to infection. Thus, all 6 ferrets produced a significant febrile response; virus was recovered from nasal washings, taken 3 days after challenge, in titres comparable to that found in unimmunized animals; and all the ferrets responded by producing both serum and nasal antibody. These results indicate that a single immunizing dose of killed A2/Hong Kong vaccine did not confer any degree of immunity to these ferrets against virulent virus challenge.

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