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Immunity to influenza in ferrets

VII. Effect of previous infection with heterotypic and heterologous influenza viruses on the response of ferrets to inactivated influenza virus vaccines

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SUMMARY

Normal ferrets did not produce serum antibody following immunization with 200 i.u. of inactivated A/Hong Kong/68 influenza virus vaccine and were found to be susceptible to subsequent challenge infection with A/Hong Kong/68 virus. High titres of virus were recovered from nasal washings collected 3 days after infection, serum antibody was produced, increased nasal protein was detected and HI antibody was detected in nasal washings. Ferrets infected with influenza virus A/PR/8/34 7 weeks before immunization with inactivated A/HK/68 virus did, however, produce serum HI antibody to A/HK/68 virus. This antibody conferred partial immunity to challenge infection with A/HK/68 virus, as shown by decreased titres of virus in nasal washings and reduced levels of nasal protein. Previous infection of ferrets with influenza virus B/Ann Arbor/66 did not result in the production of serum antibody to A/HK/68 virus following immunization with A/HK/68 vaccine and the animals were completely susceptible to subsequent challenge infection with A/HK/68 virus. Differences in the amount of nasal protein and nasal antibody produced after A/HK/68 infection were also found in ferrets previously infected with either A/PR/8/34 or B/AA/66 virus, compared with normal ferrets.

INTRODUCTION

Influenza in ferrets closely resembles that in man, and the animals are therefore useful as an experimental model in which to study the disease (Smith, Andrews & Laidlaw, 1933; Haff, Schriver, Engle & Stewart, 1966). Attempts to immunize ferrets with inactivated influenza virus vaccines against a challenge infection have, however, been unsuccessful (Potter *et al.* 1972). Antibody was only produced in the serum of animals given vaccine together with adjuvant, but this antibody failed to give the same degree of resistance to challenge infection as equal titres of antibody produced as a result of live, homologous infection (Potter, McLaren & Shore, 1973).

The ferrets used in the above studies differed from man in that they had no history of previous infection with influenza viruses. In the present study we report the effect of previous infection with either an influenza B virus or a heterotypic

influenza A virus on the response of ferrets to subsequent immunization with inactivated influenza virus A/Hong Kong/68 vaccine. In addition, the response of the ferrets to subsequent infection with influenza A/Hong Kong/68 virus is also reported.

MATERIALS AND METHODS

Viruses

Influenza viruses B/Victoria/98926/70, A/PR/8/34 (H0N1) and A/Hong Kong/68 (H3N2) were obtained from Dr G. C. Schild, World Influenza Centre, Mill Hill, London.

Viruses were grown in 10-day embryonated hen's eggs incubated for 72 hr. at 33° C, and stored at −70° C. A pool of A/HK/68 virus was also grown in duck eggs in a similar manner.

A pool of a cold adapted strain of B/Ann Arbor/66 virus grown in hen's eggs, was obtained from the Wellcome Research Laboratories, Beckenham.

Vaccines

Inactivated influenza virus A/HK/X31/68 vaccine containing 200 International Units (i.u.) per 0·5 ml. was kindly supplied by Dr D. Breeze, Evans Medical Ltd., Speke. This virus is antigenically similar to A/HK/68 (Kilbourne *et al.* 1971).

Animals

Young albino ferrets were kindly supplied by Wellcome Research Laboratories, Beckenham. The animals were immunized against canine distemper virus a number of weeks before the beginning of the experiment.

Experimental design

Groups of ferrets were infected intranasally with either A/PR/8/34 or B/AA/66 virus. Blood samples were taken by cardiac puncture before and 5–7 weeks after infection. Half of each group of convalescent animals were then inoculated intramuscularly with 200 i.u. of A/HK/X31/68 vaccine; a group of normal ferrets was also inoculated with the same vaccine.

Blood samples were taken from some of the ferrets 6 days after immunization, and 5 weeks after immunization all animals were bled and a nasal wash specimen was collected. The ferrets, together with a group of non-infected, non-vaccinated animals, were then challenged with approximately $10^{7\cdot0}$ EID50 of A/HK/1/68 influenza virus, which had had two passages in monkey kidney cells followed by four passes in eggs, inoculated intranasally under light ether anaesthesia. Nasal washings for virus isolation were collected 3 days after challenge infection, and further specimens were collected for protein determination and antibody studies on subsequent alternate days, as described previously (Potter *et al.* 1972). A further blood sample was taken three weeks after challenge.

Table 1. *Response of ferrets to immunization with 200 i.u. of A/HK/68 vaccine*

Ferret no.	Primary infection	Change in serum HI titre after immunization*		
		A/HK/68	A/PR/8/34	B/Vic
326	B/AA/66	—†	—	30–30
327		—	—	40–< 10
328		—	—	60–30
330		—	—	60–20
324	A/PR/8/34	< 10–160	960–240	—
329		< 10–120	1920–480	—
331		< 10–60	640–240	—
333		< 10–120	960–480	—
368	Nil	—	—	—
370		—	—	—
371		—	—	—

* Titre before immunization – titre 5 weeks after immunization.
† < 10–< 10.

Virus isolation

The titre of virus in unconcentrated nasal washings collected three days after infection was determined by infectivity titrations in 10-day eggs as described previously (Potter *et al.* 1972).

Protein estimations

The protein content of ten-fold concentrated nasal washings was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Serological tests

Haemagglutination inhibition (HI) tests. HI tests on sera and ten-fold concentrated nasal washings were carried out as described previously (Potter *et al.* 1972), but with an incubation period of 40–60 min between the addition of virus and erythrocytes.

Neutralization tests. The titre of neutralizing antibody in ten-fold concentrated nasal washings was measured using the allantois-on-shell technique (Fazekas de St Groth, Withell & Lafferty, 1958).

RESULTS

Response to immunization

None of the normal ferrets nor those animals infected with B/AA/66 5 weeks previously produced detectable titres of serum HI antibody to A/HK/68 virus after inoculation with 200 i.u. of A/HK/68 vaccine (Table 1).

In contrast, all of the ferrets which had been infected with A/PR/8/34 influenza virus seven weeks previously produced serum HI antibody in response to immunization with A/HK/68 vaccine (Table 1). The titres of antibody ranged from 60 to 160 5 weeks after immunization, although sera taken from two of these animals 6 days after immunization showed nearly the same titre of antibody as at 5 weeks.

Table 2. *Response of ferrets to infection with A/HK/68 virus after infection with heterotypic and heterologous influenza viruses*

		Response to A/HK/68 infection						
			Change in serum HI titre*			Change in nasal HI titre†		
Ferret no.	Primary infection	Virus yield (log_{10}EID50/ml)‡	A/HK/68	A/PR/8/34	B/Vic	A/HK/68	A/PR/8/34	B/Vic
317	B/AA/66	5·5	< 10–640	—‖	40–< 10	< 5–80	—‖	—
318		5·16 (5·41)§	< 10–640	—	120–40	< 5–120	—	—
322		5·5	< 10–480	—	10–< 10	< 5–40	—	—
325		5·5	< 10–960	—	30–20	< 5–60	—	—
319	A/PR/8/34	4·16	< 10–960	320–320¶	—	—	—	—
320		4·50	< 10–1280	480–640	—	< 5–7·5	—	—
321		4·83 (4·41)	< 10–640	240–240	—	—	—	—
323		4·16	< 10–480	960–240	—	—	—	—
352	Nil	5·16	< 10–> 5120	—	—	< 5–30	—	—
359		4·50	< 10–960	—	—	< 5–80	—	—
363		5·16 (4·91)	< 10–1920	—	—	< 5–80	—	—
367		4·83	< 10–960	—	—	< 5–30	—	—

* Titre before infection – titre 3 weeks after infection.
† Titre before infection – peak titre after infection.
‡ Titre of virus in nasal washings collected 3 days after challenge infection.
§ Mean titre of group.
‖ < 10–< 10 (serum); < 5–< 5 (nasal washings).
¶ Died 7 days after challenge.

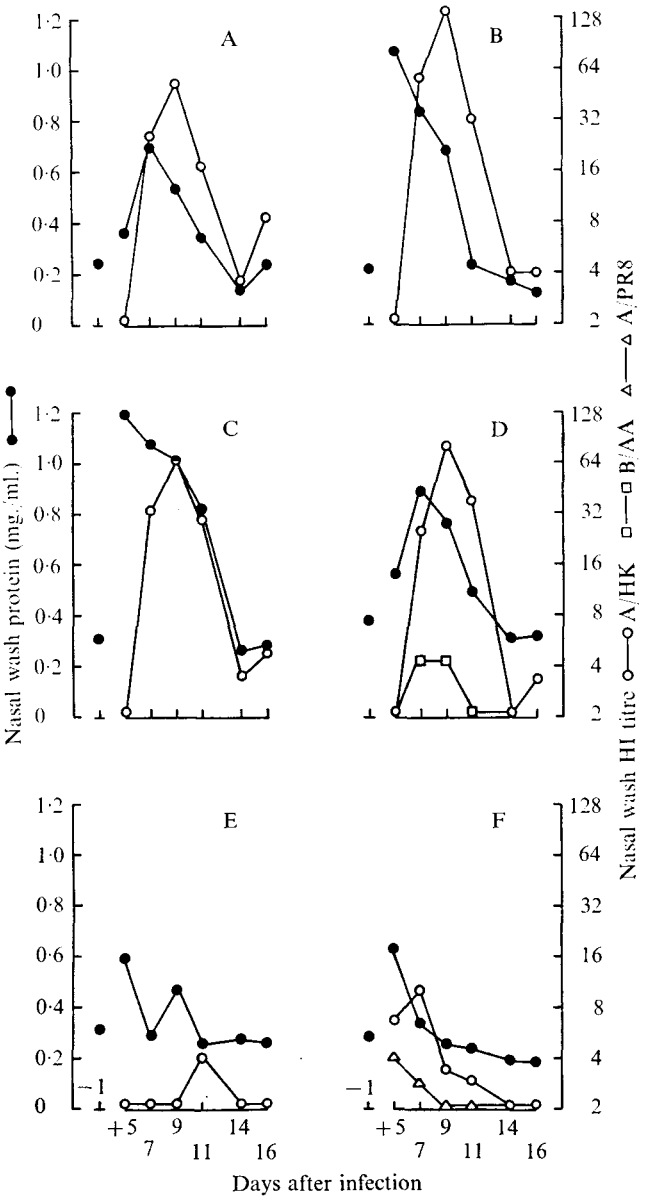

Fig. 1. Protein concentration and HI antibody titres in tenfold concentrated ferret nasal washings collected before and 5–16 days after intranasal infection with influenza virus A/Kong Kong/1/68. A. Normal ferrets. B. Normal ferrets immunized with 200 i.u. of inactivated A/HK/68 virus vaccine five weeks before challenge infection. C. Ferrets infected with B/Ann Arbor/66 influenza virus 10 weeks before challenge infection. D. Ferrets infected with B/AA/66, immunized with 200 i.u. of inactivated A/HK/68 virus vaccine 5 weeks later and then challenged with A/HK/68 virus after a further 5 weeks. E. Ferrets infected with A/PR/8/34 influenza virus 12 weeks before challenge infection. F. Ferrets infected with A/PR/8/34, immunized with 200 i.u. of inactivated A/HK/68 virus vaccine 7 weeks later and then challenged with A/HK/68 virus after a further 5 weeks.

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Similar antibody titres were measured using both hen and duck-egg grown A/HK/68 virus. No increase in antibody titres to A/PR/8/34 virus was observed after immunization with A/HK/68 vaccine.

Response to challenge infection with A/Hong Kong/68 virus

Normal ferrets

Four normal ferrets were infected by intranasal inoculation of approximately 10^7 EID 50 of A/HK/1/68 virus. Virus was recovered from nasal washings collected on day 3 after infection from all animals in the group (titres $10^{4.50}$–$10^{5.16}$ EID 50/ml., geometric mean titre (gmt) = $10^{4.9}$ EID 50/ml.) and the animals all produced serum HI antibody (Table 2). After infection, a 2–3-fold increase in protein concentration was detected in nasal washings, with maximum concentrations usually present on day 7 (mean = 0·7 mg/ml.). HI antibody was also found in nasal wash specimens, but the maximum titres of HI antibody (1/30–1/80) occurred on day 9, with only one out of four ferrets producing nasal antibody after day 11 (Fig. 1).

Ferrets previously infected with influenza virus B/AA/66

The effect of previous infection with influenza virus B/AA/66 on the response to A/HK/68 challenge infection 10 weeks later was studied in four ferrets. After A/HK/68 infection, virus was recovered from nasal washings collected on day 3 from all animals in the group (gmt = $10^{5.41}$ EID 50/ml.) and serum HI antibody to A/HK/68 virus was produced by all animals (Table 2). No boost in the serum HI titre to B/AA/66 virus was observed. The animals produced threefold increased amounts of protein in their nasal washings, with highest levels on day 5 (mean = 1·2 mg/ml.). Antibody to A/HK/68 was detected in nasal washings collected 7 days and later after infection, with maximum titres on day 9 (Fig. 1).

Ferrets previously infected with influenza virus A/PR/8/34

Ferrets infected with A/PR/8/34 virus were challenged with heterotypic A/HK/68 virus 12 weeks later. The animals were susceptible to the second infection, as shown by recovery of virus from nasal washings (gmt = $10^{4.4}$ EID 50/ml.) and the production of serum HI antibody to A/HK/68 virus (Table 2). However, after A/HK/68 infection, the concentration of protein in nasal washings increased only twofold, to a maximum mean concentration of 0·6 mg./ml. on day 5 (Fig. 1). HI antibody to A/HK/68 virus was detected in the nasal washing of only one ferret (F. 320), and on only one occasion, in this group of animals (Table 2, Fig. 1).

Response to challenge infection with A/Hong Kong/68 virus following virus infection and immunization

Normal ferrets immunized with A/HK/68 vaccine

None of the three ferrets produced serum HI antibody after inoculation with 200 i.u. of A/HK/68 vaccine and all were susceptible to challenge infection with A/HK/68 virus. Thus, virus was recovered from nasal washings collected 3 days after infection (gmt = $10^{5.05}$ EID 50/ml.) and all the animals produced serum HI antibody to A/HK/68 virus (Table 3). The animals also responded by producing

Table 3. *Response of ferrets to infection with A/HK/68 virus after immunization with* 200 *i.u. of A/HK/68 vaccine*

Ferret no.	Primary infection	Response to A/HK/68 infection: Virus yield (log_{10}EID50/ml)	Change in serum HI titre*: A/HK/68	A/PR/8/34	B/Vic	Change in nasal HI titre†: A/HK/68	A/PR/8/34	B/Vic
326	B/AA/66	5·16	< 10–1280	—§	30–< 10	< 5–30	—§	—
327		4·83	< 10–960	—	< 10–10	< 5–60	—	—
328		4·16 (4·83)‡	< 10–640	—	30–60	< 5–80	—	< 5–10
330		5·16	< 10–960	—	20–15	< 5–160	—	—
324	A/PR/8/34	4·50 (3·25)	160–640	240–240	—	< 5–7·5	—	—
329		2·83	120–960	480–240	—	—	—	—
331		3·50	60–1280	240–240	—	< 5–30	< 5–7·5	—
333		2·16	120–480	480–240	—	< 5–7·5	< 5–5	—
368	Nil	5·5 (5·05)	< 10–640	—	—	< 5–30	—	—
370		5·5	< 10–2560	—	—	< 5–320	—	—
371		4·16	< 10–960	—	—	< 5–60	—	—

* Titre before infection – titre 3 weeks after infection.
† Titre before infection – peak titre after infection.
‡ Mean titre of group.
§ < 10–< 10 (serum); < 5–< 5 (nasal washings).

a fivefold increase in nasal wash protein concentration, with a maximum mean concentration of 1·06 mg./ml. on day 5, and high titres of nasal HI antibody to A/HK/68 (Table 3, Fig. 1). The nasal antibody was first detectable on day 7, with maximum titres (1/30–1/320) on day 9, and was usually absent after day 11.

Ferrets immunized with A/HK/68 vaccine after infection with B/AA/66 virus

Ferrets previously infected with B/AA/66 virus failed to produce serum HI antibody to A/HK/68 after immunization with 200 i.u. of A/HK/68 vaccine and were found to be susceptible to challenge infection with A/HK/68 virus. Virus was recovered from nasal washings from all the animals in this group (gmt = $10^{4\cdot83}$ EID 50/ml.) and they all produced serum antibody to A/HK/68 virus (Table 3). No increases in B/AA/66 serum HI titres were observed after the challenge infection. The concentration of protein in nasal washings increased threefold after infection, with maximum values seven days after infection (Fig. 1). HI antibody to A/HK/68 virus was found in nasal washings from all the ferrets in this group, with highest titres (1/30 to 1/160) on day 9 (Fig. 1, Table 3). Antibody to B/AA/66 virus was also detected in nasal washings collected on days 7 and 9 from one animal (F. 328).

Ferrets immunized with A/HK/68 vaccine after infection with A/PR/8/34 virus

All four ferrets previously infected with A/PR/8/34 virus produced serum HI antibody to A/HK/68 when immunized with 200 i.u. of A/HK/68 virus vaccine. The animals were, however, infected after challenge with A/HK/68 virus. Thus, reduced titres of virus were recovered from nasal washings (range = $10^{2\cdot16}$–$10^{4\cdot50}$ EID 50/ml., gmt = $10^{3\cdot25}$ EID 50/ml.) and fourfold or greater rises in serum HI titres to A/HK/68 were measured. No increases in A/PR/8/34 serum HI titres were observed after challenge infection. A twofold increase in protein concentration of nasal washings was measured on day 5, but the concentration fell to pre-infection values by nine days after infection (Fig. 1). Only three out of four ferrets produced detectable titres of A/HK/68 antibody in nasal washings collected after A/HK/68 infection, with peak titres on day 5 for two animals and day 7 for the other ferret (F. 331). Two of the ferrets also had low titres (1/5 and 1/7·5) of A/PR/8/34 HI antibody in nasal washings collected 5 and 7 days after A/HK/68 challenge infection (Table 3).

DISCUSSION

Ferrets previously infected with either influenza virus A/PR/8/34 or B/AA/66 were as susceptible to challenge infection with A/HK/68 virus as normal ferrets, as measured by titre of virus recovered from nasal washings and production of serum HI antibody. The heterotypic protection produced by live influenza virus infection of mice, as measured by reduction in lung consolidation (Schulman & Kilbourne, 1965), was not observed in the experiment. However, the interval between the two infections in mice was only 4 weeks; in the present study the interval was 10–12 weeks. Other observations in this laboratory suggest that heterotypic immunity can be demonstrated in ferrets infected with a second virus 3 weeks after the first infection but that it disappears by 10 weeks after the first infection.

The local responses to challenge infection of the ferrets were found to be altered by previous infection in the present study. Thus, ferrets previously infected with B/AA/66 produced more nasal protein; an average peak concentration of 1·2 mg./ml. of protein was found in nasal washings from these animals after A/HK/68 infection, compared with a peak concentration of 0·7 mg./ml. produced by normal ferrets after infection. The cause and significance of this increased amount of protein is not known, nor is it clear whether it is associated with more severe local symptoms, such as increased nasal congestion. The protein found in nasal washings from B/AA/66 infected ferrets is probably produced locally and does not arise by transudation of serum proteins across the nasal epithelium, since the titres of HI antibody in nasal washings were similar to those in normal animals infected with A/HK/68. Similarly, no B/AA/66 antibody was detected in the nasal washings of B/AA/66-convalescent ferrets after A/HK/68 challenge infection, although high titres were present in their sera. In contrast, heterotypic infection with A/PR/8/34 virus resulted in the almost complete absence of HI antibody to A/HK/68 virus in nasal washings collected after the challenge infection. The protein concentration of nasal washings was, however, similar in A/PR/8/34-immune ferrets compared with normal animals, although the peak was reached earlier.

A striking result of previous infection of ferrets with A/PR/8/34 virus was the response of the animals to immunization with 200 i.u. of inactivated A/HK/68 virus vaccine. These animals produced serum HI antibody to A/HK/68, in contrast to normal or B/AA/66-infected ferrets which did not produce detectable serum HI antibody after immunization. Challenge infection of these ferrets showed that immunization had produced partial immunity to A/HK/68 infection, as shown by a 10–1000-fold reduction in the titres of virus recovered from nasal washings. The titre of A/HK/68 HI antibody in nasal washings was also reduced in these ferrets; however, this may have been a result of the previous A/PR/8/34 infection, since non-vaccinated A/PR/8/34-convalescent animals also had lower titres of nasal wash antibody than normal ferrets infected with A/HK/68 virus. The source of the low titres of A/PR/8/34 HI antibody in the nasal washings from vaccinated A/PR/8/34-infected ferrets is not known. Although all nasal wash specimens were negative when tested for occult blood, transudation of some antibody from the serum cannot be completely discounted (Shore, Potter & McLaren, 1972).

The results indicate that previous infection with a heterotypic influenza A virus has 'primed' the immunologic system of the animal to respond to subsequent immunization with A/HK/68 vaccine. Other studies in ferrets and hamsters have shown that an HI antibody response to inactivated influenza virus vaccine can be potentiated by previous infection with a wide range of heterotypic influenza A viruses (McLaren & Potter, 1973; Potter, Jennings, Marine & McLaren, 1973; Jennings & Potter, 1973). The mechanism involved in this priming is not known, but may involve the trapping of common carrier proteins by cells primed by the initial infection (Fazekas de St Groth & Webster, 1966; Webster, 1966; Dixon & Maurer, 1955). The nature of the hypothetical carrier protein is not yet known, but it may be one of the antigens common to influenza A viruses, such as the matrix

antigen or the ribonucleoprotein. Alternatively part of the haemagglutinin molecules, other than the haemagglutinating site, might be acting as a carrier protein.

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