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Immunity to influenza in ferrets

XI. Cross-immunity between A/Hong Kong/68 and A/England/72 viruses: serum antibodies produced by infection or immunization

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SUMMARY

The degree of immunity due to cross-reactions between antibody to influenza virus A/Hong Kong/1/68 and A/England/42/72 was studied in ferrets. Ferrets were immunized with the viruses by either live infection or by inoculation with inactivated virus vaccines. The vaccines were given with Freund's incomplete adjuvant or were given to ferrets previously infected with influenza virus A/PR/8/34. As a result of these immunizations the animals all produced similar titres of serum HI antibody to the immunizing virus, although the degree of cross-reaction with the other virus strain was variable. After immunization the animals were challenged by infection with an A/Eng/42/72-like virus and their degree of immunity was measured. It was found that the greatest immunity was in ferrets previously infected with the homologous A/Eng/42/72 virus. Animals previously infected with A/HK/68 virus also showed a measurable degree of immunity to A/Eng/42/72 infection, and this was greater than that found in animals given inactivated virus vaccines. The immunity produced by the vaccines was approximately equal, regardless of which vaccine or method of immunization was used. Thus, live infection produced a more effective, broader immunity than did the use of inactivated virus vaccines.

INTRODUCTION

The ferret provides a useful model for the study of influenza, since the disease in this animal resembles that of man (Smith, Andrews & Laidlaw, 1933; Haff, Shriver, Engle & Stewart, 1966; Potter *et al.* 1972*a*). After influenza A virus infection, virus can be recovered from nasal washings, and specific antibody is found in both serum and nasal washings. In addition, the infection can cause a febrile reaction and increased concentrations of protein can be found in nasal washings. After the primary infection the animals are completely immune to challenge infection with the homologous virus for 5 weeks or more, as indicated by failure to recover virus from nasal washings, and the absence of further antibody production (Francis & Stuart-Harris, 1938; Potter *et al.* 1972*a*). However, immunization with inactivated virus vaccine did not produce the same degree of immunity to challenge infection as found after infection (Potter, Shore, McLaren

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& Stuart-Harris, 1972*b*). Serum HI antibody was only produced in normal ferrets given the vaccine together with adjuvants (Potter *et al.* 1972*b*) or in ferrets which had been previously infected with a heterotypic influenza A virus (McLaren & Potter, 1974).

The present study examines the degree of cross-immunity between influenza viruses A/Hong Kong/68 and A/England/42/72; this latter virus is a variant of A/HK/68 virus (Schild *et al.* 1973) which caused epidemic infection in England in the winter of 1972–3, although at this time the population had relatively high titres of serum HI antibody to A/HK/68 which cross-reacted with A/Eng/72 virus (Pereira *et al.* 1972). The antibody response of ferrets was studied after infection with either A/HK/68 or A/Eng/72 virus, or after immunization with either A/HK/68 or A/Eng/72 vaccine with Freund's incomplete adjuvant, or by inoculation with vaccine of animals previously infected with influenza virus A/PR/8/34 (McLaren & Potter, 1974). The immunity of the animals was then tested by challenge infection with influenza virus A/Mill Hill/72.

MATERIALS AND METHODS

Viruses and virus vaccines

Influenza viruses A/PR/8/34 (H0N1), A/Hong Kong/1/68 (H3N2), A/England/42/72 (H3N2), A/Mill Hill/1/72 (H3N2) and recombinant virus MRC-2 (A/PR/8/34 × A/Eng/42/72 H3N2) were all obtained from Dr G. C. Schild (World Influenza Centre National Institute for Medical Research, London); the last two strains are antigenically similar to A/Eng/42/72. The viruses were grown in the allantoic cavity of hen's eggs incubated for 48–72 hr. at 33° C.

Inactivated A/Aichi/68 virus vaccine containing 300 CCA/ml. was kindly supplied by Dr Hennessen (Behringwerke AG, Marburg Lahn). Formalin/β-propiolactone inactivated A/Eng/42/72 virus vaccine, containing 600 i.u./ml., was obtained from BDH Pharmaceuticals Ltd., London.

Experimental design

Groups of ferrets were infected intranasally with approximately 10^6 EID50 of either A/Eng/72 or A/HK/68 virus; infection was confirmed by re-isolation of virus from nasal washings collected 3 days after infection and by the production of serum HI antibody. Groups of ferrets, which had been infected with A/PR/8/34 virus 7 weeks previously, were inoculated intramuscularly with a 0·5 ml. volume of either 300 CCA of inactivated A/Aichi/68 virus vaccine or with 300 i.u. of inactivated A/Eng/72 virus vaccine. Further groups of normal ferrets were inoculated with the same doses of vaccine mixed with an equal volume of Freund's incomplete adjuvant.

Eight to nine weeks after the A/Eng/72 or A/HK/68 infection, or 5 weeks after immunization with the inactivated vaccines, all the ferrets, together with a group of normal ferrets, were challenged by intranasal infection with approximately $4 \times 10^{7 \cdot 0}$ EID50 of influenza virus A/Mill Hill/1/72. Nasal washings for virus isolation were collected on the 2nd day after infection, and nasal washings for

protein and antibody measurements on day 3 and subsequent alternate days, as described previously (Potter *et al.* 1972*a*). Serum specimens were collected from each ferret before and 15 days after challenge infection.

Virus isolation

The isolation of virus from nasal washings was carried out as described previously (Potter *et al.* 1972*a*).

Protein estimation

The protein concentration of 10-fold concentrated nasal washings was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Haemagglutination inhibition (HI) tests

HI tests on sera and concentrated nasal washings were carried out as described previously (Potter *et al.* 1972*a*), but with an interval of approximately 60 min between the addition of virus and fowl erythrocytes. Recombinant virus MRC-2 was used in HI tests for antibody to A/Eng/42/72 since this virus was less sensitive than the other strains of this virus to the non-specific inhibitors present in ferret sera.

RESULTS

Serum antibody response of ferrets to infection or immunization with influenza viruses

Sera collected from ferrets 8–9 weeks after infection with either A/Eng/72 or A/HK/68 virus showed high titres of HI antibody to both viruses (Table 1). Titres were usually highest for the homologous infecting virus, and the degree of cross-reaction was variable. Thus, for ferrets infected with A/Eng/72 virus, the serum HI titres were higher to the homologous virus in three animals, but higher to the heterotypic A/HK/68 virus in one ferret (Table 1). For ferrets infected with A/HK/68 virus homologous and heterotypic serum HI antibody titres were similar (Table 1).

Ferrets infected with influenza virus A/PR/8/34 and then immunized 7 weeks later with 300 CCA of A/HK/68 vaccine or 300 IU of A/Eng/72 vaccine produced serum HI antibody to the vaccine virus (Table 1). Serum HI antibody titres to the immunizing virus ranged from 1/480 to 1/1920 for A/Eng/72-immunized ferrets and 1/240 to 1/5120 for ferrets given A/HK/68 vaccine. In both groups of ferrets, HI titres were highest against the immunizing virus, while the titres of antibody cross-reacting with the heterotypic virus were 1·5- to 8-fold lower.

Ferrets immunized with 300 CCA of A/HK/68 vaccine or 300 i.u. of A/Eng/72 vaccine, both given with Freund's incomplete adjuvant, also produced high titres of serum HI antibody (Table 1). Antibody titres to the immunizing virus ranged from 1/320 to 1/4800 for ferrets inoculated with A/Eng/72 vaccine, and from 1/120 to 1/3840 for animals given A/HK/68 vaccine. A varying degree of cross-reactivity was found between antibody to the immunizing virus and the heterotypic virus; in some cases the antibody titres to both viruses were similar.

Table 1. *Response of ferrets to infection or immunization with A/Eng/42/72 or A/HK/1/68 viruses or virus vaccines*

Ferret no.	Treatment	Serum HI antibody response to* A/Eng/72	A/HK/68
375	A/Eng/42/72 infection	< 10–1920	< 10–240
376		< 10–1600	< 10–2400
377		< 10–3840	< 10–960
378		< 10–1280	< 10–320
379	A/HK/1/68 infection	10–320	< 10–240
380		15–600	10–800
381		< 10–640	< 10–960
382		< 10–1200	< 10–2400
407	A/PR/8/34 infection	15–1600	20–1280
408	A/Eng/72 vaccine	< 10–480	< 10–320
409		< 10–960	< 10–120
410		15–1920	< 10–1280
411	A/PR/8/34 infection	< 10–30	15–240
412	A/HK/68 vaccine	15–960	30– > 5120
413		< 10–480	< 10–1920
414		< 10–120	10–320
401	A/Eng/72 vaccine with	< 10–3200	< 10–1920
402	Freund's incomplete	< 10–4800	10–6400
403	adjuvant	< 10–320	< 10–40
		15–1280	< 10–320
399	A/HK/68 vaccine with	< 10–240	10–3840
400	Freund's incomplete	< 10–240	< 10–480
405	adjuvant	< 10–240	< 10–120
406		< 10–960	< 10–1280

* Antibody titre before infection/immunization–antibody titre 8–9 weeks after infection, or 5 weeks after immunization.

Response of ferrets to challenge infection with influenza virus A/Mill Hill/1/72

Normal ferrets

Four normal ferrets were infected by intranasal inoculation of approximately $4 \times 10^{7.00}$ EID50 of influenza virus A/Mill Hill/1/72. High titres of virus were recovered from nasal washings collected on the 2nd day after infection from all four ferrets; the titres of virus in these specimens ranged from $10^{4.50}$–$10^{6.16}$ EID50/ml. (geometric mean titre (gmt) = $10^{5.24}$ EID50/ml.). The animals produced high titres of serum HI antibody to A/Eng/72 virus, with lower titres of antibody to A/HK/68 virus (Table 2). Following infection, the mean concentration of protein in nasal washings from the four ferrets increased more than threefold with peak levels present on day 7 (Fig. 1). HI antibody was also detected in nasal washings from all four ferrets, with peak antibody titres to A/Eng/72 of 1/10 to 1/160 occurring on day 11; HI antibody titres to A/HK/68 virus parallelled those to A/Eng/72, but were lower (Fig. 1).

Table 2. *Response of ferrets to A/Mill Hill/1/72 infection after previous infection with influenza A virus*

Ferret no.	First infection	Virus isolation (log_{10} EID 50/ml.)		Response to challenge infection with A/Mill Hill/1/72: Change in serum HI titre* A/Eng/72	Change in serum HI titre* A/HK/68	Change in nasal HI titre† A/Eng/72	Change in nasal HI titre† A/HK/68
383	Nil	5·16		< 10–5120	< 10–960	< 5–40	< 5–10
396	Nil	6·16	(5·24)	< 10–3840	< 10–160	< 5–80	< 5–15
397	Nil	5·16		< 10–5120	< 10–120	< 5–160	< 5–60
398	Nil	4·50		15–5120	15–1920	< 5–10	—‡
375	A/Eng/72	< 0·7		1920–5120	240–480	< 5–7·5	< 5–7·5
376	A/Eng/72	3·16	(1·28)	1600–12800	2400–12800	< 5–30	< 5–20
377	A/Eng/72	< 0·7		3840–1920	960–120	—	—
379	A/HK/68	4·5		320–> 5120	240–> 5120	—	< 5–7·5
380	A/HK/68	4·16	(3·28)	600–6400	800–12800	—	< 5–5
381	A/HK/68	3·83		640–> 5120	960–5120	—	< 5–10
382	A/HK/68	< 0·7		1200–9600	2400–12800	< 5–15	< 5–20

* Antibody titre before challenge infection–antibody titre 15 days after infection.
† Antibody titre before challenge infection–peak antibody titre after infection.
‡ — = < 5–< 5.

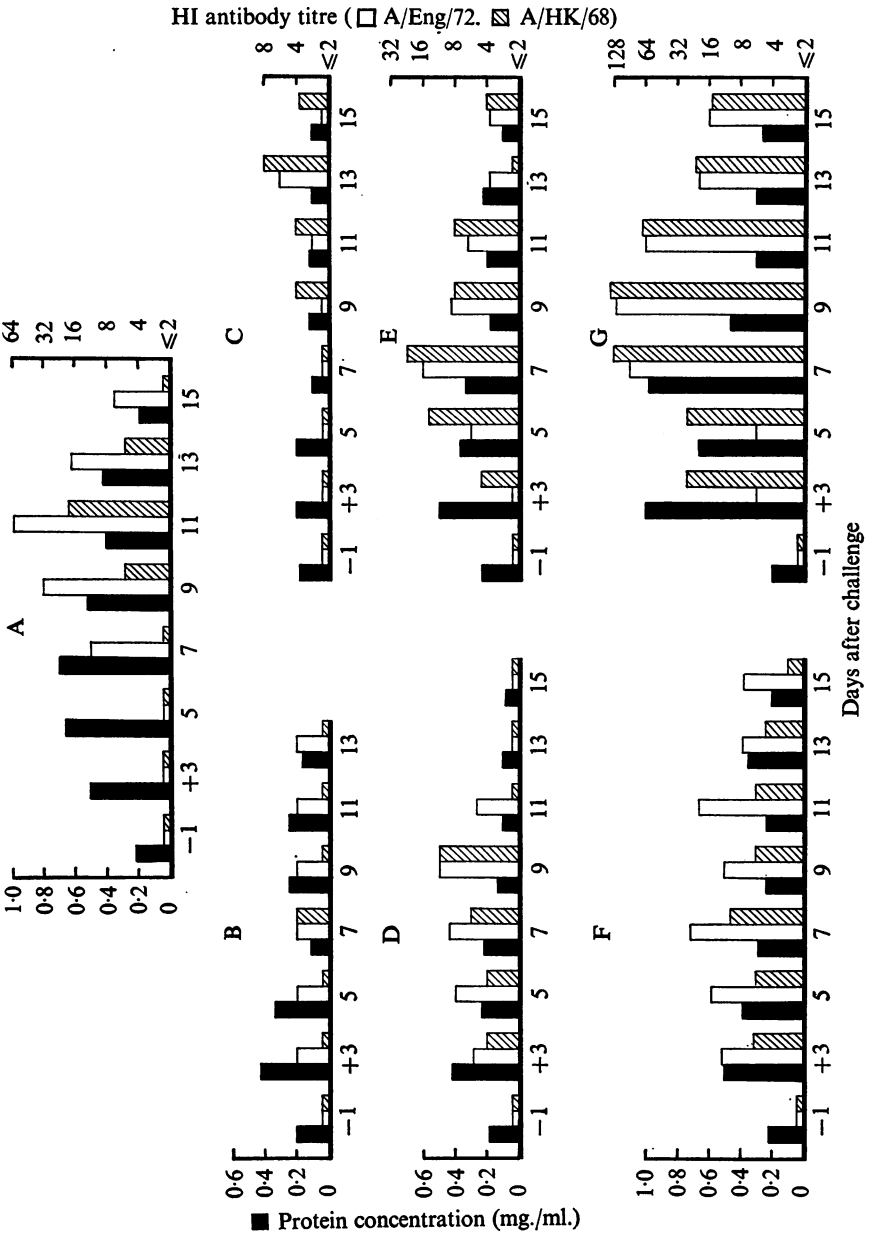

Fig. 1. Nasal response of ferrets to challenge infection with influenza virus A/Mill Hill/1/72. A, Normal ferrets; B, ferrets previously infected with A/Eng/42/72; C, ferrets previously infected with A/HK/68; D, ferrets previously infected with A/PR/8/34 then immunized with A/Eng/72 vaccine; E, ferrets previously infected with A/PR/8/34 then immunized with A/HK/68 vaccine; F, ferrets immunized with A/Eng/72 vaccine in Freund's incomplete adjuvant; G, ferrets immunized with A/HK/68 vaccine in Freund's incomplete adjuvant.

Ferrets previously infected with A/Eng/72 influenza virus

Three ferrets which had been infected with A/Eng/72 virus 10 weeks previously were challenged by intranasal inoculation of approximately $4 \times 10^{7.0}$ EID50 of influenza virus A/Mill Hill/1/72. After infection, virus was re-isolated from nasal washings taken on the 2nd day from only one of the three ferrets (Table 2). A significant increase in serum HI titre occurred in one of the animals, and two of the ferrets produced detectable levels of HI antibody in nasal washings collected after challenge infection. The mean concentration of protein in nasal washings increased approximately twofold after infection, but soon fell to pre-infection levels (Fig. 1).

Ferrets previously infected with A/HK/68 influenza virus

Four ferrets which had been infected with A/HK/68 virus 8 weeks previously were challenged by infection with A/Mill Hill/72 virus. Virus was recovered from nasal washings from three of the animals, and a four-fold or greater increase in serum HI titre was found for all four animals (Table 2). Similar increases in serum HI antibody were measured to both A/Eng/72 and A/HK/68 viruses. HI antibody to A/Eng/72 was detected in the nasal washings collected after challenge infection from only one of the four ferrets in this group; virus was not recovered from nasal washings from this ferret. In contrast, all the ferrets in this group produced detectable levels of HI antibody to A/HK/68 in nasal washings, with the maximum, mean HI titre occurring on day 13. No significant increases in the concentration of nasal wash protein were measured in specimens from these animals (Fig. 1).

Ferrets immunized with either A/Eng/72 or A/HK/68 virus vaccines

Regardless of the method of immunization, all the ferrets which had been given either A/HK/68 or A/Eng/72 vaccine were subsequently infected by the A/Mill Hill/72 challenge virus (Table 3). Thus, virus was recovered from nasal washings collected from all the ferrets, although the geometric mean titres of virus recovered from each group was measurably lower than from normal ferrets infected with the same virus. The differences in the titres of virus recovery for the different groups of immunized ferrets were not significant. All the animals showed an increase in serum HI antibody titre to A/Eng/72 virus following infection; however, for some of the animals given A/Eng/72 vaccine, either with adjuvant or after A/PR/8/34 infection, the rise in antibody titre was less than fourfold (Table 3).

For ferrets infected with A/PR/8/34 virus before immunization, the peak concentrations of protein and titres of HI antibody present in nasal washings collected after challenge were lower than those found after challenge of normal ferrets. In addition the peak concentrations of protein and HI antibody titres occurred 2–4 days earlier than was seen in normal ferrets after challenge infection (Fig. 1). The group of ferrets immunized with A/Eng/72 vaccine in Freund's incomplete adjuvant had concentrations of nasal wash protein similar to those found in ferrets given the same vaccine dose in saline after A/PR/8/34 infection; however, significantly higher titres of HI antibody to A/Eng/72 were produced in nasal washings from the former group (Fig. 1). In both groups of animals, the

Table 3. *Response of ferrets to A/Mill Hill/1/72 infection following immunization*

Ferret no.	Immunization	Virus isolation (log_{10} EID 50/ml.)		Response to challenge infection with A/Mill Hill/1/72: Change in serum HI titre* A/Eng/72	Change in serum HI titre* A/HK/68	Change in nasal HI titre† A/Eng/72	Change in nasal HI titre† A/HK/68
407	A/PR/8 infection	4·83	(4·33)	1600–4800	1280–6400	—‡	—
408	A/Eng/42/72 vaccine	4·50		580–> 5120	320–960	< 5–7·5	—
409		3·50		960–3840	120–240	< 5–30	< 5–10
410		4·50		1920–> 5120	1280–> 5120	< 5–40	< 5–30
411	A/PR/8 infection	4·16	(4·41)	30–> 5120	240–> 5120	—	—
412	A/HK/68 vaccine	3·50		960–> 5120	> 5120–> 5120	< 5–60	< 5–80
413		4·83		480–> 5120	1920–> 5120	—	—
414		5·16		120–> 5120	320–> 5120	< 5–5	< 5–20
401	A/Eng/42/72 vaccine with	4·83	(4·08)	3200–4800	1220–3840	< 5–30	< 5–15
402	Freund's incomplete	4·16		4800–38400	6400–19200	< 5–30	< 5–10
403	adjuvant	3·83		320–5120	40–320	< 5–30	< 5–5
404		3·50		1280–3840	320–640	< 5–40	< 5–10
399	A/HK/68 vaccine with	4·50	(3·92)	240–> 5120	480–> 5120	< 5–120	< 5–160
400	Freund's incomplete	4·16		240–5120	480–> 5120	< 5–60	< 5–120
405	adjuvant	4·50		240–> 5120	120–> 5120	< 5–320	< 5–320
406		2·5		960–> 5120	1280–> 5120	< 5–30	< 5–30

* Antibody titre before challenge infection–antibody titre 15 days after infection.
† Antibody titre before challenge infection–peak antibody titre after infection.
‡ —, < 5–< 5.

HI antibody titres to A/Eng/72 virus in nasal washings were higher than the titres to A/HK/68 virus.

Ferrets previously immunized with A/HK/68 vaccine in adjuvant had a more pronounced nasal response after challenge infection than any of the other groups of ferrets. Thus a mean peak concentration of protein of 1·0 mg./ml. was found in nasal washings collected on day 3, and high titres of HI antibody (1/30–1/320) were found in nasal washings, with peak titres on days 7 and 9 (Fig. 1). Antibody titres were similar to both A/Eng/72 and A/HK/68 viruses. All of the ferrets in this group showed a fourfold or greater rise in A/Eng/72 serum HI titre after challenge infection (Table 3).

DISCUSSION

Immunity to A/Eng/72 virus was produced in ferrets by infection with live virus or by immunization with inactivated virus vaccines. The results confirm earlier observations that live infection gives a more complete and broader immunity than does immunization, although similar serum HI antibody titres are produced by each procedure (Potter *et al.* 1972*b*). Thus, ferrets infected with influenza virus A/Eng/72 8 weeks before challenge with the similar virus A/Mill Hill/72 were found to be almost completely immune to the challenge infection. Virus was re-isolated in low titre and a significant rise in serum HI antibody titre to A/Eng/72 virus was obtained in only one of three ferrets after infection. The successful re-infection of this animal may be due to the large amount of virus (10^7 EID50) used for the challenge; previous attempts to re-infect ferrets with 10^6 EID50 of influenza virus A/HK/68 5–10 weeks after primary infection with the same virus were not successful (Potter *et al.* 1972*a*). Alternatively the A/Mill Hill/72 virus strain may be more virulent for ferrets than the A/Eng/72 virus. Ferrets were also infected with A/HK/68 virus before challenge with A/Mill Hill/72 virus. The first infection produced relatively high titres of cross-reacting serum HI antibody to the challenge virus, but lower titres than seen after A/Eng/72 infection; the A/HK/68-convalescent ferrets were partially immune to A/Mill Hill/72, but the immunity was not as great as found in A/Eng/72-convalescent animals.

Serum HI antibody to A/Eng/72 virus was also produced in ferrets by immunization. Previous reports have shown that ferrets only respond to conventional doses of influenza vaccines if the vaccine is given with adjuvant (Potter, McLaren & Shore, 1973), or if the animal has previously been infected with a heterotypic influenza A virus (McLaren & Potter, 1974). After immunization by either of these methods with A/Eng/72 or A/HK/68 vaccine, the ferrets produced serum HI antibody titres similar to those found in ferrets previously infected with live virus; however, these ferrets did not have the same degree of immunity to A/Mill Hill/72 challenge infection as that produced after infection. Thus, although ferrets previously infected with A/HK/68 virus had lower cross-reacting serum antibody titres to A/Eng/72 than did ferrets immunized with A/Eng/72 vaccine in adjuvant, they were significantly more resistant to A/Eng/72 challenge infection than were the immunized animals. It was also observed that the degree of

immunity to challenge infection was the same for ferrets whether they were given A/Eng/72 or A/HK/68 vaccine. Previous studies in mice have also shown that the immunity resulting from live infection is broader in activity than that produced by vaccines, and can give partial protection against heterotypic influenza viruses (Oakley & Warrack, 1940; Schulman & Kilbourne, 1965; Werner, 1966; Schulman, 1967) but the reason for the greater immunity is not clear. In ferrets it may be due to the wide range of activity reported for their nasal antibody (Haff & Pinto, 1973) which is produced after infection but not after immunization. Alternatively infection may be more effective at stimulating cellular immunity than immunization with inactivated vaccines.

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