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Sequential infection or immunization of ferrets with a series of influenza A (H3N2) strains

(Report to the Medical Research Council's Sub-Committee on Influenza Vaccines (CDVIP/IV))

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SUMMARY

Previous studies of boys at Christ's Hospital school have indicated that annual immunization with influenza virus vaccines did not significantly reduce the total incidence of influenza infection compared to unimmunized subjects. In view of the implications of this result, a similar study was conducted in ferrets to clarify these findings. Groups of ferrets were immunized or infected with a series of influenza A (H3N2) viruses over an 18-month period, and the immunity to subsequent live virus challenge was measured after each virus or vaccine exposure. The results indicated that live virus infection gave a more solid immunity than immunization with inactivated vaccine; and the serum haemagglutination-inhibiting antibody response was greater following immunization than following infection. In addition, differences in immunity could not be explained by measurements of cross-reacting and specific antibody, since the incidence of these antibodies was similar in both infected and immunized animals. The results do not suggest an explanation for the different levels of immunity induced following infection or immunization or the results obtained from the Christ's Hospital study. However, the relative contribution of various immune responses to virus or virus antigen is discussed, and it is suggested that the difference in immunity may lie in the ability of live virus infection to stimulate local antibody.

INTRODUCTION

The results from several studies have indicated that inactivated influenza vaccines induce serum haemagglutination-inhibition (HI) antibody and significant immunity to subsequent influenza virus infection in the majority of vaccinees

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(Mostow *et al.* 1969; Nicholson *et al.* 1979; Jennings, Potter & Massey, 1981; Potter, 1982). The original whole virus vaccines were relatively reactogenic, and have been widely replaced by aqueous subunit vaccines prepared from highly purified virus particles, and these vaccines are equally immunogenic in periods of antigenic drift (Rymer *et al.* 1966; Gross *et al.* 1977; Miles *et al.* 1982). In contrast, improvements in vaccines and the accumulated knowledge of the responses to immunization have not provided completely satisfactory influenza vaccines, and a significant number of vaccinees remain susceptible to challenge by natural virus infection (Hobson, 1972; Potter, 1982). Studies with live attenuated influenza virus vaccines indicate that these may induce a more solid immunity to challenge (Beare *et al.* 1968; Freestone *et al.* 1972). However, this remains theoretical, since live virus vaccines are not available for large-scale testing, and the thesis has not been tested under natural conditions. Thus the present recommendation is for annual immunization for 'at risk' subjects using an inactivated influenza virus vaccine containing haemogglutinin (HA) and neuraminidase (NA) antigens of current epidemic strains. This policy has been questioned as a result of data from a long-term study carried out at Christ's Hospital school, England. In three influenza epidemics occurring in 1972, 1974 and 1976 protection against infection was only apparent in boys immunized for the first time with a vaccine homologous to the epidemic strain; later immunization with vaccines of the same serotype did not increase immunity; the total influenza experience was not diminished by immunization; and infection induced a more solid immunity than immunization (Smith & Davies, 1977; Hoskins *et al.* 1979).

The implications of the above study are important, and it is unfortunate that similar studies were not carried out elsewhere and at the same time to confirm or challenge the results. As the epidemiological factors of the study cannot be repeated, an attempt to study the findings further was carried out in a ferret model system, in which influenza virus infection is similar to that in man (Potter & Oxford, 1977). The ferrets were infected or immunized with a series of influenza A (H3N2) viruses at 3 to 6-month intervals, and measurements of serum antibody and immunity to heterologous virus challenge were made at various times. The study was specifically aimed at the relative immunity which followed immunization or live virus infection, and the induction of specific and cross-reacting serum antibodies following sequential exposure to influenza A (H3N2) virus antigens.

MATERIALS AND METHODS

Viruses and virus vaccines

Influenza viruses A/Hong Kong/68 (H3N2), A/Port Chalmers/73 (H3N2), A/Texas/77 (H3N2), A/Bangkok/79 (H3N2) and A/FM/1/47 (H1N1) were originally obtained from Dr G. C. Schild, National Institute for Biological Standardization and Control, Holly Hill, London. Seed viruses were inoculated into 10-day embryonated hen's eggs by the allantoic route at a $10^{3\cdot0}$ dilution. After incubation for 48 h at 33 °C, egg fluids were harvested and stored at −80 °C. The virus pools were titrated by the allantois-on-shell (AOS) method (Fazekas de St Groth, Witchell & Lafferty, 1958), and the 50% egg-bit infectious dose ($EBID_{50}$) was calculated according to the method of Reed & Muench (1938).

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Monovalent, inactivated whole-virus vaccines in saline were prepared by Evans Medical, Speke, England. These were from pools of whole virus which were to be used in commercial, polyvalent vaccines and marketed as Fluvirin. The vaccines were standardized according to the HA content in international units (i.u.).

Experimental design

Adult ferrets aged 4–6 months and weighing 500–600 g were housed in individual cages for 7 days prior to experimentation. During this time nasal washes and rectal temperatures were taken to establish normal values for each animal. After this time, all ferrets were primed by intranasal inoculation with $10^{5\cdot0}$ $EBID_{50}$ of influenza A/FM/1/47 (H1N1) virus in 0·2 ml volume of phosphate-buffered saline (PBS), pH 7·4. Blood samples were taken by cardiac puncture before and 2 weeks after infection and tested for HI antibody to the homologous virus to demonstrate successful infection. The reason for primary infection was to mimic the situation of past infection which could be assumed to have occurred in the boys at Christ's Hospital school, and because prior exposure of live virus infection is a requirement for a serum antibody response to inactivated vaccine (Potter *et al.* 1973; Jennings, Potter & McLaren, 1974).

One month following primary infection with influenza virus A/FM/1/47, a further blood sample was collected from each ferret by cardiac puncture. Forty-eight hours later, half the animals were inoculated intranasally with $10^{5\cdot0}$ $EBID_{50}$ of influenza virus A/Hong Kong/68 in 0·2 ml PBS. The remaining ferrets were each inoculated intramuscularly with 400 i.u. of inactivated monovalent A/Hong Kong/68 vaccine in 0·5 ml. The temperature response, virus replication, nasal protein and antibody responses to virus infection were monitored, and this monitoring was repeated for infected animals following each subsequent inoculation of ferrets with live virus.

Three months after immunization or infection with influenza A/Hong Kong/68 virus, 10 ml serum samples were obtained by cardiac puncture from each animal. Forty-eight hours later half the ferrets previously immunized with live virus and half the group previously immunized with inactivated vaccine, together with the group of three control ferrets, were each inoculated intranasally with $10^{5\cdot0}$ $EBID_{50}$ of influenza A/Port Chalmers/73 virus; the remaining animals were immunized intramuscularly with 400 i.u. of monovalent inactivated influenza A/Port Chalmers/73 vaccine in 0·5 ml.

Six months after immunization or infection with influenza A/Port Chalmers/73 virus, all animals were again bled by cardiac puncture, and half the ferrets in each of the four groups, together with three control ferrets, were each inoculated intranasally with $10^{5\cdot0}$ $EBID_{50}$ of A/Texas/77 virus; the remaining animals were immunized intramuscularly with 400 i.u. of monovalent inactivated A/Texas/77 vaccine in 0·5 ml.

Six months after immunization or infection with A/Texas/77 vaccine, animals were again bled, and all the animals in each of the eight groups together with three control animals were inoculated intranasally with $10^{5\cdot0}$ $EBID_{50}$ of influenza A/Bangkok/79 virus. All animals were bled 3 weeks later, at which time the experiment was terminated.

Effects of virus infection

(*a*) *Temperature.* Rectal temperatures were taken daily for 3 days prior to virus infection, twice daily for 4 days following virus inoculation, and thereafter daily for 3 days.

(*b*) *Virus isolation.* Nasal washings were collected from each ferret on days 1–7 following nasal infection by dropwise instillation and recovery of 5·0 ml of PBS containing 2% bovine serum albumin as described previously (Potter & Oxford, 1977). The specimens were stored at −70 °C prior to titration for infective virus by the allantois-on-shell method.

(*c*) *Nasal wash protein and neutralizing antibody.* Nasal washes were collected from ferrets on alternate days from days 5 to 15: these washings were collected from 10 ml PBS inoculated dropwise intranasally into the ferrets. The specimens were then concentrated tenfold by dialysis against 30% carbowax. Each specimen was tested for protein content (Lowry *et al.* 1951), and for neutralizing antibody (Fazekas de St Groth, Witchell & Lafferty, 1958).

(*d*) *Assessment of responses to challenge in ferrets.* The responses of control ferrets to influenza virus infection described above was used as a baseline to determine the effects of the same viruses on immunized or infected animals. The febrile response was judged as: similar to that seen in control ferrets, demonstrably lower than for control animals, significantly elevated above the baseline or no febrile response, and these were recorded as 3, 2, 1 and 0, respectively. Peak virus titres detected in nasal washings were assessed as: similar to that of control ferrets, significantly lower than for control animals, virus recovered but at low titres, and no virus recovered, and these results were also recorded as 3, 2, 1 and 0, respectively. Similar criteria and scoring were applied to nasal protein and nasal antibody response.

Serological methods

(*a*) Haemagglutination-inhibition tests. HI tests were performed in microplates as described previously (Jennings *et al.* 1974). Before testing, serum specimens were incubated for 18 h at 37 °C with four volumes of cholera filtrate (Wellcome Reagents) and subsequently heated at 56 °C for 1 h. The antibody titres were expressed as the highest dilution which caused a 50% reduction in virus haemagglutination.

(*b*) *Single radial haemolysis tests.* Analysis of cross-reactive (CR) and strain-specific (SS) anti-haemagglutinin antibody content of ferret sera was by absorption experiments (Schild *et al.* 1977) followed by single radial haemolysis (SRH) tests (Oxford, Yetts & Schild, 1982). Sera were heat-inactivated (50 °C for 30 min) and 20 μl of sera were incubated for 30 min at room temperature with either phosphate-buffered saline (mock adsorption) or with each of the following viruses: A/Hong Kong/68, A/Port Chalmers/73, A/Texas/77 and A/Bangkok/79 (purified virus containing 10 μg protein/ml). The quantity of virus required to remove all CR and SS antibody was found by previous experiment. Strain-specific antibody to A/Hong Kong/68 virus was assayed by absorption of the sera with one of the three other viruses and testing on SRH plates containing A/Hong Kong/68 virus; SS antibody to A/Port Chalmers/73, A/Texas/77 or A/Bangkok/

79 viruses was assayed by similar procedures. A serum was judged to possess CR antibody if there was SRH activity after mock absorption which could be completely removed by virus absorption.

RESULTS

Virus infection of control ferrets

(*a*) *Temperature.* Infection of control, non-immunized and non-infected ferrets with influenza A viruses induced a marked febrile response; virus pools were selected for use by this criterion, since not all pools induce this reaction consistently. Temperatures increased by $\geqslant 1{\cdot}0$ °C above the baseline and to a level of $\geqslant 40{\cdot}0$ °C at 2 days following infection. Subsequently, the temperatures declined, but with some animals a second increase in temperature occurred on days 3–4 post inoculation. After this time temperatures fell to normal values (Fig. 1*a*). The above statements are true for all control ferrets infected with influenza A/Hong Kong/68, A/Texas/77 and A/Bangkok/79 viruses; however, infection with influenza A/Port Chalmers/73 virus induced a temperature rise of $\geqslant 1{\cdot}0$ °C in all ferrets, but in some animals a peak value of over $\geqslant 40$ °C was not observed.

(*b*) *Virus replication.* The geometric mean titres (GMT) of virus present in nasal washings collected from ferrets from days 1 to 7 post inoculation with influenza A/Hong Kong/68 virus are shown in Fig. 1*b*. Virus was detected 24 h after inoculation and rose to peak titres on day 3. Subsequently, virus titres declined, and by day 7 no virus was detected. A similar response was also seen in animals

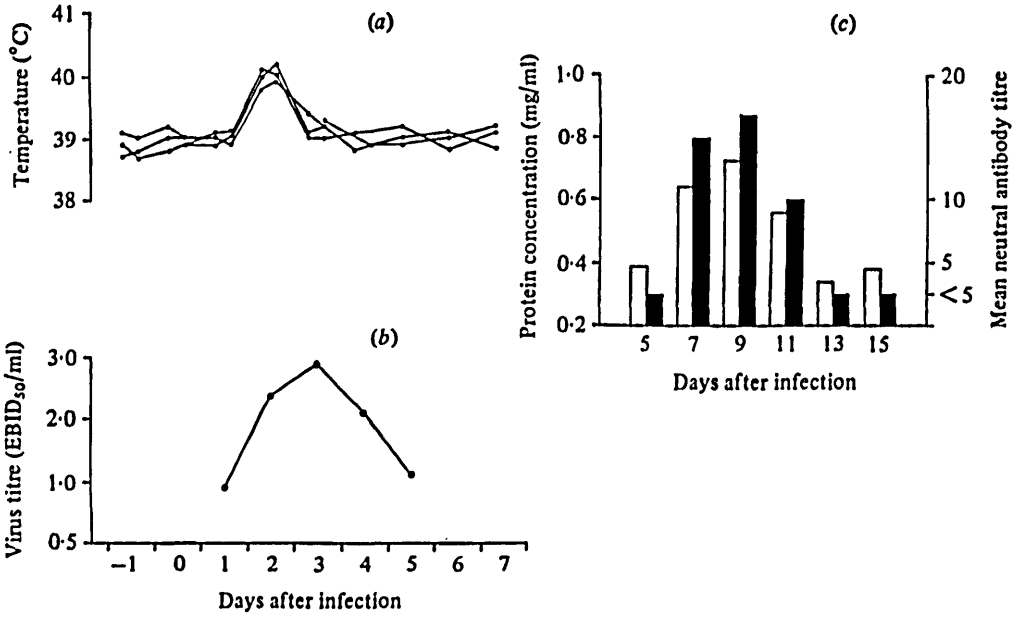

Fig. 1. (*a*) Rectal temperature of ferrets infected intranasally with $10^{5{\cdot}0}$ $EBID_{50}$ of influenza A/Hong Kong/68 virus. (*b*) Geometric mean titres of virus ($EBID_{50}$/ml) present in nasal washings collected from days 1–7 after virus infection. (*c*) Concentration of protein (mg/ml) (□) and titre of neutralizing antibody present (■) in ×10 concentrated nasal washings collected on days 5–15 after virus infection.

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infected with the other influenza viruses used in the study. Thus, virus was always detected at low titres 24 h after inoculation, and titres increased subsequently. Peak titres were seen on day 2 following infection with influenza virus A/Texas/77, but on day 3 for all the other viruses used in the present study.

(*c*) *Nasal washings*. The concentrations of protein present in nasal washings on days 5–15 following inoculation with influenza A/Hong Kong/68 virus are shown in Fig. 1*c*. Relatively low concentrations were found on day 5, but the concentration increased twofold in specimens taken on days 7 and 9; values had fallen by day 11, and on days 13–15 the concentrations were similar to those found on day 5. Compared to the values obtained on day 5 post inoculation, a twofold increase in protein concentrations on days 7–9 was found following infection with all the influenza viruses used in the present study; and in each case values had resumed normal concentration by days 13–15 post inoculation.

(*d*) *Nasal wash neutralising antibody*. Nasal washings collected from days 5–15 post inoculation were concentrated tenfold and titrated for neutralizing antibody. The results obtained for nasal washings from ferrets infected with influenza A/Hong Kong/68 virus are shown in Fig. 1*c*. Antibody was not detected in specimens taken 5 days post inoculation, but was detected in specimens taken 7–11 days post infection. No antibody was detected in specimens taken after this time. Antibody was detected in nasal washings taken on days 7 and 9 post inoculation for all viruses used in the present study, but with A/Port Chalmers/73 virus antibody titres were relatively low, and antibody was not detected in specimens collected on day 11 post inoculation.

Virus infection of ferrets previously infected or immunized with influenza virus or vaccine

The results of assessing the parameters of infection obtained for the different groups of ferrets together with their vaccine or infection history are shown in Fig. 2. For each group of ferrets the four parameters of reaction to infection are shown, and a mean value of 0, 1, 2 or 3 assigned to each of the parameters. The sum total of values is given for each group of animals.

The results indicate that ferrets previously infected with A/Hong Kong/68 virus produced a score of 2 following challenge with A/Port Chalmers/73 virus, whilst animals previously immunized with A/Hong Kong/68 vaccine and then challenged with live A/Port Chalmers/73 virus gave a score of 5. Thus infection induced a more solid immunity to heterologous challenge than immunization. For the animals infected with A/Texas/77 virus, the highest score was recorded for ferrets immunized with A/Hong Kong/68 and A/Port Chalmers/73 virus, and lowest for ferrets previously infected with A/Port Chalmers/73 virus. Again, the results indicate a more solid immunity following infection than following immunization. Finally, when all eight groups of animals were infected with A/Bangkok/79 virus, lower scores were seen for animals previously infected with live virus than those previously immunized with virus vaccine. The animals immunized sequentially with A/Hong Kong/68, A/Port Chalmers/73 and A/Texas/77 virus vaccines gave a score of 5 when challenged with A/Bangkok/77, while animals sequentially infected with the three viruses had a value of 1; and animals given a mixed history of infection and immunization tended to give results intermediate

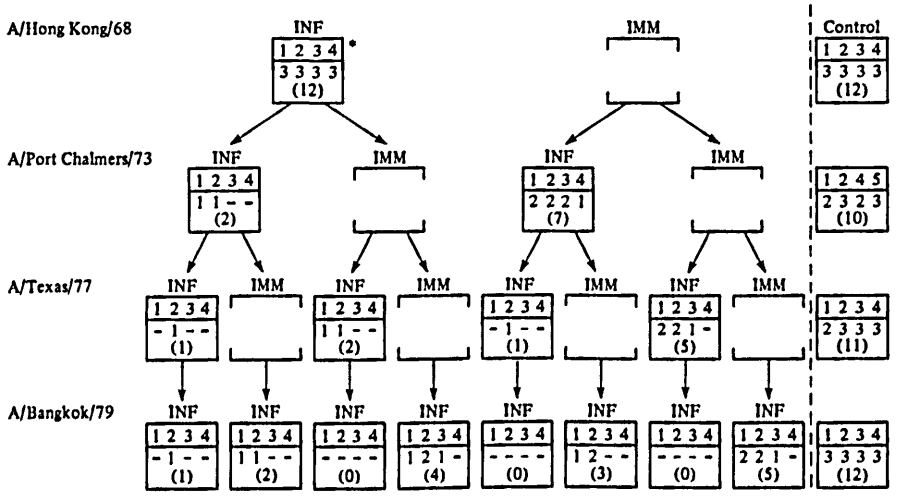

Fig. 2. Each block is for the groups of ferrets either infected (INF) or immunized (IMM) with the virus given in the left column. In each block the top line numbers are: 1, temperature response; 2, virus replication; 3, increase in nasal wash protein; 4, local neutralizing antibody response. The second line gives the score (0–3) for each of the responses to infection (see Materials and Methods). The figure in parentheses is the total score of the responses shown in the second line.

between those given above. Although the number of animals used in the final groups given A/Bangkok/79 virus were necessarily small due to the size and complexity of the study, a trend can be seen from the results (Fig. 2). Infection induced a more solid immunity than immunization, but infection with a less closely related virus does not afford the same protection.

Serum HI antibody responses

Serum specimens from each ferret were collected 24–48 h prior to infection or immunization with each of the influenza A viruses used in the present study and hence the pre- and post-infection/immunization specimens were taken 3–6 months apart. Each serum specimen was titrated for HI antibody against all four viruses used in the study, and the GMT calculated for each group of animals. The results of assessing HI antibody against the A/Port Chalmers/73 virus are shown in Fig. 3. Following infection with the A/Hong Kong/68 virus, the ferrets showed a relatively small HI antibody response to A/Port Chalmers/73 virus. In contrast, the serum antibody response to immunization with killed homologous vaccine was greater. Subsequent infection of A/Hong Kong/68-infected animals with A/Port Chalmers/73 virus induced no significant increase in serum HI antibody to A/Port Chalmers/73 virus, but a significant increase was found following immunization of such ferrets. Immunization with A/Hong Kong/68 virus followed by infection or immunization with A/Port Chalmers/73 virus induced a significant increase in serum antibody against A/Port Chalmers/73 virus (Fig. 3), although these animals were relatively susceptible to infection (Fig. 2).

The serum HI antibody response to A/Port Chalmers/73 virus following infection or immunization with A/Texas/77 virus showed a threefold increase for

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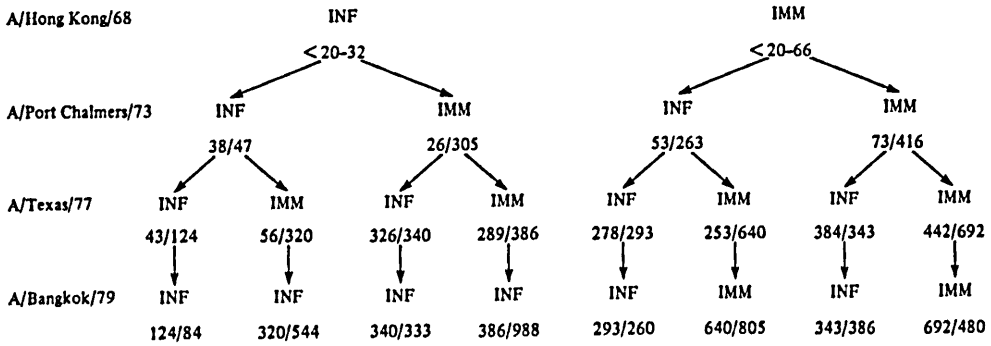

Fig. 3. Serum HI antibody titre (GMT) to influenza virus A/Port Chalmers/73 virus following infection (INF) or immunization (IMM) with virus shown in the left-hand column. Figures are the pre- and post-serum titres.

animals previously infected with either A/Hong Kong/68 or A/Port Chalmers/73 viruses, but no significant increase for animals previously immunized with either of these two viruses. A fourfold increase in HI titre was seen for ferrets infected with A/Hong Kong/68 and A/Port Chalmers/73 virus and subsequently immunized with A/Texas/77 virus. Finally, no significant response to the A/Port Chalmers/73 virus occurred following infection with A/Bangkok/79 regardless of the preceding sequence of infections and/or immunizations. These results are typical of those obtained for HI antibody against influenza A/Hong Kong/68, A/Texas/77 and A/Bangkok/79 viruses (data not shown).

The results of serum HI antibody responses to all four viruses used in the present study are summarized in Table 1. Following infection with A/Hong Kong/68 virus, relatively low titres of antibody to homologous virus were detected; lower titres were detected against A/Port Chalmers/73 and A/Texas/77 viruses, but no antibody was detected to the A/Bangkok/79 virus. Following immunization with A/Hong Kong/68 virus, higher titres of homologous antibody were detected, HI antibody was detected against A/Port Chalmers/73 and A/Texas/77 viruses, but again there was no detectable antibody against A/Bangkok/79 virus. In the subsequent three columns of Table 1 are the changes in serum HI antibody titre (GMT) to the four viruses used in the study, together with the number of fold increase in titre. The results indicate higher serum HI antibody responses following immunization with vaccines followed by infection with the homologous virus; thus, following infection with A/Hong Kong virus, the response to A/Port Chalmers/79 infection was an insignificant (twofold) increase in the HI antibody, but animals immunized after an initial A/Hong Kong/68 infection showed a ninefold increase in antibody. Again, animals infected with A/Texas/77 and A/Port Chalmers/73 viruses and subsequently infected with A/Texas/77 showed little change in A/Texas/77 titre, but a fourfold increase occurred following immunization with A/Texas/77 vaccine. This trend in results can be seen in the other animal groups (Table 1). Thus the response of ferrets to immunization with killed vaccine invariably produced a higher serum HI antibody response to both homologous and heterologous virus than occurred following virus infection.

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Table 1. *Geometric mean titres and fold increases in serum HI antibody following immunization or infection with influenza A viruses*

	Serum HI antibody titres to influenza virus:							
A/Hong Kong/68		A/Port Chalmers/73		A/Texas/77		A/Bangkok/79	
INF	H 63 (12*)	INF	H 148 (2)	INF	H 162 (1)	INF	H 240 (1·5)
	P 32 (6)		P 47 (1·5)		P 124 (2·5)		P 84 (–)
	T 20 (4)		T 30 (1·5)		T 86 (1·5)		T 114 (1)
	B < 20		B < 20		B 32 (3)		B 122 (4)
				IMM	H 184 (1)	INF	H 260 (1)
					P 320 (6)		P 544 (1·5)
					T 246 (4)		T 280 (1)
					B 77 (7)		B 723 (9)
		IMM	H 373 (6)	INF	H 284 (–)	INF	H 230 (–)
			P 305 (9)		P 340 (1·0)		P 333 (1)
			T 80 (6)		T 248 (5)		T 420 (2)
			B 15 (1·5)		B 74 (5)		B 182 (2)
				IMM	H 422 (7)	INF	H 612 (1·5)
					P 386 (1)		P 988 (3)
					T 360 (6)		T 580 (2)
					B 96 (6)		B 390 (4)
IMM	H 174 (35)	INF	H 221 (1)	INF	H 380 (1·5)	INF	H 320 (–)
	P 66 (12)		P 263 (5)		P 293 (1·5)		P 260 (–)
	T 15 (3)		T 132 (4)		T 151 (1)		T 140 (–)
	B < 20		B < 20 (–)		B 80 (8)		B 262 (3)
				IMM	H 648 (3)	INF	H 480 (–)
					P 640 (2·5)		P 805 (1·5)
					T 220 (4)		T 302 (1·5)
					B 173 (17)		B 227 (1)
		IMM	H 477 (3)	INF	H 512 (1)	INF	H 420 (–)
			P 416 (6)		P 343 (–)		P 386(1)
			T 167 (6)		T 686 (4)		T 420 (1)
			B 47 (5)		B 208 (1·5)		B 260 (1)
				IMM	H 411 (–)	INF	H 320 (–)
					P 692 (1·5)		P 480 (–)
					T 370 (2·5)		T 310 (–)
					B 85 (2)		B 372 (4)

The serum HI antibody titres (GMT) to: H, A/Hong Kong/68; P, A/Port Chalmers/73; T, A/Texas/77 and B, A/Bangkok/79 virus are shown for ferrets infected (INF) or immunized (IMM) with the virus denoted at the head of each column. Fold increases in the titres are given in parentheses.

Comparison of single and repeat immunizations

When ferrets were infected with A/Bangkok/77 virus, it was possible to compare the efficacy of a single A/Texas/77 immunization with that of repeated immunizations, and the results are shown in Table 2. Temperature responses, virus replication, nasal wash protein and local neutralizing antibody responses were assessed as described. In addition, the serological responses to infection were graded from 0 to 3 according to the magnitude of HI antibody rise. In each case, the responses of immunized ferrets to infection were compared with those of control non-immunized ferrets. Ferrets receiving three immunizations had not

Table 2. *Efficacy of single and multiple immunizations against A/Bangkok/79 infection of ferrets*

Number of immunizations	Immunizing* virus	Previous H3N2 infection (+ or −)	Temperature		Responses to A/Bangkok/79 infection: Virus replicaton		N/W Protein		Local Neut Ab		Serum** HI Ab		Total	
			C	I***	C	I	C	I	C	I	C	I	C	I
1	TX	+	3	1	3	2	3	0	3	0	3	3	16	6
2	PC/TX	+	3	1	3	2	3	1	3	0	3	3	15	7
2	HK/TX	+	3	1	3	2	3	0	3	0	3	1	15	4
3	HK/PC/TX	−	3	2	3	2	3	1	3	0	3	1	15	6

HK, A/Hong Kong/68; PC, A/Port Chalmers/73; TX, A/Texas/77; **, HI antibody responses to A/Bangkok/79 virus assessed as follows: 1, 1-fold rise; 2, 2- to 3-fold rise; 3, 4-fold rise; *** C, control ferrets; I, immunized ferrets.

Table 3. *Serum cross-reacting and specific antibody to influenza virus following sequential infection or immunization*

Serum antibody titres to influenza virus							
A/Hong Kong/68		A/Port Chalmers/73		A/Texas/77		A/Bangkok/79	
INF	CR —	INF	CR 9/10 (90)*	INF	CR 4/4 (1·0)	INF	CR 4/4
	S/H —		S/H 1/10 (10)		S/H —		S/H 3/4
	S/P —		S/P 5/10 (50)		S/P 3/4		S/P 4/4
	S/T —		S/T —		S/T 4/4		S/T 4/4
	S/B —		S/B —		S/B —		S/B —
				IMM	CR 4/4	INF	CR 5/5
					S/H 2/4		S/H 5/5
					S/P 4/4		S/P 5/5
					S/T 4/4		S/T 5/5
					S/B —		S/B 4/5
		IMM	CR 5/8 (56)	INF	CR 4/4	INF	CR 4/4
			S/H —		S/H —		S/H 4/4
			S/P 3/9 (33)		S/P 4/4		S/P 4/4
			S/T —		S/T 4/4		S/T 3/4
			S/B —		S/B —		S/B 1/4
				IMM	CR 5/5	INF	CR 5/5
					S/H —		S/H 4/5
					S/P 4/5		S/P 5/5
					S/T 3/5		S/T 5/5
					S/B —		S/B 2/5
IMM	CR 12/22 (55)	INF	CR 5/8 (63)	INF	CR 3/3	INF	CR 3/3
	S/H 4/22 (18)		S/H 1/8 (13)		S/H 3/3		S/H 3/3
	S/P —		S/P 1/8 (13)		S/P 3/3		S/P 2/3
	S/T —		S/T —		S/T 1/3		S/T 3/3
	S/B —		S/B —		S/B —		S/B —
				IMM	CR 4/4	INF	CR 3/3
					S/H 3/4		S/H 3/3
					S/P 4/4		S/P 3/3
					S/T 3/4		S/T 3/3
					S/B —		S/B —
		IMM	CR 10/11 (91)	INF	CR 5/5	INF	CR 5/5
			S/H 2/11 (18)		S/H 5/5		S/H 5/5
			S/P 2/11 (18)		S/P 3/5		S/P 5/5
			S/T —		S/T 3/5		S/T 5/5
			S/B —		S/B —		S/B 2/5
				IMM	CR 4/4	INF	CR 3/3
					S/H 4/4		S/H 3/3
					S/P 4/4		S/P 3/3
					S/T 4/4		S/T 3/3
					S/B —		S/B 1/3

The incidence of cross-reacting (CR) and specific antibody to influenza A/Hong Kong/68 (S/H), A/Port Chalmers/73 (S/P), A/Texas/77 (S/T) and A/Bangkok/79 (S/B) is shown. The antibody positive sera/number tested (% positive) is given: percentages are not given for the small groups of ferrets. The columns are headed for the virus used to infect animals (INF), or to immunize with inactivated vaccine (IMM).

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been previously infected with influenza A (H3N2) virus, whereas other immunized ferrets had been previously exposed to one or more influenza H3N2 infections. The result of this comparison is that no difference could be detected in the protection given by one, two or three immunizations.

Cross-reacting and strain-specific antibody responses

All serum samples from ferrets sequentially immunized or infected with the four influenza A viruses used in the present study were tested for cross-reacting (CR) and strain-specific (SS) serum antibody to A/Hong Kong/68, A/Port Chalmers/73, A/Texas/77 and A/Bangkok/79 viruses. The results are summarized in Table 3. Following immunization with A/Hong Kong/68 vaccine, 12 of 22 (55%) ferrets developed Cr antibody, and 4 (18%) developed SS antibody to the immunizing virus: no SS antibody to other viruses was detected. In contrast, neither CR nor SS antibody to A/Hong Kong/68 virus was detected following infection with homologous virus. HI antibody studies indicated that animals infected with A/Hong Kong/68 virus developed relatively low titres of HI antibody, compared to animals immunized with A/Hong Kong/68 vaccine; it remains possible that the low antibody responses to virus infection seen in HI tests could not be detected by the method used to identify CR and SS antibody. Following infection with A/Port Chalmers/73 virus, the majority of ferrets in all four groups developed CR and SS antibody to both A/Hong Kong/68 and A/Port Chalmers/73 viruses (Table 2). The increasing acquisition of CR and SS antibody continued following subsequent infection or immunization with A/Texas/77 and A/Bangkok/79 viruses. No SS antibody was detected in any serum sample prior to immunization or infection with that virus; in some cases SS antibody was detected to an earlier virus following subsequent infection or immunization with a later virus; and the incidence of CR and SS antibody was similar in all groups irrespective of the sequence of infection or immunization (Table 3).

DISCUSSION

The basis of immunity to influenza virus infection has been studied by many workers in the past, but the results have not given a complete understanding of which responses determine the immune state, and the relative contributions of each immune reaction. However, the titre of serum HI antibody has been shown to relate directly to immunity (Hobson, Beare & Ward-Gardner, 1972; Greenberg, Couch & Kasel, 1974; Potter & Oxford, 1979). In addition, the level of serum anti-neuraminidase antibody (Murphy, Kasel & Chanock, 1972; Rott, Becht & Orlich, 1974), the presence of local antibody in the respiratory secretions. (Potter *et al.* 1975; Couch, 1984) and cell-mediated immune responses (Potter & Oxford, 1979; McMichael *et al.* 1983) may also contribute to the immunity. Since inactivated influenza vaccines induce serum HI antibody, and the titre following vaccination can be directly related to immunity, inactivated vaccines have been developed against influenza infection through the last decade (Potter, 1982). However, the level of immunity achieved with inactivated vaccines has remained disappointingly low (Hobson, 1972), and live vaccines have been shown to produce a more solid immunity than killed vaccines despite the induction of lower titres of serum HI antibody (Beare *et al.* 1968). In addition, studies at Christ's Hospital

school following annual, sequential immunization with inactivated vaccines revealed that the sum total of influenza virus infections over several years was not significantly different for immunized and non-immunized persons. This finding brings the recommendation for annual immunization against influenza for 'at risk' people into question.

Since the studies carried out at Christ's Hospital school were not repeated elsewhere, and the epidemiological features relevant at the time will not recur, no confirmatory data will be forthcoming. In order to gain some insight into the Christ's Hospital experience (Hoskins *et al.* 1979), this study was set up to mimic the events but using ferrets. The viruses used were the same and in the same sequence that caused epidemics in man between 1968 and 1979, and ferrets have been shown to be a good model of human influenza virus infection (Potter & Oxford, 1977). On the other hand, the experiment was carried out over a 2-year period, rather than the longer natural time of the Christ's Hospital study; and ferrets, although a good model for human influenza, are not an exact one (Potter & Oxford, 1977).

The results of the present study confirm that infection of ferrets by influenza viruses induced lower levels of serum HI antibody and a more solid immunity to subsequent challenge than immunization with killed virus vaccines, and this is similar to the results of studies in man (Beare *et al.* 1968). Furthermore, the present results indicate that this observation can be made at each step in a sequence of virus infection or immunization followed by heterologous virus challenge. These results parallel in ferrets the observations made at Christ's Hospital school (Hoskins *et al.* 1979).

In contrast, the results of the study from Christ's Hospital indicated that a single dose of vaccine induced a significant immunity to the most up-to-date strain, and that further vaccination did not increase the level of protection. In the present study, the level of immunity was similar following both single and multiple immunization, and this result does not agree with the human study. Thus, although the ferret may represent a good model for influenza in man, this confirms that it is not an exact model and data from ferret studies cannot be assumed to parallel human experience.

One possible explanation for these findings was that infection induced a different quality of HI antibody than immunization. SS antibody is significantly more efficient in virus neutralization than CR antibody, and infection may preferentially induce SS antibody (Schild *et al.* 1977; Oxford *et al.* 1981). Since no reason for the differences in immunity to challenge could be found in studies of the HI antibody response to infection or immunization of ferrets, all sera were examined for SS and CR antibody. The majority of ferrets developed CR antibody following immunization or infection; SS antibody was only found following infection or immunization with homologous virus; and the incidence of SS antibody was similar in the groups of ferrets regardless of their past history of infection or immunization. Thus no evidence was found to suggest that the induction of SS antibody was more common following virus infection, and the findings do not indicate that the more solid immunity to challenge virus infection following live virus infection compared to immunization was related to preferential production of SS antibody.

The results of the present experiments confirm some of the findings of the

Christ's Hospital study, but provide no basis for explaining these observations. The indications are that live virus infection induced some immune response not found following immunization, but this was not found in the serum antibody response. Previous studies have shown that infection and immunity induce similar levels of cellular immunity (Ennis *et al.* 1981); hence the differences in immunity cannot be easily explained by differences in the cellular immune response. Studies have shown that greater local antibody responses in ferrets and man occur following infection as compared to immunization (Potter & Oxford, 1977), and this may provide the explanation. However, local antibody responses of both species are ephemeral, and are probably no longer present at the time of subsequent virus infection.

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