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IMMUNIZATION OF FERRETS AGAINST INFLUENZA: A COMPARISON OF KILLED FERRET GROWN AND EGG GROWN VIRUS

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Summary.—Both killed unadjuvanted ferret and egg grown A/Moscow/1019/65 (H_2N_2) influenza virus failed to immunize ferrets against challenge with homologous virus; the preparations were given in 2 doses, 2 weeks apart, distributed intranasally, intramuscularly and intraperitoneally. However, small doses (<2 HA units) of both preparations induced immunity in ferrets previously "primed" with a live heterologous virus (A/FM/1/47 (H_1N_1)) according to the method of Potter *et al.* (1973*a*, *b*). Although no difference in immunizing activity was detected between ferret and egg grown virus, the former induced greater HI titres than the latter. There was no correlation between HI titres in serum and protection to challenge; in fact, some protection seemed to be afforded by the " priming " virus in the absence of HI antibody to the challenge virus.

These results are discussed in relation to the possibility that a previously unrecognized antigen different from haemagglutinin and neuraminidase may contribute to immunity to influenza.

CLINICAL trials and animal experiments indicate clearly that live influenza virus vaccines produce a better and more prolonged immunity than the more safely used, dead preparations (Freestone *et al.*, 1972; Potter *et al.*, 1972*a*, *b*; Kurimura, Hirano and Okuno, 1972). Furthermore, there is increasing evidence, again both from clinical trials and animal experiments, that the haemagglutinin is not the sole immunizing antigen and may be even less important in immunity than formerly supposed (Tyrrell, 1969; Hobson *et al.*, 1972; Potter *et al.*, 1972*a*, *b*; Freestone *et al.*, 1972). These results could be due to influenza virus replicating within animal tissues, producing an antigenic viral component or product which is modified or not produced in the conventional egg grown virus, or is removed or destroyed during purification of killed vaccines. The facts that influenza virus glycoproteins are different when the virus is grown in different hosts (Compans *et al.*, 1970; Haslam *et al.*, 1970), that the extent of cleavage of the haemagglutinin molecule into polypeptides HA_1 and HA_2 is host–cell dependent (Kilbourne *et al.*, 1972; Lazarowitz, Compans and Choppin, 1971, 1973) and that a non-structural protein (Lazarowitz, Compans and Choppin, 1971; Klenk, Scholtissek and Rott, 1972; Skehel, 1972) and cell surface antigen(s) (Hahon and Eckert, 1972) is induced by viral multiplication underline these possibilities.

In the bacterial field, similar situations exist, namely that some live vaccines are better than killed preparations, and important immunizing antigens were first

recognized by examining bacteria and their products, isolated directly from infected animals without subculture, for immunizing activity (Smith, 1968, 1973). This paper describes a similar investigation with influenza virus. Virus and any possible non-structural viral products were obtained from infected ferrets as a crude homogenate of infected nasal turbinates. After inactivation, its immunizing potency and capacity to induce antibody was compared with that of inactivated egg grown virus; 2 test systems were used. Attempts were made to immunize first normal ferrets with unadjuvanted killed preparations, a notoriously difficult task (Potter *et al.*, 1972*a*, *b*), and second ferrets that had previously been " primed " by a live heterologous influenza strain (having a different H and N serotype to that of the immunizing and challenge virus) according to the method of Potter *et al.* (1973*a*, *b*). These workers have shown that prior infection of hamsters with heterotypic influenza A viruses potentiates the serum HI antibody response to killed unadjuvanted A/Hong Kong (H_3N_2) virus vaccines. Similar results were observed with ferrets (McLaren and Potter, 1973) and, in addition, these ferrets were immune to A/Hong Kong challenge (McLaren and Potter, 1974). The results are interesting with respect to all the questions posed above.

MATERIALS AND METHODS

Viruses.—Influenza viruses A/Moscow/1019/65 (H_2N_2) and A/FM/1/47 (H_1N_1) were supplied by the Wellcome Research Laboratories, Beckenham, Kent and Dr G. S. Schild, National Institute for Medical Research, Mill Hill, London respectively. Seed stocks ($10^{7\cdot3}$ $EBID_{50}$/ml, A/Moscow/1019/65; $10^{6\cdot8}$ $EBID_{50}$/ml, A/FM/1/47) were prepared by intra-allantoic inoculation of 10-day old embryonated hen's eggs and incubating at 37° for 48 hours. These stocks were stored in liquid nitrogen. Working stocks ($10^{8\cdot4}$ $EBID_{50}$/ml, 3840 HA/ml, A/Moscow/1019/65; $10^{8\cdot1}$ $EBID_{50}$/ml, 1920 HA/ml, A/FM/1/47) were obtained from allantoic fluids of 10-day old embryonated eggs inoculated with 10 $EBID_{50}$ of seed virus and incubated at 37° for 48 hours. These working stocks of egg grown virus were stored at −70°. The viruses are abbreviated to A/Mosc. (H_2N_2) and A/FM (H_1N_1) throughout the text.

Ferret grown virus (A/Mosc.) was prepared as follows: ferrets were inoculated with 10^6 $EBID_{50}$ of egg grown virus and 3 days later the ferrets were sacrificed; nasal turbinates were removed and macerated for 30 seconds in 3 ml of Hank's medium containing antibiotics (crystamycin, 0·16 mg/ml; acromycin, 0·025 mg/ml; mycostatin, 100 u/ml) using a Sorvall Omnimixer with external cooling. The nasal turbinates from 120 infected ferrets were collected to provide 360 ml of the vaccine preparations which had $\simeq 10^5$ $EBID_{50}$/ml (<5 HA/ml); gross debris was not removed by low speed centrifugation, which might remove cell-surface virus induced antigens.

Infectivity assays.—These were performed using the egg-bit assay of Fazekes de St Groth and White (1958) or embryonated hen's eggs as described by Sweet, Stephen and Smith (1974).

Haemagglutination (HA) and haemagglutination inhibition (HI) tests.—These were carried out and the HA unit defined as described previously (Sweet *et al.*, 1974).

Removal of nonspecific inhibitors from nasal turbinate homogenates before haemagglutination assays.—Nasal turbinate homogenates were incubated for 18 hours at 37° with 4 volumes of a filtrate from *Vibrio cholerae* (Burroughs Wellcome Limited) and subsequently heated at 56° for 1 hour to inactivate the filtrate. Haemagglutination inhibition titres of 1 : 8 to 1 : 16 in fresh homogenates were removed completely by this treatment.

Inactivation of virus.—Egg- and ferret grown virus was treated with 0·05% formaldehyde for 24 hours at 37° which destroyed the infectivity, as indicated by double egg passage, without affecting the haemagglutination titre. The latter could only be determined on the egg grown virus since the haemagglutination titre of the ferret grown virus (<5 HA/ml) was too low to be measured.

Vaccine tests.—Adult female ferrets (600–1000 g) were obtained from A. S. Roe, Little

Fakenham, Norfolk, and housed individually. Groups (3–5) were immunized under anaesthesia (veterinary Nembutal, Abbott, i.p., *ca* 0·5 ml/kg body weight) with different concentrations of killed egg- or ferret grown A/Mosc. (H_2N_2) virus or with live A/FM (H_1N_1) virus followed by killed egg- or ferret grown A/Mosc. (H_2N_2) virus. In the former regimen, ferrets were given 0·5 ml (0·25 ml into each nostril) intranasally (i.n.), 1 ml (0·5 ml in each of 2 distinct sites) intramuscularly (i.m.) and 6 ml intraperitoneally (i.p.) on Day 0; 14 days later the dose was repeated. In the latter regimen, ferrets were infected i.n. (0·5 ml) with A/FM (H_1N_1) virus and 5 weeks later vaccinated with killed egg- or ferret grown virus as described in the first regimen. Control animals received Dulbecco A (PBSA) solution. The ferrets were challenged i.n. 5 weeks post-vaccination with 10^6 $EBID_{50}$ (0·5 ml) of egg grown A/Mosc. (H_2N_2) virus. After 3 days the ferrets were killed and the virus infectivity in homogenates of the nasal turbinates was determined as described for ferret grown virus preparation. Under these conditions, the virus titres in homogenates from unimmunized ferrets were remarkably constant, a mean of $10^{5\cdot6}$ $EBID_{50}$/ml with a standard deviation of $\pm 10^{1\cdot0}$ being obtained in experiments with 41 ferrets.

Nasal washings were collected from ferrets immunized by the first regimen (Potter *et al.*, 1972*a*) before vaccination, 3 days after vaccination for checking the absence of virus infectivity and on Days 7, 9 and 11 after vaccination for antibody determinations. Blood specimens for antibody titrations were collected from the jugular vein (Bergman, Lodmell and Hadlow, 1972) 1 to 3 days before vaccination and challenge and by cardiac puncture 3 days post-challenge (*i.e.* at time of sacrifice). For ferrets immunized by the second regimen, nasal washings and blood specimens were collected before and after receiving the killed vaccine, as described above. In addition, nasal washings (and blood samples) were collected before infection with the priming strain, 3 days post-infection for infectivity determinations and on Days 7, 9 and 11 for antibody determinations.

RESULTS

Haemagglutination titre of the ferret grown virus

The ferret grown virus preparations contained infectivities of $\simeq 10^{5\cdot6}$ $EBID_{50}$/ml and no measurable HA (<5 HA u/ml) but this may have been masked by nonspecific inhibitors of haemagglutination, present to titres of 1 : 8–1 : 16 in homogenates. However, after treatment of the latter with *V. cholerae* filtrate (Materials and Methods) no HA activity was detectable even when the treated material was concentrated (5-fold) to its original volume by ultrafiltration (Amicon Corporation, Lexington, Massachusetts). In control experiments, the haemagglutination activity of egg grown A/Mosc. (H_2N_2) virus remained unaffected by treatment with *V. cholerae* filtrate and was restored by this treatment after being completely eliminated by prior mixing with uninfected nasal turbinate homogenate. Thus ferret grown virus contained <5 HA/ml and the total dosage (7·5 ml) was <37·5 HA.

Vaccination with killed ferret- or egg grown A/Moscow/1019/65 (H_2N_2) virus and challenge with live A/Moscow/1019/65 (H_2N_2) virus

Response to vaccination.—Nasal washings taken 3 days after each vaccinating dose were not infective, thus checking the inactivation procedure, and nasal washings taken on Days 7, 9 and 11 after the second dose of vaccine contained no detectable HI antibody after concentrating 10-fold.

Response to challenge.—Virus was isolated from all ferrets (Table I), indicating no substantial immunity had been achieved by killed egg- or ferret grown virus. However, in contrast to the control animals, all groups of immunized ferrets showed significant, albeit low, levels of serum HI antibody 3 days post-challenge.

TABLE I.—*Challenge with Influenza Virus A/Moscow/1019/65 (H_2N_2) of Ferrets Vaccinated with Two Doses (Two Weeks Apart) of Killed Egg- and Ferret Grown A/Moscow/1019/65 (H_2N_2)*

Ferret No.	Quantity of antigen in each dose	Response 3 days after challenge: Infectivity in nasal turbinates ($\log_{10}$ $EBID_{50}$/ml)	Response 3 days after challenge: Serum HI titre
35	28,800 HA(Egg)	4·9	64
36		4·8	16
37		5·8	16
38		5·0	32
39		4·1	32
40	2880 HA(Egg)	4·8	16
41		4·9	<8
42		4·6	32
43		5·1	32
44		5·7	16
45	288 HA(Egg)	4·8	8
46		4·9	16
47		6·8	ND
48		5·1	8
49		4·7	16
50	28·8 HA(Egg)	5·1	16
51		5·5	<8
52		4·8	16
53	<37·5 HA(Ferret)	5·2	16
54		5·1	8
55		4·7	8
56		4·6	8
57		5·0	16
58	PBSA Controls	5·4	<8
59		5·0	<8
60		4·9	<8
61		5·5	<8

ND. Not Done.

Vaccination with killed ferret- or egg grown A/Moscow/1019/65 (H_2N_2) virus following " priming " with live A/FM/1/47 (H_1N_1) virus: subsequent challenge with A/Moscow/1019/65 (H_2N_2) virus

Response to " priming " with A/FM/1/47 (H_1N_1) infection.—Nasal washings taken 3 days after inoculation showed that all ferrets were infected with the " priming " virus; titres of $10^{1·8}$–$10^{4·8}$ $EBID_{50}$/ml (mean $10^{3·4}$ $EBID_{50}$/ml) were obtained. Nasal washings taken 7, 9 and 11 days after infection contained HI antibody to the A/FM (H_1N_1) strain (mean 15, range 8–64) and in 6 of the 35 infected ferrets there was HI activity to A/Mosc. (H_2N_2) virus (mean 8, range 8–16). Thirty-one to 33 days after infection ferrets (only 5 groups examined) had serum HI antibody only to A/FM (H_1N_1) virus.

Response to subsequent vaccination with killed egg- and ferret grown A/Moscow/1019/65 (H_2N_2) virus.—Nasal washings taken 3 days after each immunizing dose contained no infective virus. Nasal washings taken on Days 7, 9 and 11 after the second immunizing dose contained no detectable HI antibody either to the " priming " strain or to the immunizing strain.

TABLE II.—*Response of "Primed" Ferrets to Immunization with 2 Doses (2 Weeks Apart) of Killed Egg- and Ferret grown A/Moscow/1019/65 (H_2N_2) Influenza Virus and Subsequent Challenge with A/Moscow/1019/65 (H_2N_2) Influenza Virus*

Ferret No.	Antigen in each dose	Response to immunization with killed A/Moscow/1019/65 (H_2N_2). Change in serum HI antibody* to		Response 3 days after challenge with A/Moscow/1019/65 (H_2N_2)		
				Infectivity in nasal turbinates ($log_{10}EBID_{50}$/ml)	Change in serum HI antibody† to	
		A/FM/1/47 (H_1N_1)	A/Moscow/1019/65 (H_2N_2)		A/FM/1/47 (H_1N_1)	A/Moscow/1019/65 (H_2N_2)
62	28,800 HA(Egg)	–256	–521	<1·5	256– 512	512– 64
63		–512	–256	<1·5	512–1024	256– 64
64		–256	–128	<1·5	256– 512	128– 32
65		–1024	- 512	<1·5	1024–1024	512– 64
66	2880 HA(Egg)	–512	–256	<1·5	512–1024	256– 64
67		–256	–128	<1·5	256–1024	128– 64
68		–256	–128	<1·5	256–1024	128– 32
69		–128	– 64	<1·5	128– 256	64- 32
82	288 HA(Egg)	–128	–128	<1·5	128– 256	128–128
83		–128	– 64	<1·5	128– 256	64– 16
84		–256	– 64	<1·5	256– 512	64– 32
86		–512	– 64	<1·5	512– 512	64– 32
70	28·8 HA(Egg)	256–128	<8– 16	<1·5	128–1024	16– 32
71		512–512	<8– 16	<1·5	512– 512	16– 16
72		1024–256	<8- 32	<1·5	256– 512	32– 16
73		512–128	<8 - 16	⩽2·9	128– 512	16– 16
87		1024–512	<8– 32	<1·5	512–1024	32– 32
114	0·93 HA(Egg)	256–256	<8–<8	4·4	256– 256	<8–<8
115		256–128	<8–<8	<1·5	128– 256	<8– 16
117		256–128	<8– 16	<1·5	128– 256	16– 32
118		512–256	<8–<8	⩽1·9	256– 256	<8– 8
74	<37·5 HA(Ferret)	256–256	<8– 64	<1·5	256– 512	64– 64
75		128–512	<8–128	<1·5	512– 512	128– 16
76		256–128	<8–128	<1·5	128 - 512	128– 64
77		512–256	<8–256	<1·5	256– 512	256– 64
89		512–256	<8– 64	4·33	256–1024	64– 32
119	<1·25 HA(Ferret)	512–128	<8–<8	<1·5	128– 256	<8– 16
120		512–128	<8– 16	<1·5	128– 128	16– 16
121		256–128	<8– 16	4·5	128– 64	16– 8
122		256–ND	<8–ND	<1·5	ND– 256	ND– 16
123		128–256	<8– 16	⩽3·7	256– 128	16– 16
78	PBSA	512–256	<8–<8	<1·5	256–1024	<8–<8
79		512–512	<8–<8	4·3	512– 512	<8–<8
80		256– 64	<8-<8	3·3	64– 512	<8–<8
81		2048–256	<8–<8	⩽2·8	256– 512	<8–<8
90	PBSA	–<8	–<8	4·6	<8– <8	<8–<8
91		–<8	–<8	4·9	<8– <8	<8–<8
92		–<8	–<8	5·6	<8– <8	<8–<8
93		–<8	–<8	5·4	<8– <8	<8–<8

* Change from pre- to post-immunization titre.
† Change from pre- to post-challenge titre.
ND. Not Done.
Ferrets 90, 91, 92 and 93 were uninfected controls.

The titres of HI antibody to the " priming " virus in all ferrets at 33 days after the second immunizing dose remained virtually unaffected (Table II). All doses of killed egg- and ferret grown virus induced HI antibody to A/Mosc. (H_2N_2) virus in " primed " ferrets, a normal dose/response result being observed. Ferret grown virus (<37·5 HA) produced titres comparable to HI titres induced by egg grown virus doses approximately 100-fold greater (2880 HA). As little as 2 HA egg- or ferret grown virus induced some HI antibody in at least one ferret of each group.

Response to challenge.—All ferrets immunized with 28,800, 2880 and 288 HA of egg grown A/Mosc. (H_2N_2) virus were immune, as demonstrated by the failure to isolate virus from the nasal turbinates. Four of 5 ferrets immunized with 28·8 HA egg grown virus were protected, as were 4 of 5 ferrets immunized with ferret grown virus (<37·5 HA). Lower doses of egg- (0·93 HA) and ferret grown virus (<1·25 HA) also showed some protection. Control ferrets inoculated with PBSA only were completely susceptible. However, ferrets infected with A/FM (H_1N_1) virus but not receiving killed A/Mosc. (H_2N_2) virus were partially protected from live challenge with A/Mosc. (H_2N_2) virus despite the absence of detectable serum and nasal HI antibody to the latter virus.

HI antibody titres to A/Mosc. (H_2N_2) virus were reduced 3 days post-challenge in all immunized ferrets except those immunized with low doses of virus, whereas the antibody titres to the A/FM (H_1N_1) virus remained unaffected.

DISCUSSION

The results reported in this communication are in general agreement with the observations of Potter and his co-workers (Potter *et al.*, 1972*a*; McLaren and Potter, 1973, 1974). However, 3 new observations have been made. Firstly, "primed " ferrets not vaccinated with killed A/Mosc. (H_2N_2) virus were partially immune to challenge with A/Mosc. (H_2N_2) influenza virus in the absence of detectable HI antibody. Secondly, ferret grown virus (HA) stimulated the production of HI antibody titres which were comparable to those induced by egg grown virus (HA) doses approximately 100-fold greater. Thirdly, HI antibody titres to the challenge A/Mosc. (H_2N_2) virus were reduced in vaccinated " primed " ferrets 3 days after challenge.

The failure of Potter *et al.* (1972*a*) to immunize ferrets by intramuscular inoculation of killed influenza virus, as measured by resistance to challenge and production of serum HI antibody, has been confirmed in experiments using 3 simultaneous routes for vaccination. Ferrets immunized intranasally, intramuscularly and intraperitoneally with 2 large doses (28,800 HA) of unadjuvanted egg grown A/Mosc. (H_2N_2) virus given 2 weeks apart were susceptible to challenge with homologous virus. Similar observations were made in ferrets receiving low doses of ferret- or egg grown virus. An additional interesting observation showed that vaccinated ferrets, in contrast to unvaccinated ferrets, had some serum HI antibody 3 days post-challenge, indicating some response to the primary vaccination. In this respect there was no difference between egg- and ferret grown virus.

Prior infection of ferrets with A/FM (H_1N_1) influenza virus potentiated the serum HI antibody response to killed unadjuvanted A/Mosc. (H_2N_2) virus. This agrees with the observations made in hamsters by Potter *et al.* (1973*a*, *b*) and in

ferrets by McLaren and Potter (1973, 1974). As little as 1 HA of egg grown A/Mosc. (H_2N_2) virus produced HI antibody after " priming " with the heterotypic A/FM (H_1N_1) virus (Table II). The more striking result, however, was that ferret grown virus (<37·5 HA) produced HI titres much greater than those induced by a similar quantity of egg grown virus (28·8 HA); the titres were comparable to that produced by 2880 HA of egg grown virus. There are at least 3 explanations for this result. First, the antigenicity of the HA was increased by homologous *in vivo* passage, second that crude nasal turbinates have a nonspecific adjuvant effect, and third the HA is slowly released (and hence perhaps the HI response is maintained) from specific receptors present in the crude tissue preparations. Despite this difference in HI response, there was no significant difference in protection to challenge evoked by similar doses of ferret- (<1·25 HA) and egg grown virus (0·93 HA). Similar observations regarding the lack of correlation of HI antibody with protection have been observed in human volunteers (Freestone *et al.*, 1972; Hobson *et al.*, 1972). Cross-reacting nasal HI antibody may be important (Waldman, Wigley and Small, 1970; Haff and Pinto, 1973), but we detected this antibody only in a few ferrets (6 of 35) on Days 7, 9 and 11 after the " priming " infection with A/FM (H_1N_1) virus; it was undetectable 33 days after infection or later.

Infection with A/FM (H_1N_1) influenza virus provided some protection against the totally heterotypic A/Mosc. (H_2N_2) virus in the absence of detectable HI antibody to the challenge virus (ferrets 78–81, Table II). McLaren and Potter (1974) failed, in their studies, to detect heterotypic immunity over a similar time period to that used in our experiments (12 weeks). They did, however, demonstrate heterotypic immunity in ferrets challenged 3 weeks after the first infection. Heterotypic protection has been observed in several studies with mice (Kurimura *et al.*, 1971, 1972; Masurel, Baars and Frankena, 1973; Schulmån and Kilbourne, 1965).

The conclusion of these findings, by ourselves with regard to HI and by others with regard to HI and NI, is that serum and nasal anti-haemagglutination and anti-neuraminidase (NI) antibodies are poor indices of immunity to influenza. Some other hitherto unknown antigen may contribute to immunity against influenza. Kurimura *et al.* (1973) have demonstrated cross-immune reactions between A_1 (H_1N_1) and A_2 (H_2N_2) influenza viruses due to cell surface antigens (unrelated to HA or neuraminidase) produced by viral multiplication in tissue culture. Recently, Masurel *et al.* (1973) have demonstrated heterotypic immunity in mice to A/Equi/Miami/1/63 (Heq 2 Neq 2), after immunization with killed A/Hong Kong/1/68 (H_3N_2) virus, in the absence of detectable haemagglutination- or neuraminidase inhibiting antibodies and they suggest that an unknown antigenic component (mice protecting antigen) may be responsible. Possibly immunological mechanisms other than serum or nasal HI or anti-neuraminidase antibody may play a role, such as IgE antibody attached to cells in the respiratory tract or cell mediated immunity (Freestone *et al.*, 1972).

A final interesting observation with regard to serum HI responses was that in the " priming " experiments HI antibody titres to A/Mosc. (H_2N_2) virus were reduced 3 days after the homologous challenge, whereas titres to the " priming " A/FM (H_1N_1) virus remained unaffected. One possible explanation for this could be reaction of the antibody with the challenge virus escaping from the infected nasal turbinates into the vascular system, either directly or possibly by tissue

damage in the turbinates resulting from hypersensitivity reaction in immune ferrets. Alternatively, a local hypersensitivity reaction in the nasal mucosa may lead to exudation of serum antibody on to the infected nasal epithelium, thereby affording protection in a manner similar to that thought to occur in immunity to helminths (Murray, 1972).

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