Mechanism of Immunity to Influenza: Maternal and Passive Neonatal Protection Following Immunization of Adult Ferrets with a Live Vaccinia–Influenza Virus Haemagglutinin Recombinant but Not with Recombinants Containing Other Influenza Virus Proteins

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SUMMARY

Neonatal ferrets are protected against infection with influenza virus by milk-derived anti-influenza virus IgG after suckling on an immune mother. Live vaccines protect better than killed vaccines despite their stimulation of lower maternal haemagglutination-inhibiting antibody levels. This suggests that antibody to virus proteins other than the haemagglutinin may also be involved. To investigate this, adult ferrets were immunized intradermally with live vaccinia–influenza virus recombinants each expressing one of the 10 influenza virus polypeptides. Adult ferrets immunized with a recombinant expressing the H3 haemagglutinin were completely protected, and also passively protected their offspring, against a live challenge with clone 7a of the reassortant influenza virus A/Puerto Rico/8/34-A/England/939/69 (H3N2), immunity being mediated by IgG antibody. However, ferrets immunized similarly with recombinants expressing the H1 haemagglutinin, neuraminidase (N1 or N2), polymerases (PB1, PB2 or PAC), matrix protein (M1 or M2), nucleoprotein (NP) or non-structural proteins (NS1 or NS2) were completely susceptible to the influenza virus.

INTRODUCTION

Immunity to influenza virus infection is subtype-specific and antibody-mediated, whereas cell-mediated immunity is broadly cross-reactive and probably aids in the recovery from infection (Askonas et al., 1982; Ada et al., 1983; Ada & Jones, 1986). The haemagglutinin (HA) is the major immunogen for eliciting humoral immunity; field and volunteer studies showed that resistance to infection correlated with serum anti-HA antibody levels (Potter, 1982) and also that passive transfer of anti-HA antibody, both polyclonal and monoclonal, protected mice against challenge inoculation (Askonas et al., 1982). Similar studies indicate that antibodies to neuraminidase (NA) play a secondary role, reducing virus yield and cell-to-cell spread. Anti-matrix protein (M1) and anti-nucleoprotein (NP) antibodies, although produced during infection, do not neutralize infectivity in vitro or passively immunize in vitro (Cretescu et al., 1978; Virelizier et al., 1979). Also, animals immunized with highly purified M1 and NP proteins are fully susceptible (Oxford & Schild, 1976; Becht et al., 1979). The antibody response in man to other viral proteins is unknown (Ada & Jones, 1986). With regard to antigens mediating cellular immunity, influenza infection of mice elicits cytotoxic T cells capable of recognizing at least eight of the 10 influenza virus-encoded proteins (HA, NA, M1, NP, the polymerases PAC, PB1 and PB2 and the non-structural protein, NS1; Ada & Jones, 1986; Bastin et al., 1987; Bennink et al., 1987). However, the important antigen mediating recovery appears to be NP with some contribution from M1, PB2, NS1 and HA (Yewdell et al., 1985; Taylor & Askonas, 1986; Gotch et al., 1987; Wysocka & Bennink, 1988).

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Our previous studies (Husseini et al., 1984; Sweet et al., 1987) examining the role of live and killed vaccines in inducing passive immunity in neonatal ferrets, indicated that immunity was type-specific and acquired as milk-derived anti-influenza IgG (Sweet et al., 1987b). However, higher levels of maternal haemagglutination-inhibiting (HI) antibody were required to protect after immunization with killed vaccines compared to live infection. One explanation for these observations is that influenza virus proteins additional to the HA are involved in humoral immunity; we have investigated this in the present study by immunizing animals with live vaccinia–influenza virus recombinants each expressing one of the 10 influenza virus polyepitides. Initially, protection was assessed in both the upper and lower respiratory tracts of adult ferrets and finally any protective proteins were also assessed in the neonatal model.

METHODS

Influenza viruses and their assay. Clone 7a (H3N2; virulent) of the reassortant influenza virus A/Puerto Rico/8/34-A/England/939/69 was prepared as described previously (Sweet et al., 1974a, b; Matsuyama et al., 1980). Influenza virus A/Northern Territories/60/68 (A/NT) was obtained from Dr P. A. Reay, Sir William Dunn School of Pathology, University of Oxford, U.K. A seed stock was prepared by intratracheal inoculation of 10-

day-old embryonated hen’s eggs and incubation at 37 °C for 48 h. Working stocks were obtained from allantoic fluids of 10-day-old embryonated eggs inoculated with 10̇̇ egg infectious doses (EIDso) of seed virus and incubated at 37 °C for 24 h. The method of preparation of high titre stocks of virus has been described (Gould et al., 1972). Stocks, stored at –70 °C, were subsequently assayed using the egg-bit or egg-techniques (Sweet et al., 1974a) and titres were expressed in 50% egg-bit infectious doses (EBIDso) or EIDso.

Vaccinia–influenza virus recombinants. These were kindly supplied by Dr P. A. Reay (vacc–N2) and Dr B. Moss, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A. and Dr G. L. Smith, Department of Pathology, Virology Division, Cambridge University, U.K. (vacc–H3, vacc–H1, vacc–N1, vacc–PB1, vacc–PB2, vacc–M2, vacc–NS1 and vacc–NS2). They were supplied with titres of 7 × 10̇̇ to 3.7 × 10̇̇ p.f.u./ml suitable for the immunization of ferrets. The parental origin for the majority (H1, PB1, PB2, PAC, NP, M1 and NS1) of recombinant influenza virus genes was A/Puerto Rico/8/34 (H1N1) (A/PR/8); the H3 gene was derived from A/NT (H3N2), the N1 gene from A/Cambridge/1/46 (H1N1), the NS2 and M2 genes from A/Udorn/72 (H3N2) (Gotch et al., 1987) and the N2 gene from A/NT (H3N2) (Webster et al., 1988).

Ferrets, their immunization and challenge. Adult male ferrets, obtained from A. S. Roe, Barnham, Norfolk, were shaved at the back of the neck and immunized with three intradermal inoculations administered over a 6 week period at 3-weekly intervals, of 0.2 ml of phosphate-buffered saline (PBS) containing 10̇̇ p.f.u. of vaccinia virus recombinant. Three weeks after the final inoculation, they were challenged intranasally under ether anaesthesia with 10̇̇ EBIDso influenza virus clone 7a (H3N2). Adult female ferrets, mated as described previously (Sweet et al., 1977), were inoculated similarly to give passive immunity to their offspring but the timing of the challenge varied because of the difficulty of timing mating and birth with the immunizing schedule. One-day-old ferrets were challenged intranasally with 10̇̇ EBIDso of clone 7a as described previously (Sweet et al., 1987a).

Estimation of immune protection. Adult animals were nasally washed (Toms et al., 1977) for virus determination on days 1 to 3 post-challenge and killed on the 3rd day by intraperitoneal inoculation of 1 mg Sagatal (May and Baker). Lungs were removed and homogenized in 13 ml of Hanks’ balanced salt solution (Gibco), supplemented with 0.01 g/ml bovine serum albumin (BSA), 100 units/ml penicillin G (Sigma) and 100 μg/ml streptomycin (Sigma) using a Sorvall Omnimixer as described previously (Sweet et al., 1977). One-day-old ferrets were killed by pentobarbitone overdose 4 days after challenge and virus titres in nasal turbinates and lungs were determined as described previously (Sweet et al., 1987a).

Antibody tests. Sera, from adult ferrets under ether anaesthesia, were collected by cardiac puncture before immunization and/or challenge and tested for anti-influenza HA antibodies using the HI test as described previously (Basarab & Smith, 1969). Class-specific and total anti-influenza virus antibodies to each influenza virus polypeptide, in sera and in 10-fold concentrated nasal washes (concentrated by dialysis against sucrose and PBS), were assayed by ELISA using class-specific anti-ferret immunoglobulins prepared as described previously (Sweet et al., 1987b) and a rabbit antiserum prepared to whole serum. Total anti-vaccinia virus antibodies in ferret sera were measured similarly.

The ELISA was performed essentially as described previously (Sweet et al., 1987b) with the following modifications. Influenza viruses to be used as the capture antigen (clone 7a, A/NT, A/PR/8) were purified as described by Coates et al. (1986) and disrupted by the addition of an equal volume of 5% (w/v) sodium deoxycholate (Sigma) and incubation at 37 °C for 1 min. When the vacc–M1 recombinant was used as the capture antigen, cell-grown virus was disrupted as above but for 5 min at 37 °C. In each case the detergent was removed by dialysis against PBS for 3 days with daily changes of buffer. Two μg of viral protein (clone 7a, A/NT, A/PR/8) or
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the equivalent of $10^7.3$ p.f.u. (vacc–M1) in 0.2 M-sodium carbonate buffer pH 9.5, was added to each well in a flat-bottomed microtitre plate (Nunc Immuno Plate 1) and incubated at 4 °C for 18 h. After washing and blocking non-occupied sites with BSA, doubling dilutions (100 μl) of the test sera were added to each well and the plate was re-incubated for 1 h at 37 °C. After washing, 100 μl of the monospecific (IgA, IgG or IgM) or 100 μl of the polyspecific (IgA, IgG and IgM) anti-ferret serum in PBS–Tween–BSA was added to each well and the plate was incubated for 1 h at 37 °C. After washing, 100 μl of peroxidase-labelled goat anti-rabbit (IgG and total immunoglobulin) or goat anti-guinea-pig (IgM and IgA) was added. Following incubation at 37 °C for 1 h, the plate was washed, 100 μl of OPD reagent [34 mg O-phenylenediamine, 20 μl 30 vol. H2O2, 100 ml citrate phosphate buffer (0.05 M-citric acid, 0.1 M-disodium hydrogen orthophosphate, pH 5.0)] was added and the plate incubated at 37 °C for 30 min before the reaction was stopped by the addition of 50 μl 12% (v/v) H2SO4. The absorbance was read at 492 nm on a Titertek Multiskan MCC ELISA plate reader. Results are expressed as the maximum dilution giving a transmission value of 1.

Since whole 'split' virus was used for the antigen, the specificity of the antibody response was determined by inhibition of the reaction with anti-H3 (clone HC3; ELISA titre 1/8000, HI titre 1/6400), anti-N2 (clone NC81; ELISA titre 1/8000) and anti-NP (ELISA titre 1/204800) monoclonal antibodies by adding 100 μl of the relevant monoclonal antibody and incubating for 1 h at 37 °C at the step immediately prior to adding the test sera. These monoclonal antibodies were kindly supplied by Alan Douglas, World Influenza Centre, NIMR, London, U.K.

RESULTS

Determination of optimum dosage from serum antibody levels

In preliminary studies, groups of two or three adult ferrets were immunized intradermally with one, two or three doses of $10^8$ p.f.u. vacc–H3 and bled 3 weeks after each dose. Serum HI antibody levels to clone 7a were low (20 to 80) after a single dose, increasing to 80 to 160 and 320 to 640 after two and three doses respectively. HI antibody levels were similar against A/NT, the virus that provided the H3 gene in vacc-H3. Consequently, three doses of the vaccinia recombinant were used in subsequent experiments.

Virus titres in nasal washes and lung homogenates of adult ferrets immunized with
vaccinia–influenza virus recombinants

As previously described (Toms et al., 1976, 1977; Smith & Sweet, 1988), virus levels in nasal washes of unimmunized adult ferrets inoculated intranasally with $10^6$ EBID50 clone 7a reached a peak 24 h post-infection at about $10^6$ EBID50, declining over the next 2 days (Table 1). Lung titres were high at day 3 (Toms et al., 1976; Sweet et al., 1981). Ferrets immunized with three doses of vacc–H3 produced good serum HI antibody levels (320 to 640) to clone 7a and A/NT and, when challenged, no virus was shed in nasal washings during the 3 days and on day 3 no virus was isolated from the lungs, indicating complete and rapid protection (Table 1). Protection was serotype-specific because animals immunized with vacc–H1, exhibiting a good serum HI antibody response (512 to 1024) to A/PR/8 (H1N1), were completely susceptible (Table 1). Vaccination with all other vaccinia virus recombinants, including vacc–N2, failed to protect (Table 1).

Virus titres in nasal turbinates and lungs of newborn ferrets born to mothers immunized with
one, two or three doses of vacc–H3

As described previously (Husseini et al., 1983, 1984; Coates et al., 1984) 1-day-old (newborn) ferrets born to unimmunized mothers were completely susceptible to infection, producing high virus titres in the lungs and nasal turbinates at 4 days post-infection; lung titres were approximately 10-fold greater than in nasal turbinates (Table 2). One mother immunized with a single dose of vacc–H3 produced a low serum HI antibody titre of 1:80 and her kittens were completely susceptible. Two inoculations of vacc–H3 induced slightly higher HI antibody levels (80 to 160) in vaccinated mothers but gave no more protection. However, three doses of vacc–H3 induced H1 antibody levels of 1:640 which completely protected both the upper and lower respiratory tracts of newborn ferrets suckled by these mothers. Thus, three doses of vacc–H3 were equally effective in producing passive and active protection in newborn and adult ferrets respectively.
Table 1. Mean total virus titres in nasal washes and lung homogenates at various days post-intranasal (p.i.) challenge with 6.0 log<sub>10</sub> EBID<sub>50</sub> clone 7a (H3N2) of adult ferrets immunized with three intradermal inoculations of various vaccinia–influenza virus recombinants

<table>
<thead>
<tr>
<th>Immunizing antigen*</th>
<th>Number of animals</th>
<th>HI titres in serum</th>
<th>Nasal washes (days p.i.)</th>
<th>Lungs (days p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Clone 7a (H3N2)</td>
<td>A/NT (H3N2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/PR/8 (H1N1)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Nil</td>
<td>4</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–H3</td>
<td>3</td>
<td>320–640</td>
<td>320–640</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–H1</td>
<td>3</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–N2</td>
<td>2</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–N1</td>
<td>2</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–NP</td>
<td>3</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–M1</td>
<td>3</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–M2</td>
<td>3</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–PB1</td>
<td>3</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–PB2</td>
<td>3</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–PAC</td>
<td>3</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–NS1</td>
<td>3</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc–NS2</td>
<td>3</td>
<td>&lt;10</td>
<td>ND†</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* 10<sup>6</sup> p.f.u. of recombinant virus given intradermally at 3 weekly intervals.
† Virus titres in nasal washes are expressed in log<sub>10</sub> EBID<sub>50</sub>, those in lung homogenates as log<sub>10</sub> EID<sub>50</sub>.
§ ND, Not determined.
$\pm$ S.E.M., Standard error of the mean, given in parentheses.

Table 2. Mean total virus titres in nasal turbinates and lungs at 4 days post-intranasal challenge with 1.0 log<sub>10</sub> EBID<sub>50</sub> clone 7a (H3N2) of newborn ferrets born to and suckling on mothers immunized intradermally with one, two or three doses of vacc–HA

<table>
<thead>
<tr>
<th>Number of mothers; HI titres to clone 7a (H3N2)</th>
<th>Total number of newborn ferrets (no. of litters)</th>
<th>Mean total virus titre (log&lt;sub&gt;10&lt;/sub&gt;EBID&lt;sub&gt;50&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;20</td>
<td>3 (1)</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>2 (1)</td>
</tr>
<tr>
<td>2</td>
<td>80–160</td>
<td>9 (3)</td>
</tr>
<tr>
<td>3</td>
<td>640</td>
<td>7 (2)</td>
</tr>
</tbody>
</table>

This protection contrasts with that elicited by killed virus for, as described previously (Sweet et al., 1987a), three doses of 100, 100 and 25 μg respectively of killed virus given at weekly intervals with hydrogel to pregnant mothers produced maternal HI antibody titres of 2560 to 10240 but only partial protection of their offspring. Titres in nasal turbinates and lungs were 3.59 (S.E.M. 0.56) and 2.14 (S.E.M. 0.14) respectively 4 days post-challenge (Sweet et al., 1987a). A single dose (100 μg) of killed vaccine in hydrogel elicited HI antibody levels similar to three doses of vacc–H3 (320 to 640) but with little or no protection: titres were 5.46 (S.E.M. 0.49) and 3.34 (S.E.M. 0.46) respectively for nasal turbinates and lungs at 4 days (Sweet et al., 1987a).

Nature of the antibody mediating protection

To investigate the antibody classes found in the serum, an ELISA was used utilizing whole detergent 'split' virus as the capture antigen (see Methods). Anti-influenza virus antibodies of all three classes were found in sera of adult ferrets immunized with vacc–H3 (Table 3). Antibody was primarily IgG, its titre being 64 to 256 times greater than for IgA, and 500 to 1000 times greater than for IgM. Responses to vacc–N2, vacc–NP and vacc–M1 were similar and again predominantly IgG (Table 3). Differences in levels of response to individual influenza virus
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Table 3. Total and class-specific ELISA antibody titres of ferret adult sera immediately prior to challenge following intradermal immunization with three doses of recombinant vaccinia–influenza viruses containing various influenza virus genes

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Clone 7a (H3N2)</th>
<th>A/PR/8 (H1N1)</th>
<th>Vacc-M</th>
<th>Total antibody titres</th>
<th>Class-specific antibody titres to clone 7a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>16–32</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Vacc-H1</td>
<td>&lt;10</td>
<td>2560–5120</td>
<td>512–1024</td>
<td></td>
<td>ND†</td>
</tr>
<tr>
<td>Vacc-N2*</td>
<td>4096–10240</td>
<td>&lt;10</td>
<td>2048</td>
<td>2560–5120</td>
<td>10–20</td>
</tr>
<tr>
<td>Vacc-N1</td>
<td>&lt;10</td>
<td>640</td>
<td>512–2048</td>
<td></td>
<td>ND†</td>
</tr>
<tr>
<td>Vacc-NP</td>
<td>512–1024</td>
<td>ND</td>
<td>256–1024</td>
<td></td>
<td>ND†</td>
</tr>
<tr>
<td>Vacc-M1</td>
<td>1024–2560</td>
<td>ND</td>
<td>256–2048</td>
<td></td>
<td>ND†</td>
</tr>
<tr>
<td>Vacc-M2</td>
<td>&lt;10</td>
<td>ND</td>
<td>512–2048</td>
<td></td>
<td>ND†</td>
</tr>
<tr>
<td>Vacc-PB1</td>
<td>&lt;10</td>
<td>ND</td>
<td>1024</td>
<td>ND</td>
<td>ND†</td>
</tr>
<tr>
<td>Vacc-PB2</td>
<td>&lt;10</td>
<td>ND</td>
<td>512–2048</td>
<td></td>
<td>ND†</td>
</tr>
<tr>
<td>Vacc-PAC</td>
<td>&lt;10</td>
<td>ND</td>
<td>1024</td>
<td>ND</td>
<td>ND†</td>
</tr>
<tr>
<td>Vacc-NS1</td>
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<td>ND</td>
<td>512–1024</td>
<td></td>
<td>ND†</td>
</tr>
<tr>
<td>Vacc-NS2</td>
<td>&lt;10</td>
<td>ND</td>
<td>256–2048</td>
<td></td>
<td>ND†</td>
</tr>
</tbody>
</table>

* Similar levels of antibody were detected when A/Northern Territories/60/68 (H3N2) was used as the capture antigen.
† ND, Not determined.

Table 4. Class-specific ELISA antibody titres to clone 7a in adult ferret nasal washings and sera prior to and 3 weeks after a dose of vacc–H3 given at 3 weekly intervals

<table>
<thead>
<tr>
<th>Sample</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal washings†</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* The results are the range of responses of three animals vaccinated intradermally with 10⁶ p.f.u. of vacc–H3 at 3 weekly intervals, the response being measured 3 weeks after each dose.
† Nasal washings were concentrated 10-fold prior to the ELISA but titres are expressed per 100 µl of original sample.

proteins probably reflect their relative amounts and availability in the 'split' virus since responses to vaccinia virus were similar (Table 3). No responses were detected to vacc–H1 or vacc–N1 when clone 7a (H3N2) or A/NT (H3N2) was utilized as the capture antigen, illustrating the specificity of the assay. Responses to both the H1 and N1 glycoproteins were detected, however, when A/PR/8 was used as the capture antigen (Table 3). No responses were detected to vacc–M2, vacc–PB1, vacc–PB2 or vacc–PAC, presumably reflecting their low amount in virions used for antibody capture, or to vacc–NS1 or vacc–NS2 because of their absence from virions; anti-vaccinia virus responses were evident in these animals (Table 3).

Thus, immunization with vacc–H3 protected both the upper and lower respiratory tracts of newborn and adult ferrets. Since it has been reported previously (Small et al., 1985) that scarification of mice with a vaccinia–influenza virus recombinant encoding the H1 gene did not induce local antibody or protect the upper respiratory tract of mice against intranasal challenge, nasal and serum class-specific antibody responses to vacc–H3 were examined following one, two and three doses of vaccine using the ELISA. Low levels of anti-influenza IgG (but not IgA or IgM) antibody were detected in sera but not nasal washes 3 weeks after one dose of vaccine (Table 4). Serum IgG antibody levels increased by eight- to 16-fold following the second dose
and by a further 16- to 32-fold following the third dose. Nasal antibody was detected only after two vaccine doses and then at barely detectable levels of only anti-influenza IgG (Table 4); these levels increased 16- to 32-fold following the third dose. Since antibody in nasal washes was apparently entirely IgG and increased proportionately with serum IgG, it was more likely that it was derived from serum than produced locally in the respiratory tract.

DISCUSSION

Different vaccinia recombinant viruses, each expressing a single foreign antigen, have been used to protect experimental animals from a variety of viral diseases (reviewed in Mackett & Smith, 1986). In particular, various animal species have been immunized with vaccinia virus recombinants containing HA genes of both type A and type B influenza viruses; these recombinants elicited HI antibody titres which were protective in some cases (Smith et al., 1983; Small et al., 1985; Andrew et al., 1987; Rota et al., 1987). Similarly, birds inoculated with a vaccinia–N2 recombinant were protected against the lethal consequences of the influenza virus challenge although still developing disease (Webster et al., 1988), whereas vaccinia–NP recombinants did not elicit protective immunity (Andrew et al., 1987). These results agree well with previous observations with purified influenza virus proteins and monospecific antibodies, both monoclonal and polyclonal (see Introduction). The present study has confirmed these results but has extended the work to include vaccinia virus recombinants encoding one each of all seven other influenza virus genes.

Interestingly, only recombinants encoding the HA gene protected adult ferrets directly or newborn ferrets passively against intranasal challenge. Protection appeared to be predominantly IgG-mediated. Unlike the studies of Small et al. (1985) with mice, intradermal vaccination of ferrets protected the nasal epithelium and lung and induced antibody locally in the nose. Again, this was predominantly, if not solely, IgG, confirming previous studies (Shore et al., 1972), which demonstrated that ferret nasal antibody had a sedimentation coefficient of 7S (IgG). It is not certain whether this antibody is produced locally or results from exudation of serum antibody onto infected nasal epithelium. It has been argued by some that at least some of the nasal IgG is produced locally in the ferret (Shore et al., 1972; McLaren & Butchko, 1978) suggesting that intradermal vaccination of ferrets, unlike mice, may induce local antibody production in the nasal tract. However, in the case of maternal vaccination with vacc–H3, producing passively immunized newborn ferrets with protected upper and lower respiratory tracts, protection is mediated here by serum IgG (Sweet et al., 1987b). Also, levels of nasal IgG antibodies increased in parallel with serum IgG antibodies (Table 4).

Surprisingly, vaccination with vacc–N2 did not mediate protection although it induced a good serum antibody response. Neither was there any evidence of a reduced virus yield within 3 days following the challenge. Apparently the major contribution of anti-neuraminidase immunity is to modify disease. Schulman et al. (1968) established that the immunization of mice with isolated N2 neuraminidase modified the severity of infection, reduced titres in the lung, and caused lung lesions to be less severe. Similarly, chickens immunized by scarification of the comb with vacc–N2 developed the disease but survived lethal infection (Webster et al., 1988). Therefore, immunity to neuraminidase may manifest itself after the 3 days examined here but it would not have modified the disease which, in the ferret, is essentially over within this time. Supporting this suggestion is the lack of effect on recovery within the 3 day period of immunity raised to vacc–NP, although it is well established that cell-mediated immunity directed at the NP aids recovery (Ada & Jones, 1986).

With regard to the use of vaccinia recombinants as vehicles for vaccination, it is disappointing to find that three doses were necessary to raise a protective immune response in ferrets. HI antibody levels induced by a single dose of vaccinia were low but comparable with those observed in studies for other animal species (Smith et al., 1983; Boyle et al., 1986; Coupar et al., 1986). Further doses increased HI antibody levels about 10-fold confirming that revaccination can result in the boosting of antibody levels (Mackett et al., 1985; Perkus et al., 1985). The low responses may derive from poor expression of cloned genes in vaccinia virus although these
recombinants expressed well in vitro (Smith et al., 1987) under the control of the P7.5 promoter. This promoter has been shown to be better than some vaccinia virus promoters for expressing foreign genes (Coupar et al., 1986).

Alternatively, these recombinants may replicate poorly in ferrets as they do in mice since they are all thymidine kinase-negative, which markedly attenuates their replication in vivo (Buller et al., 1985). Indeed, there is no formal proof that the recombinants replicated in the majority of ferrets because only in one animal did a lesion typical of a poxvirus occur. In addition, the inoculum was not purified virus and contained free influenza virus proteins produced together with vaccinia virus. Nevertheless, indirect evidence argues in favour of a replicating virus inducing the immunity in these experiments. Firstly, it is difficult if not impossible to immunize ferrets with killed vaccines in the absence of adjuvant (Potter et al., 1972a, b; Sweet et al., 1974c). Secondly, the levels of antibody which are elicited by three doses of vacc–H3 do not protect when these result from vaccination with non-replicating influenza virus (Sweet et al., 1987b).

The main conclusion from these and previous studies is that IgG antibody to influenza virus HA alone can protect. Apparently, no immune response to any other influenza virus protein contributes to protection against infection. However, caution is needed in making this latter conclusion since a role for host responses to proteins other than HA in recovery from infection cannot be excluded. The results showed an antibody response could be detected against N2, NP and M1 as well as HA, whereas none could be detected against M2, PB1, PB2, PAC, NS1 or NS2. Although we stated in Results that this is most probably due to the limitations of the antigens used for the ELISA and not to lack of expression of the cloned genes nor an ability of ferrets to respond to these proteins the latter explanations cannot be discounted. If immunity to HA alone is involved in protection, then no satisfactory explanation exists as to how live vaccines, which induce lower HI antibody levels than killed vaccines, nevertheless offer better protection. One possibility is that inactivation of the virus destroys key epitopes on the haemagglutinin required for eliciting protective immune responses. This might result in the production of non-neutralizing or inappropriate antibodies as has been suggested to occur for the killed respiratory syncytial virus vaccine (Murphy et al., 1986). Alternatively, it is possible that live vaccines induce antibodies which, although less effective at inhibiting virus attachment to red blood cells and perhaps susceptible epithelial cells, are nevertheless better at neutralizing virus infectivity or taking part in other antibody-mediated immune mechanisms. Several lines of evidence support this suggestion. Firstly, IgG (and IgA), unlike IgM and secretory IgA, apparently neutralizes infectivity subsequent to attachment and internalization since IgG-neutralized virus can still attach to and enter cells (Taylor & Dimmock, 1985a, b; Dimmock, 1987). Secondly, a comparison of IgG:HI ratios in the present study for serum samples following immunization with vacc–H3 and live infection shows a ratio of 0.5:1 to 1:1 for one dose of vacc–H3, 2:1 to 16:1 for two doses, 8:1 to 16:1 for three doses and 40:1 to 320:1 for clone 7a infection. Since the three-dose regimen and live infection protects whereas one or two doses of vacc–H3 do not it seems that much anti-influenza IgG is produced during protective immunization which does not take part in the HI activity of the serum. Furthermore, IgG2a antibodies are preferentially elicited by infections with a wide variety of viruses (Coutelier et al., 1987) including influenza (Balkovic et al., 1987; Hocart et al., 1988) but the subclass distribution of antibodies may be influenced by the physical form in which the viral protein antigen is presented to the immune system (Balkovic et al., 1987). Clearly, differential induction of the IgG subclasses can affect the level of protection since the various subclasses exhibit differences in their ability to activate complement and to bind to Fc receptors on macrophages and neutrophils (Spiegelberg, 1974). However, the relative contributions of such activities compared to virus neutralization in a protective immune response is unknown as is the efficiency of neutralization of the virus by the individual subclasses and awaits further work.

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