Role of Milk-derived IgG in Passive Maternal Protection of Neonatal Ferrets against Influenza

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SUMMARY

Neonatal ferrets are protected against infection with influenza virus by colostral and milk-derived anti-influenza virus IgG after suckling on an immune mother. The levels of IgG elicited and then transmitted to neonates were similar when mothers were immunized by either live infection or killed vaccines. Maternal anti-influenza virus IgA and IgM appears not to cross the neonatal gut epithelium although both are present in maternal serum and milk.

Influenza of previously healthy adults is usually a mild self-limiting infection (Stuart-Harris & Schild, 1976; Sweet & Smith, 1980). However, in young children it has been implicated in bronchiolitis, croup, pneumonia, febrile convulsions and the sudden infant death syndrome (Stuart-Harris & Schild, 1976; Laraya-Cuasay et al., 1977; Paisley et al., 1978; Kim et al., 1979; Glezen, 1980; Murphy et al., 1981; Murphy & Webster, 1985; Zink et al., 1987). As a possible model for human infant infection, 1 day old (newborn) ferrets were inoculated intranasally with a virulent clone 7a (H3N2) of the reassortant influenza virus, A/Puerto Rico/8/34-A/England/939/69 (Collie et al., 1980). This infection proved more severe than in human babies being invariably fatal. There was severe involvement of the upper respiratory tract and some neonates died apparently from obstruction of the airways while others succumbed to uncomplicated influenza pneumonia (Collie et al., 1980).

Despite their high susceptibility to lethal infection with clone 7a, newborn ferrets can be protected by immune components acquired from mothers immunized 3 weeks previously with the same or serologically related strains (Husseini et al., 1984). Similarly, protection was provided by maternal vaccination with formalin-inactivated virus (Sweet et al., 1987). This protection was subtype-specific and therefore probably antibody-mediated. It was also at least partly milk-derived, since neonates born to non-immune mothers and fostered on immune mothers were protected (Husseini et al., 1984). However, the class of the mediating antibody was unknown. Furthermore, a transplacental component mediating protection could not be excluded since neonates born to immune mothers and fostered 24 h later onto non-immune mothers also exhibited some protection (Husseini et al., 1984). This paper provides answers to both of these questions, i.e. the class of antibody mediating protection and its route of transmission.

Clone 7a (H3N2; virulent) and its attenuated parent strain A/Puerto Rico/8/34 (H1N1) were prepared as described previously (Matsuyama et al., 1980; Sweet et al., 1974a, b) and assayed using the egg-bit technique (Sweet et al., 1974a). Titres are expressed in 50% egg-bit infectious doses (EBID$_{50}$). Formalin-inactivated preparations of the reassortant virus, A/Puerto Rico/8/34-A/Hong Kong/68 (H3N2) (strain X-31 of Kilbourne, 1969), containing 2.7 to 3.0 mg protein/ml, were supplied by Dr G. Appleyard of Wellcome Research Laboratories, Beckenham, U.K. X-31 is antigenically indistinguishable from clone 7a as determined by haemagglutination-inhibition and neutralizing antibody tests (G. Appleyard, personal communication).
Adult ferrets, obtained from A. S. Roe (Barnham, U.K.), were mated as described previously (Sweet et al., 1977) and immunized by (i) intranasal infection with $10^6$ EBID$_{50}$ clone 7a (H3N2), (ii) intranasal infection with $10^6$ EBID$_{50}$ A/Puerto Rico/8/34 (H1N1), or (iii) subcutaneous inoculation of three doses of 100 $\mu$g, 100 $\mu$g and 25 $\mu$g of formalin-inactivated X-31 (H3N2) given at weekly intervals following a 'priming' nasal infection with $10^6$ EBID$_{50}$ A/Puerto Rico/8/34 (Sweet et al., 1987). A further group included unimmunized controls. Protocols (i) and (ii), shown previously to be equally effective in protecting neonates (Husseini et al., 1984; Sweet et al., 1987), were chosen to establish whether similar mechanisms of protection applied to both killed and live vaccine regimens. Prior infection with a serologically heterotypic influenza A virus was adopted as this 'primes' or potentiates the serum antibody response to killed vaccines (McLaren & Potter, 1973; Sweet et al., 1974c).

To obtain sera, maternal ferrets were bled by cardiac puncture under ether anaesthesia whereas neonatal ferrets were bled similarly after death following intraperitoneal injection of 0.1 ml of Sagatal; all samples were obtained at 6 days post-partum. Near-term foetal and maternal sera were also collected following sacrifice of pregnant ferrets 2 days before the expected date of parturition (ferret gestation = 42 ± 2 days). The sera of all kits in each litter were pooled. Milk was collected 6 days post-partum from anaesthetized (0.44 ml Sagatal/kg body weight) maternal ferrets injected intramuscularly with 4 IU of synthetic human Pitocin (Sigma) prior to milking (Coe & Race, 1978; Schoknecht et al., 1985). All samples were stored at -20 °C prior to analysis.

Class-specific anti-influenza virus antibodies in sera and milk were assayed by an antibody capture ELISA using class-specific anti-ferret immunoglobulins prepared as follows. Ferret serum was precipitated at 50% saturation with ammonium sulphate. The precipitate was dissolved and dialysed against Dulbecco's phosphate-buffered saline A (PBS). It was then immunoelectrophoresed against rabbit anti-whole ferret antisera and the resultant precipitin lines were identified as IgA, IgG and IgM by comparison with the reactions of class-specific goat anti-human immunoglobulins (Sigma) or rabbit anti-ferret immunoglobulins (kindly supplied by Dr D. D. Porter, Department of Pathology, UCLA School of Medicine, Los Angeles, Ca., U.S.A.). Precipitin lines for each antibody class (IgA, IgG and IgM) were cut out, homogenized in PBS and pooled to provide stock immunoglobulin complexes. These were used to immunize rabbits (for anti-ferret IgG) or guinea-pigs (for anti-ferret IgA and IgM). Each animal received three injections containing the equivalent of two precipitin lines at each injection; the first and second injections were given 2 weeks apart subcutaneously with Freund's complete adjuvant, the third was given by a similar route but without adjuvant. Poor responses were obtained with ferret IgM and IgA complexes in rabbits but satisfactory sera were obtained in guinea-pigs. The animals were bled, sera prepared and immunoglobulins precipitated with 50% saturated ammonium sulphate. The precipitate was dissolved in and dialysed against PBS.

To remove cross-reactivity, it was then adsorbed with Sepharose-linked ferret immunoglobulins (IgA, IgG and/or IgM), prepared by the method of Johnstone & Thorpe (1982). Ferret immunoglobulins for cross-linkage to Sepharose were prepared by Sephacryl S-300 (Pharmacia) column filtration and DEAE-cellulose (Whatman) anion exchange chromatography. Anti-ferret IgG was then adsorbed with ferret IgG light chains, prepared from the purified ferret IgG by the method of Coe (1972), to render it heavy chain-specific; anti-ferret IgA was adsorbed with ferret IgG and IgM and anti-ferret IgM with ferret IgG and IgA. The antisera were then tested for specificity by immunoelectrophoresis against rabbit anti-whole ferret serum and shown to be monospecific.

The ELISA assay was performed as follows. One-hundred $\mu$l of the monospecific anti-ferret serum (IgA, IgG or IgM), diluted in PBS (see below), was added to each well in a flat-bottomed microtitre plate (Nunc Immuno Plate 1) and incubated at 4 °C for 18 h. Excess fluid was removed and the plate washed three times by filling the wells with PBS-Tween-bovine serum albumin (PBS, 0.01% v/v Tween 20, 0.01% w/v BSA) with 5 min incubation between washes. Two hundred $\mu$l of 2% (w/v) BSA in PBS was then added to each well and the plate incubated at 37 °C for 1 h. Wells were washed three times as above. Then doubling dilutions (100 $\mu$l) of the test samples (maternal serum, neonatal serum, foetal serum or milk) were added to each well and
Table 1. Anti-clone 7a (H3N2) IgG, IgA and IgM antibody titres of ferret adult sera and milk and neonatal sera 6 days post-partum following immunization of mothers by various protocols

<table>
<thead>
<tr>
<th>Immunizing protocol*</th>
<th>Sample</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 7a (H3N2)</td>
<td>Maternal serum (3)</td>
<td>51,200–102,400</td>
<td>80–160</td>
<td>20–40</td>
</tr>
<tr>
<td>intranasal live infection</td>
<td>Milk (3)</td>
<td>400–1600</td>
<td>20–40</td>
<td>10</td>
</tr>
<tr>
<td>X-31 (H3N2); killed vaccine (sub-cutaneous with adjuvant and priming)</td>
<td>Maternal serum (3)</td>
<td>12,800–51,200</td>
<td>80–160</td>
<td>10–80</td>
</tr>
<tr>
<td>PR/8 (H1N1) intranasal live infection</td>
<td>Maternal serum (1)</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Nil</td>
<td>Maternal serum (1)</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* For details of immunizing protocols, see Sweet et al. (1987).
† For details of method of analysis see text.

the plate re-incubated for 1 h at 37 °C. After washing three times as above, 100 μl of a suspension of purified clone 7a, prepared as described by Gould et al. (1972) and containing 2 μg viral protein in PBS–Tween–BSA, was added. The plate was re-incubated for 1 h at 37 °C and washed three times as before. Then, 100 μl of anti-haemagglutinin (X-31) mouse monoclonal antibody (haemagglutination inhibition titre, 6400; kindly supplied by Alan Douglas, World Influenza Centre, NIMR, London, U.K.) in PBS–Tween–BSA was added and the plate incubated at 37 °C for 1 h. After washing, 100 μl of peroxidase-labelled goat anti-mouse serum (Sigma) was added. Following incubation at 37 °C for 1 h, the plate was washed three times, 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·2H₂O, pH 5.0)] was added and the plate was incubated at 37 °C for 30 min before the reaction was stopped by addition of 50 μl 12% (v/v) H₂SO₄. The plate 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 1.0. When comparing results using different monospecific anti-ferret sera, each component in the reaction mixture, except the test sample, must be used in excess. Hence, dilutions of the components were tested against a known positive high titre serum and dilutions chosen at least twofold in excess of that giving the maximum response. These were 1:100 for rabbit anti-ferret IgG, 1:10 for guinea-pig anti-ferret IgA and IgM, 1:500 for anti-X-31 mouse monoclonal antibody and 1:1000 for goat anti-mouse peroxidase-labelled conjugate.

Antibodies of all three classes were found in the sera of maternal ferrets immunized either by live infection with clone 7a (H3N2) or with three doses of adjuvanted inactivated X-31 vaccine (H3N2) [following ‘priming’ with A/Puerto Rico/8/34 (H1N1)] (Table 1). Antibody was primarily IgG, its titres being 300 to 1000 times greater than for IgA antibodies and 1000 to 5000 times greater than for IgM antibodies. All three antibody classes were also present in milk, although at much lower levels than in sera; and again the predominant antibody was IgG (Table 1). No significant differences (P>0.05) were observed in IgG, IgA or IgM titres for samples from animals immunized by live infection or formalin-inactivated virus. Anti-influenza virus activity was not detected in samples from unimmunized animals or those immunized with a serologically unrelated strain of influenza virus (A/Puerto Rico/8/34; H1N1) (Table 1).

The sera of neonates born to mothers immunized by live infection or killed vaccine showed similar amounts of anti-influenza virus IgG but anti-influenza virus IgA and IgM could not be detected (Table 1). Neonatal IgG titres were 5 to 10% of maternal serum IgG titres and 200 to 300% of milk titres. IgG antibodies were thus selectively transported by the neonatal gut and
Table 2. Anti-clone 7a (H3N2) IgG, IgA and IgM antibody titres in pre-term maternal and foetal serum in mothers immunized by infection with 10^6 EBID_{50} clone 7a (H3N2)

<table>
<thead>
<tr>
<th>Sample (no. of animals)</th>
<th>Range of antibody titres*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>Maternal serum (3)</td>
<td>20480-40960</td>
</tr>
<tr>
<td>Pre-term foetal serum (3)t</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* For details of method of analysis see text.
† Serum of two to eight kits in each litter pooled.

concentrated in the serum. Antibody was not detected in the sera of neonates born to control animals (Table 1).

Foetal sera from kits of near-term mothers, immunized by live clone 7a infection, showed no detectable anti-clone 7a IgG, IgA or IgM activity (Table 2).

In summary, all detectable maternal anti-influenza virus immunoglobulin transfer was post-partum confirming the view of Husseini et al. (1984) that immunity was milk-derived. These findings agree well with those of others for respiratory syncytial virus-infected ferrets (Suffin et al., 1979) and mink (Porter, 1965; Coe & Race, 1978).

Maternal serum and milk contains anti-influenza virus IgG, IgA and IgM with IgG predominating. Neonatal serum contains only anti-influenza virus IgG at concentrations higher than those in milk. The absence of anti-influenza virus IgA and IgM in neonatal serum may be due to their relatively low concentrations in maternal serum and milk, but is more likely to be due to preferential uptake of IgG within the neonatal gut. The gastrointestinal tract of infant ferrets selectively takes up IgG over a fivefold concentration gradient whereas IgA and IgM, although present, were not shown to be concentrated (Suffin et al., 1979). Similarly, in the neonatal mouse gut, receptors exist for IgG transport (Guyer et al., 1976) and transport in the neonatal rat is specific for IgG (MacKenzie, 1984).

Selective transport of IgG across the intestine largely explains a previous observation in ferrets. Immunization of mothers by live infection or killed vaccine did not always fully protect the nasal turbinates of newborn ferrets, whereas the lung was invariably protected (Husseini et al., 1984; Sweet et al., 1987). This differential protection is probably due to different components of the immune response being effective at different sites, e.g. IgA in nasal epithelium and IgG in the lung. It is generally accepted that locally produced antibody, predominantly IgA but including IgG, is important in protection of the upper respiratory tract of humans (Ada & Jones, 1986) and prevention of nasal infection is a function of local immunity in the ferret (Barber & Small, 1978). In contrast, the most likely mediator of resistance in the lung during human influenza is IgG, derived entirely or partly from serum (Couch et al., 1984). This is consistent with the greater proportion of IgG relative to IgA found in lower respiratory tract secretions compared to those in the nasopharynx (Reynolds et al., 1978). Similarly, serum antibody, probably predominantly IgG (see Table 1), prevented viral pneumonia (Loosli et al., 1953) but not tracheitis (Ramphal et al., 1979) or rhinitis (Kris et al., 1985) in adult mice. IgG in passively administered immune maternal serum abolished respiratory syncytial virus replication in the lungs of neonatal cotton rats but not in nasal epithelium (Wong & Ogra, 1986). Thus, the differential protection probably results from the selective ability of IgG to protect the lung rather than the nasal turbinates.

Do these observations have relevance to human influenza? Obviously caution is needed in any extrapolation from animal studies to humans since in ferrets, as in many other animal species, IgG is transmitted from mother to offspring almost entirely via the milk, while in humans transfer of immunity is mainly transplacental (Hayward, 1983) and adsorption of colostral antibody and other immune factors in the gut of humans may be less efficient (Soothill, 1983). Thus, the passive transfer of maternal antibody to influenza virus occurs transplacentally in humans (Masurel et al., 1978; Sumaya & Gibbs, 1979) and such acquired anti-influenza IgG has been associated with less severe influenza disease in the human infant (Puck et al., 1980).
Nevertheless, whilst the route of transmission may be different in ferrets and humans the mechanism (IgG) appears to be similar and thus the effectiveness of the various vaccine protocols and the differential protection of the upper and lower respiratory tracts may well apply to human as well as ferret infants.

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REFERENCES


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