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Role of maternal immunity in the protection of newborn ferrets against infection with a virulent influenza virus

R. H. HUSSEINI, C. SWEET, H. OVERTON & H. SMITH *Department of Microbiology, University of Birmingham, Birmingham*

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Summary. Intranasal infection of newborn ferrets with a virulent strain of influenza virus invariably resulted in their deaths following virus replication to high titre in both lung and nasal turbinates (Collie *et al.*, 1980). However, a similar challenge of newborn ferrets born to mothers immunized by infection with virulent or attenuated viruses resulted in complete protection; no virus replicated in their lungs and little or no virus was isolated from their nasal turbinates. Protection appeared to be antibody-mediated since it was subtype-specific and milk-derived since newborn ferrets born to non-immune mothers but fostered onto immune mothers exhibited a similar level of protection to neonates born to and suckled by immune mothers.

INTRODUCTION

Influenza in humans is usually a relatively mild, self-limiting disease (Sweet & Smith, 1980). Nevertheless, it can cause significant problems in both the old and the very young. In infants it has been implicated in bronchiolitis, croup, pneumonia, febrile convulsions and the sudden infant death syndrome (Laraya-Cuasay *et al.*, 1971; Paisley *et al.*, 1978; Kim *et al.*, 1979; Glezen, 1980; Murphy *et al.*, 1981). Similarly, infection of adult ferrets with influenza virus produces a mild transient infection while infection of newborn (1 day old) ferrets leads to a severe respiratory illness which is invariably fatal (Collie *et al.*, 1980; Husseini *et al.*, 1983).

An approach to protecting human babies against such a potentially lethal respiratory infection is the transfer of maternal immune components either via the placenta or in the colostrum or milk. The passive transfer of maternal antibody to influenza virus occurs in humans (Masurel *et al.*, 1978; Sumaya & Gibbs, 1979), in pigs (Renshaw, 1975) and in mice (Reuman *et al.*, 1983), and transplacentally acquired anti-influenza IgG may be associated with less severe influenzal disease in the human infant (Puck *et al.*, 1980). In addition, while transfer of IgG is mainly transplacental in humans (Hayward, 1983) breast feeding protected against respiratory syncytial virus (RSV) infection (Downham *et al.*, 1976). Similarly, breast feeding protected against RSV infection in ferrets (Suffin *et al.*, 1979) and against influenza in mice (Reuman *et al.*, 1983).

In the present study, the role of maternal immunity in the protection of newborn ferrets against fatal influenza infection has been investigated as a possible model of what may happen in humans.

MATERIALS AND METHODS

Influenza viruses and their assay

Clones 7a (virulent) and 64d (attenuated) of the recombinant influenza virus A/PR/8/34-A/Eng-

Correspondence: Dr C. Sweet, Department of Microbiology, University of Birmingham, Birmingham B15 2TT.

land/939/69 (H3N2) and the attenuated parent strain A/PR/8/34 (H1N1) were prepared as described previously (Matsuyama *et al.*,1980; Sweet, Stephen and Smith, 1974a,b) and assayed using the egg-bit technique (Sweet *et al.*, 1974a).

Ferrets and their inoculation

Adult ferrets were obtained from A.S. Roe, Little Fakenham, Norfolk. They were mated as described by Sweet, Toms & Smith (1977). Non-Immune 3-week pregnant (i.e. mid-gestation; see Sweet *et al.*, 1977) ferrets were inoculated intranasally (under ether anaesthesia) with 0·5 ml (0·25 ml per nostril) of phosphate-buffered saline (Dulbecco A; PBS) containing 6·0 $\log_{10}$ 50% egg-bit infectious doses ($EBID_{50}$) of the virulent or attenuated recombinant clones or the A/PR/8/34 parent virus. Respiratory infection of pregnant ferrets at this stage of gestation has no detrimental effects on fetal development (Sweet *et al.*, 1977). Ferrets which failed to give birth 3 weeks after inoculation, because they were pseudopregnant, or whose litter died soon after birth because of poor maternal care, were mated again to give birth 14 weeks after their initial inoculation with virus.

Challenge of newborn ferrets and collection of respiratory tissues

One day old (newborn) ferrets were inoculated intranasally (without anaesthesia) with 0·05 ml of PBS containing *ca* 1·0 $\log_{10}$ $EBID_{50}$ clone 7a (Collie *et al.*, 1980). At 4, 6 or 8 days p.i. the newborn ferrets were killed by intraperitoneal (i.p.) injection of 0·1 ml Sagatal (May and Baker Ltd, Dagenham). The lungs and nasal turbinates were removed and homogenized in 3 ml of Hank's balanced salt solution (Gibco, Glasgow), supplemented with 0·01 g/ml bovine serum albumin, 100 U/ml Penicillin G (Sigma, London) and 100 μg/ml streptomycin (Sigma, London), using a Sorvall Omnimixer as described previously (Sweet *et al.*, 1977).

Treatment of newborn ferrets with antibiotics

To prevent possible bacterial superinfection which may occur during the course of the experiments (Husseini *et al.*, 1983), newborn ferrets were injected i.p. with a solution of penicillin G (100 mg/kg) and gentamicin (Kirkby-Warrick pharmaceuticals Ltd, Mildenhall, Suffolk) (15 mg/kg) twice daily. The treatment continued until the animals died or were killed.

Fostering of newborn ferrets

One day after birth, about half the newborn ferrets born to a non-immune ferret were transferred to a mother immune to clone 7a and *vice versa*. This meant that the newborn ferrets had suckled from their natural immune or non-immune mother prior to fostering. The procedure was repeated for two other pairs of immune and non-immune mothers. To facilitate fostering, newborn ferrets were thoroughly rubbed against the foster mother to acquire her scent. Within 3 hr of fostering the newborn ferrets were challenged with 1·0 $\log_{10}$ $EBID_{50}$ clone 7a and at 4 and 6 days p.i. they were killed and the respiratory tissues removed and assayed for virus as described above.

Haemagglutination-inhibition (HI) tests

Sera, collected from female ferrets by cardiac puncture before infection and/or challenge of newborn ferrets with clone 7a, were tested for anti-influenza antibodies using the HI test as described previously (Basarab & Smith, 1969).

RESULTS

Virus titres in nasal turbinates and lungs of newborn ferrets born to immune and non-immune mothers

Ferrets born to unimmunized mothers and challenged with clone 7a were completely susceptible and, as described previously (Coates *et al.*, 1984; Husseini *et al.*, 1983), produced high titres of virus in both the upper and lower respiratory tracts, titres in the lung exceeding those in the nasal turbinates by approximately 10-fold on each day examined (Table 1). In addition, the newborn ferrets were small and looked ill, seven of 27 animals dying during the course of the experiments. In contrast, ferrets born to mothers immunized with the virulent clone 7a (H3N2) were completely protected, looking healthy and strong and none died up to 8 days post-infection (Table 1). No virus was recovered from their lungs and in only the occasional animal was virus, of low titre ($\leqslant$3·3 $\log_{10}$ $EBID_{50}$ on day 4; $\leqslant$2·5 $\log_{10}$ $EBID_{50}$ on day 6), isolated from the nasal turbinates. Immunization with the attenuated clone 64d (H3N2) induced a similar level of protection (Table 1). However, ferrets born to mothers immunized with A/PR/8/34 (HlNl) were as susceptible to infection with clone 7a as newborn ferrets of non-immune mothers (Table 1).

Table 1. Mean total virus titres in nasal turbinates and lungs at different days post intranasal inoculation (dpi) with 1·0 $\log_{10}$ $EBID_{50}$ clone 7a of newborn ferrets born to and suckling from immune or non-immune mothers

Virus used to infect (immunize) mother	Immune status of mothers HI titres in serum to:		Total number of newborn ferrets (No. of litters)	Mean total virus titre ($\log_{10}$ $EBID_{50}$) in:					
				Nasal turbinates (SEM) (d.p.i.)			Lungs (SEM) (d.p.i.)		
	Clone 7a (H3N2)	PR/8 (H1N1)		4	6	8	4	6	8
—	< 10	< 10	27 (5)	4·7 (0·2)	4·4 (0·2)	3·9 (0·2)	5·7 (0·3)	5·6 (0·1)	5·0 (0·5)
7a	320–1280	< 10	21 (3)	⩽1·8*	⩽1·7*	< 1·6	< 1·6	< 1·6	< 1·6
64d	1280–5120	< 10	16 (4)	⩽2·1†	< 1·6	< 1·6	< 1·6	< 1·6	< 1·6
PR/8	< 10	320–2560	21 (5)	5·4 (0·1)	5·0 (0·3)	3·7 (0·4)‡	5·9 (0·2)	5·7 (0·3)	5·3 (0·3)‡

* One of eight newborn ferrets had detectable virus (⩽3·3 $\log_{10}$ $EBID_{50}$ at 4 d.p.i.; ⩽2·5 $\log_{10}$ $EBID_{50}$ at 6 d.p.i.).

† One of five newborn ferrets had detectable virus (4·2 $\log_{10}$ $EBID_{50}$).

‡ Two of six newborn ferrets, both from the same litter, had no detectable virus in their lungs and low titres only (⩽2·2 and ⩽2·6 $\log_{10}$ $EBID_{50}$) in their nasal tubinates.

Protection of newborn ferrets born to mothers immunized 14 weeks previously

Serum HI antibody titres to clone 7a of mothers had dropped 8–64-fold from 2560 to 5120 at 3 weeks to 80 to 640 at 14 weeks. Nevertheless, of 19 newborn ferrets born to three immune mothers, all were protected from lung infection with clone 7a since no virus was isolated from their lungs from 4 to 8 days p.i. However, virus was consistently isolated from their nasal turbinates, although of lower titre than from newborn ferrets of non-immune mothers; thus, the mean total virus titres were 3·9 (SEM: 0·5), 2·9 (0·3) and $\leqslant 1{\cdot}9$ (0·2) $\log_{10}$ $EBID_{50}$ at days 4, 6 and 8 respectively.

Virus titres in respiratory tissues of fostered newborn ferrets

In a separate series of experiments the effect of fostering newborn ferrets onto immune or non-immune mothers prior to virus challenge was examined. In agreement with the above experiments (Table 1), 11 ferrets born to three non-immune mothers were completely susceptible while those (five) born to three immune mothers were completely protected from lung infection and, in general, showed no or reduced replication in the upper respiratory tract (Table 2). Similarly, seven ferrets born to and suckled by the three non-immune mothers for 1 day prior to fostering to the immune mothers exhibited no virus replication in their lungs and only occasional infection in the upper respiratory tract (Table 2). Seven ferrets born to and suckled by the three immune mothers prior to fostering to the non-immune mothers also exhibited complete protection of their lungs but, in contrast, virus replicated in the nasal turbinates of the majority of the animals reaching titres similar to those found in the natural newborn ferrets of non-immune animals.

DISCUSSION

That maternal immunity can play a role in protecting the newborn ferret against challenge with a virulent influenza virus has been clearly demonstrated. Ferrets born to non-immune mothers suffered a severe and generally fatal infection when inoculated with clone 7a (Collie *et al.*, 1980; Coates *et al.*, 1984; Husseini *et al.*, 1983). However, prior infection of mothers 3 weeks' *prepartum* resulted in the birth of newborn ferrets which were completely protected from infection, no virus being shed from the lower respiratory tract and little or no virus from the upper respiratory tract. Mothers infected with an attenuated virus clone 64d of the same serotype as clone 7a (H3N2) also conferred complete protection on their offspring. The immunity appears to be subtype-specific since another type-A influenza virus, A/PR/8/34, of different subtype (HlNl) failed to confer any protection.

These results suggest that the immunity is likely to be mediated by antibody rather than cytotoxic T cells since the latter are generally able to protect against all

Table 2. Effects of immunization of the natural or foster mother on the outcome of infection in newborn animals following intranasal challenge with 1·0 $\log_{10}$ $EBID_{50}$ clone 7a

Immune status (HI titre) of:		No. of animals infected / No. of animals examined in:			
		Nasal turbinates (d.p.i.)		Lungs (d.p.i.)	
Natural mother	Foster mother	4	6	4	6
Non-immune (<10)	—	6/6 (5·6±0·2)*	5/5 (5·0±0·2)	6/6 (6·1±0·2)	5/5 (5·7±0·3)
Immune (320–1280)	—	2/3 (3·7±1·0)	0/2 (<1·6)	0/3 (<1·6)	0/2 (<1·6)
Non-immune (<10)	Immune (320–1280)	1/4 (4·3)	1/3 (4·0)	0/4 (<1·6)	0/3 (<1·6)
Immune (320–1280)	Non-immune (<10)	3/4 (5·5±0·2)	2/3 (4·8±0·5)	0/4 (<1·6)	0/3 (<1·6)

* Mean total virus titres in $\log_{10}$ $EBID_{50}$±SEM for infected animals.

type-A viruses (McMichael *et al.*, 1982). In general, of 12 mothers examined, there was a good correlation between maternal serum HI titre and the level of protection in their offspring. All mothers, regardless of their serum HI titre (80-5120), produced litters which were completly resistant to lung infection. Four mothers with serum HI titres of 2560 or greater conferred almost complete protection on the nasal turbinates of their offspring, only one of 16 (6%) neonates exhibiting virus replication in the upper respiratory tract. With lower maternal serum HI titres (320-1280) more neonates became infected (seven of 32; 22%) in the upper respiratory tract and with HI titres of 80, 11 of 13 neonates (85%) became infected.

Suffin *et al.* (1979) have demonstrated that infant ferrets are born with extremely low immunoglobulin levels in serum and the vast majority of maternal antibody is transferred *postpartum*, transmucosal uptake of IgG by the infant ferret occurring over the first 30 days of life. Our results with influenza virus accord with these observations. The immunity is probably derived from the mother during suckling since 1 day old ferrets born to non-immune mothers and fostered on immune mothers showed a similar level of protection to ferrets born to and suckled by immune mothers; virus replicated at reduced levels in the nasal turbinates of the occasional animal but not at all in the lungs.

The differential protection of the upper and lower respiratory tract was interesting. Newborn ferrets were often not fully protected from virus replication in the nasal turbinates whereas the lung was invariably protected by maternal transfer of immunity. Similar observations have been made following influenza virus infection of infant (6 day old) mice (Reuman *et al.*, 1983). However, these observations contrast with experiments in adult ferrets where passively administered antibody failed to prevent influenza virus shedding from the upper respiratory tract (Barber & Small, 1978) and in neonatal ferrets (3 days old) infected with RSV no differential protection was observed (Suffin *et al.*, 1979). Mothers with high levels of immunity protected both the upper and lower respiratory tracts of their offspring against RSV challenge whereas mothers with low levels of immunity protected neither tract. The reasons for these differences are not known. The lack of protection of the infant ferret lung against RSV infection may relate to differences in protective mechanisms; protection against RSV is believed to be non-antibody-mediated (Suffin *et al.*, 1979) whereas protection against influenza appears to correlate with serum antibody levels (Reuman *et al.*, 1983). The differential protection of the upper and lower respiratory tract of neonatal animals against influenza virus may result from selective transport of antibody to the lung. It does appear to relate both to the level of maternal antibody (see above) and to the duration of suckling. Ferrets born to immune mothers with high HI titres but fostered to non-immune mothers prior to infection showed complete protection of their lungs but virus replication in their upper respiratory tracts was similar to that of newborn ferrets born to non-immune mothers. Thus, passive transfer of antibody for 1 day prior to challenge was not sufficient to protect their nasal turbinates. However, colostral transfer of antibody to newborn ferrets born to non-immune mothers but fostered on immune mothers did protect their upper respiratory tracts because there is little replication until the 3rd or 4th day following inoculation (Collie *et al.*, 1980; Coates *et al.*, 1984).

Many have argued for the beneficial effect of breast feeding as opposed to artificial (bottle) feeding for the protection of the newborn infant (Grulee, Sanford & Herron, 1934; Robinson, 1951; Paine & Coble, 1982; Downham *et al.*, 1976). Our results using ferrets and others from experiments with mice (Reuman *et al.*, 1983) support the suggestion that vaccination of prospective human mothers against influenza prior to or even during pregnancy (since there appears to be no detrimental effects on the developing fetus: Mackenzie & Houghton, 1974), followed by breast feeding, especially in the first few months of life may be beneficial to their offspring. However, caution is needed in extrapolating from animal studies to humans since significant transplacental passage of IgG occurs in humans and adsorption of colostral antibody and other immune factors in the gut of humans may be much less efficient (Soothill, 1983).

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