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# Stimulation of rotavirus IgA, IgG and neutralising antibodies in baboon milk by parenteral vaccination

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*A rhesus rotavirus vaccine adjuvanted with ISCOMs was injected intramuscularly to 5 pregnant baboons, with repeated doses 1–2 and 14 weeks after delivery. Maternal blood and milk samples and blood samples from their babies were collected at 2-weekly intervals until 26 weeks after parturition. Samples were assayed for rotavirus antibodies by ELISAs and neutralisation tests. Vaccination produced statistically significant increases in maternal serum IgG and neutralising antibodies, and in milk IgA, IgG, and neutralising antibodies. Control baboon mothers sampled from 12 weeks after delivery had lower serum and milk antibody titres, but responded to vaccination at 16 weeks by producing a similar antibody profile in serum and milk to those previously vaccinated. Because of the endemic nature of human rotaviral infections, similar maternal vaccinations have potential as a means of increasing milk antibodies to a level at which they may be protective to infants.*

**Keywords:** Rotavirus; passive immunisation; maternal vaccination

Rotavirus is the most important cause of severe diarrhoea in children world-wide<sup>1</sup>, and is of similar significance as a cause of neonatal diarrhoea in many domesticated animal species<sup>2</sup>. Development of a rotaviral vaccine has been accorded high priority by WHO, with the emphasis on live vaccines for oral delivery to the young child. This has stimulated considerable research, using particularly rotaviruses of animal origin or genetic reassortant viruses of mixed animal–human strains. The principal problems encountered have been: the serotypic specificity of the immune response, necessitating the inclusion of multiple serotypes; and the difficulty in achieving the appropriate balance between attenuation and ability to replicate effectively in the intestine<sup>3</sup>. To date, safe and effective vaccines for children are not available.

The situation for animal rotaviral vaccines is rather more complex. The first modified live calf rotavirus vaccines had been developed by 1973<sup>4</sup>, but contemporary comparative trials consistently failed to demonstrate protection<sup>5,6</sup>. As a result, the more popular approach in cattle has been through maternal parenteral vaccination, stimulating the secretion of specific antibodies in colostrum and milk<sup>7</sup> to take

advantage of the fact that rotavirus antibody present in the lumen of the intestine is an effective mediator of protection<sup>8,9</sup>. Not all vaccines based on this concept have proven efficacious, perhaps due to insufficient antigen or poor adjuvantation; but several effective and commercially successful vaccines for cattle do exist, and have been proven in the field over a number of years<sup>10–13</sup>. The development of rotaviral vaccines for pigs is some years behind that for cattle, and at this stage mixed results have been obtained<sup>14,15</sup>. A potentially significant advantage of maternal vaccination is the heterotypic nature of the immune response of adults with wide previous exposure to endemic rotaviral infections. Children and young animals encountering their first rotavirus infection mount an immune response that is largely serotype-specific in terms of *in vitro* assays and *in vivo* protection<sup>16–18</sup>. By contrast, adults with endemic exposure are stimulated by infection or vaccination to produce immune responses to a wide range of serotypes<sup>16,17,19</sup>. This heterotypic response is of sufficient magnitude to obviate the need for multi-serotype vaccines in maternal vaccination schedules, and successful cattle vaccines containing typically one strain of virus confer protection against the multiple strains present in the field. Additional areas where parenteral maternal vaccination has potential advantages are in the provision of protection to the neonate, and in safety through the use of inactivated vaccine.

It was against this background that we decided to undertake an experiment to attempt to manipulate the antibody response to rotavirus in the milk of a non-human primate, to ascertain if some of these potential

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advantages of passive immunisation through the mother could be realised in a species phylogenetically related to man.

## MATERIALS AND METHODS

### Animals

A group of pregnant multiparous Olive Baboons (*Papio anubis*, postdelivery weight range 13–19 kg) maintained at the Institute of Primate Research (IPR) facility was used. The 5 principals receiving rotavirus vaccine were housed in a group cage with the 3 controls, and after delivery their babies remained with them until weaned at the termination of the experiment (6 months). All animals in this colony had serum antibodies to rhesus rotavirus by virus neutralisation assay, indicating previous natural intestinal exposure. Animals were sedated by intramuscular injection of 1.5 ml mixture (7:3) of 100 mg/ml ketamine hydrochloride (Parke Davis) and 2% xylazine (Bayer) prior to handling. This study was reviewed and approved by the Institute (IPR) Scientific Resources Evaluation and Review Committee.

### Vaccine

Simian rotavirus RRV (strain MMU18006, lot RRV-2, cultivated in rhesus monkey diploid cells DBS-FRHL2) was produced by DynCorp for trials in man and kindly provided by Dr Lou Potash. This virus was passaged once in MA104 cells, and the harvest extracted with 1,1,2-trichlorotrifluoroethane (GPR, BDH). The infectivity titre of the extracted culture was  $10^{6.55}$  fluorescent focus units (ffu)/ml, with a protein concentration of 5.7 mg/ml. Mock-infected MA104 cultures were prepared similarly, and had no detectable infectivity with a protein content of 5.6 mg/ml. Both vaccine and placebo were adjuvanted by adding 400 µg/ml of ISCOM matrix<sup>20</sup>, manufactured by Iscotec and kindly provided by Dr B. Sundquist. These vaccines were stored for up to 3 months at 4°C, and subsequently at -70°C for up to a further 3 months. They were administered by deep intramuscular injection, without any evidence of local reaction or discomfort.

### Vaccination and sampling schedules

The principals were vaccinated initially at 3–7 weeks before parturition, and received second and third vaccine doses 1–2 and 14 weeks after delivery, respectively. Blood for serum was collected at initial vaccination, and then after delivery at 2-week intervals until weaning at approximately 7 months. On each occasion after delivery, milk was also collected and the babies bled for serum. Sampling of the controls was not initiated until 12 weeks after delivery, after which point samples were collected from mothers and babies as for the vaccinees. The controls received an initial vaccine dose at 16 weeks after delivery.

### ASSAYS

Isotype-specific ELISAs utilised microtitre plates (Nunc Maxisorb) coated with a rabbit anti-rotavirus capture serum, RRV-2 and negative MA104 as antigens, the test and control samples, horseradish peroxidase-conjugated rabbit anti-monkey IgG (Sigma) or rabbit anti-human

IgA (Dako), with the colour developed by adding substrate  $H_2O_2$  and *o*-phenylenediamine dihydrochloride to the bound peroxidase. The reaction was stopped with 2 M  $H_2SO_4$ , and the absorbance measured at 490 nm. Net absorbance was calculated by subtracting the values in the negative antigen wells from the corresponding RRV-2 wells. A pool consisting of postvaccination serum from 3 baboons was assayed at eight 2-fold dilutions on each plate for both the IgG and IgA ELISAs, and the net absorbance as a function of arbitrary antibody units was fitted to a lin-log sigmoid curve. The pool was used at initial dilutions of 1/2000 and 1/50 for the IgG and IgA ELISAs, respectively. In each case this produced curve fits of  $r > 0.95$  and at the final dilution there was no difference between the absorbances with the positive and negative antigens. The resulting parameters were then used to extrapolate antibody units for each of the test samples, taking into consideration the relative dilution factor at which each sample was tested.

Virus neutralising assays to rotaviruses RRV-2 and Wa (as a typical human virus of serotype G1P8) were performed in microtitre plates and the endpoints were determined by 60% reduction in ffu.

### Statistical analysis

Comparisons between groups were made by two-sample *t*-tests using Minitab software.

## RESULTS

### Maternal serum antibody response

The group of 5 pregnant baboons responded to the initial vaccination with mean increases in serum antibody titre by 2 weeks after delivery of 25-, 12- and 16-fold when assayed by the IgG ELISA, the homotypic RRV-2 VNT and the heterotypic Wa VNT, respectively (Table 1). These increases were significant ( $p < 0.05$ ). There was no significant serum IgA antibody response ( $< 3$ -fold,  $p > 0.1$ ). These elevated IgG and VN titres showed small further increases to the repeated immunisations at 2 and 14 weeks post-delivery, but only the response to RRV VNT at the 14-week boost reached significance ( $p < 0.05$ ). Otherwise titres were maintained at approximately constant levels throughout lactation. The increases observed after 22 weeks in IgA and Wa titres were not significant ( $p > 0.05$ ). The serum titres of the control group vaccinated for the first time at 16 weeks after parturition showed significant ( $p < 0.05$ ) increases in IgG and both homotypic and heterotypic VN but not IgA titres.

### Maternal milk antibody response

Although trends are apparent, it is not possible to be certain about the effects of vaccination on milk antibody profiles in early lactation as due to unforeseen problems the control animals were not sampled until 12 weeks after delivery. However, from 12 weeks onwards direct comparisons are possible.

Mean IgG ELISA titres in the vaccinated group varied only within narrow limits throughout lactation (Table 2), and these titres were very low at about 1/400th of the serum titres. However, during the period from 12 to 14 weeks the vaccinated animals had a mean

**Table 1** Mean antibody titres in sera of baboon mothers

Assay	Group	Titres at time (weeks) after parturition													
		-3-7	2	4	6	8	10	12	14	16	18	20	22	24	26
IgG ELISA	Vaccinees	↓ 390	↓ 9750	15066	3090	8870	6053	4571	↓ 4246	↓ 6408	6674	6657	5297	5636	5000
	Controls							352	339	230	8093	5496	3967	2693	11272
IgA ELISA	Vaccinees	536	1888	1510	845	1245	789	701	726	357	346	536	617	1778	1641
	Controls							817	303	330	718	637	494	597	558
VNT RRV	Vaccinees	70	845	1810	1280	905	845	845	640	1689	1469	1280	845	1114	1279
	Controls							25	32	101	1614	2032	1279	403	1016
VNT Wa	Vaccinees	15	243	453	113	538	139	106	80	279	160	92	121	211	279
	Controls							6	5	5	160	40	25	13	101

Solid arrows—time of vaccination of the principals  
 Open arrows—time of vaccination of the controls

IgG titre of 18, while all observations from the controls were <10, indicating a significant boost from vaccination ( $p < 0.01$ ). The controls responded rapidly to vaccination at 16 weeks with a significant ( $p < 0.01$ ) increase in IgG titre to approximately the same level as the vaccinated group. The response of the already-vaccinated group from a titre of 16 to 38 was also significant ( $p < 0.05$ ).

The IgA titre in the colostrum samples taken in the first 2 weeks of lactation was high, at a ratio of 1.4 to the concurrent serum titres. The value halved over the following 2 weeks, and was then maintained at this level. By 12–14 weeks into lactation, these titres were 17-fold higher than those of the controls ( $p < 0.01$ ). Subsequent vaccination of the controls at 16 weeks significantly ( $p < 0.05$ ) elevated their milk IgA titres to levels similar to those of the vaccinees. The already-vaccinated animals had no milk IgA response to the repeated immunisation at 14 weeks.

The mean VN titres to RRV in the milk of the immunised animals showed a similar pattern, with initial colostrum titres falling by approximately half and then remaining constant for the rest of the lactation. By 3 months into lactation these titres were not significantly higher than those in the controls. The increase in the control titres to RRV was not significant.

The mean VN titres to Wa remained low (<10) throughout.

**Serum antibody in the infants**

Maternally derived antibodies were detected in the sera of the babies from the immunised mothers by IgG ELISA, and VNTs (Table 3). No transmission of IgA had taken place. The VN titres waned to undetectable levels by 20 weeks after birth. The lack of any response in the babies probably indicates that rotavirus did not circulate in the colony during the period of observation.

**DISCUSSION**

This experimental vaccination was successful in raising circulating IgG antibodies with virus-neutralising activity in all vaccinated animals. However, of greater significance was the observation that this simple parenteral immunisation also stimulated increases in IgA production by the mammary glands. Due to a fault in experimental procedures milk was not collected from the control group until 12 weeks into lactation, so an accurate definition of the extent to which IgA antibodies in early lactation were raised cannot be made. However, by the period of 12–16 weeks when comparable samples were obtained, IgA, IgG, and RRV VN antibodies were enhanced in the immunised group. The potential for successful immunisation even during lactation was shown after vaccination of the control group at 16 weeks, which produced increases particularly in IgA titres.

**Table 2** Mean antibody titres in baboon milk

Assay	Group	Titre at time (weeks) after parturition													
		2	4	6	8	10	12	14	16	18	20	22	24	26	
IgG ELISA	Vaccinees	↓ 23	26	20	24	19	19	↓ 16	↓ 38	48	39	28	35	34	
	Controls						<10	<10	<10	36	30	24	18	73	
IgA ELISA	Vaccinees	1690	579	698	546	535	413	480	394	598	463	592	403	537	
	Controls						33	19	29	423	269	343	345	1088	
VNT RRV	Vaccinees	57	28	34	17	15	15	10	23	26	35	35	106	40	
	Controls						4	10	10	13	20	20	16	20	
VNT Wa	Vaccinees	<5	<4	8	8	9	8	8	7	9	10	10	3	8	
	Controls						3	4	3	4	3	3	5	6	

Solid arrows—time of vaccination of the principals  
 Open arrows—time of vaccination of the controls

**Table 3** Mean antibody titres in sera from infant baboons

Assay	Group	Titres at age (weeks)									
		2	4	6	8	10	12	14	16	18	20
IgG ELISA	Vaccinate	4335	4467	1738	1169	671	414	296	166	155	156
	Control						552	<100	<100	<100	<100
VNT RRV	Vaccinate	557	453	226	135	53	30	30	13	13	<10
	Control						<10	<10	<10	<10	<10
VNT Wa	Vaccinate	92	67	40	24	17	23	10	<10	<10	<10
	Control						<10	<10	<10	<10	<10

IgA ELISA titres were in all samples <10

Animal studies have been invaluable in demonstrating an intimate relationship between antibody production in the mammary gland and prior intestinal antigen exposure, thus making the approach of stimulating milk antibody production specific to endemic enteric infections particularly appropriate. Many of the key features of this relationship were elucidated in studies on transmissible gastroenteritis (TGE) of swine, a severe coronaviral enteritis<sup>21</sup>. Secretory IgA is the dominant immunoglobulin isotype in postcolostral sows' milk, and more than 90% of this SIgA is locally produced by mammary gland plasma cells whose precursors originated in the intestinal tract and migrated through the so-called gut-mammary axis. Enhancement of effective antibody secretion in milk is dependent on prior stimulation of local intestinal immunity, and in herds with endemic TGE parenteral vaccination of sows leads to boosting of milk SIgA and improved protection of piglets fed with this milk<sup>22</sup>.

As rotaviruses are endemic in swine, it could be expected that parenteral rotaviral vaccination of sows would similarly lead to boosting of milk SIgA titres, and this has been confirmed<sup>2, 21</sup>. This passive immunisation of the piglets fed with this milk delayed the onset and shortened the duration of both rotavirus shedding and diarrhoea.

Comparisons with ruminant species are less instructive, as in these animals the major immunoglobulin in colostrum is IgG1 which is selectively transported from serum, and postcolostral milk has only very low levels of immunoglobulin again predominantly of IgG1 class<sup>2, 23</sup>. However, in spite of these apparently unpromising obstacles to rotavirus passive immunisation, increasing the titre of the IgG1 colostrum and milk antibody response of cows by vaccination remains the sole substantial efficacious and commercially successful development in rotavirus immunoprophylaxis<sup>2, 12</sup>.

The efficient transfer *in utero* of immunoglobulins from human mother to foetus contrasts with the almost totally agammaglobulinaemic state of newborn ungulates. This is compensated for in ungulates by an early colostrum immunoglobulin content that is largely IgG. However, by the postcolostral stage human milk has an Ig profile typical of that of a monogastric animal, with IgA predominating<sup>24</sup>. Additionally, in man as in animals, the precursors of mammary gland plasmocytes originate in the intestinal tract, and migrate to the mammary gland<sup>25</sup>. As would be anticipated, this leads to the production in milk of IgA specific to rotavirus but not to endemic but nonintestinal pathogens such as herpes simplex virus<sup>26</sup>.

Records of attempts to stimulate increased secretion of specific IgA in lactating women are scanty, but the evidence suggests that the occurrence or otherwise of prior intestinal antigen exposure is a key factor in determining the lacteal response to parenteral vaccination. In a classic experiment, Svennerholm and colleagues<sup>27</sup> gave a subcutaneous injection of whole-cell cholera vaccine to both Swedish and Asian women. The Swedes were anticipated to have had no previous cholera exposure, and indeed had low or negative pre-existing antibody titres. The Asian women were from an endemic area where natural exposure could be anticipated. Although vaccination stimulated increases in serum IgG in both groups, only the mothers with prior intestinal exposure produced significant titres of milk IgA antibodies. Subsequently a prospective study confirmed that protection against symptomatic cholera infection in sucking infants was associated with higher levels of antibody in breast milk<sup>28</sup>.

There are few systematic records of rotaviral milk antibody profiles throughout lactation. A majority of women secrete antibody detectable by both IgA ELISAs and VN assays in the first week of lactation; a lower proportion of individuals continue to secrete antibody for many months, and occasional increases in titre can be observed presumably in response to intestinal infection<sup>29-31</sup>.

As the protective role of breastfeeding in childhood rotaviral diarrhoea is at best controversial, the potential for passive immunisation by maternal vaccination has received scanty attention. Protection against rotaviral infection in the immediate postnatal period has been demonstrated<sup>32</sup>. Further into lactation, any effect is clearly limited, with at best suggestions that the clinical course but not the infection rate may be modified by milk antibody titres<sup>33</sup>, or that higher milk antibody titres may interfere with vaccine take<sup>34</sup>. More commonly it has not been possible to demonstrate a protective effect of human milk against rotaviral diarrhoea<sup>35, 36</sup>. However, a more optimistic assessment of the potential for passive immunisation through maternal immunisation can be drawn from consideration of animal experiments and artificial feeding of infants: (1) while 'natural' titres of antibody in the milk of endemically infected cows and sows are also not protective, they can be boosted through maternal vaccination to levels which are demonstrably protective against field infections<sup>2</sup>; (2) passive protection is an outcome of the maintenance of an adequate concentration of antibody in the intestine over a period of time, and is crucially dependent on titre<sup>37</sup>; (3) the protective potential of ingested antibody has been amply demonstrated in infants<sup>38-40</sup>. It therefore seems

reasonable to postulate that if milk antibody titres could be sufficiently stimulated by vaccination a protective effect may ensue.

ISCOM matrix was selected as an appropriate adjuvant because of its suitability for a wide range of species including primates. Furthermore the activity of ISCOM matrix is immunologically well characterised, and it can be prepared with virtually non-toxic Quillaja components even when given at high doses<sup>41-46</sup>.

These preliminary results from a very simple experiment in endemically infected baboons show that in a nonhuman primate a rotavirus vaccine adjuvanted with ISCOMs was safe, and effective in raising serum antibody IgG and VN titres to both vaccine and heterologous rotaviruses. Significant IgA and VN antibody titres were also present in milk at titres higher than those in endemically infected but unvaccinated controls. The IgA response suggests local antibody production in the mammary gland, which was elicited by the prior intestinal exposure of these animals to rotavirus antigens. VN titres were not raised as consistently as IgA titres, and attempts to use more sensitive neutralisation assays such as plaque reduction or epitope blocking using neutralising monoclonal antibodies<sup>47</sup> would be warranted. Further studies are necessary to optimise this enhancement of lacteal antibodies, and to investigate their protective potential. One potential disadvantage of this approach could be the loss of the protective effect that neonatal rotaviral infection has on subsequent childhood infection<sup>48</sup>. However, it is more likely that passive ingestion of antibody would allow limited replication of virus in a subclinical infection thus allowing development of immunity, rather than conferring complete protection against infection<sup>21</sup>. In view of the difficulties in developing effective oral immunisation for children in developing countries, it is also relevant to assess the significance of these results for human vaccination.

## ACKNOWLEDGEMENTS

Dr Lou Potash of DynCorp kindly provided the RRV-2 vaccine virus, through the good offices of Dr A. Z. Kapikian. ISCOM matrix was provided by Dr B. Sundquist of Iscotec. The care and animal handling was expertly provided by Drs N. Hahn and J. Nyaundi. This study was supported in part by the Scottish Office Agriculture and Fisheries Department and the Moredun Foundation.

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