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Homotypic and heterotypic serum and milk antibody to rotavirus in normal, infected and vaccinated horses.

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ABSTRACT

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The homotypic and heterotypic antibody response to rotavirus was determined in three pony mares and their foals. The normal concentrations of anti-rotavirus antibodies in mares' milk and mares' and foals' serum over the first 10 weeks post-partum were measured using IgA, IgG and rotavirus serotype-specific enzyme linked immunosorbent assays. Experimental infection of the foals with serotype 3 equine rotavirus produced a rapid, serotype-specific response which peaked 10 days after infection and a slower heterotypic response which peaked 32 days later. In contrast, vaccination of the mares with an inactivated, adjuvanted serotype 6 bovine rotavirus produced a heterotypic response similar to that of the homotypic response in both serum and milk, although the predominant response in serum was IgG, while in milk it was IgA. These results suggest that non serotype-restricted passive protection of foals against rotavirus may be achieved by parenteral vaccination of mares.

INTRODUCTION

Absolute concentrations of the various immunoglobulin isotypes in mares' milk have been established (Rouse and Ingram, 1970; Pahud and Mach, 1972; McGuire and Crawford, 1973) and IgA shown to be predominant. However, there is no information on the specific antibody titres to endemic enteric pathogens or other antigens, beyond the colostral period.

Rotaviruses are important enteric pathogens of many species and have been shown to cause diarrhoea in foals (Kanitz, 1976; Imagawa et al., 1984; Higgins et al, 1988). Surveys have implicated rotavirus as the cause of diarrhoea in between 10% and 60% of cases in foals (Conner and Darlington, 1980; Gillespie, et al., 1984; Herbst et al., 1987; Dwyer et al., 1988; Browning et al., unpublished data). Effective vaccination against rotavirus has been achieved

routinely in cattle, by passive immunisation through dam vaccination (Snodgrass, 1986; McNulty and Logan, 1987). However, the protection is mediated by IgG₁, the dominant immunoglobulin in the ruminant milk (Fahey et al. 1981), which is derived by selective transfer from the circulating pool, rather than by local synthesis in the mammary gland as in monogastric animals like the horse (Lascelles, 1977). Rotavirus vaccination has not been assessed in mares.

The purpose of this work was three-fold—to establish the normal titres of anti-rotavirus antibody in mare milk and mare and foal serum throughout the first 10 weeks post-partum; to examine the response of the foal to infection; and to examine the response to intramuscular vaccination of the mare. The responses were assessed by enzyme-linked immunosorbent assays (ELISAs) which detected anti-rotavirus IgG, anti-rotavirus IgA and anti-rotavirus antibody specific for serotype 2, 3 or 6 rotaviruses.

MATERIALS AND METHODS

Animals

Three pregnant Welsh Mountain pony mares were housed in separate boxes and allowed to foal normally. Each foal was kept in the same box as its dam throughout the experiment. Sera were collected from the mares at about 3 months and 3 weeks pre-partum and at 21, 42 and 63 days post-partum. Sera were collected from the foals at about 12 hours post-partum, then at weekly intervals thereafter. Milk samples were taken daily from 1 day pre-partum to 21 days post-partum and thereafter at weekly intervals. An equal quantity of milk from each side of the udder was pooled and whey prepared by centrifugation in a microcentrifuge at 15 000 g for 5 minutes.

Infection of foals with rotavirus

At 10 weeks of age each foal was infected with equine rotavirus following the method of Higgins et al. (1988). The foals were separated from their dams for 24 hours pre-inoculation and 24 hours post-inoculation, and each animal received sequentially, by stomach tube, 50 ml of 0.9 M NaHCO₃, a faecal suspension containing equine rotavirus, then 200 ml of phosphate buffered saline. The faecal rotavirus was a serotype 3 virus detected in a diarrhoeic foal. After infection of the foals their faeces were screened daily for 10 days for the presence of rotavirus, utilising polyacrylamide gel electrophoresis (PAGE), with silver staining for detection of viral double stranded RNA (Herring et al., 1982). The mares' milk and the mares' and foals' sera were sampled on the day of inoculation, than at 3, 6, 10, 14 and 21 days post-inoculation.

Vaccination of mares

Twentyone days after experimental infection of her foal, each mare was inoculated intramuscularly with 1 ml of a commercially available bovine rotavirus vaccine containing rotavirus strain UK, serotype 6 (Rotavec-K99*). Mares' milk and mares' and foals' sera were sampled at 3, 6, 10, 14, 21, 35, 49 and 63 days after vaccination.

Enzyme-linked immunosorbent assays

Anti-rotavirus antibodies in milk and sera were estimated using five different assays—anti-rotavirus IgA- and IgG-specific ELISAs; and rotavirus monoclonal antibody epitope blocking ELISAs specific for antibody to serotypes 2, 3 or 6. In all five ELISAs one well margin was left around the outside of the plates, these wells being filled with 100 μ l of phosphate buffered saline–0.05% Tween-20 (PBS–Tw20) during incubation steps to eliminate edge effects. Plates were coated with the respective antigen or antibody diluted in 15 mM Na_2CO_3 –35 mM NaHCO_3 buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed three times with PBS–Tw20 between each stage of the assays. The final incubation stage in all assays was with 100 μ l freshly prepared peroxidase substrate solution (110 μ M orthophenylenediamine–0.024% H_2O_2 –22 mM citric acid–51 mM Na_2HPO_4 per well at room temperature. The reactions were stopped by the addition of 50 μ l 2M H_2SO_4 per well and the optical density at 492 nm measured.

Isotype-specific ELISAs

Rotavirus-specific IgA and IgG ELISAs were performed essentially as described by McLean et al. (1980) and Bishop et al. (1984). Antigen was prepared by ultracentrifugation (80 000 g for 1 hour at 4°C) of clarified (5000 g for 30 minutes at 4°C) lysates of MA104 cell cultures and resuspended in 5 ml of 20 mM Tris–HCl (pH 7.5). Each assay was performed in duplicate Nunc-Immuno Polysorp plates, one coated with antigen from cells infected with equine rotavirus strain H-2 (Hoshino et al., 1983), the other with antigen from mock infected cells. Each sample was diluted two-fold or greater in 0.1 M Tris–HCl–0.5N NaCl–1 mM EDTA–2% BSA–3% Triton-X-100–3% Tween-20 (pH 7.4) (Shafren et al., 1989) and tested in triplicate. Each plate also contained a five-step, two-fold dilution series of a standard sample. The samples were incubated at 37°C for 2 hours. After washing, rabbit anti-horse IgG (H+L) or IgA (Fc)** antiserum diluted in PBS–Tw20 was added and incubated for 1 hour at 37°C. In the penultimate stage goat anti-rabbit IgG conjugated to horseradish peroxidase, diluted in PBS–Tw20, was added and incubated for 1 hour at 37°C.

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The mean OD_{492} of each sample was calculated and compared to the plot of the standard dilution series to determine the amount of anti-rotavirus antibody in the sample, assuming the standard contained 10 000 units. If a sample fell outside the range of the standard dilution series it was retested at a different dilution. No non-specific reactions were detected on the control antigen plates.

Monoclonal antibody epitope-blocking ELISAs

These assays were performed essentially as described by Shaw et al (1987). The reagent diluent was PBS-Tw20 with 2% foetal calf serum. Nunc-Immuno Maxisorp plates were coated with rabbit anti-rotavirus IgG. After washing, a lysate of MA104 cells infected with rotavirus, diluted 1:4, was added and incubated at 37°C for 3 hours. Human rotavirus DS-1 was used in the serotype 2 specific assay, rhesus rotavirus in the serotype 3 assay and bovine rotavirus UK in the serotype 6 assay (Hoshino et al., 1984). This was followed by a five-step, two-fold dilution series of each sample, performed in duplicate; the sixth row on the plate contained diluent alone and served as a negative control. After 1 hour at 37°C, the plates were washed and a serotype 2-specific neutralising monoclonal antibody (2F1, Shaw et al., 1987), a biotinylated serotype 3-specific neutralising monoclonal antibody (4F8, Shaw et al., 1986) or a biotinylated serotype 6-specific neutralising monoclonal antibody (UK/7 Snodgrass et al., 1990) was added to each of the respective assays. After 1 hour at 37°C the plates were washed and either rat monoclonal antibody against mouse IgG* or avidin**, conjugated to horseradish peroxidase added for 1 hour at 37°C.

In an extension of these assays used to examine further the nature of the response in the foals, parallel assays were performed using either rhesus rotavirus or UK rotavirus as the target antigen, and dilutions of foal sera taken post inoculation used to block a biotinylated, cross-neutralizing monoclonal antibody, 57-8 (Mackow et al., 1988).

The mean OD_{492} of each dilution step was used to plot a curve for each sample and the result expressed as the reciprocal of the dilution calculated to give an OD_{492} of 50% that of the mean of the negative control wells. Each time the test was performed a standard serum was included and used to compare results obtained in different tests.

RESULTS

Antibody in normal milk and sera

The results from these three animals are expressed as geometric means in Fig. 1. The plots for the first 10 weeks show the pattern that would be ex-

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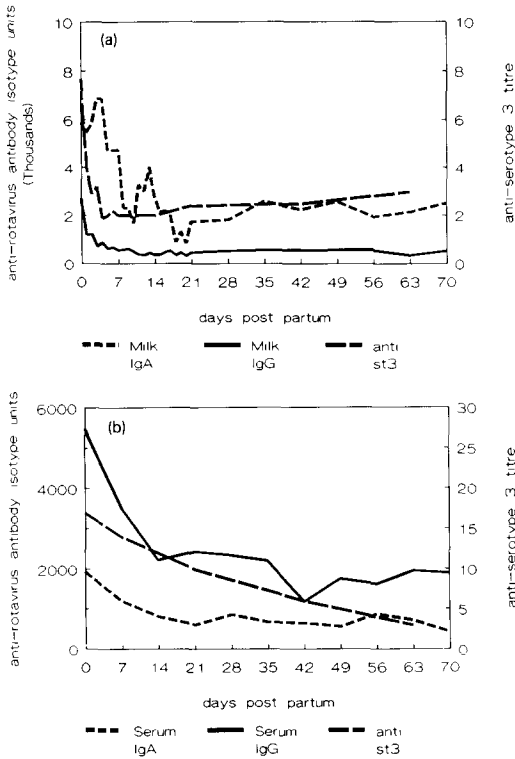


Fig. 1. Anti-rotavirus antibody in normal mares' milk (a) and foals sera (b) during the first 10 weeks post partum. The geometric mean (GM) titres of three animals are shown for rotavirus-specific IgA, IgG and serotype 3 rotavirus-specific antibody. Samples from mares were collected daily from days 0 to 21, then on days 28, 35, 42, 49, 56, 63 and 70. Samples from foals were collected on days 0, 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70. Log standard errors (SE) for IgA and IgG were less than 16% of the log mean GM while those for anti-serotype 3 were less than 50% of the log GM.

pected from published reports of total concentrations of immunoglobulins in foal sera and mares' milk (Fig. 1). Colostral titres of anti-rotavirus antibody were very high but dropped rapidly within 2 days post-partum. IgG and anti-serotype 3 antibody titres remained stable for the next 10 weeks but, after the initial drop, IgA declined slowly until about 3 weeks post-partum, then remained constant for the next 7 weeks. Anti-rotavirus antibody titres in the mares sera remained essentially constant from 3 months pre-partum until 10 weeks post-partum (data not shown).

Response of foals to infection

Only one of the three foals showed clinical signs of disease following experimental infection, manifested by inappetance, abdominal pain, reduced gut

sounds, and constipation. This foal was also the only one which excreted detectable amounts of rotavirus—from day 2 to day 7 post-infection. All foals showed a similar immunological response to infection as measured by the anti-serotype 3 ELISA, with a rise in titre detectable by the 6th day, which peaked on the 10th day, then dropped to about half the peak titre by day 14, and by day 21 dropped further to a titre which was maintained for the next 9 weeks (Fig. 2a). Both IgG and IgA titres rose in all three foals, but responses were less regular than those against the serotype 3 (Fig. 2b). Two of the three mares showed a serum and a milk response following infection of their foals although the serological response was less marked than that of the foals (Figs. 3 and 4).

Response of mares to vaccination

The mares' response to vaccination was much more dramatic than their response to the infection they acquired from their foals. Serum responses were

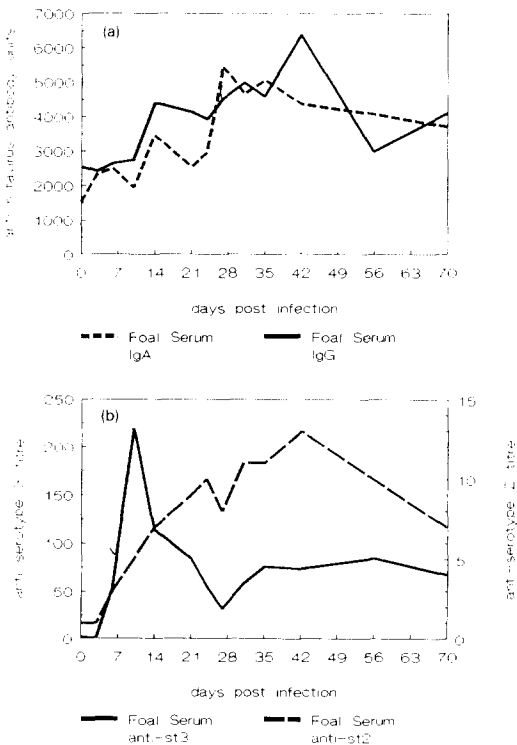


Fig. 2. Antirotavirus antibody in foals' sera following infection with a serotype 3 equine rotavirus. GM titres of three foals are shown for rotavirus-specific IgA and IgG (a) and serotype 3 and serotype 2 rotavirus-specific antibody (b). Samples were collected on days 0, 3, 6, 10, 14, 21, 28, 35, 42, 56 and 70. The log SE for IgA and IgG were within 10% of the log GM, while those for anti-serotypic antibody were within 50% of the log GM.

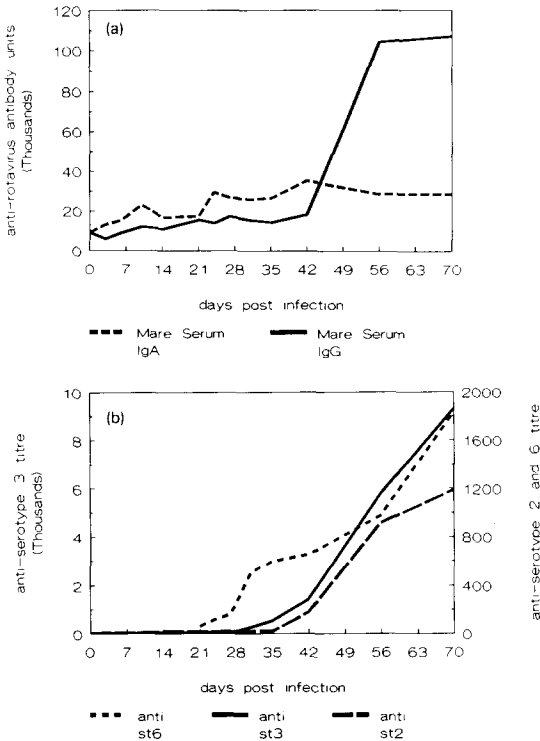


Fig. 3. Anti-rotavirus antibody in mares' sera following infection of their foals (day 0) and intramuscular vaccination with serotype 6 bovine rotavirus in mineral oil (at day 21). GM titres of three mares are shown for rotavirus-specific IgA and IgG (a) and serotype 6, serotype 3 and serotype 2 rotavirus-specific antibody (b). Samples were collected on days 0, 3, 6, 10, 14, 21, 24, 27, 31, 35, 42, 56 and 70. Log SE for IgA and IgG were within 10% of the log GM, while those for anti-serotypic antibody were within 16% of the log GM after day 21.

seen by 10 days post vaccination but the titres continued to rise for the next 6 weeks (Fig. 3). Milk titres did not rise until 14 to 63 days post-vaccination (Fig. 4).

Homotypic and heterotypic responses

The kinetics of the serotype-specific responses in the foals were significantly different from those seen in the mares. The response of the foals was more restricted to the inoculated serotype, and the heterotypic response to serotype 2 peaked much later than the homotypic response (Fig. 2b). In contrast the serotype 2, 3 and 6 responses of the mares were essentially similar, although the serotype 6 response appeared to begin earlier than the heterotypic response (Fig. 3b).

The parallel cross-reactive epitope-blocking assays using monoclonal antibody 57-8, are compared in Fig. 5. The geometric mean titres of the foals sera

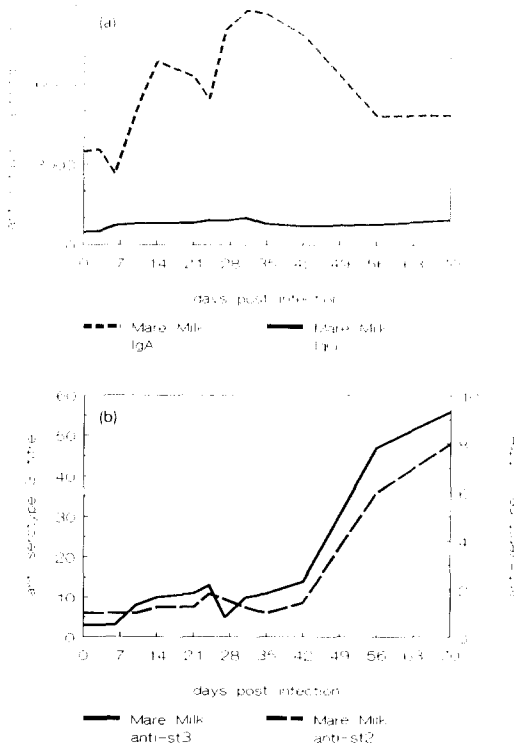


Fig. 4. Anti-rotavirus antibody in mares' milk following infection of their foals (day 0) and vaccination with serotype 6 bovine rotavirus in mineral oil (at day 21). GM titres of 3 mares are shown for rotavirus specific IgA and IgG (a) and serotype 3 and serotype 2 rotavirus-specific antibody (b). Samples were taken on days 0, 3, 6, 10, 14, 21, 24, 27, 31, 35, 42, 56 and 70. Log SE for IgA and IgG were within 12% of the log GM, while those for anti-serotypic antibody were within 50% of the log GM on days 56 and 70.

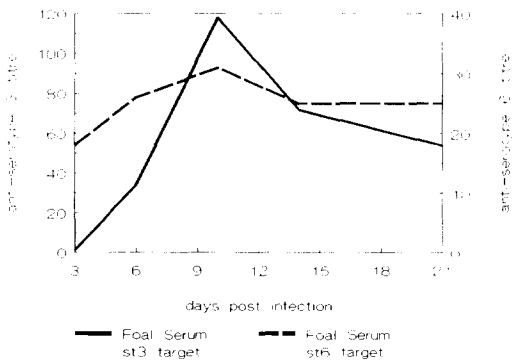


Fig. 5. Comparative epitope blocking assays with foal sera following infection with serotype 3 equine rotavirus. GM titres of three foals are shown for determinations of ability of sera to block the epitope of the cross-reactive monoclonal antibody 57/8 on either a serotype 3 rotavirus or a serotype 6 rotavirus target. Samples were measured on days 3, 6, 10, 14 and 21. Log SE for serotype 3 target were within 32% of the log GM, while those for serotype 6 target were within 13% of the log GM.

from day 3 to day 21 post inoculation are shown, with either a serotype 3 (rhesus rotavirus) or a serotype 6 (UK bovine rotavirus) as a target antigen. The response measured using a serotype 3 target mirrors that seen using the homotypic assay (Figure 2b), with a sharply defined peak at day 10. In contrast, the response as determined using a serotype 6 target is more similar to that seen in the heterotypic, serotype 2 assay, although a slight peak at day 10 is evident.

DISCUSSION

This study reports the first recorded observations of antibody titres in mares' milk to a specific antigen throughout lactation. Although the observations are not significantly different from what would have been predicted from studies of absolute concentrations of the various antibody isotypes in milk, they nevertheless confirm the relative predominance of IgA in milk, and its probable importance in passive protection of the sucking foal. The observation that anti-rotavirus antibody titres in milk vary little from 7 to 70 days post partum suggests that a single measurement during this period would be a reliable prediction of titres throughout lactation. Similarly, detection of rises throughout this period would indicate a response to infection or vaccination.

The predictable decay of maternally derived antibody was observed in the foals' sera throughout the first 9 weeks post partum. Of the three assays, the monoclonal antibody epitope-blocking ELISA gave the most significant exponential regression ($R^2 = 56\%$) and the calculated half life (23 days), which presumably reflects all antibody isotypes, was similar to that determined for passively acquired IgG antibody in other studies (Reilly and Macdougall, 1973; MacDougall and Dunlop, 1974).

During the development of the isotype-specific assays used in this study a variety of formats was used to reduce non-specific binding of equine immunoglobulins. The optimal method described reduced background to an acceptable level for IgG and IgA ELISAs, but no method could be found to reduce non-specific IgM binding. Similar assays on human sera have been shown predominantly to detect antibody to the inner capsid protein (VP6) of rotavirus, rather than that to the outer capsid proteins, VP7 and VP4 (Kalica, et al., 1981). The epitope-blocking assay has been shown to correlate better with serum neutralisation tests (Shaw et al., 1987) and also to be serotype specific (Shaw et al., 1987; Beards and Desselberger, 1989). On the basis of this study, the immune response of mares and foals to rotavirus is more reproducibly assessed by the epitope-blocking assay. The assay could be adapted to field trials to determine the incidence of clinical and subclinical infections and the predominant serotype responsible.

The serum responses in the foals following infection were quite rapid. Although it is possible that the responses were anamnestic, prior exposure seems

unlikely as the foals' sera were monitored weekly up to the time of infection, and antibody titres did not rise throughout the pre-infection period. Experience with experimental infection of 2-day old, specific pathogen-free foals (Browning et al., unpublished data) suggest that the primary humoral immune response can be detected at 6 days post inoculation in animals known to be naive.

The earlier peak of serum anti-rotavirus IgA, in comparison to that of IgG has been observed in human adults (Bernstein et al. 1989), although the peak responses occurred somewhat earlier than was observed in the foals. Of greater interest is the response as measured by the monoclonal antibody blocking ELISAs. In all three animals a peak response to the infecting serotype was observed 10 days after infection. The heterotypic response (that is, that directed against the serotype 2 virus) had a less pronounced and later peak, with a smaller rise in titre (14 fold, compared to 220 fold). Such a sensitive assay has obvious utility in epidemiological investigations—a sharply defined peak response would enable an accurate estimate of the date of infection in individual animals and the infecting serotype could also be established. Additionally, the assay seems sufficiently specific and sensitive to investigate the epidemiology of subclinical rotavirus infections, both in terms of incidence and prevalent serotypes.

The mares also showed a serum response following infection of their foals but the response was much less marked, and there was little difference between the kinetics of the homotypic and heterotypic responses. Virus excretion must have occurred in all foals although double stranded viral RNA was detected in only one.

It is notable that the milk antibody response appears to be predominantly IgA, while the serum response is predominantly IgG-associated, suggesting that local mammary gland production of IgA may have been stimulated (Fig. 4a). Montgomery et al. (1978) examined the effect of vaccination on IgA in mares' milk during the first 3 days post partum and showed a response to oral and intraductal inoculation, but not to parenteral inoculation. However it is unlikely that the mares had been exposed to the antigen, dinitrophenylated type III pneumococcus, prior to the study. In studies with transmissible gastroenteritis virus, Saif and Bohl (1982) have shown that intramammary vaccination of seronegative sows produces primarily an IgG response in milk, while in a seropositive sow the response was predominantly IgA. Saif (1985) has also shown that intramuscular inoculation of a seropositive sow with attenuated rotavirus at 4 days post partum was able to induce a neutralising antibody response in milk. Svennerholm et al. (1980) similarly demonstrated that milk secretory IgA can be boosted in Pakistani women with a single subcutaneous inoculation of cholera vaccine, but that a primary response could not be induced in the milk of Swedish women. Thus, the response of mares to a single inoculation with a rotavirus vaccine is consistent

with responses observed in other monogastric animals upon parenteral vaccination with an antigen which they have encountered enterically.

One point of concern in other studies is that the milk antibody response in sows to attenuated transmissible gastroenteritis virus and to attenuated rotavirus vaccination declined before the end of lactation (Saif and Bohl, 1982; Saif, 1985). This weak and transient response is similar to that observed in the mares that acquired infections from their foals. However the inclusion of an adjuvant (in our study mineral oil) in the vaccine prevented any decline in the milk antibody response up to 10 weeks after vaccination.

It should be noted that the vaccine strain used was a serotype 6 bovine rotavirus, but was able to stimulate antibody to serotype 3, the dominant equine rotavirus serotype (Browning et al., unpublished data) as well as to serotype 2 which has not been found in horses. Such a heterotypic response in experienced animals has been noted in other species (Brussow et al., 1988; Saif, 1985) and enables the use of a single serotype vaccine to protect against multiple serotypes. Heterotypic responses are minimal in naive animals (Snodgrass et al., 1984, Estes et al., 1989) and serotypic diversity has been a barrier to successful active vaccination against rotavirus (Chanock et al., 1988). Although these results are promising, further work is necessary to establish a viable vaccine for use in mares.

It will be necessary to establish whether vaccination prepartum will raise titres of antirotavirus antibody throughout the lactation period of the mare, and whether an alternative adjuvant can be found, as the vaccine used in this study causes severe reactions in some mares (Stoneham, personal communication). Our study does suggest that, should a decline occur following vaccination prepartum, a boosting inoculation in late lactation is feasible. It is also important to ensure that the lactogenic response induced by vaccination is not dependent upon recent enteric exposure to rotavirus as occurred with the mares in this study.

The difference in the kinetics of the serotype 2 and 3 responses in apparently naive foals suggests that although there is a slight heterotypic response, it is fundamentally different from the homotypic response. The comparison of the kinetics of the ability of the foals' sera to block a cross-reactive epitope on both serotype 3 and 6 rotaviruses provides some indication of the spectrum of the epitope-blocking assay, and also demonstrates the complexity of the humoral response to rotaviruses. It is apparent from a comparison of the parallel assays that the epitope-blocking assay, not unexpectedly, detects not only antibody able to bind to the monoclonal antibody's target epitope, but also that binding to the domain surrounding that epitope. Otherwise, the two assays would be similar, and that using the serotype 3 rotavirus target would not correspond to the homologous assay so closely. However, the slight peak observed at day 10 using a serotype 6 target suggests a specific response to the cross-reactive epitope, albeit much less pronounced than that to homotypic

epitopes. Application of these parallel assays and similar methods using other monoclonal antibodies may help elucidate why responses to homotypic epitopes predominate in naive animals, while responses to heterotypic epitopes are more apparent in immune animals.

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