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Immunoglobulin and Specific Antibody Responses to *Rhodococcus* (*Corynebacterium*) *equi* Infection in Foals as Measured by Enzyme-Linked Immunosorbent Assay

SHINJI TAKAI,* SHINICHIRO KAWAZU, AND SHIRO TSUBAKI

Department of Animal Hygiene, School of Veterinary Medicine and Animal Science, Kitasato University, Towada, Aomori, 034, Japan

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Humoral immune response to intestinal *Rhodococcus* (*Corynebacterium*) *equi* in horses was studied by enzyme-linked immunosorbent assay. Anti-*R. equi* immunoglobulin M (IgM), IgG, and IgA antibodies were demonstrated in the healthy horse population. Adult horse levels of anti-*R. equi* IgM and IgG antibodies were reached by 5 to 9 weeks of age in two healthy newborn foals. *R. equi* was recovered from the foals in the range of 10^3 to 10^4 per g of intestinal contents. A 1-week-old foal was infected with *R. equi* by mouth daily for 9 weeks. The foal did not show any clinical signs of illness. Anti-*R. equi* IgM antibody values in the foal increased about 5 to 8 weeks after initial inoculation, similar to the naturally occurring immune response to intestinal *R. equi*. There were differences among the antibody responses to *R. equi* in healthy horses, foals with suspected infection, and infected foals. These results suggest that exposure to *R. equi* is widespread in the horse population and that intestinal *R. equi* is the most important source of antigenic stimulation for a naturally occurring immune response in horses.

Rhodococcus (*Corynebacterium*) *equi* is an important pathogen which causes a suppurative pneumonia and enteritis in foals (1, 6, 15, 26, 28, 34). Although infection is thought to occur at a very young age, the foal does not show evidence of clinical signs until 1 to 6 months of age (6, 7, 15, 26, 28, 34). The natural route of *R. equi* transmission is not known but is generally regarded as inhalation and ingestion (1, 7, 15, 28). *R. equi* is known to be a saprophyte with widespread distribution in domestic animals and their soil environments (2, 3, 21, 24, 32, 35).

In the natural state, *R. equi* seems to be a common inhabitant of the intestine of horses, and there may well be natural immunization from this organ (8, 10, 23, 33). Results of the lymphocyte stimulation test by Prescott et al. (23) indicated the great extent to which an immune response to *R. equi* develops with age in the horse population; it was evident after about month 2 of life. Surveys of mares by intradermal skin and indirect immunofluorescence tests for serum antibody indicated that most mares had been exposed to *R. equi* and had mounted both an antibody and a cell-mediated immune response by adulthood (33). Using a more sensitive enzyme-linked immunosorbent assay (ELISA) technique for the detection of antibody, Hietala et al. (10) and Takai et al. (29) demonstrated a consistent anti-*R. equi* immunoglobulin G (IgG) response in a healthy horse population. Recently, we showed that *R. equi* was found in the feces of foals during week 1 of life and the highest numbers were present during the first 8 weeks (31). Intestinal *R. equi* is considered to be the most important source of antigenic stimulation for the immune response of foals, providing a small but unremitting source of antigen (21, 23, 33). It is well known that some natural antibodies reacting with certain indigenous bacteria are induced by those bacteria in their natural habitat in the alimentary tract (4, 5, 27). Although considerable information regarding the epidemiology of *R. equi* in horses has accumulated (1–3, 6, 15, 21, 26, 32, 34, 35), only limited attention has been given to the immunological responses of foals to their own intestinal *R. equi*.

The purposes of this study were to evaluate anti-*R. equi* IgM, IgG, and IgA antibodies in horses that were naturally and experimentally exposed to the bacterium and to determine the extent of formation of natural antibodies from intestinal *R. equi* in foals.

MATERIALS AND METHODS

Bacteria. *R.* (*Corynebacterium*) *equi* ATCC 6939, which was provided by Ryo Yanagawa, Hokkaido University, Hokkaido, Japan, was used for antigen preparation and experimental infection.

Immune serum. Immune serum against ATCC 6939 was prepared by intravenous inoculation of a healthy yearling foal with killed *R. equi* ATCC 6939 (approximately 10^{10} bacteria). To determine the optimum conditions of the assay, preimmune serum was used as the negative control and immune serum collected at 2 weeks after inoculation was used as the positive control.

Serum from healthy horses and foals. Serum samples were randomly collected from 177 healthy mares and stallions between 4 and 13 years of age from 23 farms, 48 healthy yearling foals from 30 farms, and 34 healthy foals between 1 and 6 months of age from 5 farms in Aomori prefecture, Japan.

Two thoroughbred foals, born slightly weak, were obtained at 3 and 4 weeks of age. Weekly serum samples were collected from these foals for 7 weeks. Sera were stored at −20°C until assayed.

Serum of healthy newborn foals. Serum samples were collected via the umbilical cords of 49 healthy newborn foals from three farms. Sera were stored at −20°C until assayed.

Serum of infected foals. Serum samples were collected from five infected foals. A diagnosis of *R. equi* infection was made by recognition of lesions in the lungs and intestines and by bacteriologic isolation and identification of *R. equi*.

* Corresponding author.

TABLE 1. Age-related changes in humoral immune response to *R. equi* in horses between 0 and 13 years of age

Age (yr)	No. of horses tested	ELISA OD_{492}[a]		
		IgM	IgG	IgA
0[b]	49	0.040 ± 0.027	0.010 ± 0.018	0.007 ± 0.015
1	34	0.131 ± 0.078	0.077 ± 0.057	0.005 ± 0.009
2	48	0.248 ± 0.072	0.094 ± 0.033	0.050 ± 0.031
4	8	0.163 ± 0.087	0.054 ± 0.073	0.050 ± 0.026
5	19	0.181 ± 0.054	0.031 ± 0.037	0.055 ± 0.020
6	23	0.197 ± 0.065	0.047 ± 0.050	0.054 ± 0.027
7	20	0.189 ± 0.086	0.059 ± 0.056	0.064 ± 0.035
8	22	0.194 ± 0.075	0.036 ± 0.055	0.060 ± 0.032
9	21	0.158 ± 0.047	0.048 ± 0.066	0.059 ± 0.029
10	12	0.210 ± 0.086	0.082 ± 0.091	0.073 ± 0.031
11	19	0.206 ± 0.057	0.050 ± 0.044	0.064 ± 0.019
12	17	0.153 ± 0.053	0.024 ± 0.035	0.062 ± 0.029
13	16	0.212 ± 0.067	0.039 ± 0.047	0.068 ± 0.025

[a] Mean ± standard deviation.
[b] At birth.

Serum samples were also collected from 11 foals with suspected *R. equi* infection. Sera were stored at −20°C until assayed.

Antigen. Antigen was prepared from *R. equi* ATCC 6939 as described previously (29). Briefly, *R. equi* ATCC 6939 was grown on yeast extract-casein-cystine agar. Bacteria were harvested after 2 days of incubation at 37°C. *R. equi* (2 g [wet weight]) was suspended in 10 ml of 0.0125 M sodium phosphate buffer (pH 7.4) containing 0.1% (wt/vol) Tween 20, incubated at 37°C for 90 min in a water bath with agitation, and centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant was used as an antigen. Antigen protein content was determined by the method of Lowry et al. (16) and adjusted to 1.0 μg/ml in carbonate-bicarbonate buffer (pH 9.6) containing 0.02% NaN_3 before use.

ELISA. The ELISA used was based on that of Takai et al. (29). Optimal dilutions of reagents were obtained by checkerboard titrations. Plates were washed four times between each step with phosphate-buffered saline solution (pH 7.2) containing 0.02% Tween 20 (200 μl per well). Tween 20 antigen was used to coat micro-ELISA plates. The plates were incubated at 4°C for ≥16 h in a humid box. Horse sera were inactivated at 56°C for 30 min, diluted in phosphate-buffered saline (pH 7.2) containing 10% fetal bovine serum, and added to the wells after washing. The plates were incubated at 37°C for 60 min. Anti-horse IgG (prepared in rabbits, H- and L-chain specific; Cappel Laboratories, Cochranville, Pa.), anti-horse IgM (prepared in rabbits, μ-chain specific; Cappel Laboratories), and anti-horse IgA (prepared in rabbits, α-chain specific; Cappel Laboratories) were added to each plate, and the plates were incubated at 25°C for 30 min. Horseradish peroxidase conjugated to anti-rabbit IgG (prepared in sheep; Cappel Laboratories) was added to each plate, and the plates were incubated at 25°C for 30 min. After the addition of substrate (0.4 mg of *o*-phenylenediamine dihydrochloride and 0.2 μl of H_2O_2 per ml in citric acid-Na_2HPO_4 buffer solution [pH 4.8]), the plates were incubated at 25°C for 20 min. The reaction was stopped with 100 μl of 3 N H_2SO_4. Optical density (OD) was measured by a multiscan ELISA reader at 492 nm. Two controls were used: conjugate added to antigen-coated wells without serum (ODs were always close to O) and sera in plates coated with solutions that were used in the preparation of antigen (low OD, usually <0.1); the low ODs were substracted from the ODs obtained with antigen-coated plates. ODs are expressed as the means of two determinations.

Experimental infection. A 1-week-old thoroughbred foal was used for experimental infection. This foal did not receive colostrum because of the death of her dam after delivery. The foal was obtained at 2 days of age and was fed with baby formula during the experiment. *R. equi* was not isolated from the feces before experimental infection. In the experimental infection, the foal was given 100 ml of a suspension of 1×10^9 to 5×10^9 *R. equi* in baby formula daily for 9 weeks. Blood and feces were collected at 4-day intervals during the experiment. Quantitative *R. equi* culture of the feces was done as described previously (29).

Statistical analysis. Results were expressed as the mean ± standard deviation and compared by Student's *t* test.

RESULTS

Age-related changes in equine humoral immune response to *R. equi*. Serum antibody to *R. equi* was measured by ELISA with anti-horse IgM, IgG, or IgA as secondary antibodies. Serum samples collected from 308 healthy horses between 0 and 13 years of age were assayed. The results were analyzed according to age and are shown in Table 1. The foals had no anti-*R. equi* IgM, IgG, and IgA antibodies at birth. ELISA ODs for anti-*R. equi* IgM, IgG, and IgA antibodies increased after birth and reached their maximum values at 2 years of age. There were small increases and decreases in the ELISA ODs for the sera of horses 4 to 13 years of age.

Serum samples from two foals, 3 and 4 weeks old, which already had *R. equi* in their feces, were collected weekly for 7 weeks. Anti-*R. equi* IgM and IgG antibodies of the foals gradually increased week by week and reached the levels found in adult horses (Table 2). The IgM antibody value was always higher than the IgG antibody value. During the experiment, anti-*R. equi* IgA antibody was not detected until the foals were 9 weeks old. *R. equi* was recovered from the foals in the range of 10^3 to 10^4 per g of intestinal contents. No pathological changes were found in the two foals at necropsy.

Humoral immune response of a foal to *R. equi* given by mouth. To examine the immune response of a foal in relation to fecal *R. equi*, a 1-week-old formula-fed foal was given *R. equi* ATCC 6939 (approximately 10^9 bacteria) by mouth daily for 9 weeks. The increases in anti-*R. equi* IgM, IgG, and IgA antibody values are shown in Fig. 1. The IgM antibody value slowly increased for 4 weeks after initial inoculation and increased markedly about 5 to 8 weeks after initial inocula-

TABLE 2. Age-related changes in humoral immune response to *R. equi* in two newborn foals

Age (wk)	ELISA OD_{492}					
	Foal 1			Foal 2		
	IgM	IgG	IgA	IgM	IgG	IgA
1	NT[a]	NT	NT	NT	NT	NT
2	NT	NT	NT	NT	NT	NT
3	0.082	0.066	0	NT	NT	NT
4	0.100	0.098	0	0.072	0.024	0
5	0.143	0.113	0	0.143	0.038	0
6	0.155	0.124	0	0.197	0.053	0
7	0.155	0.145	0	0.281	0.155	0.029
8	0.201	0.142	0	0.285	0.213	0
9	0.204	0.170	0	NT	NT	NT

[a] NT, Not tested.

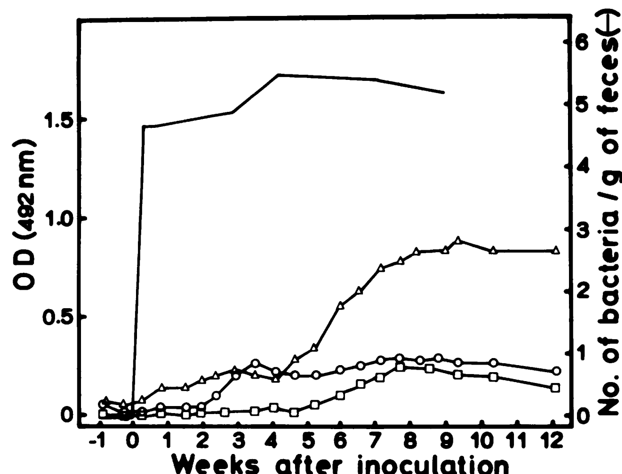

FIG. 1. *R. equi*-specific IgM (△), IgG (○), and IgA (□) antibodies as measured by ELISA in a foal given *R. equi* by mouth daily for 9 weeks. The solid line indicates the number of *R. equi* per gram of feces.

tion. The IgG antibody value increased about 2 to 4 weeks after initial inoculation and remained at that level throughout the remainder of the experiment. On the other hand, the IgA antibody value did not change until week 5 after initial inoculation, and then it gradually increased during weeks 5 through 8 after initial inoculation.

R. equi was recovered from the feces on day 2 after inoculation and then continuously during the experiment. The number of *R. equi* in the feces during the experiment ranged from 4 to 5 ($\log_{10}$) (Fig. 1).

Comparison of immune response to *R. equi* among normal horses, foals with suspected *R. equi* infection, and foals naturally infected with *R. equi*. The mean ODs of anti-*R. equi* IgM, IgG, and IgA antibodies were compared among normal horses, foals with suspected *R. equi* infection, and foals naturally infected with *R. equi* (Table 3). The mean value of anti-*R. equi* antibody in foals with suspected infection was significantly higher than that of normal horses, but the mean value of anti-*R. equi* IgM antibody in infected foals was not significantly higher than that of normal horses. The mean values of anti-*R. equi* IgG antibody in foals with suspected infection and infected foals were significantly higher than that of normal horses. On the other hand, the mean values of anti-*R. equi* IgA antibody in normal horses, foals with suspected infection, and infected foals were almost the same.

DISCUSSION

In the present study, anti-*R. equi* IgM, IgG, and IgA antibodies obtained in equine sera indicated that exposure to *R. equi* is widespread in the horse population and that intestinal *R. equi* might be the most important source of antigenic stimulation for a naturally occurring immune response in horses. Results of a lymphocyte stimulation test indicated the great extent to which an immune response to *R. equi* develops with age in the horse population (8, 9, 22, 23, 29, 33). An ELISA for the detection of antibody to *R. equi* also demonstrated a consistent *R. equi*-specific antibody response in healthy horses (10, 29). In recent reports, most horses tested harbored *R. equi* in their intestines (21, 24, 32, 35). It is believed that *R. equi* is widespread and may be part of the normal equine fecal flora (21, 24, 32, 35). Wilks et al. (33) noted that a high reactor rate to intradermal skin and indirect immunofluorescence tests for serum antibody in mares and foals on all stud farms surveyed in Australia agreed with reports of the frequent isolation of *R. equi* in fecal and soil samples (35). Berg and Savage (4) suggested that some natural antibodies reacting with certain indigenous bacteria are induced by those bacteria in the natural habitat in the alimentary tracts of mice. Sharpe et al. (27) showed that sera of ruminants and a horse contained relatively high titers of agglutinating antibodies against strains of anaerobic bacteria isolated from the bovine rumen. The consistent presence of antibodies against *R. equi* in the horse population suggests that *R. equi* colonizes the intestine, where constant or recurring antigenic stimulation could occur.

Some investigators believe that natural antibodies are products of the physiological development of the serum globulin (5). Others believe that natural antibodies are the result of inapparent antigenic stimulation (5). In the two newborn foals, anti-*R. equi* IgM and IgG antibody values increased markedly from 4 to 8 weeks of age. *R. equi* was found in the feces during the first weeks of life, and the organisms continued to be present in the feces during the entire observation period. In a recent study, we showed that the highest numbers of *R. equi* were present in the feces of foals during the first 8 weeks of life, which coincides with the age when the foals are most liable to *R. equi* infection (31). Therefore, the high concentration of these organisms might evoke a strong antigenic stimulus in foals. McGuire et al. (19) suggested that foals should have significant quantities of serum immunoglobulins owing to synthesis after 2 months of age, even if maternal transfer had not occurred. It seems that natural antibodies to *R. equi* in foals might be the result of inapparent antigenic stimulation. The IgM antibody value was always higher than the IgG value in the two foals. Rejnek et al. (25) showed that newborn horses had both IgM and IgG. The amount of IgM was significantly higher than the amount of IgG in all serum samples tested (25). The quantitative relation of IgG and IgM in newborn horse sera was the opposite of that found in the sera of adult horses (25). Anti-*R. equi* IgM antibody was not detected in newborn foals; therefore, IgM antibody detected in this study was synthesized as the result of a primary response to *R. equi* in

TABLE 3. Comparison of immune response to *R. equi* among normal horses, foals with suspected *R. equi* infection, and foals naturally infected with *R. equi*

Horses	No. of horses tested	ELISA OD_{492}[a]					
		IgM	*P*	IgG	*P*	IgA	*P*
Normal horses	264	0.183 ± 0.072		0.051 ± 0.063		0.061 ± 0.028	
Foals with suspected infection	11	0.287 ± 0.135	<0.05	0.796 ± 0.312	<0.01	0.060 ± 0.059	NS[b]
Infected foals	5	0.254 ± 0.206	NS	1.100 ± 0.524	<0.01	0.080 ± 0.067	NS

[a] Mean ± standard deviation.
[b] NS, Not significant.

the foals. To explain the local immune response of the intestinal tract of foals to *R. equi*, the immunoglobulins of the intestinal tract must be investigated in further studies.

A 1-week-old foal was infected with 1×10^9 to 5×10^9 *R. equi* by mouth daily for 9 weeks. The production of anti-*R. equi* IgM antibody developed fairly slowly until 4 weeks after initial inoculation and then increased rapidly until 9 weeks after initial inoculation. The foal shed 10^4 to 10^5 *R. equi* in the feces during the experiment, which was coincident with the highest number of *R. equi* in the feces of normal foals during the first 8 weeks of their lives (31). Sharpe et al. (27) described the agglutination titers in bovine sera against anaerobic rumen bacteria and showed that the antibodies to the rumen organisms were highly specific and of the IgM type. In the present study, the anti-*R. equi* IgG antibody of the 1-week-old foal increased slightly but not to the level of the five infected foals, and the foal did not show any clinical signs of illness. This might be partly explained by the virulence of the strain used (30), the mode of oral infection (14), and the susceptibility of the foal (19). McGuire et al. (19) indicated that failure of passive transfer of antibody in foals does not invariably result in infection. The production of anti-*R. equi* specific IgM antibody in the foal seems to be a condition similar to the naturally occurring immune response to intestinal *R. equi*.

In the present study, passive transfer of anti-*R. equi* antibodies was not detected in the foals. Hietala et al. (10) demonstrated passive transfer of anti-*R. equi* IgG antibody and the temporal development of a specific response in foals after the first 5 to 6 months of life by ELISA. The importance of transfer of colostral immunoglobulins in newborn foals has long been recognized (13, 18, 19). Antibodies and other macromolecular proteins are absorbed principally during the first 24 h of life and in negligible amounts thereafter (11). Three foals used in this study were considered to have failed to obtain or absorb colostral immunoglobulins because of the delay or failure of suckling. Two deleterious effects can be associated with the transmission of passive antibody (13). First, the transfer of maternal antibody can be the cause of immunological incompatibility hemolytic disease. Second, active immunization of the young foal may be hindered or prevented if specific maternal antibodies are present. Active immune response to *R. equi* in foals might be due to the failure of passive antibody transfer. Jeffcott (12) showed that in colostrum-deprived foals autogenous γ-globulin was first detected after 2 weeks of life and the γ-globulin level then rose fairly steeply until 7 to 8 weeks and remained at that level until 1 year of age. Although the ability to produce a primary response to a few antigens may not be fully developed until a few days after birth, foals are born with a reasonably competent lymphoid immune system (12, 17). The lymphoid system of newborn foals initiates primary response to pathogens encountered (12, 17).

The mean values of anti-*R. equi* IgG antibody in foals with suspected infection and infected foals were strikingly higher than those of normal horses. On the other hand, there were no significant differences between normal horses and infected foals in the mean values of anti-*R. equi* IgM and IgA antibodies. Nakazawa (20) demonstrated precipitating antibodies against concentrated culture supernatants of *R. equi* in the 7S globulin fraction and against *R. equi* sonic extracts in both 7S and 19S globulin fractions in naturally infected foals. There is little doubt that there are differences in the quality and quantity of *R. equi* antigens which evoke the different immune responses of normal horses and foals infected with *R. equi*.

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